KU Leuven

Biomedical Sciences Group

Faculty of Medicine

Department of Public Health and Primary Care

KU LEUVEN

DOCTORAL SCHOOL
BIOMEDICAL SCIENCES

External and Internal Drivers of Unhealthy Ageing

Yuling YU

Supervisor:	Prof. Dr. Tim S. Nawrot
Co-supervisors:	Prof. Dr. Karel Allegaert
	Prof. Dr. Peter Verhamme
Mentor:	Prof. Dr. Jan A. Staessen
Chair examining committee:	Prof. Dr. Peter Hoet
Chair public defence:	Prof. Dr. Jeroen Vanoirbeek
Jury members:	Prof. Dr. Chantal Mathieu
	Prof. Dr. Maarten Naesens
	Prof. Dr. Marc De Buyzere
	Prof. Dr. Tine Willum Hansen

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical Sciences

March 2025

Table of contents

Acknowledgements **VII**

List of abbreviations **XI**

Chapter 1 Introduction **1**

Chapter 2 Unhealthy ageing in relation to lead exposure

Part 1 Two-year responses of renal function to first occupational lead exposure **35**

Part 2 Blood pressure and renal function responses in workers exposed to lead for up to six years **79**

Part 3 Health risks related to environmental and occupational lead exposure **113**

Chapter 3 Unhealthy ageing in relation to urinary proteomic profiling

Part 1 Osteoporosis in relation to a bone-related ageing biomarker derived from the urinary proteomic profile: a population study **139**

Part 2 OSTEO18, a novel urinary proteomic signature, associated with osteoporosis in heart transplant recipients **183**

Part 3 Urinary proteomic signature of mineralocorticoid receptor antagonism by spironolactone: evidence from the HOMAGE trial **223**

Chapter 4 Unhealthy ageing in relation to clinical risk factors

Part 1 Blood pressure trajectory from birth to preschool- and school-age: evidence from ENVIRONAGE birth cohort **261**

Part 2 Blood pressure and cardiovascular risk in relation to birth weight and urinary sodium: an individual-participant meta-analysis of European family-based population studies **291**

Part 3 Racial and regional disparities in the risk of non-communicable disease between sub-Saharan Black and European White patients **335**

Chapter 5 General Discussion **383**

Summary **409**

Samenvatting **415**

摘要 **421**

Curriculum Vitae **425**

List of publications **427**

Acknowledgements

My doctoral training has been an amazing journey, with lots of challenges, setbacks and successes. Throughout this journey, I have always appreciated and cherished the encouragement and support from all the best people around me. I am so fortunate to have my supervisors, colleagues, family and friends, who have accompanied and supported me unconditionally.

I am deeply grateful to my promotor, Prof. Tim S. Nawrot for his endless guidance and support. Not only his expertise in epidemiology and insightful feedback improved my research and manuscripts, but his encouragement also built my confidence and helped me to get through difficulties. Moreover, I would like to express my sincere gratitude to my co-promotors, Prof. Karel Allegaert and Prof. Peter Verhamme. Prof. Allegaert always responded to my questions promptly and provided me with helpful suggestions. Prof. Verhamme always inspired me in face-to-face meetings and taught me how to compile my findings into a good story. Very importantly, I deeply appreciate my mentor, Prof. Jan A. Staessen, for starting my journey in KU Leuven, and his unwavering support all the time. He provided me with numerous suggestions during regular meetings and followed my progress meticulously. His expertise, rigorousness and persistence had a lot of influence on me.

I would like to thank my jury members in the thesis examination committee. As internal jury members, Prof. Chantal Mathieu and Prof. Maarten Naesens provided insightful comments and suggestions at every evaluation milestone, which deepened my critical thinking, enriched my scientific understanding, and shaped my research better. I would like to express my gratitude to Prof. Marc De Buyzere and Prof. Tine Willum Hansen, for accepting to be my external jury members and to comment on my thesis. I would like to thank Prof. Peter Hoet for tracking my progress throughout my doctoral training and as the delegate of the thesis advisory committee to read my thesis. I would also like to thank Prof. Jeroen Vanoirbeek for accepting to chair my public defence.

I am grateful to all my colleagues and co-authors for their support and collaborations. Special thanks to Prof. Stefan Janssens and Prof. Johan Van Cleemput for supporting my research work in UZ Leuven. Special thanks to Prof. Harald Mischak and Dr. Agnieszka Latosinska for supporting my research and sharing their knowledge during my lab visit in Mosaiques Diagnostics GmbH, Germany. I appreciate Prof. Yan Li from Shanghai Institute of Hypertension, China, for her insightful comments on my manuscripts. I also appreciate my colleagues Dr. Dewei An and Dr. Dongyan Zhang, for the scientific collaboration and companionship in Leuven. Besides, I truly appreciate Dr. Dries S.

Martens, my colleague from Hasselt University, for sharing his expertise and knowledge with me and encouraging me all the time. I thank Babangida Chori for working together with me for more than two years.

As a part of the research unit Environment and Health, I have learned a lot about environmental and occupational health sciences. I appreciate my colleagues, Prof. Steven Ronsmans, Dr. Manosij Ghosh, Dr. Katrijn Broothaerts, Dr. Nirosha Ganesan, Dr. Lisa Janssen, Rani Claus, Frauke Lemaire, Aala Azari, Wai Kei Chu, Thomas Celis, Nora Marain, Evi De Ryck, Sacha Reep, Pirawan Wangupadcha, who have accompanied me throughout my journey and brought professional knowledge. In addition to work, I will always remember the joyful moments, such as the homemade Indian and Persian dinners, Belgian potluck, football games, the visits to Leuven Kermis and Christmas market, and other interesting team buildings.

During my first stay in Leuven, I gained generous support from my colleagues in the Department of Cardiovascular Sciences. I deeply thank Prof. Zhenyu Zhang for her kind help both at work and in life. I appreciate Ms. Lutgard Thijs for the suggestions on statistical analysis. I thank my peer colleagues, Prof. Jesus Melgarejo, Dr. František Sabovčik and Dr. Dongmei Wei for their companionship in Leuven and during international conferences.

Importantly, the research and fieldwork experience in Guangdong Provincial People's Hospital and Guangdong Cardiovascular Institute, China, are the foundations of my scientific career. I would like to thank my supervisor during my master's training, Prof. Yingqing Feng, who has always cared about my study and life, and had international calls to provide guidance. I deeply thank Dr. Yuqing Huang for his scientific support. I cherish the long-standing friendship with my peer colleagues, Jiayi Huang, Lin Liu, Chaolei Chen, and wish them a successful career!

I would like to express my gratitude to all my friends at home and abroad, as they are an indispensable part of my life. In particular, it was a pleasure to share the lunch hours in Alma Gasthuisberg together with Xin Jin and Ji Ma, whose humour is the best seasoner. Moreover, it is unforgettable that Zuodong Zhao and I supported each other during the chilly winter when we were writing our dissertations. During my spare time, I also enjoyed travelling, hiking and learning English with Jingyan Zhang. I thank my lovely neighbours, Jie Zhang, Jeffee Hsiung, Weili Chen, Qiao Sun, who always offered lots of delicious food. In addition, I have encountered many good friends in Leuven: Fatemeh Rafaapoor, Marin Inoue, Qingqing Wang, Dandan Peng, Junyu Chen, Congrong Wang, Bowen Wang, Shanping Ji, Weiyang Kang, Zhixuan Zhang, Shikang Zhao, Hao Yu, Peng Nie, Shuncong Wang, Miaomiao Liu, Yuansheng Luo, Xinyuan Dang, Yanmei Feng,

Xiongwei Yin, Yulin Zhou, and the countless others. Outside of Leuven, I thank my roommates in university, Joyce Cheung, Jiayi Luo, Mi Zhang, with whom I took the anatomy course, learned experimental skills, studied overnight for the exams, and shared the most memorable moments in the medical school. Especially, I thank my best friends, Baozhu Chen, Meijuan Chen, Yingyin Huang, Kailin Liang, and cherish our long-lasting friendship since middle school.

Last but not least, I deeply appreciate my family, especially my parents, Cuiping Ye and Liangming Yu, and my grandparents, who have provided me with unconditional support since I was born, and educated me to be a better person. Heartfelt thanks to my cousin, Xiaoying Ye, for her sweet phone calls from home, and best wishes to her future! I am very grateful to my boyfriend, Josse Cottenier, who is always there to support and comfort me without any strings. Additionally, I deeply thank Josse's family for bringing me familial warmth and joy in this beautiful country.

Thank you and best wishes to you all!

Yuling Yu

Leuven, 16 February 2025

List of abbreviations

AAP	American Academy of Pediatrics
ACR	albumin-to-creatinine ratio
AUC	area under curve
BL	blood lead
BMD	bone mineral density
BMI	body mass index
BP	blood pressure
BW	birth weight
CE-MS	capillary electrophoresis coupled to mass spectrometry
CBLI	cumulative blood lead index
CI	confidence interval
CITP	carboxyterminal telopeptide of collagen I
CKD	chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
COL1A1	the α1 chain of collagen type 1
CV	cardiovascular
CVD	cardiovascular disease
DALY	disability-adjusted life-year
DBP	diastolic blood pressure
dp-ucMGP	desphospho-uncarboxylated matrix Gla protein
ECM	extracellular matrix
EPA	Environmental Protection Agency
eGFR	estimated glomerular filtration rate
eGFRcc	eGFR derived from both serum creatinine and serum cystatin C
eGFRcrt	eGFR derived from serum creatinine
eGFRcys	eGFR derived from serum cystatin C
ENVIRONAGE	Environmental Influence on Early Ageing
EPA	Environmental Protection Agency
EPOGH	European Project on Genes in Hypertension
ESRD	end-stage renal disease

FLEMENGHO	Flemish Study on Environment, Genes and Health Outcomes
FRS	Framingham 10-year risk score
FU	Follow-up
GBD	Global Burden of Disease
HASD	difference between body height and arm span
HASR	body-height-to-arm-span-ratio
HDL	high density lipoprotein
HF	Heart failure
HOMAGE	Heart “Omics” in Ageing Study
HTx	heart transplant
HR	hazard ratio
HRV	heart rate variability
IGF2	insulin-like growth factor II
IQR	interquartile range
ITT	intention-to-treat
LVD	left ventricular dysfunction
MAP	mean arterial pressure
MGP	Matrix Gla protein
MR	mineralocorticoid receptor
NCD	non-communicable diseases
NCV	nerve conduction velocity
NHANES	National Health Examination Survey
OR	odds ratio
OSHA	Occupational and Health Administration
OSTEO18	an 18 peptide multidimensional osteoporosis UPP biomarker
PDR	the 5th-95th percentile data range
PI	the 5th-95th percentile interval
PICP	carboxyterminal propeptide of procollagen I
PIGR	polymeric immunoglobulin receptor
PP	per-protocol
QALY	quality-adjusted life-year

RAAS	renin-angiotensin-aldosterone system
RF	renal function
ROC	receiver operating characteristic curve
SBP	systolic blood pressure
SD	standard deviation
SDI	social-demographical index
SE	standard error
SES	socioeconomic status
SPHERL	Study for Promotion of Health in Recycling Lead
UNAK	urinary sodium-to-potassium ratio
UVNA	24-h urinary sodium excretion
UVCD	24-h urinary cadmium excretion
UPP	urinary proteomic profiling
UPPost-age	an aging-related biomarker indicative of osteoporosis
UPRIGHT-HTM	Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform
uPROPHET	Urinary Proteomics in Predicting Heart Transplantation
UVNA	urinary sodium excretion
XRA	dual energy X ray absorptiometry

Chapter 1

Introduction

Ageing is a natural and inevitable course of life. As defined by World Health Organisation, healthy ageing, also known as optimal lifecourse, is the process of developing and maintaining the functional ability that enables wellbeing in older age [1]. The epidemiological transition, as defined by the Global Burden of Disease (GBD) consortium, is a global demographic revolution characterised by long life expectancy, which comes at a cost of lengthier disability-adjusted life-years and lower quality of life [2], due to unhealthy ageing [3]. Epidemiology is the science that studies the patterns, causes, and effects of risk factors and disease in populations or patient cohorts. Over the past decades, population scientists have been dedicated to investigating on age-related manifestations and exploring therapeutic interventions to decelerate, stop or even reverse unhealthy ageing [4]. Although these studies primarily focused on the functional decline and adverse health outcomes in middle and late adulthood [4], unhealthy ageing already started *"silently"* at the very early stages of life [5-7], and progresses throughout the whole human life span [8].

Risk factors differentiate unhealthy ageing from the average lifecourse with effect modifications driven by both external and internal drivers (**Figure 1.1**). External drivers are the exogenous exposure relevant to human health in the working and living environment. Worldwide attention increasingly revolves around the theme of environmental pollutants and their health effects [9]. Lead, which is a ubiquitous heavy metal in this class, was previously reported, at excessive exposure levels, to affect multiple organs, including cardiovascular [10,11], nervous [12] and renal [11,13] systems, thereby increasing morbidity and mortality [14,15]. Because of the tightening legislations, in developed countries, blood lead levels fell drastically over the past decades [9,16,17]. Nevertheless, environmental exposure to lead and other toxic pollutants remains a matter of concern for unhealthy ageing [9].

Internal drivers involve underlying disease mechanisms, which could be reflected by the urinary proteome, showing molecular pathogenic processes. Nowadays, researchers embrace the wave of new technologies and are increasingly making use of "*omics*", such as genetics, proteomics, epigenetics, metabolomics [18], which allow to identify age-related disease at the molecular level and prior to the clinical stage [19,20].

Clinical risk factors find their origins in both internal and external environment, and are operative throughout the human life span starting already "*in utero*", continuing in the perinatal period of life onto childhood [6] and adulthood, until old and very old age [21]. Demographic characteristics, perinatal condition, physiological features and life style are common clinical risk factors. From an epidemiological perspective, the assessments on

clinical risk factors could provide explanatory evidence to internal disease mechanisms and predict the progression of ageing in different life trajectories.

Currently, health care resources are for over 90% spent in treating established diseases, whereas prevention of diseases is much more cost effective [22]. Health strategies lag behind, possibly due to the latency of risk factors and imperceptibility of susceptible individuals, so that preventive and therapeutic interventions could not be timely employed. From this point of view, my doctoral dissertation focused on the external and internal drivers of unhealthy ageing in widely different settings ranging from children to adults, from general population to patients, and from healthy preindustrial individuals to the workers with occupational lead exposure (**Figure 1.1**). The ultimate goal is to emphasise the importance of timely prevention and intervention ahead of disease development, thereby reaching an optimal lifecourse (**Figure 1.1**).

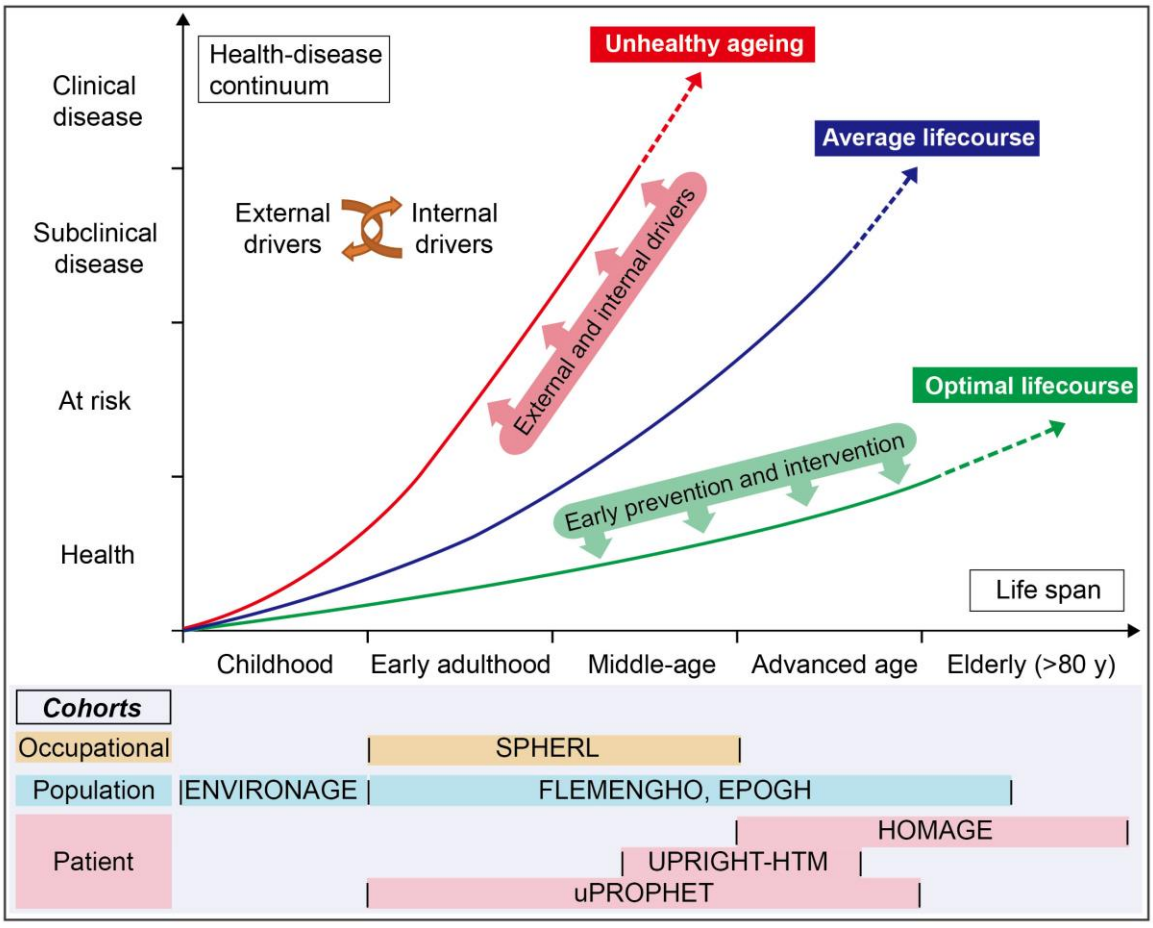


Figure 1.1 Lifecourse and health-disease continuum.
The figure shows three lifecourse trajectories: the lifecourse of individuals with unhealthy ageing (red), the average lifecourse in general population (blue), and the optimal lifecourse (green). External and internal factors drive the average lifecourse from healthy to unhealthy ageing. Early prevention and intervention should be provided to improve lifecourse trajectory. The occupational, population and patient cohorts in this dissertation cover the whole human life span and health-disease continuum (see ***Chapter 1.3***).

1.1 External and internal drivers

External and internal drivers are the risk factors increasing the likelihood to develop *"at risk"* status, subclinical or clinical disease. These ageing precursors are not simply additive, but reinforce one another. **Figure 1.2** provides an overview of the external and internal drivers assessed in this dissertation.

1.1.1 Lead exposure

Lead (Pb; atomic number: 82) is a cumulative heavy metal with high toxicity. Among toxic metals threatening human health, lead is the dominating contaminant in this class. Sources of lead exposure are ubiquitous in the environmental and occupational settings. Historically, lead exposure originated from pyrolytic zinc ore refinement, leaded paints, as additive to fossil fuels and drinking water distributed via leaded pipes [23]. Coarse and fine particulate containing lead is a prime source of exposure in western countries, while the gastrointestinal route is an exposure vector due to the environmental contamination of food or drinking water in middle- and low-income countries [23,24]. The toxicokinetics and pathophysiology of lead are described in ***Chapter 2.3***. In the past, the adverse health effects caused by the high lead exposure included renal function decline [13,25,26], hypertension [10,11], impairment of neurocognition, especially in young children [27-30], and dysfunction of central and peripheral nervous system [12].

In the past decades, the tightening legislation in developed countries resulted in a decreased blood lead (BL) level in general population. The National Health Examination Survey (NHANES) demonstrated that mean BL levels among American adults dropped from 13.1 µg/dL in NHANES II (1976–1980) [31] to 2.76 µg/dL in NHANES III (1988–1994) [31], and further to 1.64 µg/dL in NHANES IV (1999–2002) [17,32]. In the light of the falling environmental lead exposure [17,31,32], agencies, such as the National Toxicology Program [33] and the Environmental Protection Agency (EPA) [34,35] reviewed the literature in weight-of-the-evidence analyses [36], and proposed that BL levels of 5 µg/dL or lower might be associated with adverse health effects [33-36]. In particular, the workers in the battery manufacture and recycling plants are exposed to a high contamination of lead [37], but given the current legislation protecting workers, whether the long-term occupational lead exposure causes adverse health outcomes remains unclear.

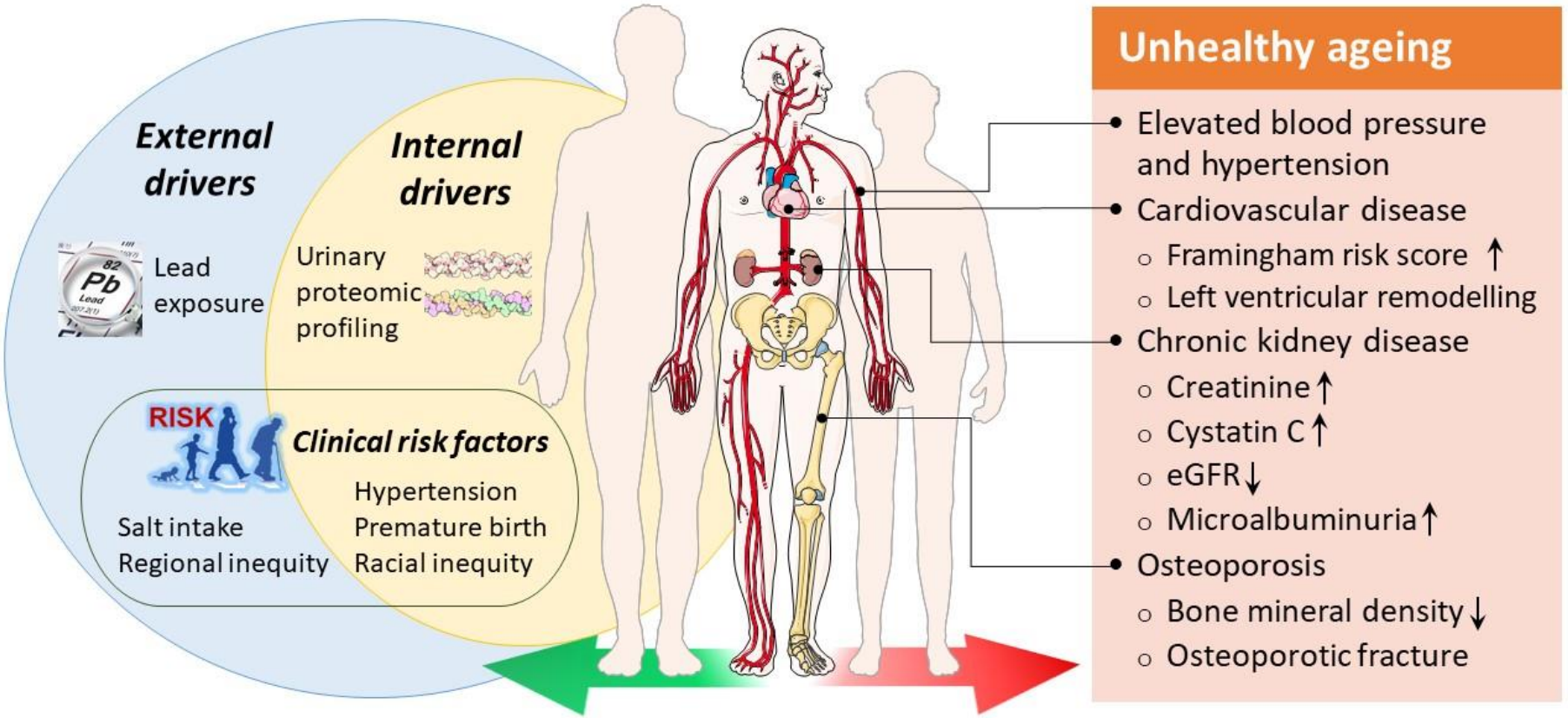


Figure 1.2 External and internal drivers leading to unhealthy ageing.
Abbreviation: eGFR, estimated glomerular filtration rate.

The Study for Promotion of Health in Recycling Lead (SPHERL; **Figure 1.3**) [38] provided the opportunity to test the association between health outcomes and occupational lead exposure among young healthy workers without known lead exposure. This study was conducted under the regulations issued by Occupational and Health Administration (OSHA). Prior to lead exposure, the mean BL level was 4.50 μg/dL in the young newly employed workers (mean age, 28.6 years), which was comparable to the BL level exposed to the natural source, and was not significantly associated to any of the functional decline in cardiovascular system [39-41], brain [41] or kidneys [42]. During my first stay as visiting scholar in the Research Unit of Hypertension and Cardiovascular Epidemiology, Department of Cardiovascular Sciences, KU Leuven (2019-2020), I assessed the longitudinal outcomes of SPHERL using data collected up to the second year of follow-up. On average, BL increased significantly at follow-up. The associations of blood pressure changes or the incidence of hypertension [43], neurocognition [44] and autonomous nervous system function [45] with BL did not reach significance in the workers following two-year occupational lead exposure. Building on this line of research, in ***Chapters 2.1*** and ***2.2***, I further investigated the co-primary endpoints, blood pressure (BP) and renal function, in relation to BL by extending the follow-up visit for up to six years.

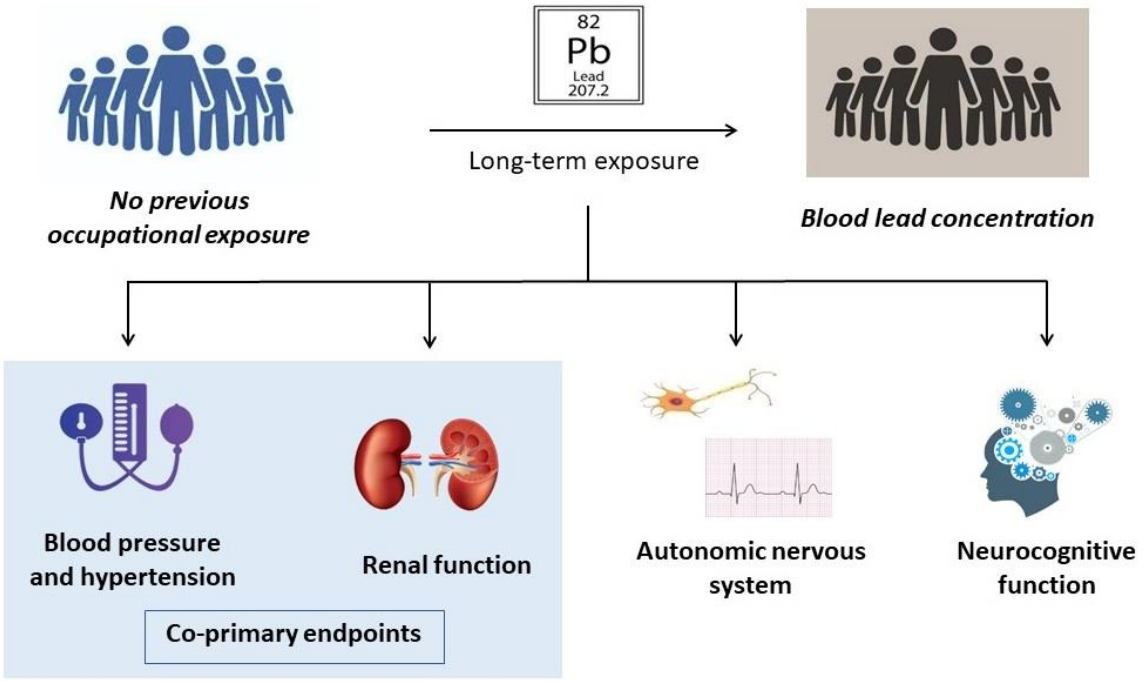


Figure 1.3 Study for Promotion of Health in Recycling Lead.

1.1.2 Urinary proteomic profiling

Urine contains over 21,000 endogenous peptides, which are generated along the nephron or which pass through the glomerular sieve into the tubular fluid and ultimately appear in the urine [46,47]. Among all the urinary peptides, about 5,000 are sequenced, allowing the identification of the parental proteins (**Figure 1.4**) [20]. Moreover, 60% of the sequenced peptides are collagen fragments [48]. Collagen is the main component of extracellular matrix (ECM), supporting the biological structures and maintaining the function of heart, bones, skin, blood vessels and other organs [49-51]. Other urinary peptides, for instance, derived from matrix Gla protein (MGP) [20] and mucin-1 subunit α [52], are biomarkers of unhealthy ageing. The urinary proteomic profiling (UPP) therefore provides a body-wide molecular signature of disease silently processing from early asymptomatic stage [19,20] to established disease.

Rather than chronological age, ageing could be more precisely reflected by biological age, as estimated by UPP [20]. A previous study in 778 randomly recruited Flemish individuals demonstrated that chronological age was significantly associated with 210 sequenced peptides, mainly showing downregulation of collagen fragments [20].

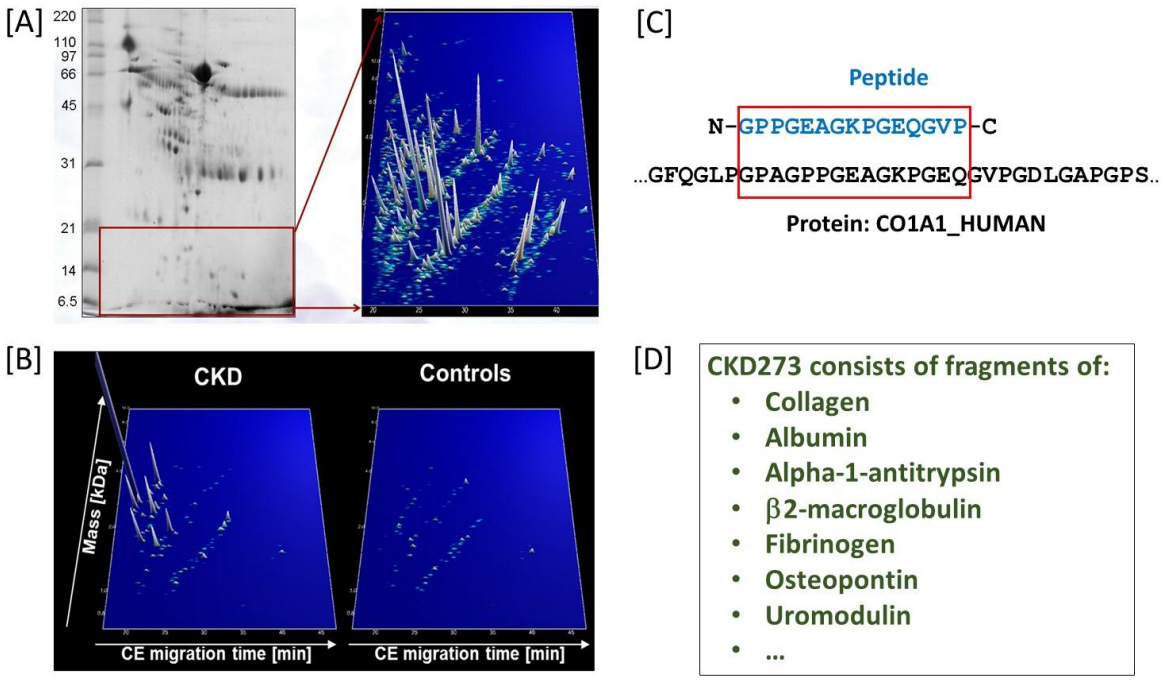


Figure 1.4 Urinary proteomic profiling and CKD273. Urinary proteomic is captured by capillary electrophoresis coupled to mass spectrometry (CE-MS, panel A). According to the molecular mass and CE migration time, UPP signals are captured, thereby allowing the differentiation between the CKD cases and controls (panel B). The sequenced peptides allow the identification of parental protein (panel C). CKD273 consists of 273 peptides differentiating CKD cases and controls (panel D). Abbreviations: CKD, chronic kidney disease; CE, capillary electrophoresis; UPP, urinary proteomic profiling.

UPP-age, as derived by the trained model relating chronological age to the UPP, was significantly associated with various risk markers of cardiovascular, metabolic and renal disease and inflammation [20]. Moreover, independent of chronological age, UPP-age was a predictive biomarker of ageing-disabilities, total and cardiovascular mortality [20].

Besides, CKD273 (**Figure 1.4**) [53,54] and HF1 [55,56], are the multidimensional classifiers indicative for chronic kidney disease (CKD) and diastolic left ventricular dysfunction (LVD), consisting of 273 and 85 urinary peptides, respectively. The thresholds indicating increased risk are >0.154 for CKD273 and ≥-0.350 for HF1 in normalised units. CKD273 and HF1 were validated and certified as *in-vitro* diagnostics, and are being applied in clinical practice.

Despite the explosion of *"omics"* studies, the molecular mechanisms of some ageing-related diseases and their treatments were not fully understood. UPP provides the possibility to identify the population *"at risk"* of unhealthy ageing (***Chapters 3.1*** and ***3.2***), and as a step to investigate novel and personalised therapeutics (***Chapter 3.3***). In this dissertation, UPP signature was captured by capillary electrophoresis coupled to mass spectrometry (CE-MS), as described in detailed in ***Chapter 3***.

1.1.3 Clinical risk factors

Clinical risk factors are arise through the interplay between external and internal drivers of ill health. In this dissertation, BP is both a risk factor and a health outcome (**Figure 1.2**), which is introduced in ***Chapter 1.2.1***. Herein, we introduce other clinical risk factors investigated in this dissertation.

1.3.3.1 Birth weight

According to the World Health Organization [57,58], the United Nations Children's Fund [57], and the US Centers for Disease Control and Prevention [59], birth weight was categorised as extremely low, very low, low, normal and high birth weight, respectively (**Table 1.1**). High birth weight, mainly driven by maternal diabetes, maternal obesity and large for gestational age, is a forerunner of obesity [60] and type-1 diabetes [61], which are important determinants of morbidity and mortality in the adulthood. Conversely, according to the developmental origins of disease hypothesis [62], undernutrition and an adverse intrauterine environment, on the background of genetic factors, slows foetal growth as an adaptive response that favours short-time survival at the expense of a higher susceptibility to disease later in life [63]. Furthermore, because incomplete nephron endowment [64], compared to the controls born at term, very low birth weight predisposes to hypertension. Eleven-year old children born with extremely low birth weight, compared with controls born at term, were 10 times more likely to have

hypertension with preservation of the inverse association of plasma renin activity with BP [65]. Worldwide, more than 20 million infants, representing 15.5% of births, have a low birth weight, among which developing regions has a double prevalence than developed regions [57]. Regarding to this health challenge occurring at birth, investigations on birth weight and its health effects in later life are urgently needed. ***Chapter 4.1*** addressed the course of BP from birth to 4-6 years and to 9-11 years, accounting for birth weight. ***Chapter 4.2*** explored how birth weight modify the association between cardiovascular (CV) risk, BP and salt intake.

Table 1.1 Categories of birth weight

Category	Birth weight, g
Extremely low	<1000
Very low	1000 - 1499
Low	1500 - 2499
Normal	2500 - 4000
High	>4000

1.3.3.2 Salt intake and urinary sodium excretion

Sodium is an essential nutrient, which is involved in multiple physiological processes along with potassium and other cations [66]. Approximately 80% of the sodium in the glomerular filtrate is reabsorbed in the proximal tubules, thereby maintaining serum osmolarity within normal range. Endocrine, immune and neural mechanisms support the maintenance of the sodium metabolism [66]. Under normal physiological conditions, the 24-h urinary sodium excretion (UVNA) reflects the amount of dietary sodium intake [66].

The level of sodium intake affects the renin-angiotensin-aldosterone system (RAAS), and the increased renin levels are associated with increased CV risk. However, the association between health and sodium is likely to be J-shaped (**Figure 1.5**) [67,68]. The evidence of BP and CV risk in relation to sodium in humans originates from population-based studies [69] and randomised clinical trials of interventions on dietary salt intake [70,71]. According to a systematic literature review [66], the dietary sodium consumption in moderate range (2.3-4.6 g/day; 1-2 teaspoons of salt) is not associated with CV risk, while BP and the risk of CV complications increased when sodium intake exceeded 5 g/day. In an individual-participant meta-analysis of European population studies [72], per 2.3 g higher UVNA was merely associated with 0.4 mm Hg higher systolic BP per year, and not with increased risk of hypertension or major CV events. As mentioned in ***Chapter***

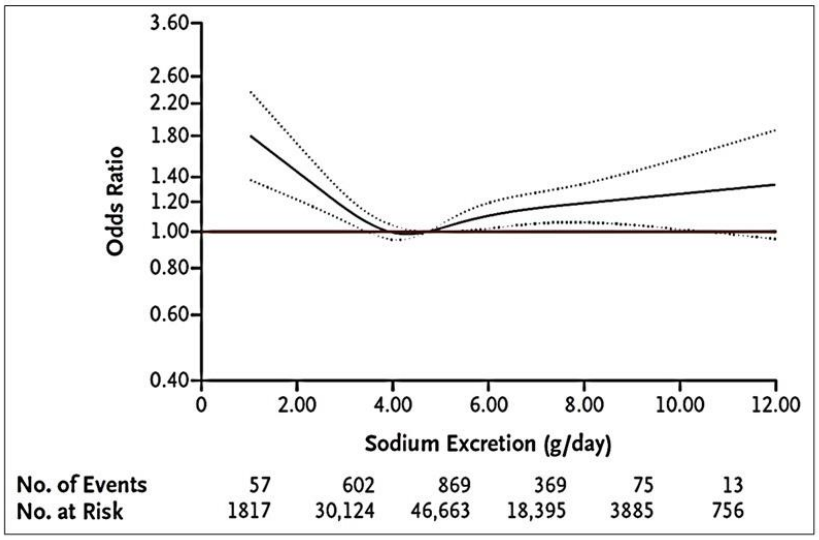


Figure 1.5 Estimated sodium excretion and risk of major cardiovascular events. The association between the risk of cardiovascular events and urinary sodium excretion is J-shaped. (Reproduced with permission from *N.Engl.J.Med.* 2014;371:612-623.)

1.3.3.1, low birth weight predisposes to hypertension due to incomplete nephron endowment [64]. Guyton's model [73] hypothesised that a smaller number of nephrons necessitates a higher BP to excrete sodium. Therefore, considering the interaction of birth weight and UVNA, we aimed to explore whether the associations of UVNA with BP and the risk hypertension, death and a composite CV endpoint were modified by birth weight in ***Chapter 4.2***. In the population-based studies conducted in Belgium and other European countries, we assessed UVNA in 24-hour urine samples, which is widely accepted as the most reliable approach.

1.3.3.3 Racial and regional inequity

Racial and regional inequity represent not only the differences of environmental and innate risk factors, but also lead to discrepancies in genetics, epigenetics, socioeconomical status, education and health resource allocation. According to the GBD report from 1990 to 2017 [74], the healthy life expectancy increased with a steeper gradient (10.5 *vs* 3.8 years) in the countries with low social-demographical index (SDI) than those with middle and high SDIs. However, the total healthy life expectancy is still far lower in the low SDI countries [74]. In particular, sub-Saharan countries face persisting health challenges, such as infectious diseases, malnutrition, and child and maternal mortality, and more recently the growing burden of non-communicable diseases (NCD) [75,76]. Hypertension, obesity, and type-2 diabetes are the drivers of the NCD in burden sub-Saharan Africa [77,78], where population health indicators generally remain behind most other low- and middle-income countries [76,79].

In the United States, the racial disparity between Blacks and Whites in the disease burden due to cardiovascular (CVD) and chronic kidney (CKD) disease is well documented [80-82]. Among all racial groups, African-Americans of all ages have the highest hypertension prevalence [80,83], and sub-Saharan African ancestry apparently plays a pivotal role in the susceptibility to CVD and CKD [84-86]. Given that the American studies are confounded by genetic admixture [80-83], in ***Chapter 4.3***, we aimed to compare the CVD and CKD predisposition of Blacks "*born and living in sub-Saharan Africa*" with European Whites.

1.2 Unhealthy ageing

Unhealthy ageing is characterised as the status with risk or development of subclinical and clinical disease, coupled with comorbidities, functional decline and low quality of life. The outcomes indicative of unhealthy ageing differ across distinct age strata and different populations with specific characteristics. In this dissertation, we focused on the adverse outcomes affecting the heart, kidneys and bones (**Figure 1.2**).

1.2.1 Blood pressure and hypertension

Hypertension is the world's number one killer and the strongest modifiable cardiovascular risk factor associated with cardiac renal and cerebrovascular complications. Notably, hypertension is a *"silent"* killer, because target organ damages silently occur after a long asymptomatic course. The global prevalence of hypertension ranks high among NCDs, accounting for 4% in children younger than 19 years [87] and 30-45% in adults [70]. The Lancet Commission on Hypertension suggested to tackle prevention and treatment of elevated BP with a lifecourse approach in consideration of the early lifetime programming, the long-term benefits and changing priorities with ageing [8]. In line with this goal, this dissertation addressed blood pressure and hypertension in both children and adults.

1.2.1.1 Children

In the early 20th century, pioneering investigators [88,89] reported that the average BP level increases with age. In children, this increase is marked for systolic BP (SBP) and lasts until the age of 20 years with an average increment of 2 mm Hg per year in boys and 1 mm Hg per year in girls [90]. This rise in diastolic BP (DBP) is less pronounced [90]. BP in children is mainly determined by genetic factors, prematurity (e.g., low birth weight), body size development, peripheral arterial resistance, endocrine and renal dysfunction, and unhealthy life style [90]. However, only since 1977, BP measurement became routine in paediatric care [91]. In 2004, the publication of the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents [92]

emphasised the importance of BP management in children. In 2017, the American Academy of Pediatrics (AAP) guideline [93] updated the definition of BP and hypertension categories in children based on the sex-, age-, and height-specific BP percentiles.

"Tracking" is the stability of rank of an individual in the BP distribution over time [90], while *"horse-racing"* is the correlation of the higher BP in later life with the faster BP increase in childhood [90,94]. BP tracking across life plays an important role in predicting whether children will become hypertensive adults and develop its complications [95]. Previous studies indicated that children with higher BP levels are more likely to develop persistent hypertension [96,97], arterial stiffness [96] and left ventricular hypertrophy [96] in adulthood. Moreover, autopsy [98] and imaging [99] studies also reported that young patients with hypertension are more likely to experience accelerated vascular ageing and CV target organ damage [98,99]. Diabetes [87], obesity [100,101], metabolic syndrome [97], CKD [102,103] and obstructive sleep apnoea [104] are the common comorbidities of hypertension in children. To the best of our knowledge, no investigation assessed BP from birth to childhood. To fill this knowledge gap, in ***Chapter 4.1***, we explored the BP trajectory from birth to preschool- and school-age with BP measured in a healthy Flemish birth cohort [105].

1.2.1.2 Adults

According to World Health Organisation, hypertension affects 1.28 billion adults aged 30-79 years worldwide, of which two-third are living in low- and middle-income countries [106]. The global age-standardised average prevalence of hypertension in adults was 34% in men and 32% in women [107]. In general, SBP increases with age at least until the eighth

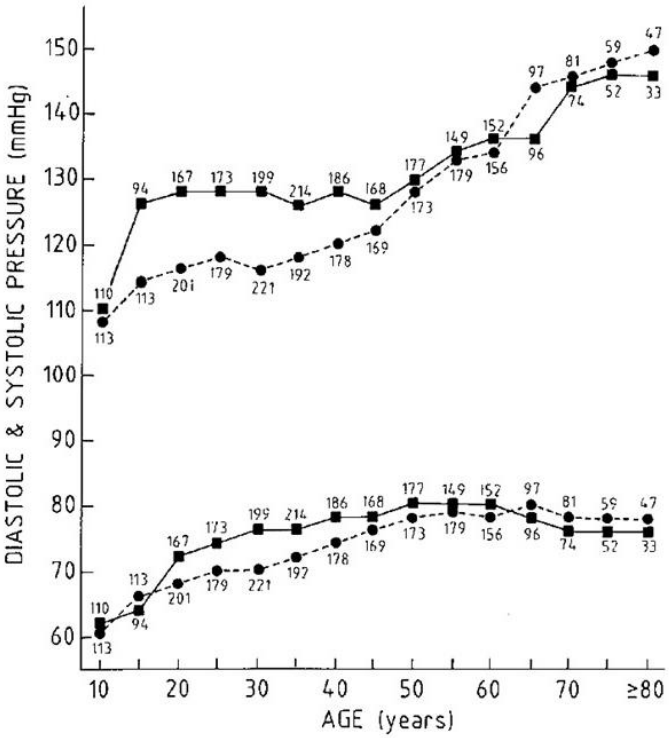


Figure 1.6 Systolic and diastolic blood pressure in 5-year age classes in a representative sample (n=4202) of the population of five Belgian districts. In each subject, five blood pressure readings were obtained at home visit and were averaged for this presentation. For each sex and age group, the number of subjects contributing to the mean is given. (Reproduced with permission from *J Hypertens*. 1990; 8: 393-405.)
■, Males (n=2044); ●, females (n=2158).

decade of life, while DBP rises only until the age of 50 years, and thereafter either levels off, or even slightly decreases (**Figure 1.6**) [108]. Along with advancing age, the prevalence of hypertension progressively increases, and amounts to over 60% in the population older 60 years [109].

Hypertension is both an adverse outcome and a risk factor of CVD. Essential hypertension is a pathophysiological disorder caused by dysregulated physiological mechanisms (e.g., RAAS) or modified by environmental factors (e.g., environmental pollutants) and lifestyle (e.g., smoking, alcohol, diet) [106,110]. More importantly, numerous studies reported a continuous log-linear relationship of BP and the risk of stroke, coronary heart disease, heart failure (HF) and the development and progression of CKD, irrespective of age and ethnic groups [111]. A meta-analysis including 61 prospective studies demonstrated that each increase of 20 mm Hg in SBP or 10 mm Hg in DBP is associated with approximately twofold mortality rate in stroke or coronary artery disease [112]. Another participant-level meta-analysis in 17,921 participants (51.3% women, mean age 54.2 years) from 17 population cohorts, demonstrated that the rates of CV events by BP levels increased significantly from the youngest (≤60 years) to the oldest (>80 years) age groups (**Figure 1.7**) [113]. Therefore, In this dissertation, we assessed BP as one of the co-primary endpoints in relation to occupational lead exposure among young workers in ***Chapter 2.2***. We also explored the association of BP and CV risk with birth weight and UVNA in European general population in ***Chapter 4.2***. Moreover, BP was also assessed as a common risk factor of CVD in other chapters of this dissertation.

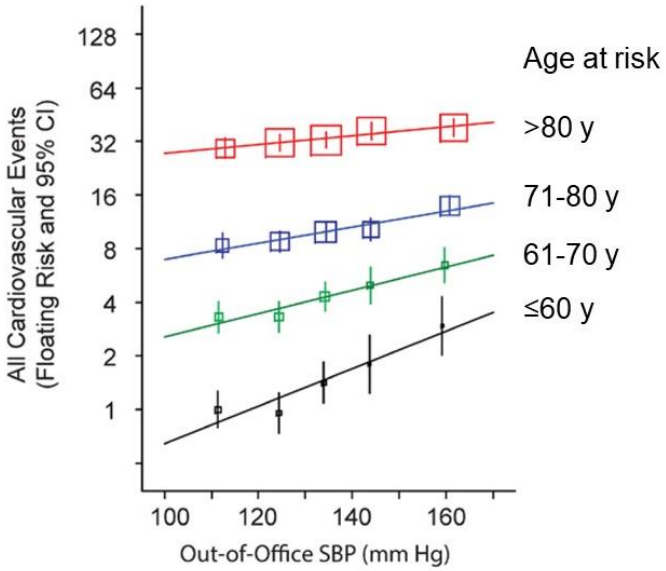


Figure 1.7 Cardiovascular events by age-at-risk groups and categories of out-of-office blood pressure. Point estimates and 95% CIs for the floating absolute risks were plotted along the vertical axis. The size of the squares is proportional to the inverse the variance of each hazard ratio. Risk estimates were stratified by cohort and adjusted for sex, body mass index, serum cholesterol, smoking and drinking, antihypertensive drug treatment, and history of diabetes mellitus and cardiovascular disease. The categories plotted along the horizontal axis are <120, 120–129, 130–139, 140–149, and ≥150 mm Hg for SBP. Log-linear relations were fitted for each age group for out-of-office SBP. Abbreviations: SBP, systolic blood pressure; CI, confidence interval. (Reproduced with permission from *Hypertension*. 2019;74:1333-1342.)

1.2.2 Cardiovascular disease

CVD refers to a group of disorders of the heart and blood vessels, such as myocardial infarction, heart failure and stroke [80]. Chronic CVD progressively develops over lifecourse. In addition to genetic factors [114] and ageing, the development and progression of CVD are driven by modifiable risk factors, such as BP, diabetes, lifestyle and dietary. Since 1990, the total number of DALYs due to ischaemic heart disease and stroke has risen steadily, reaching 182 million and 143 million in 2019, respectively [115]. CVD remains the most common cause of mortality, responsible for approximately 18.6 million deaths worldwide in 2019 [115].

Left ventricular fibrosis is a common pathophysiologic feature in heart disease. Activation of the mineralocorticoid receptor (MR) initiates a cascade of molecular events leading to cell growth and inappropriate expansion and disorganisation of the ECM in the myocardium [116], which is a hallmark of HF, irrespective of its cause [117]. The ECM mainly consists of collagen, of which the α1 chain of collagen type 1 (COL1A1) is the predominate component [49,50]. The mineralocorticoid antagonist, spironolactone, is an important treatment of HF. Spironolactone causes a fall in serum carboxyterminal propeptide of procollagen I (PICP), a rise in serum carboxyterminal telopeptide of collagen I (CITP) and a fall in the PICP/CITP ratio [118]. PICP and CITP are circulating biomarkers of COL1A1 synthesis and degradation, respectively (**Figure 1.8**) [118,119]. In ***Chapter 3.3***, we analysed the between-group differences in the UPP and the associations of urinary with serum fibrosis markers by spironolactone in the patients at high risk of developing HF.

As recommended by American Heart Association and European Society of Cardiology [120,121], CVD risk algorithms and intervention strategies were well established and widely utilised in clinical practice. The Framingham Heart Study investigators developed a sex-specific CVD risk prediction model. The Framingham 10-year risk score (FRS) is derived from age, total serum cholesterol, high density lipoprotein (HDL) cholesterol, SBP, antihypertensive treatment status (yes vs. no), smoking (yes vs. no) and diabetes (yes vs. no) [122]. Multiple studies applied the FRS in White individuals [122,123]. Subsequently, the FRS was also further validated in African-Americans [124,125] in the Jackson Heart Study [125] and a multi-ethnic study [124]. Using this convenient tool, in ***Chapter 4.3***, we compared the CVD and CKD risk of the Blacks “born and living in” sub-Saharan Africa to European Whites.

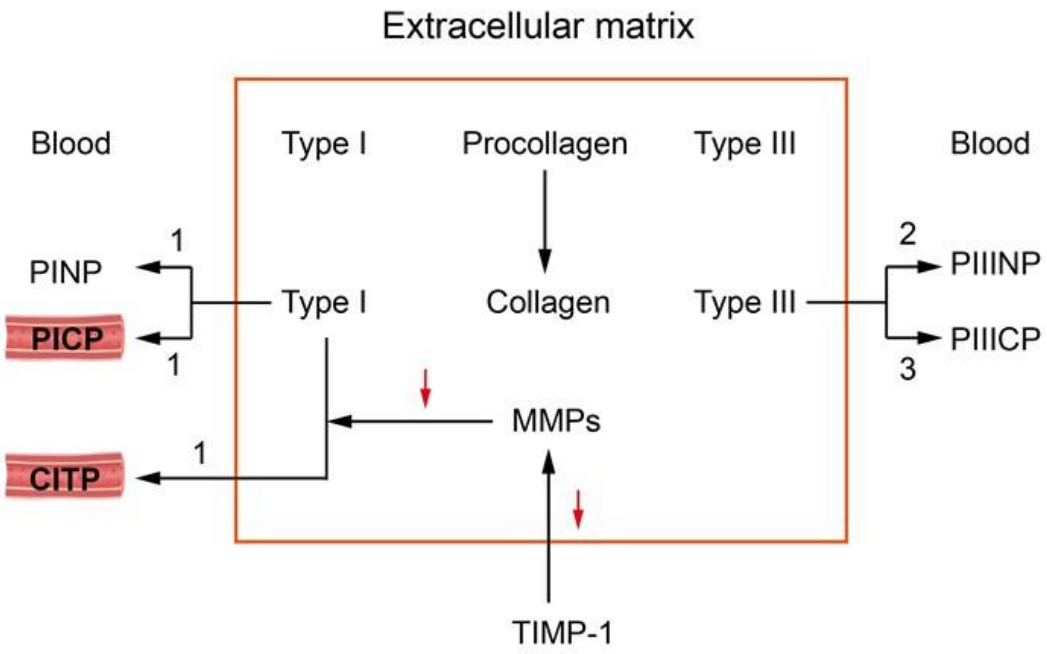


Figure 1.8 Circulating biomarkers of collagen turnover.
Procollagen type-I amino-terminal propeptide (PINP) and procollagen type-I carboxy-terminal propeptide (PICP) are released during conversion of procollagen type-I to collagen type-I and carboxyterminal telopeptide of type-I collagen (CITP) during the degradation of collagen type-I by matrix metalloproteinases (MMPs), which are inhibited by tissue inhibitor of the matrix metalloproteinase type-I (TIMP1). Procollagen type-III amino-terminal propeptide (PIIINP) and procollagen type-III carboxy-terminal propeptide (PIIICP) are released during conversion of procollagen type-III to collagen type-III. Bracketed numbers indicate the stoichiometric ratio. PICP and CITP, which were analysed in the current HOMAGE Trial dataset, are serum markers of collagen type-I synthesis and degradation, respectively. PIIINP is an indirect indicator of collagen III synthesis, because cleavage at the amino-terminus proceeds at a relatively slow rate and, thus, partially processed procollagen molecules remain bound to the surface of collagen type-III fibres. (Reproduced with permission from *JACC* 2015;65:2449-2456.)

1.2.3 Chronic kidney disease

In 2017, GBD investigators reported 697.5 million (95% uncertainty intervals: 649.2-752.0) cases of all-stage CKD worldwide, resulting in a global prevalence of 9.1% (8.5%-9.8%) [126]. By 2021, an international joint statement demonstrated that 850 million people suffer from kidney disease [127]. CKD was responsible for 41.5 million disability-adjusted life-years (DALYs), and the cause of mortality in 1.43 million people [127]. Most of CKD death were observed in the low SDI countries [127]. In addition, previous African-American studies demonstrated that Black patients experience approximately twice the risk of end-stage renal disease (ESRD) compared to White patients, after accounting for socioeconomic and clinical risk confounders [82,128]. Renal function declines faster after the onset of CKD in Black patients, which might be attributed to the high-risk apolipoprotein L1 gene variants [129]. Focusing on the racial and regional disparities between sub-Saharan Africa and Europe, we evaluated renal function of the patients with more than five CVD risk factors and categorised their CKD grade by eGFR and albuminuria in ***Chapter 4.3***.

1.2.4 Osteoporosis

Osteoporosis is an age-related skeletal disorder characterised by the loss of bone mass (**Figure 1.9**), which is caused by increased osteoclast-stimulated bone resorption and inhibition of osteoblast-stimulated bone formation, and leading to the fragility of bone tissue and high risk of fractures [130]. Bone mineral density (BMD) detected by dual energy X-ray absorptiometry (XRA) is the gold standard in the diagnosis of osteoporosis, because the BMD at the lumbar spine and hip closely correlate with bone strength and predicts the risk of fracture. [131,132] The methods of BMD and its diagnostic criteria are described in ***Chapter 3.2***. In addition, body-height-to-arm-span-ratio (HASR) is an anthropometric measurement reflecting the osteoporotic compression of the vertebrae [133,134]. Because body height and arm span are approximately equal in young adults, HASR reflects the age-related loss of body height, with arm span as reference unaffected by ageing (**Figure 1.9**).

Based on NHANES 2005-2010, the prevalence of osteoporosis in American adults aged 50 years or older was 10.3%, which is equivalent to 10.2 million people at risk [135]. Elderly people, especially menopausal women, experience a higher risk of osteoporosis and osteoporotic fracture [135,135-137]. Furthermore, organ transplantation recipients are prone to develop osteoporosis as a consequence of corticosteroid treatment, and of the renal dysfunction induced by the direct renal toxicity of immunosuppressive drugs and the indirect renal damage cause by hypertension [138].

Because osteoporosis and its fractures subsequently worsen the quality of life [139] and represent high socio-economic burden [136,140,141], screening and prevention should be employed in a timely manner [142]. To focus on different populations, we firstly generated an UPP ageing biomarker for osteoporosis risk prediction in general population in ***Chapter 3.1***, and subsequently developed a multidimensional UPP biomarker indicative of osteoporosis in heart transplantation recipients in ***Chapter 3.2***.

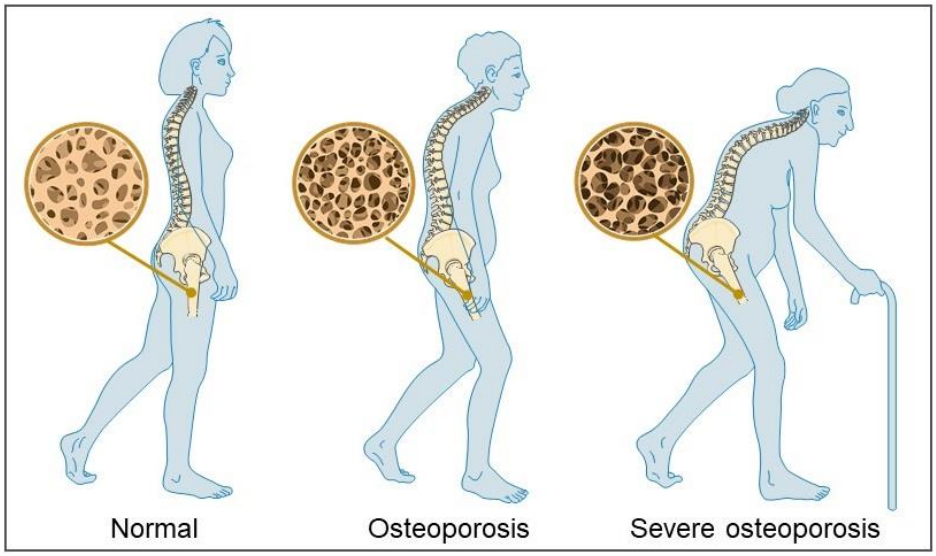


Figure 1.9 Progression of osteoporosis.
The progression of osteoporosis leads to the loss of bone mass. Decrease in body height reflects the osteoporotic compression of the vertebrae.

1.3 Study participants

To investigate various spectrum of unhealthy ageing, we conducted our research in an occupational cohort (***Chapters 2.1 and 2.2***), population cohorts (Children: ***Chapter 4.1***; Adults: ***Chapters 3.1 and 4.2***) and patient cohorts (***Chapters 3.2, 3.3 and 4.3***). The description of these study cohorts are summarised in **Table 1.3**. The occupational cohort provided the opportunity to evaluate the potential adverse health effects in relation to BL under contemporary exposure levels (***Chapters 2.1 and 2.2***). The population and patient cohorts allowed to investigate unhealthy ageing as reflected by urinary proteomic profiling (***Chapters 3.1, 3.2 and 3.3***) or as attributable to clinical risk factors (***Chapters 4.1, 4.2 and 4.3***). The study cohorts in this dissertation cover the whole human life span and the continuum from health to being at risk status and to end-stage disease (**Figure 1.1**). All study protocols complied with the Declaration of Helsinki [143] and were approved by the institutional or competent ethics committees. All participants provided written informed consent.

Table 1.3 Study cohorts (starts)

Cohort	Description
Occupational cohort	
SPHERL *(Chapters 2.1 and 2.2)*	**Study for Promotion of Health in Recycling Lead** was conducted at lead recycling plants in the United States. Newly hired workers (n=506) without previous occupational lead exposure were recruited. Blood lead was measured prior to occupational lead exposure and at follow-up for up to 6 years. The co-primary endpoints were blood pressure and renal function. This is a *"real world experiment"*, conducted under OSHA regulations for the protection of workers.
Population cohorts	
ENVIRONAGE *(Chapter 4.1)*	**Environmental Influence on Early Ageing** is a birth cohort with prospective epidemiological follow-up visits from the newborn stage. This cohort includes 1300 healthy mother-infant pairs in Limburg, Belgium. The recruitment is still ongoing. The gestational age of neonates ranged from 35 to 42 weeks. At birth, the age of 4-6 years and 9-11 years, the children had blood pressure and other phenotypes measured. ENVIRONAGE is designed to obtain evidence on the interactions of environmental exposures with processes of ageing.
FLEMENGHO *(Chapters 3.1, 4.2)*	**Flemish Study on Environment, Genes and Health Outcomes** is a large-scale family-based study (n=3343) focusing on the epidemiology of chronic age-related disease in relation to genetic and environmental determinants. UPP was measured in 850 participants in 2005-2010 and repeated in the same individuals in 2009-2013. Birth weight was collected by a validated questionnaire. Urinary sodium excretion were measured in 24-h urine samples. Follow-up of fatal and non-fatal outcomes is available until 31 December 2019.
EPOGH *(Chapter 4.2)*	**European Project on Genes in Hypertension** recruited participants from 1999 until 2001. The investigators applied the same protocol, questionnaires and follow-up procedures, as used in FLEMENGHO. Birth weight, 24-h urinary sodium excretion and other risk factors were collected in individuals randomly recruited in the Russian Federation, Poland, the Czech Republic, Italy and Belgium (n=1369).

Table 1.3 Study cohorts (ends)

Cohort	Description
Patient cohorts	
HOMAGE *(Chapter 3.3)*	**Heart "Omics" in Ageing Study** was a multicentre open-label randomised clinical trial conducted in France, Italy, United Kingdom, Netherlands, Germany and Ireland. The trail included 527 patients (≥60 years), who were at high risk of developing heart failure. Patients were randomised to spironolactone 25-50 mg/d on top of usual therapy or usual therapy alone and were followed up for 9 months. The UPP and serum biomarker of fibrosis were measured to quantify changes in the myocardial extracellular matrix.
UPRIGHT-HTM *(Chapter 4.3)*	**Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform** is an investigator-initiated, multicentre, open-label, randomised trial with blinded endpoint evaluation designed to assess the efficacy of HTM plus UPP (experimental group) over HTM alone (control group) in guiding treatment in asymptomatic patients, aged 55-75 years, with ≥5 cardiovascular risk factors. Until now, approximately 700 participants, including sub-Saharan Blacks, European Whites and Asian, were screened, and 550 were randomised to assess risk factors.
uPROPHET *(Chapter 3.2)*	**Urinary Proteomics in Predicting Heart Transplantation** is a proof-of-concept project and a single-centre study that already led to the initial validation and clinical application of risk-carrying UPPs in heart transplantation recipients. A total of 389 patients were enrolled, followed up and had a median of 3 UPP assessments. The goal of this project is to apply UPP to help choosing treatment modalities and post-transplant management with the greatest probability of achieving long-term graft survival with a high quality of life.

Abbreviations: OSHA, Occupational Safety and Health Administration; UPP, urinary proteomic profiling; HTM, home blood pressure monitoring.

1.4 Main objectives

The main objective of this doctoral dissertation was to identify and validate external and internal drivers, which change a person's lifecourse from healthy to unhealthy ageing in the occupational, population and patient cohorts. Identification and validation of the external and internal drivers of unhealthy ageing allow the identification of susceptible and *"at risk"* individuals, and the implementation of timely disease prevention, thereby reaching an optimal lifecourse.

In ***Chapter 2***, we focused on lead exposure in an occupational setting (SPHERL) and reviewed the literature:

- *Part 1* assessed renal function in relation to the first occupational exposure to lead for two years;
- *Part 2* investigated the responses of blood pressure and renal function in workers exposed to lead for up to six years;
- *Part 3* summarised the toxicokinetics, legislation and the latest evidence focusing on hypertension and its complication in association with environmental and occupational lead exposure.

In ***Chapter 3***, we addressed osteoporosis and cardiac fibrosis by urinary proteomic profiling:

- *Part 1* constructed an ageing-related urinary proteomic biomarker indicative of osteoporosis in the general population (FLEMENGHO);
- *Part 2* developed and validated a multidimensional urinary proteomic biomarker for osteoporosis in heart transplantation recipients (uPROPHET);
- *Part 3* refined the understanding of the antifibrotic action by spironolactone using urinary proteomic signature in patients at high risk of developing heart failure (HOMAGE).

In ***Chapter 4***, we focused on traditional risk factors from childhood to adulthood:

- *Part 1* investigated the childhood blood pressure trajectory and risk of incident hypertension from birth to preschool and school-age (ENVIRONAGE);
- *Part 2* explored whether the relation of hypertension, death or cardiovascular disease with urinary sodium excretion was modified by birth weight in European general populations (FLEMENGHO and EPOGH);
- *Part 3* analysed the racial and regional disparities in the risk of cardiovascular and chronic kidney disease between sub-Saharan Black and European White patients (UPRIGHT-HTM).

References

1. Venkatapuram S, Thiyagarajan JA. The capability approach and the WHO healthy ageing framework (for the UN decade of healthy ageing). *Age Ageing.* 2023; 52: iv6-iv9.
2. GBD 2019 Investigators. Five insights from the Global Burden of Disease Study 2019. *Lancet.* 2020; 396: 1135-1159.
3. Noto S. Perspectives on aging and quality of life. *Healthcare.* 2023; 11: 2131.
4. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell.* 2013; 153: 1194-1217.
5. Martens DS, Van Der Stukken C, Derom C, Thiery E, Bijnens EM, Nawrot TS. Newborn telomere length predicts later life telomere length: tracking telomere length from birth to child- and adulthood. *EBioMed.* 2021; 63: 103164.
6. Draper CE, Yousafzai AK, McCoy DC *et al*. The next 1000 days: building on early investments for the health and development of young children. *Lancet.* 2024; 404: 2094-2116.
7. Melén E, Faner R, Allinson JP *et al*. Lung-function trajectories: relevance and implementation in clinical prctice. *Lancet.* 2024; 403: 1494-1503.
8. Olsen MH, Angell SY, Asma S *et al*. A call to action and a lifecourse strategy to address the global burden of raised blood pressure on current and future generations: the *Lancet* Commission on hypertension. *Lancet.* 2016; 388: 2665-2172.
9. GBD 2017 Risk Factor Collaborators. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risk factors or clusters of risks for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* 2018; 392: 1923-1994.
10. Nawrot TS, Thijs L, Den Hond EM, Roels HA, Staessen JA. An epidemiological re-appraisal of the association between blood pressure and lead : a meta-analysis. *J Hum Hypertens.* 2002; 16: 123-131.
11. Wedeen RP. Bone lead, hypertension, and lead nephropathy. *Environ Health Perspect.* 1988; 78: 57-60.

12. Araki S, Sato H, Yokoyama K, Murata K. Subclinical neurophysiological effects of lead : a review on peripheral, central, and autonomic nervous system effects in lead workers. *Am J Ind Med.* 2000; 37: 193-204.
13. Staessen JA, Lauwerys RR, Buchet JP *et al*. Impairment of renal function with increasing blood lead concentrations in the general population. *N Engl J Med.* 1992; 327: 151-156.
14. Schober SE, Mirel LB, Graubard BI, Brody DJ, Flegal KM. Blood lead levels and death from all causes, cardiovascular disease, and cancer : results from the NHANES III mortality study. *Environ Health Perspect.* 2006; 114: 1538-1541.
15. Lanphear BP, Rauch S, Auinger P, Allen RW, Hornung RW. Low-level lead exposure and mortality in US adults: a population-based cohort study. *Lancet Public Health.* 2018; 3: e177-e184.
16. An DW, Yu YL, Hara A *et al*. Lead-associated mortality in the US 1999-2020: a time-stratified analysis of a national cohort. *J Hypertens.* 2024; 42: 1322-1330.
17. Hara A, Thijs L, Asayama K *et al*. Blood pressure in relation to environmental lead exposure in the National Health and Nutrition Examination Survey 2003 to 2010. *Hypertension.* 2015; 65: 62-69.
18. López-Otín C, Biasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: an expanding universe. *Cell.* 2023; 186: 243-278.
19. Kistler AD, Mischak H, Poster D, Dakna M, Wüthrich RP, Andreas AL. Identification of a unique urinary biomarker profile in patients with autosomal dominant polycystic kidney disease. *Kidney Intern.* 2024; 76: 89-96.
20. Martens DS, Thijs L, Latosinska A *et al*. Urinary peptidomics to address age-related disabilities: a prospective population study with replication in patients. *Lancet Healthy Longev.* 2021; 2: e690-e703.
21. Kashtanova DA, Taraskina AN, Erema VV *et al*. Analyzing successful aging and longevity: risk factors and health promoters in 2020 older adults. *Int J Res Public Health.* 2022; 19: 8178.
22. Escobar C, Varela L, Palacios B *et al*. Costs and healthcare utilisation of patients with heart failure in Spain. *BMC Health Serv Res.* 2020; 20: 964.
23. Resongles E, Dietze V, Green DC, Weiss DJ. Strong evidence for the continued contribution of lead deposited during the 20th century to the atmospheric environment in London today. *Proc Natl Acad Sci.* 2021; 118: e2102791118.

24. Jin T, Amini H, Kosheleva A *et al*. Association between long-term exposures to airborne PM2.5 components and mortality in Massachusetts: mixture analysis exploration. *Environ Health.* 2022; 21: 96.
25. Kim R, Rotnitzky A, Sparrow D, Weiss ST, Wagner C, Hu H. A longitudinal study of low-level lead exposure and impairment of renal function. The Normative Aging Study. *J Am Med Assoc.* 1996; 275: 1177-1181.
26. Chowdhury R, Darrow L, McClellan W, Sarnat S, Steenland K. Incident ESRD among participants in a lead surveillance program. *Am J Kidney Dis.* 2014; 64: 25-31.
27. Lim SS, Vos T, Flaxman AD *et al*. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010 : a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* 2012; 380: 2224-2260.
28. Wright RO, Tsaih SW, Schwartz J *et al*. Lead exposure biomarkers and mini-mental status exam scores in older man. *Epidemiology.* 2003; 14: 713-718.
29. Lanphear BP, Hornung R, Khoury J *et al*. Low-level environmental lead exposure and children's intellectual function: an international pooled analysis. *Environ Health Perspect.* 2005; 113: 894-899.
30. Payton M, Riggs KM, Spiro A, III, Weiss S, Hu H. Relations of bone and blood lead to cognitive function: the VA Normative Aging Study. *Neurotoxicol Teratol.* 1998; 20: 19-27.
31. Pirkle JL, Brody DJ, Gunter EW *et al*. The decline in blood lead levels in the United States. The National Health and Nutrition Examination Surveys (NHANES). *J Am Med Assoc.* 1994; 272: 284-291.
32. Muntner P, Menke A, DeSalvo KB, Rabito FA, Batuman V. Continued decline in blood lead levels among adults in the United States : the National Health and Nutrition Examination Surveys. *Arch Intern Med.* 2005; 165: 2155-2161.
33. National Toxicology Program. NTP technical report on the toxicology and cardiogenesis studies of antimony triaxide in wistar han rats and B6C3F1/N mice (inhalation studies). 2017.
34. United States Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment. *Integrated Science Assessment for Lead (http://www.epa.gov/ncea/isa/lead.htm)*. Research Triangle Park, NC: 2013;

35. United States Environmental Protection Agency. *Air Quality Criteria for Lead. Volume 1 (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=158823)*. Washington DC: 2006;
36. Weed DL. Weight of evidence : a review of concept and methods. *Risk Analysis.* 2005; 25: 1545-1557.
37. Holland MG, Cawton D. Workplace lead exposure. *J Occup Environ Med.* 2010; e371-e374.
38. Hara A, Gu YM, Petit T *et al*. Study for promotion of health in recycling lead - rationale and design. *Blood Press.* 2015; 24: 147-157.
39. Yang WY, Efremov L, Mujaj B *et al*. Association of office and ambulatory blood pressure with blood lead in workers before occupational exposure. *J Am Soc Hypertens.* 2017; 12: 14-24.
40. Yu CG, Wei FF, Yang WY *et al*. Heart rate variability and peripheral nerve conduction velocity in relation to blood lead in newly hired lead workers. *Occup Environ Med.* 2019; 76: 382-388.
41. Yu CG, Yang WY, Saenen N *et al*. Neurocognitive function in relation to blood lead among young men prior to chronic occupational exposure. *Scand J Work Environ Health.* 2019; 45: 298-307.
42. Mujaj B, Yang WY, Zhang ZY *et al*. Renal function in relation to low-level environmental lead exposure. *Nephrol Dial Transplant.* 2018; 34: 941-946.
43. Yu YL, Yang WY, Thijs L *et al*. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *Hypertension.* 2020; 76: 1299-1307.
44. Yu YL, Thijs L, Saenen N *et al*. Two-year neurocognitive responses to first occupational lead exposure. *Scand J Work Environ Health.* 2020; 47: 233-243.
45. Yu YL, Thijs L, Yu CG *et al*. Two-year responses of heart rate and heart rate variability to first occupational lead exposure. *Hypertension.* 2021; 77: 1775-1786.
46. Pieper R, Gatlin CL, McGrath AM *et al*. Characterization of the human urinary proteome : a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. *Proteomics.* 2004; 4: 1159-1174.
47. Carone FA. Renal handling of proteins and peptides. *Ann Clin Lab Sci.* 1978; 8: 287-294.

48. Coon JJ, Zürbig P, Dakna M *et al*. CE-MS analysis of the human urinary proteome for biomarker discovery and disease diagnostics. *Proteomics Clin Appl.* 2008; 2: 964-973.
49. Weber KT. Cardiac interstitium in health and disease : the fibrillar collagen network. *J Am Coll Cardiol.* 1989; 13: 1637-1652.
50. Weber KT, Sun Y, Tyagi SC, Cleutjens JPM. Collagen network of the Myocardium : function, structural remodeling and regulatory mechanisms. *J Mol Cell Cardiol.* 1994; 26: 279-292.
51. Lin X, Patil S, Gao YG, Qian A. The bone extracellular matrix in bone formation and regeneration. *Front Phrmacol.* 2020; 11: 757.
52. Zhang ZY, Ravassa S, Pejchinovski M *et al*. A urinary fragment of mucin-1 subunit α is a novel biomarker associated with renal dysfunction in the general population. *KI Reports.* 2017; 2: 811-820.
53. Pontillo C, Zhang ZY, Schanstra JP *et al*. Prediction of chronic kidney disease stage 3 by CKD273, a urinary proteomic biomarker. *KI Reports.* 2017; 2: 1066-1075.
54. Tofte N, Lindhardt M, Adamova K *et al*. Early detection of diabetic kidney disease by urinary proteomics and subsequent intervention with spironolactone to delay progression (PRIORITY): a prospective observational study and embedded randomised placebo-controlled trial. *Lancet Diabetes Endocrinol.* 2020; 8: 301-312.
55. Kuznetsova T, Mischak H, Mullen W, Staessen JA. Urinary proteome analysis in hypertensive patients with left ventricular diastolic dysfunction. *Eur Heart J.* 2012; 33: 2342-2350.
56. Zhang ZY, Nkuipou-Kenfack E, Staessen JA. Urinary peptidomic biomarker for personalized prevention and treatment of diastolic left ventricular dysfunction. *Proteomics Clin Appl.* 2019; 13: e1800174.
57. Wardlaw T, Blanc A, Zupan J, Åhman E, World Health Organization, United Nations Children's Fund. *Low Birthweight - Country, Regional and Global Estimates*. United Nation's Children's Fund / World Health Organization, New York, NY, USA / Geneva Switzerland: 2004;
58. Branca F, De-Regil LM, Engesveen K *et al*. *Global nutrition targets 2025: low birth weight policy brief (WHO/NMH/NHD/14.5).* World Health Organization, Geneva, Switzerland: 2014; 1-8
59. Martin JA, Hamilton BE, Osterman MJK, Curtin SC, Mathews TJ. Births: final data for 2013. *Natl Vital Stat Rep.* 2015; 64: 1-65.

60. Schellong K, Schulz S, Harder T, Plagemann A. Birth weight and long-term overweight risk: systematic review and a meta-analysis including 643,902 persons from 66 studies and 26 countries globally. *PLoS One.* 2012; 7: e47776.
61. Cardwell CR, Stene LC, Joner G *et al*. Birthweight and the risk of childhood-onset type 1 diabetes: a meta-analysis of observational studies using individual patient data. *Diabetologia.* 2010; 53: 641-651.
62. Barker DJP, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. *Lancet.* 1993; 341: 938-941.
63. Bateson P, Barker D, Clutton-Brock T *et al*. Developmental plasticity and human health. *Nature.* 2004; 430: 419-421.
64. Luyckx VA, Perico N, Somaschini M *et al*. A developmental approach to the prevention of hypertension and kidney disease: a report from the Birth Weight and Nephron Number Working Group. *Lancet.* 2017; 390: 424-428.
65. Raaijmakers A, Zhang ZY, Claessens J *et al*. Does extremely low birth weight predispose to low-renin hypertension? *Hypertension.* 2017; 69: 443-449.
66. O'Donnell M, Mente A, Alderman MH *et al*. Salt and cardiovascular disease: insufficient evidence to recommend low sodium intake. *Eur Heart J.* 2020; 41: 3363-3373.
67. McCarron DA. Physiology, not policy, drives sodium intake. *Am J Hypertens.* 2013; 26: 1191-1193.
68. O'Donnell MJ, Yusuf S, Mente A *et al*. Urinary sodium and potassium excretion and risk of cardiovascular events. *J Am Med Assoc.* 2011; 306: 2229-2238.
69. Whelton PK, Carey RM, Aronow WS *et al*. 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA guideline for the prevention, detection, evaluation, and management of high blood pressure in adults: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol.* 2018; 71: e127-e248.
70. Williams B, Mancia G, Spiering W *et al*. 2018 ESC/ESH guidelines for the management of arterial hypertension. *Eur Heart J.* 2018; 39: 3021-3104.
71. Piepoli MF, Hoes AW, Agewall S *et al*. 2016 European Guidelines on cardiovascular disease prevention in clinical practice. The Sixt Joint Task Force for the European Society of Cardiologie and other societies on cardiovascular disease prevention in clinical practice (constituted by representatives of 10 societies and by invited experts). Developed with the special contribution of the European

Association for Cardiovascular Prevention and Rehabilitation (EACPR). *Eur Heart J.* 2016; 37: 2315-2381.

72. Stolarz-Skrzypek K, Kuznetsova T, Thijs L *et al*. Fatal and nonfatal outcomes, incidence of hypertension and blood pressure changes in relation to urinary sodium excretion. *J Am Med Assoc.* 2011; 305: 1777-1785.
73. Hall JE, Guyton AC, Brands MW. Pressure-volume regulation in hypertension. *Kidney Int Suppl.* 1996; 55: S35-S41.
74. GBD 2017 DALYs and HALE Collaborators. Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* 2018; 392: 1859-1922.
75. Agyepong IA, Sewanjamba N, Binawaho A *et al*. The path to longer and healthier lives for all Africans by 2030: the *Lancet* Commission on future health in sub-Saharan Africa. *Lancet.* 2017; 390: 2803-2859.
76. Abubakar I, Dalglish SL, Angell B *et al*. The *Lancet* Nigeria Commission: investing in health and the future of the nation. *Lancet.* 2022; 399: 1155-1200.
77. Keates AK, Mocumbi AO, Ntsekhe M, Sliwa K, Stewart S. Cardiovascular disease in Africa: epidemiological profile and challenges. *Nat Rev Cardiol.* 2017; 14: 273-293.
78. Schutte AE, Botha S, Fourie CMT *et al*. Recent advances in understanding hypertension development in sub-Saharan Africa. *J Hum Hypertens.* 2017; 31: 491-500.
79. Willett W, Rockström J, Loken B *et al*. Food in the Anthropocene: the EAT-*Lancet* Commission on healthy diets from sustainable food systems. *Lancet.* 2019; 393: 447-492.
80. Tsao CW, Aday AW, Almarzooq ZI *et al*. Heart disease and stroke statistics-2023 update: a report from the American Heart Association. *Circulation.* 2023; 147: e93-e621.
81. Jolly S, Vittinghoff E, Chattopadhyay A, Bibbins-Domingo K. Higher cardiovascular disease prevalence and mortality among younger Blacks compared to Whites. *Am J Med.* 2010; 123: 811-818.
82. Lipworth L, Mumma MT, Cavanaugh KL *et al*. Incidence and predictors of end stage renal disease among low-income Blacks and Whites. *PLoS One.* 2012; 7: e48407.

83. Nichols OI, Fuller-Rowell TE, Robinson AT, DaJuandra E, Homandberg LK. Neighborhood socioeconomic deprivation in early childhood mediates racial disparities in blood pressure in a college student sample. *J Youth Adolesc.* 2022; 51: 2146-2160.
84. Gafane-Matemane LF, Kruger R, Smith W *et al*. Characterization of the renin-angiotension-aldosterone system in young healthy black adults: the African Prospective Study on the Early Detection and Identification of Hypertension and Cardiovascular (African-PREDICT Study). *Hypertension.* 2021; 78: 400-410.
85. Parsa A, Kao L, Xie D *et al*. APOL1 risk variants, race, and progression of chronic kidney disease. *N Engl J Med.* 2013; 369: 2183-2196.
86. Masimango MI, Jadoul M, Binns-Roemer EA, David VA, Sumaili CA, Limou S. APOL1 renal risk variants and sickle cell trait associations with reducesd Kidney function in a large Congolese population-based study. *KI Reports.* 2022; 7: 474-482.
87. Song P, Zhang Y, Yu J *et al*. Global prevalence of hypertension in children: a systematic review and meta-analysis. *JAMA Pediatr.* 2019; 173: 1154-1163.
88. Judson CF, Nicholson P. Blood pressure in normal children. *Am J Dis Child.* 1914; 8: 257-278.
89. Stocks P. Blood pressure in early life: a statistical study. *London: Cambridge University Press.* 1924;
90. Hofman A. Blood pressure in children: an epidemiological approach to the aetiology of hypertension. *J Hypertens.* 1984; 2: 323-328.
91. Blumenthal S, Epps RP, Heavenrich R *et al*. Report of the task force on blood pressure control in children. *Pediatrics.* 1977; 59: 797.
92. National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents. The Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children en Adolescents. *Pediatrics.* 2004; 114: 555-576.
93. Flynn JT, Kaelber DC, Baker-Smith CM *et al*. Clinical practice guideline for screening and management of high blood pressure in children and adolescents. *Pediatrics.* 2017; 140: e20171904.
94. Fletcher CM, Peto R. The horse-racing effect. *Lancet.* 1981; 318: 467-468.
95. Azegami T, Uchida K, Tokumura M, Mori M. Blood pressure tracking from chilhood to adulthood. *Front Pediatr.* 2021; 9: 785356.

96. Liao YY, Ma Q, Chu C *et al*. The predictive value of repeated blood pressure measurements in childhood for cardiovascular risk in adults: the Hnagzhou Adolesscent Hypertension Study. *Hypertens Res.* 2020; 43: 969-978.
97. Sun SS, Grave GD, Siervogel RM, Pickoff AA, Arslanian SS, Daniels SR. Systolic blood pressure in childhood predicts hypertension and metabolic syndrome later in life. *Pediatrics.* 2007; 119: 237-246.
98. Tracy RE, Newman III WP, Wattigney WA, Berenson GS. Risk factors and atherosclerosis in youth: autopsy findings of the Bogalusa Heart Study. *J Med Sci.* 1995; 310 (suppl 1): S37-S41.
99. Urbina EM, Khoury PR, McCoy C, Daniels SR, Kimball TR, Dolan LM. Cardiac and vascular consequences of pre-hypertension in youth. *J Clin Hypertens.* 2011; 13: 332-342.
100. Sorof J, Daniels S. Obesity hypertension in children: a problem of epidemic proportions. *Hypertension.* 2002; 40: 441-447.
101. Brady TM. Obesity-related hypertension in children. *Front Pediatr.* 2017; 5: 197.
102. Flynn JT, Mitsnefes M, Pierce C *et al*. Blood pressure in children with chronic kidney disease: a report from the chronic kidney disease in children study. *Hypertension.* 2008; 52: 631-637.
103. Samuels J, Ng D, Flynn JT *et al*. Ambulatory blood pressure patterns in children with chronic kidney disease. *Hypertension.* 2012; 60: 43-50.
104. Geng X, Wu Y, Ge W *et al*. Ambulatory blood pressure monitoring in children with obstructive sleep apnea syndrome. *Pediatr Invest.* 2019; 3: 217-222.
105. Janssen BG, Madhloum N, Gyselaers W *et al*. Cohort profile: The ENVIRonmental influence *ON* early AGEing (ENVIRO*N*AGE): a birth cohort study. *Int J Epidemiol.* 2017; 46: 1386-1387.
106. Mancia G, Kreutz R, Brunström M *et al*. 2023 ESH guidelines for the management of arterial hypertension. *J Hypertens.* 2023; 41: 1874-2071.
107. NCD Risk Factor Collaboration (NCD-RisC). Worldwide trends in hypertension prevalence and progress in treatment and control from 1990 to 2019: a pooled analysis of 1201 population-representative studies with 104 million participants. *Lancet.* 2021; 398: 957-980.
108. Staessen J, Amery A, Fagard R. Editorial review. Isolated systolic hypertension in the elderly. *J Hypertens.* 1990; 8: 393-405.

109. NCD Risk Factor Collaboration (NCD-RisC). Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1497 population-based measurement studies with 19.1 million participants. *Lancet.* 2017; 389: 37-55.

110. Staessen JA, Wang J, Bianchi G, Birkenhäger WH. Essential hypertension. *Lancet.* 2003; 361: 1629-1641.

111. Zhou B, Perel P, Mensah GA, Ezzati M. Global epidemiology, health burden and effective interventions for elevated blood pressure and hypertension. *Nat Rev Cardiol.* 2021; 18: 785-802.

112. Lewington S, Clarke R, Qizilbash N, Peto R, Collins R, Prospective Studies Collaboration. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet.* 2002; 360: 1903-1913.

113. Li Y, Thijs L, Zhang ZY *et al*. Opposing age-related trends in absolute and relative risk of adverse health outcomes associated with out-of-office blood pressure. *Hypertension.* 2019; 74: 1333-1342.

114. Baccarelli A, Rienstra M, Benjamin EJ. Cardiovascular epigenetics : basic concept and results from animal and human studies. *Circulation Cardiovasc Genet.* 2010; 3: 567-573.

115. Roth GA, Mensah GA, Johnson CO *et al*. Global burden of cardiovascular disease and risk factors, 1990-2019: update from the GBD 2019 study. *J Am Coll Cardiol.* 2020; 76: 2981-3021.

116. Epstein M. A podcast discussing aldosterone and mineralocorticoid antagonists in 2021: a paradigm shift. *Diabetes Ther.* 2022; 13: 583-588.

117. Díez J. Mechanisms of cardiac fibrosis in hypertension. *J Clin Hypertens.* 2007; 9: 546-550.

118. Cleland JGF, Ferreira JP, Mariottoni B *et al*. The effect of spironolactone on cardiovascular function and markers of fibrosis in people at increased risk of developing heart failure: the heart 'OMics' in AGEing (HOMAGE) randomized clinical trial. *Eur Heart J.* 2021; 42: 684-696.

119. Ravassa S, Kuznetsova T, Varo N *et al*. Biomarkers of cardiomyocyte injury and stress identify left atria and left ventricular remodelling and dysfunction: a population-based study. *Int J Cardiol.* 2015; 185: 177-185.

120. Visseren FLJ, Mach F, Smulders YM *et al*. 2021 ESC guidelines on cardiovascular disease prevention in clinical practice. *Eur Heart J.* 2021; 42: 3227-3337.

121. Arnett DK, Blumenthal RS, Albert MA *et al*. 2019 ACC/AHA guideline on the primary prevention of cardiovascular disease: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice GUidelines. *Circulation.* 2019; 140: e596-e646.

122. D'Agostino RB, Sr., Vasan RS, Pencina MJ *et al*. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation.* 2008; 117: 743-753.

123. Cappuccio FP, Oakeshott P, Strazzullo P, Kerry SM. Application of Framingham risk estimates to ethnic minorities in the United Kingdom and implications for primary prevention of heart disease in general practice: cross-sectional population based study. *Br Med J.* 2002; 325: 1271-1277.

124. D'Agostino RB, Jr., Grundy S, Sullivan LM, Wilson P, for the CHD Risk Prediction Group. Validation of the Framingham coronary heart disease predictions scores. Results of a multiple ethnic groups investigation. *J Am Med Assoc.* 2001; 286: 180-187.

125. Fox ER, Samdarshi TE, Musani SK *et al*. Development and validation of risk prediction models for cardiovascular events in black adults: the Jackson Heart Study Cohort. *JAMA Cardiol.* 2016; 1: 15-25.

126. GBD Chronic Kidney Disease Collaboration. Global, regional, and national burden of chronic kidney disease, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* 2020; 395: 709-733.

127. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2024 clinical practice guideline for the evaluation and management of chronic kidney disease. *Kidney Intern.* 2024; 105: S117-S314.

128. Grams ME, Chow EKH, Segev DL, Coresh J. Lifetime incidence of CKD stages 3-5 in the United States. *Am J Kidney Dis.* 2013; 62: 245-252.

129. Parsa A, Kao L, Xie D *et al*. APOL1 risk variants, race, and progression of chronic kidney disease. *N Engl J Med.* 2013; 369: 2183-2196.

130. Rachner TD, Khosla S, Hofbauer LC. Osteoporosis: now and the future. *Lancet.* 2011; 377: 1276-1287.

131. Cosman F, de Beur SJ, LeBoff MS *et al*. Clinician's guide to prevention and treatment of osteoporosis. *Osteoporos Int.* 2014; 25: 2539-2381.

132. Lorentzon M, Cummings SR. Osteoporosis: the evolution of a diagnosis. *J Intern Med.* 2015; 277: 650-661.

133. Verhaar HJJ, Koele JJ, Neijzen T, Dessens JAG, Duursma SA. Are arm span measurements useful in the prediction of osteoporosis in postmenopausal women? *Osteoporos Int.* 1998; 8: 174-176.

134. Ofluoglu D, Unlu F, Akyuz G. Relationship between arm span and height in postmenopausal osteoporotic women. *Rheumatol Int.* 2008; 28: 737-741.

135. Veiras LC, Girardi ACC, Curry J *et al*. Sexual dimorphic pattern of renal transporters and electrolyte homeostasis. *J Am Soc Nephrol.* 2017; 28: 3504-3517.

136. Bruge R, Dawson-Hughes B, Solomon DH, John BW, Alison K, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. *J Bone Miner Res.* 2007; 22: 465-475.

137. Hernlund E, Svedbom A, Ivergård M *et al*. Osteoporosis in the European Union: a compendium of country-specific reports. *Arch Osteoporos.* 2013; 8: 136.

138. Kovvuru K, Kanduri SR, Vaitla P *et al*. Risk factors and management of osteoporosis post-transplant. *Medicina (Kaunas).* 2020; 56: 302.

139. Gao S, Zhao Y. Quality of life in postmenopausal women with osteoporosis: a systematic review and meta-analysis. *Qual Life Res.* 2023; 32: 1551-1565.

140. Dunnewind T, Dvortsin EP, Smeets HM *et al*. Economic consequences and potentially preventable costs related to osteoporosis in the Netherlands. *Value Health.* 2017; 20: 762-768.

141. Williams SA, Daigle SG, Weiss R, Wang Y, Arora T, Curtis JR. Economic burden of osteoporosis-related fractures in the US medicasre population. *Ann Pharmacother.* 2021; 55: 821-829.

142. GBD 2019 Fracture Collaborators. Global, regional, and national burden of bone fractures in 204 countries and territories, 1990-2019: a systematic analysis from the Global Burden of Disease Study 2019. *Lancet Healthy Longev.* 2021; 2: e580-e592.

143. World Medical Association. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. *J Am Med Assoc.* 2013; 310: 2191-2194.

Chapter 2

Unhealthy ageing in relation to lead exposure

Part 1

Two-year responses of renal function to first occupational lead exposure

Yu-Ling Yu, Lutgarde Thijs, Dong-Mei Wei, Jesus D. Melgarejo, Cai-Guo Yu, Wen-Yi Yang, Harry A. Roels, Zhen-Yu Zhang, Tim S. Nawrot, Jan A. Staessen

Previously published in:
Kidney International Report. 2022; 7:1198-1209.

Abstract

Background: Whether in developed countries lead exposure still contributes to renal impairment is debated, because blood lead (BL) is declining towards pre-industrial levels and because longitudinal studies correlating renal function and BL changes over time are scarce.

Methods: The Study for Promotion of Health in Recycling Lead evaluated the 2-year renal function responses in 251 workers (mean age, 29.7 years) transiting from environmental to occupational exposure. Main study endpoint was the estimated glomerular filtration rate (eGFR) derived from serum creatinine, cystatin C or both (eGFRcrt, eGFRcys, eGFRcc). BL was measured by inductively coupled plasma mass spectrometry (detection limit 0.5 µg/dl).

Results: Over follow-up, mean baseline BL of 4.13 µg/dl increased 3.30-fold. In fully adjusted mixed models, additionally accounting for the within-participant clustering of the 1- and 2-year follow-up data, a 3-fold BL increment was not significantly correlated with changes in eGFR with estimates amounting to -0.86 (95% CI, -2.39, 0.67), -1.58 (-3.34, 0.18) and -1.32 (-2.66, 0.03) ml/min/1.73 m^2 for eGFRcrt, eGFRcys or eGFRcc, respectively. Baseline BL and the cumulative lead burden did not materially modify these estimates, but baseline eGFR was a major determinant of eGFR changes showing regression to the mean during follow-up. Responses of serum osmolarity, urinary gravity or the urinary albumin-to-creatinine ratio were also unrelated to the BL increment. The age-related decreases in eGFRcrt, eGFRcys and eGFRcc~~,~~ were -1.41, -0.96 and -1.10 ml/min/1.73 m^2, respectively.

Conclusions: In the current study the 2-year changes in renal function were unrelated to the blood lead increase. However, given the confidence intervals around the point estimates of the changes in eGFRcc and eGFRcys, a larger study with longer follow-up is being planned.

Introduction

According to the 2017 Global Burden of Disease report [1], lead exposure causes or aggravates chronic kidney disease (CKD). From 2007 until 2017, global mortality and disability adjusted life-years (DALYs) related to lead exposure amounted to 43,000 and 56,000 deaths and 1,094,000 and 1,297,000 DALYs, respectively. However, over the same time interval, lead exposure (-27.7%) and the age-standardised mortality and DALY rates shrank by 10%. Limitations of the GBD results included: residual confounding, for instance by co-exposure to other pollutants; uncertainty about the generalisability of the association sizes; and the impossibility to account for secular trends in exposure. The latter issue is not trivial, because among American adults enrolled in consecutive cycles of the National Health and Nutrition Examination Survey (NHANES), mean blood lead (BL) dropped from 13.1 µg/dl in 1976-1980 [2] to 1.2-2.76 µg/dl in 1988-1994 [2], and further to 1.64 µg/dl in 1999-2002 [3,4], which is close to the estimated BL in pre-industrial humans (2 µg/dl), only exposed by natural sources [1].

Several studies of populations [5-10] or workers [11] reported an inverse association between eGFR and BL, but given that 70% of lead is renally excreted [12,13] cannot distinguish whether higher BL induces renal impairment or *vice versa*. Experts argue as to whether current-day lead exposure still contributes to CKD rates [14,15]. Furthermore, the high prevalence of comorbid conditions and the lack of longitudinal data associating temporal trends in renal function and BL hamper interpretation of the literature [15]. The Study for Promotion of Health in Recycling Lead (SPHERL; NCT02243904) is addressing these knowledge gaps by evaluating the 2-year responses of the estimated glomerular filtration rate (eGFR) [16,17] as primary renal endpoint in newly hired workers not occupationally exposed before. eGFR was determined not only from serum creatinine, but also from serum cystatin C, which is less vulnerable to confounding by ethnicity, sex, age, muscle mass and protein intake [18].

Methods

SPHERL is a longitudinal study of newly hired lead workers at battery manufacturing and lead recycling plants in the United States [16,17]. SPHERL complies with the Helsinki declaration for investigations in humans. The Ethics Committee of the University Hospitals Leuven (Belgium) approved the study protocol (document number, B322201421631). The health of the labour force was protected in compliance with the US Occupational Safety and Health Administration Standard (www.osha.gov/laws-regs/regulations/standardnumber/1910/ 1910.1025), which includes regular health check-ups, proper workplace ventilation, and the obligatory use of personal protective

equipment. The predefined primary renal endpoint was the eGFR response to lead exposure in newly hired workers not occupationally exposed before [16]. With 2-tailed significance and power set at 0.05 and 0.90, respectively, SPHERL was powered to detect a doubling of the age-related eGFR decline, if 250 workers would be followed up for 2 years.

Of 746 newly hired workers invited to participate, 601 (80.6%) consented. However, in the interval between consent and the planned baseline examination (median: 19 days; 5th-95th percentile interval: 9-59 days), 95 laborers left the work place or withdrew. From 25 January 2015 until 19 September 2017, 506 workers underwent the baseline examination, of whom 289 (57.1%) had at least one follow-up visit and 236 (46.6%) had two follow-up visits (**Figure 1**). Of 289 participants with at least one follow-up visit, 61 were excluded because baseline/follow-up blood lead (n=1/3), serum creatinine (n=7/1) or serum cystatin C (n=3/23) had not been measured. This left 251 workers, including 21 women, for statistical analysis. The clinical variables and the analytical methods of laboratory tests, including the measurements of renal function, haematological parameters and blood lead, the quality control of these measurements, and the supporting references are given in the Data Supplement. In short, BL was determined on whole blood by inductively coupled plasma mass spectrometry at an analytical laboratory certified for BL analysis in compliance with the provisions of the OSHA Lead Standard, 29CFR 1910.1025 (Occupational Safety and Health Administration [www.osha.gov]). The BL detection limit was 0.5 µg/dl. **Table S1** lists the formulae to estimate the glomerular filtration rate from serum creatinine, serum cystatin C or both (**Table S1**) [18]. Serum osmolality (mOsm/kg) was computed as 2 × (serum Na^+ [mmol/L]) + (blood glucose [mg/dl] / 18) + (blood urea nitrogen [mg/dl] / 2.8) [19]. Haemoglobin and the red blood cell count were measured using a fully automated analyser. From these two measurements, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, and the red cell distribution width were calculated by the device software (Data supplement, expanded method).

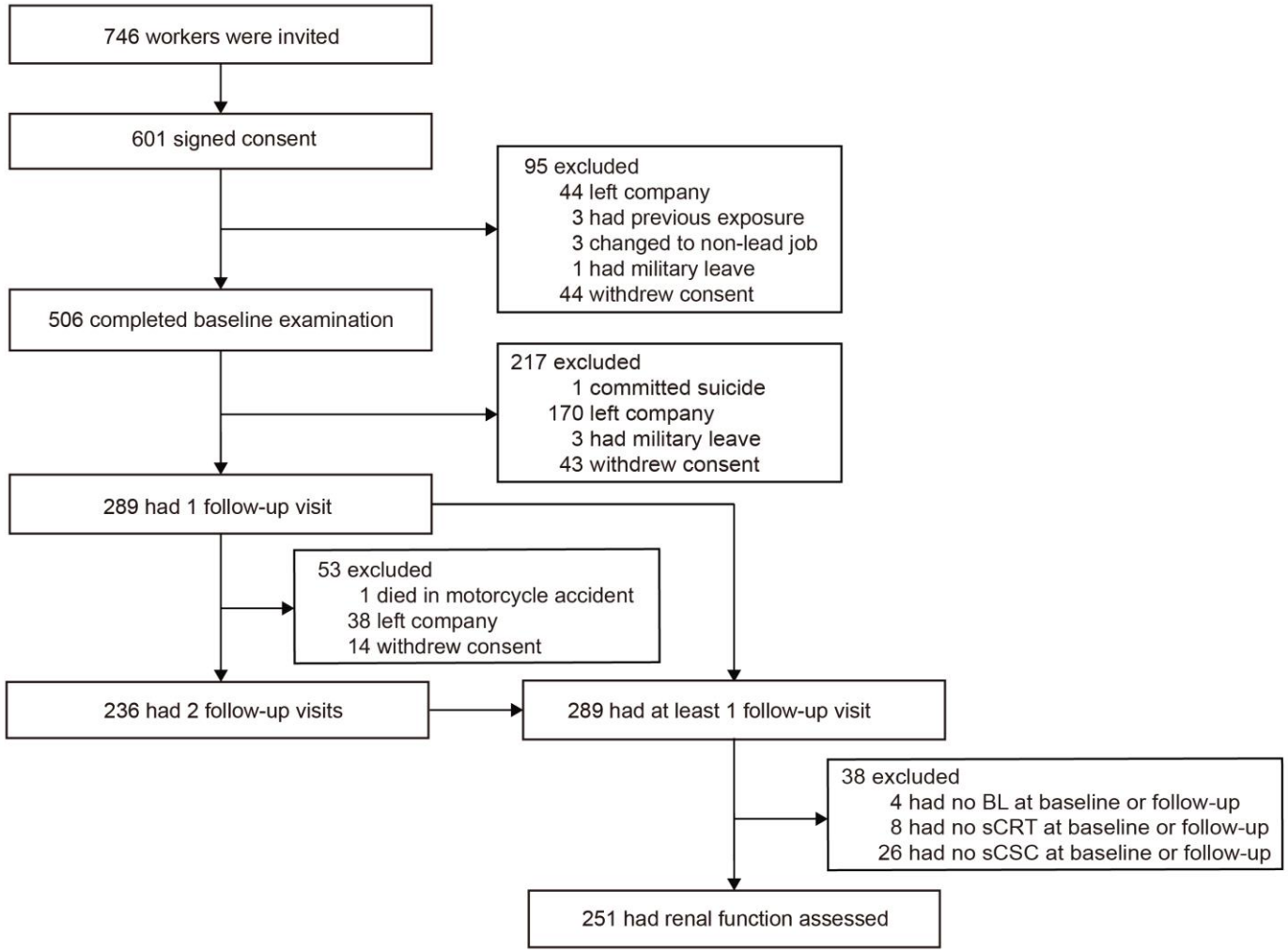


Figure 1. Chart showing the flow of study participants.

Abbreviations: BL, blood lead; sCRT, serum creatinine; sCSC, serum cystatin C.

For database management and statistical analysis, we used the SAS software, version 9.4, maintenance level 5 (SAS Institute Inc., Cary, NC). Departure from normality was evaluated by the Shapiro-Wilk statistic. We applied a logarithmic transformation (base 10) to normalise the distributions of serum cystatin C, the urinary albumin-to-creatinine ratio (ACR), γ-glutamyltransferase, and blood lead. We reported the central tendency and spread of continuously distributed variables as mean and SD or as geometric mean and the interquartile range (IQR) or the 5th-95th percentile data range (PDR) for logarithmically transformed variables. To compare means and proportions, we applied the t-statistic or ANOVA for continuous variables, and Fisher exact test for categorical variables, respectively.

In exploratory analyses, renal function changes were first assessed across quartiles of the follow-up-to-baseline BL ratio without any adjustment. In the next step of the analyses, point estimates with 95% confidence intervals (95% CI) of the association sizes between the changes in the outcome variables and in blood lead were derived from mixed models with random intercept and random slope. Unadjusted, adjusted and fully adjusted models were constructed with the follow-up-to-baseline BL ratio as the independent variable and the changes in renal function and haematological variables as the dependent variables. Irrespective of the adjustment, all models included a random effect term to account for clustering of observations within study participants, of whom 236 (94.0%) had two follow-up visits (**Figure 1**). Adjusted models accounted for age, the change of age from baseline to follow-up (equivalent to the between-visit interval), the time of day of the blood sampling (nighttime vs daytime), and the baseline renal function measurement being analysed. Fully adjusted models additionally accounted for baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes vs no), the total-to-HDL cholesterol ratio and γ-glutamyltransferase (index of alcohol intake). These covariables were considered based on previous publications [17,18,20]. In the next analysis step, adjusted mixed models were constructed to account for the combined effects of the BL change and the baseline BL on eGFR. Association sizes were expressed for a 3-fold BL increment from baseline over follow-up and for a 3-fold higher baseline BL. Finally, sensitivity analyses were conducted, excluding workers on antihypertensive drug treatment at baseline, follow-up, or both. Moreover, the influence of the lead body burden at enrolment was assessed by stratifying study participants according to the median age, the median baseline BL, and the median cumulative blood lead index (CBLI) [21]. To compute CBLI [21], age for workers leaving school at less

than, at, and above the 12th grade were assumed to be 14, 18 and 23 years; based on NHANES data (1988-1994), the corresponding blood lead levels were set at 2.2, 1.4 and 1.5 μg/dl, respectively [22].

Results

Baseline characteristics

Of 251 workers, 230 (91.6%) were men, 122 (48.6%) were white, 107 (42.6%) were Hispanic, 12 (4.8%) were Black and 10 (4.0%) had other self-reported ethnicities. At baseline, age averaged 29.7 years, body mass index 28.9 kg/m^2, mean arterial pressure 93.1 mm Hg, total and HDL serum cholesterol 171.8 mg/dl and 46.3 mg/dl, the total-to-HDL cholesterol ratio 3.91, blood glucose 94.3 mg/dl, and γ-glutamyltransferase 22.6 U/l (**Table 1**). The characteristics of the workers analysed and not analysed were broadly similar (**Table S2**).

Blood lead

Median follow-up was 2.0 years (PDR: 1.0-2.3 years). At baseline, the geometric mean BL was 4.13 μg/dl (IQR: 0.40-7.80 μg/dl; PDR: 1.00-14.8 μg/dl) and at the last follow-up 13.6 μg/dl (IQR, 10.2-22.5 μg/dl; PDR, 3.30-30.5 μg/dl; **Figure S1**). At the 1- and 2-year follow-up visits, these BL values were 14.0 μg/dl (IQR: 10.5-21.8 μg/dl; PDR: 3.70-30.4 μg/dl) and 13.5 μg/dl (IQR: 10.4-22.0 μg/dl; PDR: 3.20-30.4 μg/dl), respectively. The last-follow-up-to-baseline BL ratio averaged 3.30 (IQR: 1.91-5.72; PDR: 0.73-14.3 μg/dl; **Figure S1**). Over follow-up, participants with lower baseline blood lead experienced larger increases in the biomarker of exposure than those with higher baseline levels and *vice versa* (**Figure S2, Panel A**).

Renal function at baseline and follow-up

Serum creatinine and cystatin C averaged 0.958 mg/dl and 0.676 mg/l at baseline and increased (P<0.001) over follow-up by 0.0609 mg/dl and 0.0339 mg/l, respectively (**Table 2**). Consequently, eGFRcrt, eGFRcys and eGFRcc decreased (P<0.001) by 7.33, 4.52 and 6.44 ml/min/1.73 m^2 from their baseline level of 105.4, 125.1 and 114.7 ml/min/1.73 m^2. Over follow-up (**Table 2**), serum osmolality increased by 2.32 mOsm/kg from 286.8 mOsm/kg at baseline and urinary gravity by 0.0027 from a starting value of 1.020 (P<0.001). ACR did not significantly change from baseline to follow-up (P=0.52). To estimate the ageing effect on eGFR, age at the last follow-up was introduced in the formula to compute eGFRcrt, eGFRcys and eGFRcc from the baseline values of serum creatinine, serum cystatin C or both; the resulting estimates were -1.41, -0.96 and -1.10 ml/min/1.73 m^2, respectively.

Table 1. Baseline characteristics of 251 workers

Characteristic	N (%)	Characteristic	Mean (SD/IQR)
Male	230 (91.6)	Age, years	29.7 (9.8)
White ethnicity	122 (48.6)	Body mass index, kg/m^2	28.9 (6.1)
Hispanic ethnicity	107 (42.6)	Systolic blood pressure, mm Hg	120.0 (10.2)
Black ethnicity	12 (4.8)	Diastolic blood pressure, mm Hg	79.7 (8.8)
Other ethnicities	10 (4.0)	Mean arterial pressure, mm Hg	93.1 (8.7)
Current smokers	67 (27.0)	Total cholesterol, mg/dl	171.8 (37.8)
Alcohol intake	110 (44.4)	HDL cholesterol, mg/dl	46.3 (12.0)
Hypertension stage ≥1	125 (49.8)	Total-to-HDL cholesterol ratio	3.91 (1.3)
Hypertension stage ≥2	46 (18.3)	Blood glucose, mg/dl	94.3 (15.8)
Treated hypertension	17 (6.8)	γ-glutamyltransferase, U/l	22.6 (16.0, 33.0)
Diabetes mellitus	12 (4.8)	Blood lead, µg/dl	4.13 (2.40, 7.80)

Values are number of participants (%), arithmetic mean (SD) or geometric mean (IQR). Blood pressure was the average of five readings. Hypertension was categorised according to the 2017 ACC/AHA guideline, irrespective of treatment status (reference 36). Mean arterial pressure was diastolic blood pressure plus one third of pulse pressure. Diabetes mellitus was a self-reported diagnosis, a fasting blood glucose of ≥126 mg/dl, or use of antidiabetic drugs. To convert total or high-density lipoprotein (HDL) serum cholesterol to mmol/l, multiply by 0.0259; to convert blood glucose to mmol/l, multiply by 0.0559.

Table 2. Renal function at baseline and follow-up

Characteristic	Baseline	Year 1	Year 2	Δ (95% CI)	*P* value
Serum creatinine, mg/dl	0.958 (0.168)	1.002 (0.175)	1.015 (0.174)	0.0609 (0.0437, 0.0780)	<0.001
Serum cystatin C, mg/l	0.676 (0.106)	0.696 (0.132)	0.714 (0.161)	0.0339 (0.0187, 0.0491)	<0.001
eGFRcrt, ml/min/1.73 m^2	105.4 (17.0)	100.4 (17.3)	97.8 (16.4)	-7.33 (-8.94, -5.71)	<0.001
eGFRcys, ml/min/1.73 m^2	125.1 (13.3)	122.6 (16.6)	119.8 (19.9)	-4.52 (-6.27, -2.77)	<0.001
eGFRcc, ml/min/1.73 m^2	114.7 (14.4)	110.5 (15.2)	107.8 (16.0)	-6.44 (-7.85, -5.04)	<0.001
Serum osmolality, mOsm/kg	286.8 (3.8)	288.5 (3.9)	289.1 (4.6)	2.32 (1.67, 2.97)	<0.001
Serum sodium, mmol/l	138.3 (1.8)	139.1 (1.8)	139.3 (2.2)	1.05 (0.73, 1.37)	<0.001
Blood glucose, mg/dl	94.3 (15.8)	85.5 (16.2)	89.2 (19.0)	-5.91 (-8.32, -3.51)	<0.001
Serum insulin, U/l	7.21 (3.70, 13.2)	8.24 (4.30, 15.2)	9.24 (4.70, 18.6)	22.8 (8.77, 38.7)	0.001
Blood urea nitrogen, mg/dl	14.0 (3.5)	15.3 (4.0)	15.5 (3.7)	1.55 (1.09, 2.02)	<0.001
BUN-to-SCRT ratio	14.9 (4.1)	15.5 (3.8)	15.5 (3.7)	0.59 (0.08, 1.09)	0.023
Urinary gravity	1.020 (0.0077)	1.022 (0.0072)	1.022 (0.0069)	0.0027 (0.0016, 0.0038)	<0.001
ACR, mg/g	4.49 (2.86, 6.33)	4.29 (2.73, 6.01)	4.61 (2.86, 6.63)	2.90 (-5.80,12.4)	0.52

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both (reference 18). BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). ACR is the urinary albumin-to-creatinine ratio. Values are arithmetic mean (SD) or geometric mean (IQR). Changes from baseline to last follow-up are given with 95% confidence interval; for logarithmically transformed serum insulin and ACR changes are expressed as percentage. *P* values denote the significance of the changes from baseline to last follow-up. To convert serum creatinine to µmol/l, multiply by 88.42; to convert cystatin C from mg/l to nmol/l, multiply by 74.9; to convert blood glucose to mmol/l, multiply by 0.0559; to convert blood urea nitrogen to mmol/l, multiply by 0.3571.

At baseline and last follow-up, eGFRcrt, eGFRcys and eGFRcc were respectively 8.96 ml/min/1.73 m^2 (P<0.001), 4.68 ml/min/1.73 m^2 (P=0.004) and 7.19 ml/min/1.73 m^2 (P<0.001) lower, if determined from night shift blood samples compared with morning and evening blood samples (**Figure S3**). At baseline and follow-up, two workers had an eGFRcrt of <60 ml/min/1.73 m^2, whereas their eGFRcys and eGFRcc were above this limit. Thirteen workers (5.2%) reported a history of nephrolithiasis at enrolment, but no new or recurrent cases occurred during follow-up.

Categorical analysis

In unadjusted categorical analyses, the changes in renal function, also including serum osmolality, urinary gravity and the urinary albumin-to-creatin ratio (**Table 3**) and the haematological measurements (**Table S10**) were analysed by quartiles of the distribution of the follow-up-to-baseline BL ratio. None of these measurements showed a significant trend across increasing categories of the follow-up-to-baseline BL ratio. **Table S3** and **Table S4** exclude that the renal results given in **Table 3** might be explained by opposing trends in the baseline or follow-up data.

Mixed model regression analysis

All analyses of the relation between the changes in the renal and other outcome measurements accounted for clustering of the data within individuals (**Table 4**). Results are presented without any further adjustment, with partial adjustment for sex, enrolment age, follow-up duration, the time of day of the blood sampling (nighttime *vs* daytime), and the baseline renal function measure being analysed. Fully adjusted models additionally accounted for baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), total-to-HDL cholesterol ratio and γ-glutamyltransferase. In unadjusted (**Table 4** and **Figure 2**) and in partially and fully adjusted models (**Table 4**), the changes in serum creatinine, serum cystatin C, eGFRcrt, eGFRcys and eGFRcc were not significantly correlated with the BL changes. In fully adjusted models (**Table 4**), the association sizes for a 3-fold BL increment amounted to 0.0072 mg/dl (95% CI: -0.0081 to 0.0226 mg/dl) for serum creatinine, 0.0118 mg/L (95% CI: -0.0033 to 0.0268 mg/L) for serum cystatin C, -0.86 ml/min/1.73 m^2 for eGFRcrt (95% CI: -2.39 to 0.67 ml/min/1.73 m^2), -1.58 ml/min/1.73 m^2 (95% CI: -3.34 to 0.18 mL/min/1.73 m^2) for eGFRcys, and -1.32 ml/min/1.73 m^2 (95% CI: -2.66 to 0.03 ml/min/1.73 m^2) for eGFRcc. In fully adjusted models, a 3-fold BL increase was not significantly associated with the decreases in eGFRcrt, eGFRcys and eGFRcc, amounting to 0.86, 1.58 and 1.32 ml/min/1.73 m^2, respectively (**Table 4** and **Figure 2**).

Given the whole range of the follow-up-to-baseline BL ratio (range, 0.16-34.0; **Figure S1; Panel C**), the predicted mean changes in eGFRcrt, eGFRcys and eGFRcc amounted to -5.91 ml/min/1.73 m^2 (*P*=0.30), -3.46 ml/min/1.73 m^2 (*P*=0.082) and -5.11 ml/min/1.73 m^2 (*P*=0.045), respectively. Finally, in unadjusted and adjusted analyses, changes in serum osmolarity, its constituents, urinary gravity and ACR (**Table 4**) and in the haematological measurements (**Table S11**) were not significantly correlated with the change in BL during follow-up.

Baseline effect modifiers

In the next analysis step, with full adjustment for covariables applied, heat maps based on the fully adjusted mixed model were constructed to account for the combined effects of baseline BL and the BL change on eGFR (**Figure 3**). Baseline BL was not significantly associated with the changes during follow-up in eGFRcrt (β: -0.65 µg/dl; 95% CI: -3.03 to 1.74 µg/dl; *P*=0.59), eGFRcys (β: -2.43 µg/dl; 95% CI: -5.02 to 0.17 µg/dl; *P*=0.067) or eGFRcc (β: -1.81 µg/dl; 95% CI: -3.90 to 0.28 µg/dl; *P*=0.089). However, accounting for baseline BL made the eGFR estimates associated with the BL ratio borderline significant for eGFRcys (*P*=0.013) and eGFRcc (*P*=0.009). The predicted values in the central boxes of heat maps (**Figure 3**) were consistent with the predicted eGFRs derived from the fully adjusted model not including baseline BL: -5.81 *vs* -5.91 ml/min/1.73 m^2 for eGFRcrt (**Panel B**), -3.36 *vs* -3.46 ml/min/1.73 m^2 for eGFRcys (**Panel C**), and -4.98 *vs* -5.11 ml/min/1.73 m^2 in eGFRcc (**Panel D**). The absence of a significant interaction between baseline BL and the BL changes indicated that the effect modification of baseline BL was steady over its range (*P*-value for interaction, ≥ 0.38).

Furthermore, heat maps were constructed to assess the influence of the baseline eGFR in combination with baseline BL (**Figure S4**) or the BL change (**Figure S5**) on eGFR. Baseline eGFR was a major determinant of the eGFR changes ($P \leq 0.001$), higher baseline values being associated with more eGFR decline during follow-up, and *vice versa*, however, without significant interaction ($P \geq 0.11$) between baseline eGFR and the baseline BL or BL change (**Figure S4** and **Figure S5**).

Table 3. Renal function changes from baseline to last follow-up by quartiles of the distribution of the follow-up-to-baseline blood lead concentration ratio

Characteristic	<1.91	1.91-3.45	3.45-5.66	≥5.66	*P* value
Number in group	62	63	63	63	…
Serum creatinine, mg/dl	0.0624 (0.0275, 0.0973)	0.0833 (0.0505, 0.1160)	0.0335 (0.0072, 0.0742)	0.0643 (0.0382, 0.0903)	0.57
Serum cystatin C, mg/l	0.0221 (-0.0054, 0.0496)	0.0249 (-0.0041, 0.0539)	0.0432 (0.0075, 0.0789)	0.0451 (0.0168, 0.0734)	0.21
eGFRcrt, ml/min/1.73 m^2	-7.38 (-10.4, -4.32)	-9.33 (-12.6, -6.04)	-4.91 (-8.68, -1.14)	-7.68 (-10.3, -5.10)	0.63
eGFRcys, ml/min/1.73 m^2	-3.12 (-6.08, -0.17)	-3.70 (-7.00, -0.40)	-5.19 (-9.38, -1.00)	-6.04 (-9.45, -2.63)	0.20
eGFRcc, ml/min/1.73 m^2	-5.82 (-8.56, -3.08)	-6.91 (-9.42, -4.39)	-5.57 (-8.85, -2.28)	-7.47 (-10.1, -4.88)	0.57
Serum osmolality, mOsm/kg	2.39 (0.84, 3.95)	2.42 (1.09, 3.74)	1.99 (0.81, 3.17)	2.49 (1.38, 3.59)	0.96
Serum sodium, mmol/l	1.13 (0.33, 1.93)	0.92 (0.28, 1.56)	0.97 (0.39, 1.54)	1.17 (0.64, 1.71)	0.90
Blood glucose, mg/dl	-4.74 (-9.55, 0.06)	-5.01 (-9.20, -0.82)	-8.41 (-13.7, -3.12)	-5.47 (-10.3, -0.62)	0.61
Insulin, %	37.0 (7.84, 74.0)	8.91 (-15.6, 40.5)	19.3 (-5.67, 50.9)	28.0 (0.84, 62.6)	0.85
Blood urea nitrogen, mg/dl	1.11 (0.27, 1.96)	2.40 (1.36, 3.43)	1.46 (0.70, 2.22)	1.24 (0.24, 2.23)	0.78
BUN-to-SCRT ratio	0.31 (-0.67, 1.29)	0.95 (-0.12, 2.01)	0.99 (0.07, 1.90)	0.11 (-0.94, 1.15)	0.80
Urinary gravity,	0.0044 (0.0023, 0.0064)	0.0028 (0.0005, 0.0052)	0.0009 (-0.0014, 0.0032)	0.0027 (0.0007, 0.0047)	0.17
ACR, %	-1.54 (-18.0, 18.3)	2.00 (-15.6, 23.2)	10.6 (-7.03, 31.7)	0.85 (-13.9, 18.1)	0.71

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both (reference 18). BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). ACR is the urinary albumin-to-creatinine ratio. Within-group changes are arithmetic mean (SD) or geometric mean (IQR). For logarithmically transformed serum insulin and ACR, changes are expressed as percentage. *P* values denote the significance of the between-group differences. To convert serum creatinine to µmol/l, multiply by 88.42; to convert cystatin C from mg/l to nmol/l, multiply by 74.9; to convert blood glucose to mmol/l, multiply by 0.0559; to convert blood urea nitrogen to mmol/l, multiply by 0.3571.

Table 4. Association between changes in renal function and change in blood lead

Variable	Unadjusted		Adjusted		Fully adjusted	
	β (95% CI)	*P* value	β (95% CI)	*P* value	β (95% CI)	*P* value
Serum creatinine, $\times 10^{-2}$ mg/dl	0.28 (-1.37, 1.92)	0.74	0.74 (-0.74, 2.23)	0.32	0.72 (-0.81, 2.26)	0.35
Serum cystatin C, $\times 10^{-2}$ mg/L	0.65 (-0.89, 2.19)	0.41	1.05 (-0.42, 2.52)	0.16	1.18 (-0.33, 2.68)	0.12
eGFRcrt, ml/min/1.73 m^2	-0.43 (-2.08, 1.23)	0.61	-0.86 (-2.34, 0.61)	0.25	-0.86 (-2.39, 0.67)	0.27
eGFRcys, ml/min/1.73 m^2	-0.88 (-2.67, 0.91)	0.33	-1.42 (-3.13, 0.30)	0.11	-1.58 (-3.34, 0.18)	0.078
eGFRcc, ml/min/1.73 m^2	-0.75 (-2.21, 0.71)	0.31	-1.21 (-2.51, 0.09)	0.069	-1.32 (-2.66, 0.03)	0.055
Serum osmolality, mOsm/kg	0.08 (-0.57, 0.74)	0.80	0.02 (-0.47, 0.50)	0.95	-0.05 (-0.55, 0.46)	0.85
Serum sodium, mmol/l	0.13 (-0.19, 0.45)	0.42	0.09 (-0.16, 0.34)	0.48	0.06 (-0.20, 0.31)	0.66
Blood glucose, mg/dl	-2.19 (-4.67, 0.29)	0.084	-0.78 (-2.91, 1.34)	0.47	-0.93 (-3.07, 1.22)	0.40
Insulin, %	-4.44 (-15.4, 7.94)	0.46	-4.30 (-14.6, 7.26)	0.45	-3.75 (-13.6, 7.19)	0.48
Blood urea nitrogen, mg/dl	-0.13 (-0.63, 0.38)	0.62	-0.18 (-0.61, 0.25)	0.40	-0.13 (-0.57, 0.32)	0.57
BUN-to-SCRT ratio	-0.23 (-0.76, 0.29)	0.38	-0.40 (-0.83, 0.03)	0.067	-0.35 (-0.80, 0.09)	0.12
Urine specific gravity, $\times 10^{-2}$	-0.03 (-0.14, 0.09)	0.65	-0.03 (-0.11, 0.06)	0.54	-0.00 (-0.09, 0.08)	0.94
ACR, %	1.31 (-8.03, 11.6)	0.79	-0.37 (-8.34, 8.29)	0.93	0.23 (-7.94, 9.12)	0.96

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both (reference 18). BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). Changes in serum insulin and the urinary albumin-to-creatinine ratio are expressed as percentage differences from baseline to follow-up. Association sizes (β), given with 95% confidence interval, express the change in the dependent variable for a 3-fold increase in the blood lead concentration. Adjusted models accounted for sex, age, follow-up duration, the time of day of blood sampling (nighttime *vs* daytime), and the baseline renal function measure being analysed. Fully adjusted models additionally accounted for baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), the total-to-HDL cholesterol ratio and γ-glutamyltransferase.

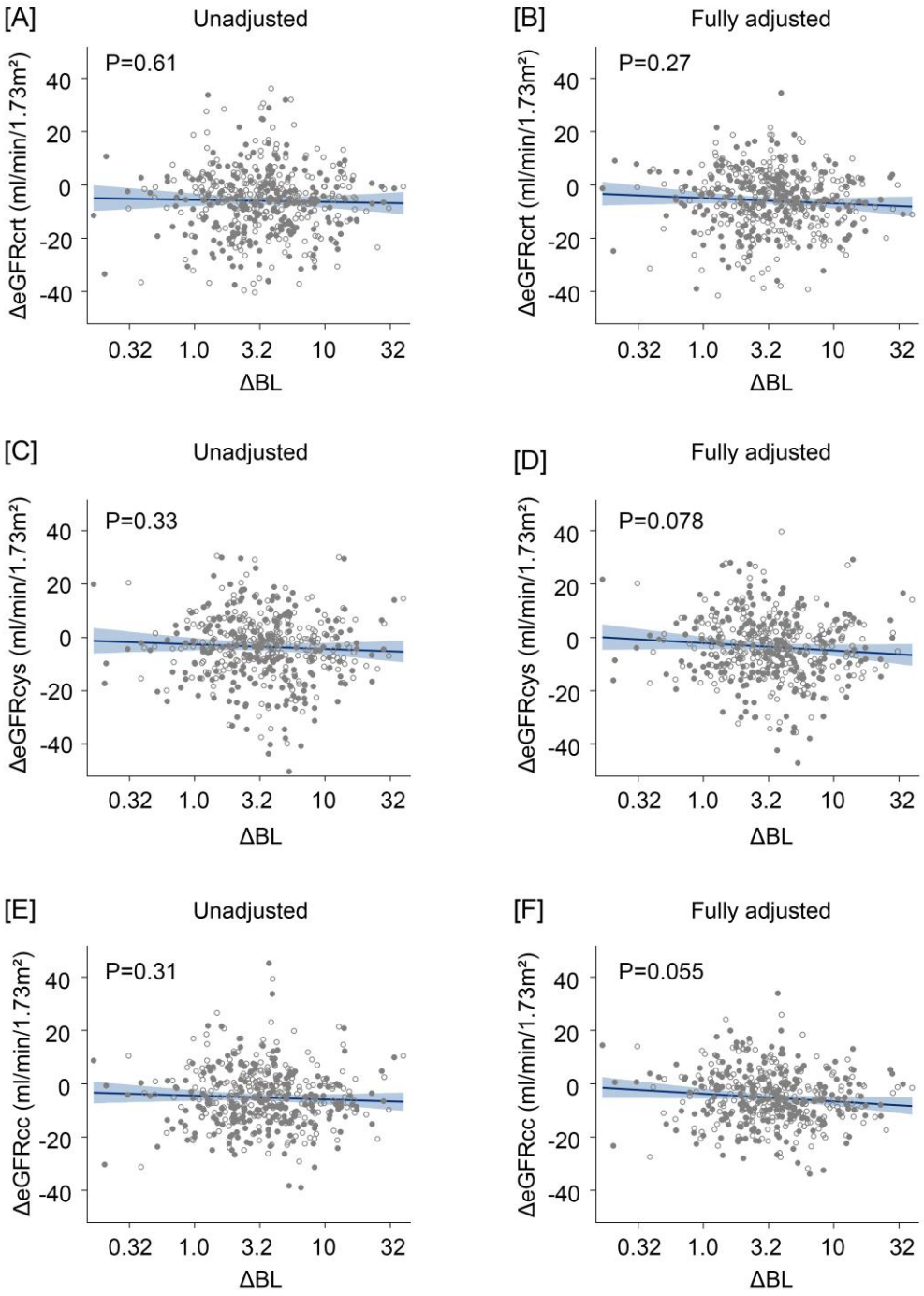


Figure 2. Association of the change in glomerular filtration rate estimated from serum creatinine (eGFRcrt), cystatin C (eGFRcys) or both (eGFRcc) with the follow-up-to-baseline blood lead concentration ratio (ΔBL). All plotted associations account for clustering of data within participants. Open symbols represent the first-year data and closed symbols second-year data. The regression line and 95% confidence interval were derived from a mixed model, unadjusted (A, C, E) or adjusted (B, D, F) for sex, age, follow-up duration, the time of day of blood sampling, the baseline eGFR being analysed, body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), the total-to-HDL cholesterol ratio, and γ-glutamyltransferase.

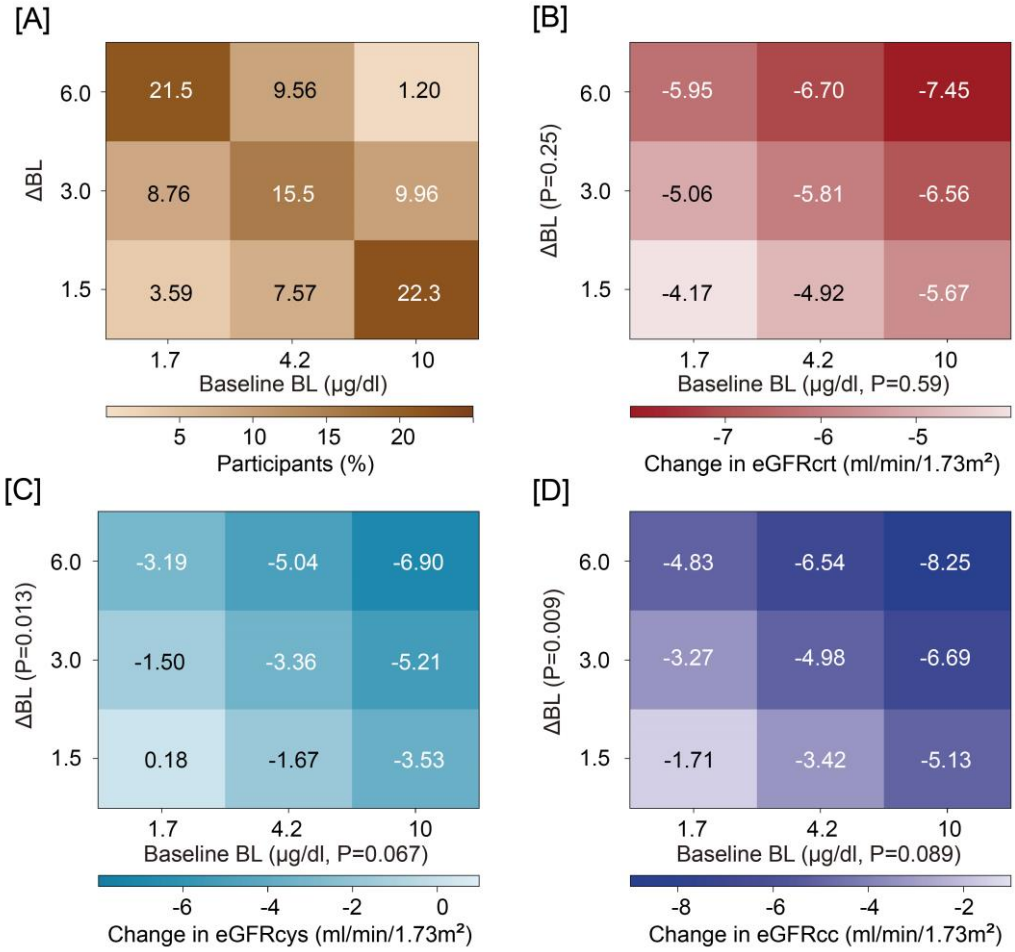


Figure 3. Heat maps relating the changes in renal function to baseline blood lead (BL) level and the follow-up-to-baseline BL concentration ratio. Participants were cross-classified by thirds of the distributions of baseline BL and the follow-up-to-baseline BL ratio. The percentage of participants contributing to each cell of the heat maps is given in panel A. Results for the glomerular filtration rate as derived from serum creatinine (eGFRcrt), serum cystatin C (eGFRcys) or both (eGFRcc) are presented in panels B, C, and D, respectively. The colour codes and numbers in the grids are the longitudinal changes in glomerular filtration as estimated from the corresponding thick marks at mean values of the covariables, including sex, age, follow-up duration, the time of day of blood sampling, (daytime *vs* nighttime), the baseline eGFR being analysed, body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), the total-to-HDL cholesterol ratio, and γ-glutamyltransferase. The *P* values for the interaction terms between baseline BL and the BL change were 0.56, 0.44 and 0.38 for eGFRcrt (B), eGFRcys (C) and eGFRcc (D), respectively.

Sensitivity analyses

Sensitivity analyses from which 28 workers on antihypertensive drug treatment at baseline (n=17), follow-up (n=28) or both (n=17) were excluded confirmed the main findings (**Table S5** *vs* **Table 4**). Furthermore, the cross-sectional relations of the renal and associated measurements with BL at baseline and at last follow-up were similar, none of the differences in the slopes reached significance ($P \geq 0.074$; **Table S6**). The β-coefficients for a 3-fold increment of BL at baseline *vs* a 3-fold increment of BL at follow-up in adjusted models were 0.26 *vs* 2.96 mg/dl for cystatin C ($P=0.13$), 1.31 *vs* -0.80 ml/min/1.73 m^2 ($P=0.29$) for eGFRcrt, -0.19 *vs* -3.36 ml/min/1.73 m^2 ($P=0.099$) for eGFRcys, and 0.63 *vs* -2.33 ml/min/1.73 m^2 for eGFRcc ($P=0.082$).

To assess the influence of the lead body burden, the 251 workers were categorised by median age (**Table S7**), median BL at enrolment (**Table S8**), or the median CBLI (**Table S9**). The multivariable-adjusted slopes of any eGFR measure did not differ, if dichotomised by baseline BL (*P* value for interaction ≥0.45, **Table S8**), but were slightly but significantly different between older and younger workers for serum sodium (0.39 *vs* -0.32 mmol/l), blood glucose (-1.91 *vs* 1.75 mg/dl) and urine specific gravity (-0.0011 *vs* 0.0011), if categorised by baseline age ($P \geq 0.002$; **Table S7**). Moreover, if categorised by median CBLI ($P \geq 0.054$; **Table S9**), the slope of eGFRcc (-0.93 *vs* -3.46 ml/min/1.73 m^2) slightly but not differed between the low *vs* high CBLI group.

Discussion

In a real-world experiment, conducted in an occupational setting, 2 years of lead exposure was not significantly associated with eGFR decline, the co-principal renal SPHERL endpoint [16,17]. In fully adjusted mixed models, accounting for covariables and the within-participant clustering of the 1- and 2-year follow-up data, a 3-fold BL increment generated estimates of the lead-related eGFR changes (**Table 4**) amounting to -0.86, -1.58 and -1.32ml/min/1.73 m^2 for eGFRcrt, eGFRcys or eGFRcc, respectively. These eGFR changes must be gauged against the expected age-related decline in eGFR, estimated in the current study to be -1.41, -0.96 and -1.10 ml/min/1.73 m^2, for eGFRcrt, eGFRcys and eGFRcc, respectively. The responses of serum osmolality, urine specific gravity, ACR (**Table 4**) and the haematological measurements (**Table S11**) were not significantly associated to the increment in lead exposure.

In addition to regression to the mean, the present study highlights the importance to account in longitudinal studies for concealed albeit not unexpected confounders and to distinguish crudely observed eGFR changes in the context of the biomarker of exposure of interest, blood lead in the current study. **Figure S3** shows that eGFRcrt, eGFRcys and

eGFRcc were respectively 8.96, 4.68 and 7.18 ml/min/1.73 m^2 lower when determined from night shift blood samples compared with morning or evening blood samples. Both serum creatinine and serum cystatin C, from which eGFR is derived, show a diurnal rhythm with little influence of meals or meat ingestion on serum cystatin C, whereas these confounders increase serum creatinine [23]. Along similar lines, during sleep, urine flow decreases and the tubular reabsorption of water increases [24,25]. The newly hired workers recruited into SPHERL not only transited from environmental to occupational exposure, but also from a sedentary to a physically demanding lifestyle. Based on published tables [26], the jobs offered to the workers required an energy expenditure of 6 to >8 metabolic equivalents defined as the amount of oxygen consumed while resting in the sitting position. Physical labour induces acute renal changes in healthy adults [27]. In young adults, exercise reduces renal plasma flow and eGFR with smaller effects on eGFRcys than on eGFRcrt [28]. Strenuous physical work is also associated with sodium and water loss through sweating and an increased respiration rate and with higher insulin sensitivity, hence increases in serum sodium and urine specific gravity, lower blood glucose, and higher serum insulin during follow-up compared with baseline (**Table 2**).

Several studies of populations [5-8,10] or workers [11] in Europe [5,8,10], the United States [6,7,11] and Taiwan [9] reported an inverse association between eGFR and BL or an increased risk of chronic kidney disease in relation to BL. In a study of randomly recruited Belgians published in 1992 [5], mean BL was 7.5 µg/dl in 1016 women and 11.4 µg/dl in 965 men; in sex-stratified analyses, a 10-fold higher BL was associated with a 13 and 10 ml/min lower creatinine clearance in women and men, respectively. However, given that approximately 70% of lead excretion occurs via the urine [12,13], the observational studies referenced above [5-11] could not ascertain the directionality of the association between eGFR and BL, reduced eGFR being associated with lesser urinary lead excretion and higher BL, or higher lead exposure as reflected by BL reducing eGFR. Lead is a cumulative contaminant, which is for 90-95% stored in bone [29,30] Bone lead, for 99% carried by red blood cells, correlates with blood lead [30,31] and explains around 20% of the variance in blood lead, depending on seasonality [30] and hormonal and other endogenous and environmental stimuli influencing the balance between bone formation and resorption [31]. Both bone lead and BL increase with age. However, the sensitivity analyses dichotomized by median age at baseline (**Table S7**), median baseline BL (**Table S8**) or the median CBLI (**Table S9**) addressed modification of the association between eGFR and BL by the lead body burden before starting a job in the lead industry. The multivariable-adjusted association sizes between changes in eGFR and in BL did not differ, if dichotomised by age or baseline BL, but were slightly but significantly higher for

eGFRcys and eGFRcc, if categorised by median CBLI. However, the categorisation by median CBLI [21] relied on extrapolation of BL at 14, 18 and 23 years of age, so that the latter findings must be carefully interpreted [22].

Among the strong points of our study are its longitudinal design with annual follow-up visits, the stringent quality control of the blood lead measurement maintained during the 2-year course of the study, and the use of serum cystatin C in addition to serum creatinine to derive eGFR. Notwithstanding these strong points, our study also has limitations. First, the attrition rate among the 506 workers who participated in the baseline examination, but defaulted from follow-up amounted to 217 (42.9%), mainly because they left employment (**Figure 1**). The small sample size and the 2-year follow-up of the current SPHERL cohort warrant a cautious interpretation of the findings. According to the published SPHERL protocol [16] the anticipated attrition rate was 50%. To meet the sample size required to address renal function as primary endpoint, 500 workers had to be enrolled, a target that was met. According to recently updated plans, consenting workers remaining employed at the study sites are now being followed up for an additional 2 years, using a simplified protocol focusing on the key traits potentially associated with chronic lead exposure, also including early markers of tubular dysfunction. Additionally, to enlarge the sample size, 200 new participants are being recruited, of whom presumably 100 will have at least one follow-up examination. Third, the healthy worker effect [32] might partially account for the nonsignificant results in relation to lead exposure in this occupational cohort with mean age of 29.7 years. The current observations should not be unthoughtfully generalised and are therefore not applicable to older individuals or patients with comorbidities, such as diabetes [33] which increases the vulnerability of renal function. Finally, co-exposure to cadmium is common in lead recycling plants. This metal accumulates in the kidneys, where its half-life exceeds 30 years [34]. Cadmium adversely affects renal tubular and glomerular function [35]. In keeping with the OSHA standard, blood cadmium levels were monitored in workers operating the blast furnaces, but not in other study participants, so that adjustment for co-exposure to cadmium was not feasible. Although residual confounding by unmeasured risk factors can never be excluded in observational studies, SPHERL did address a wide array of potential confounders.

In conclusion, in a real-world experiment, conducted in an occupational setting, there was no association between the 2-year changes in renal function [16] the co-principal SPHERL endpoint, and the increment of blood lead. However, given the confidence intervals around the point estimates of the changes in eGFRcc and eGFRcys (**Table 4**), a larger study with longer follow-up, as planned, should consolidate the current findings.

However, the current findings cast doubt on the premise that at the current environmental exposure levels, which in developed countries are much lower than in an occupational setting [15], lead exposure is still a major contributor to CKD [15]. This might not apply to patients with comorbidities, such as hypertension or diabetes, which predispose to CKD. An important concept highlighted by the SPHERL experience is that in longitudinal studies in an occupational setting not accounting for confounders, such as ageing, the diurnal variation in body functions, shift work, or the physiological changes normally associated with physically strenuous work, might lead to falsely attributing noxious effects to the pollutant under study.

Funding

The International Lead Association (www.ila-lead.org) provided an unrestricted grant to the Research Unit Hypertension and Cardiovascular Epidemiology partially supporting the data management and statistical analysis. The Non-Profit Research Association Alliance for the Promotion of Preventive Medicine, Mechelen, Belgium (URL, http://www.appremed.org) received a nonbinding grant from OMRON Healthcare Co. Ltd., Kyoto, Japan.

Conflict of interest

None of the authors declares a conflict of interest related to the contents of this article.

References

1. GBD 2017 Risk Factor Collaborators. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risk factors or clusters of risks for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* 2018; 392: 1923-1994.
2. Pirkle JL, Brody DJ, Gunter EW, et al. The decline in blood lead levels in the United States. The National Health and Nutrition Examination Surveys (NHANES). *J Am Med Assoc.* 1994; 272: 284-291.
3. Muntner P, Menke A, DeSalvo KB, et al. Continued decline in blood lead levels among adults in the United States: the National Health and Nutrition Examination Surveys. *Arch Intern Med.* 2005; 165: 2155-2161.
4. Hara A, Thijs L, Asayama K, et al. Blood pressure in relation to environmental lead exposure in the National Health and Nutrition Examination Survey 2003 to 2010. *Hypertension.* 2015; 65: 62-69.
5. Staessen JA, Lauwerys RR, Buchet JP, et al. Impairment of renal function with increasing blood lead concentrations in the general population. *N Engl J Med.* 1992; 327: 151-156.
6. Kim R, Rotnitzky A, Sparrow D, et al. A longitudinal study of low-level lead exposure and impairment of renal function. The Normative Aging Study. *J Am Med Assoc.* 1996; 275: 1177-1181.
7. Muntner P, He J, Vupputuri S, et al. Blood lead and chronic kidney disease in the general United States population: results from NHANES III. *Kidney Int.* 2003; 63: 1044-1050.
8. Åkesson A, Lundh T, Vahter M, et al. Tubular and glomerular kidney effects in Swedish women with low environmental cadmium exposure. *Environ Health Perspect.* 2005; 113: 1627-1631.
9. Lai LH, Chou SY, Wu FY, et al. Renal dysfunction and hyperuricemia with low blood lead levels and ethnicity in community-based study. *Sci Tot Environ.* 2008; 401: 39-43.
10. Harari F, Sallsten G, Christensson A, et al. Blood lead levels and decreased kidney function in a population-based cohort. *Am J Kidney Dis.* 2018; 72: 381-389.

11. Chowdhury R, Darrow L, McClellan W, et al. Incident ESRD among participants in a lead surveillance program. *Am J Kidney Dis.* 2014; 64: 25-31.

12. O'Flaherty EJ. Physiologically based methods for bone-seeking elements. IV. Kinetics of lead disposition in humans. *Toxicol Appl Pharmacol.* 1993; 118: 16-29.

13. Leggett RW. An age-specific kinetic model of lead metabolism in humans. *Environ Health Perspect.* 1993; 101: 598-616.

14. Ekong EB, Jaar BG, Weaver VM. Lead-related nephrotoxicity : a review of the epidemiologic evidence. *Kidney Int.* 2006; 70: 2074-2084.

15. Evans M, Elinder CG. Chronic renal failure from lead: myth or evidence-based fact? *Kidney Int.* 2011; 79: 272-279.

16. Hara A, Gu YM, Petit T, et al. Study for promotion of health in recycling lead - rationale and design. *Blood Press.* 2015; 24: 147-157.

17. Mujaj B, Yang WY, Zhang ZY, et al. Renal function in relation to low-level environmental lead exposure. *Nephrol Dial Transplant.* 2018; 34: 941-946.

18. Inker LA, Schmid CH, Tighiouart H, et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med.* 2012; 367: 20-29.

19. Rasouli M. Basic concepts and practical equations on osmolality: biochemical approach. *Clin Biochem.* 2016; 49: 936-941.

20. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009; 150: 604-612.

21. Roels H, Konings J, Green S, et al. Time-integrated blood lead concentration is a valid surrugate for estimating the cumulative lead dose assessed by tibial lead measurement. *Environ Res.* 1995; 69: 75-82.

22. Fadrowski JJ, Navas-Acien A, Tellez-Plaza M, et al. Blood lead level and kidney function in US adolescents. The Third National Health ans Nutrition Examination Survey. *Arch Intern Med.* 2010; 170: 75-82.

23. Hilderink JM, van der Linden N, Kimenai DM, et al. Biological variation of creatinine, cystatin C,, and eGFR over 24 hours. *Clin Chem.* 2018; 64: 851-860.

24. Sirota JH, Baldwin DS, Villareal H. Diurnal variation of renal function in man. *J Clin Invest.* 1950; 29: 187-192.

25. Dyer AR, Martin GJ, Burton WN, et al. Blood pressure and diurnal variation in sodium, potassium, and water excretion. *J Hum Hypertens.* 1998; 12: 363-371.

26. Ainsworth BE, Haskel WL, Hermann SD, et al. 2011 Compendium of Physical Activities: a second update of codes and MET values. *Med Sci Sports Exerc.* 2011; 43: 1575-1581.

27. Poortmans JR, Ouchinsky M. Glomerular filtration rate and albumin excretion after maximal exercise in aging sendentary and active men. *J Gerontol A Biol Sci Med Sci.* 2006; 61: 1181-1185.

28. Mingels A, Jacobs L, Kleijnen V, et al. Cystatin C a marker for renal function after exercise. *Int J Sports Med.* 2009; 30: 668-671.

29. Rabinowitz MB. Toxicokinetics of bone lead. *Environ Health Perspect.* 1991;91:33-37.

30. Oliveira S, Aro A, Sparrow D, et al. Season modifies the relationship between bone and blood lead levels : the Normative Aging Study. *Arch Environ Health.* 2002; 57: 466-472.

31. Korrick SA, Schwartz J, Tsaih SW, et al. Correlates of bone and blood lead levels among middle-aged and elderly women. *Am J Epidemiol.* 2002; 156: 335-343.

32. Nuyts GD, Elseviers MM, De Broe ME. Healthy worker effect in a cross-sectional study of lead workers. *J Occup Med.* 1993; 35: 387-391.

33. Tsaih SW, Korrick S, Schwartz J, et al. Lead, diabetes, hypertension, and renal function: the Normative aging study. *Environ Health Perspect.* 2004; 112: 1178-1182.

34. Nordberg GF, Kjellström T, Nordberg M. Kinetics and metabolism. In Friberg L, Elinder CG, Kjellström T, Nordberg GF (eds): *Cadmium and Health : A Toxicological and Epidemiological Appraisal. Volume I. Exposure, Dose, and Metabolism*. Boca Raton, Florida, USA, CRC Press Inc., 1985, pp 103-178.

35. Staessen JA, Lauwerys RR, Ide G, et al. Renal function and historical environmental cadmium pollution from zinc smelters. *Lancet.* 1994; 343: 1523-1527.

36. Whelton PK, Carey RM, Aronow WS, et al. 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/ APhA/ASH/ASPC/NMA/PCNA guideline for prevention, detection, evaluation, and management of high blood pressure in adults: A report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol.* 2018; 71: e127-e248.

Data supplement

Expanded methods

Clinical measurements

At the study sites, trained nurses measured the workers' anthropometric characteristics and applied current guidelines to measure office blood pressure at the brachial artery. After the workers had rested for 5 minutes in the sitting position, the nurses obtained five consecutive blood pressure readings to the nearest 2 mm Hg by auscultation of the Korotkoff sounds, using standard mercury sphygmomanometers. For analysis, the five readings were averaged. Mean arterial pressure was diastolic blood pressure plus one third of pulse pressure (the difference between systolic and diastolic blood pressure). Blood pressure was categorised according to the 2017 ACC/AHA guideline. If systolic and diastolic blood pressure were in different categories, the highest value was used to classify participants. Body mass index was body weight in kilograms divided by height in meters squared. The study nurses administered the validated [1] questionnaire at baseline and follow-up, to collect information about each worker's medical history, exposure to heavy metals, smoking and drinking habits, intake of medications and life style. Diabetes mellitus was a self-reported diagnosis, a fasting blood glucose of 126 mg/dl (7.0 mmol/l) or higher, or use of antidiabetic drugs.

Biochemical measurements

Venous blood samples were obtained after 8 to 12 hours of fasting. BL was determined on whole blood by inductively coupled plasma mass spectrometry at an analytical laboratory certified for BL analysis in compliance with the provisions of the OSHA Lead Standard, 29CFR 1910.1025 (Occupational Safety and Health Administration [www.osha.gov]). Prior to analysis, the specimens were digested with nitric acid and spiked with an iridium internal standard. The BL detection limit was 0.5 µg/dl. The accuracy of the BL tests was verified by use of proficiency samples purchased from the College of American Pathologists (CAP) and the Pennsylvania Department of Blood Lead Programs [2]. Proficiency testing was performed in six separate trial runs, including in total 30 test samples annually. All survey materials were handled in the same manner as the study samples and processed with the normal workflow utilising the same repeat/dilution protocols and calibration and quality control frequency [2]. Compliance with Clinical Laboratory Improvement Amendments (CLIA), CAP and New York State accreditation and regulatory requirements was verified routinely with test level review of the laboratory services by external auditors. Calibrators with certified accuracy (National

Institute of Standards and Technology [htpps://www.nist.gov]) were included in each batch of study samples and spanned the range of the analytical measurement range. Accuracy was evaluated on Westgard Rules [3] and defined within the total allowable error established with review of the CAP, Centers for Disease Control and Prevention, CLIA 88 [4], and OSHA guidelines. Accuracy, defined as the deviation from known BL standards ran along with the study samples, was within 10%. The bias determined according to the Bland and Altman approach [5] in 30 spilt blood samples with BL concentrations (average in duplicate samples) ranging from 0.70 to 27.9 µg/dl, was 0.08 µg/dl (95% confidence interval [CI], -0.01 to 0.18, *P*=0.07) [6]. The repeatability coefficient, defined as twice the standard deviation (SD) of the signed differences between duplicate measurements [5], was 0.52. Expressed as a percentage of mean BL or as a percentage of near maximal BL variation (four times the SD of the logarithmically transformed distribution), the repeatability coefficient was 6.7% and 1.9%, respectively. Lower values indicate better repeatability.

Total and high-density (HDL) lipoprotein serum cholesterol, serum creatinine, serum cystatin C, γ-glutamyltransferase (an index of alcohol intake), blood urea nitrogen, and blood glucose were measured by automated enzymatic methods, serum insulin by ELISA, and serum sodium by flame photometry. Serum creatinine was measured, using Jaffe's method with modifications in a single certified laboratory that applied isotope-dilution mass spectrometry for calibration [7]. eGFR was derived from serum creatinine, serum cystatin C and both, using the Chronic Kidney Disease Epidemiology Collaboration equations (**Table S1**) [8]. CKD stages were categorised according to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative guideline [9]. Serum osmolality (mOsm/kg) was computed as 2 × (serum Na^+ [mmol/L]) + (blood glucose [mg/dl] / 18) + (blood urea nitrogen [mg/dl] / 2.8) [10]. Fresh urine samples were analysed for specific gravity and the albumin-to-creatinine ratio (ACR) defined as the urinary albumin in milligrams divided by urine creatinine in grams. Over three evaluations, the laboratory obtained a proficiency score of 100% for the BL measurements and 100% for routine biochemistry.

Haematological measurements

Haemoglobin and the red blood cell (RBC) count were measured, using the fully automated UniCel DxH800 haematology analyser (Beckman Coulter, Brea, CA, USA). In the RBC chamber of the instrument, RBCs are counted and discriminated by electrical impedance as the cells are pulled through each of three sensing apertures (50 µm in diameter and 60 µm in length). Electrical pulses generated in the counting cycles are

sent to the analyser for editing, coincidence correction, and digital conversion. Two of the three RBC counts obtained must match within specified limits for the counts to be accepted by the instrument. This multiple counting procedure prevents data errors resulting from aperture obstructions or statistical outliers and allows for excellent reproducibility. The haematocrit, mean corpuscular volume (MCV), mean cell haemoglobin (MCH), and mean cell haemoglobin concentration (MCHC) are calculated from measured and derived values. MCV is obtained by multiplying a volume of blood by the proportion of blood that is cellular (the haematocrit), and dividing that product by the number of RBCs. MCH is average quantity of haemoglobin in a single RBC. MCHC is the average amount of haemoglobin per RBC, relative to the size of the cell. The RBC distribution width is derived directly from the histogram as the coefficient of variation of the RBC volume distribution.

References

1. Staessen JA, Lauwerys RR, Buchet JP *et al*. Impairment of renal function with increasing blood lead concentrations in the general population. *N Engl J Med.* 1992; 327: 151-156.
2. Centers for Disease Control and Prevention. Screening young children for lead poisoning: Guidance for state and local public health officials; Appendix C1: The Lead Laboratory (accessed 18 December 2018). 2018.
3. Westgard JO, Barry PL, Hunt MR, Groth T. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem.* 1981; 27: 493-501.
4. Centers for Disease Control and Prevention. CLIA '88 Focus on Clinic and Office Laboratories (accessed 18 December 2018). 1988.
5. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet.* 1986; 1: 307-310.
6. Yu CG, Wei FF, Yang WY *et al*. Heart rate variability and peripheral nerve conduction velocity in relation to blood lead in newly hired lead workers. *Occup Environ Med.* 2019; 76: 382-388.
7. Myers GL, Miller WG, Coresh J *et al*. Recommendations for improving serum creatinine measurement: a report from the laboratory working group of the National Kidney Disease Education Program. *Clin Chem.* 2006; 52: 5-18.
8. Inker LA, Schmid CH, Tighiouart H *et al*. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med.* 2012; 367: 20-29.

9. Levey AS, Eckardt KU, Dorman NM *et al*. Nomenclature for kidney function and disease — executive summary and glossary from a Kidney Dusease: Improving Global Outcomes (KDIGO) consensus conference. *Eur Heart J.* 2020; 41: 4592-4598.
10. Rasouli M. Basic concepts and practical equations on osmolality: biochemical approach. *Clin Biochem.* 2016; 49: 936-941.

Table S1. Chronic Kidney Disease Epidemiology Collaboration equations for estimating glomerular filtration rates derived from serum creatinine, serum cystatin C and both

Variable	Sex	Serum creatinine (mg/dl)	Serum cystatin C (mg/l)	Equation for estimating GFR
eGFRcrt	Female	≤ 0.7		$144 \times (\text{Scr} / 0.7)^{-0.329} \times 0.993^{\text{Age}}$ [× 1.159 if black]
		> 0.7		$144 \times (\text{Scr} / 0.7)^{-1.209} \times 0.993^{\text{Age}}$ [× 1.159 if black]
	Male	≤ 0.9		$144 \times (\text{Scr} / 0.7)^{-0.411} \times 0.993^{\text{Age}}$ [× 1.159 if black]
		> 0.9		$144 \times (\text{Scr} / 0.7)^{-1.209} \times 0.993^{\text{Age}}$ [× 1.159 if black]
eGFRcys	Female or male		≤ 0.8	$133 \times (\text{Scys} / 0.8)^{-0.499} \times 0.996^{\text{Age}}$ [× 0.932 if female]
			> 0.8	$133 \times (\text{Scys} / 0.8)^{-1.328} \times 0.996^{\text{Age}}$ [× 0.932 if female]
eGFRcc	Female	≤ 0.7	≤ 0.8	$130 \times (\text{Scr} / 0.7)^{-0.248} \times (\text{Scys} / 0.8)^{-0.375} \times 0.995^{\text{Age}}$ [× 1.08 if black]
			> 0.8	$130 \times (\text{Scr} / 0.7)^{-0.248} \times (\text{Scys} / 0.8)^{-0.711} \times 0.995^{\text{Age}}$ [× 1.08 if black]
		> 0.7	≤ 0.8	$130 \times (\text{Scr} / 0.7)^{-0.601} \times (\text{Scys} / 0.8)^{-0.375} \times 0.995^{\text{Age}}$ [× 1.08 if black]
			> 0.8	$130 \times (\text{Scr} / 0.7)^{-0.601} \times (\text{Scys} / 0.8)^{-0.711} \times 0.995^{\text{Age}}$ [× 1.08 if black]
	Male	≤ 0.9	≤ 0.8	$135 \times (\text{Scr} / 0.7)^{-0.207} \times (\text{Scys} / 0.8)^{-0.375} \times 0.995^{\text{Age}}$ [× 1.08 if black]
			> 0.8	$135 \times (\text{Scr} / 0.7)^{-0.207} \times (\text{Scys} / 0.8)^{-0.711} \times 0.995^{\text{Age}}$ [× 1.08 if black]
		> 0.9	≤ 0.8	$135 \times (\text{Scr} / 0.7)^{-0.601} \times (\text{Scys} / 0.8)^{-0.375} \times 0.995^{\text{Age}}$ [× 1.08 if black]
			> 0.8	$135 \times (\text{Scr} / 0.7)^{-0.601} \times (\text{Scys} / 0.8)^{-0.711} \times 0.995^{\text{Age}}$ [× 1.08 if black]

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both. To convert serum creatinine to µmol/l, multiply by 88.42; to convert cystatin C from mg/l to nmol/l, multiply by 74.9. This table is reproduced from *N Engl J Med.* 2012;367:20-29 (reference [8]).

Table S2. Baseline characteristics of workers followed up and not followed up

Characteristic	N (%)		Characteristic	Mean **(SD/IQR)**	
	Analysed	**Not analysed**		**Analysed**	**Not analysed**
Male	230 (91.6)	218 (85.5)	Age, years	29.7 (9.8)	27.8 (10.0)
White ethnicity	122 (48.6)	110 (43.1)	Body mass index, kg/m^2	28.9 (6.1)	29.0 (6.8)
Hispanic ethnicity	107 (42.6)	122 (47.8)	Systolic blood pressure, mm Hg	120.0 (10.2)	120.3 (10.5)
Black ethnicity	12 (4.8)	10 (4.0)	Diastolic blood pressure, mm Hg	79.7 (8.8)	81.1 (8.4)
Other ethnicity	10 (4.0)	13 (5.1)	Mean arterial pressure, mm Hg	93.1 (8.7)	94.2 (8.3)
Current smokers	67 (27.0)	75 (29.4)	Total cholesterol, mg/dl	171.8 (37.8)	170.6 (37.9)
Alcohol intake	110 (44.4)	100 (39.2)	HDL cholesterol, mg/dl	46.3 (12.0)	47.7 (12.3)
Hypertension stage ≥1	125 (49.8)	161 (63.1)*	Total-to-HDL cholesterol ratio	3.91 (1.3)	3.82 (1.3)
Hypertension stage ≥2	46 (18.3)	35 (13.7)	Blood glucose, mg/dl	94.3 (15.8)	92.7 (11.5)
Treated hypertension	17 (6.8)	8 (3.1)	γ-glutamyltransferase, U/l	22.6 (16.0, 33.0)	21.8 (15.0, 30.0)
Diabetes mellitus	12 (4.8)	2 (0.78)*	Blood lead, µg/dL	4.13 (2.40, 7.80)	4.04 (2.20, 8.40)

Values are number of participants (%), arithmetic mean (SD) or geometric mean (IQR). Blood pressure was the average of five readings. Hypertension was categorised according to the 2017 ACC/AHA guideline, irrespective of treatment status. Mean arterial pressure was diastolic blood pressure plus one third of pulse pressure. Diabetes mellitus was a self-reported diagnosis, a fasting blood glucose of ≥7 mmol/l, or use of antidiabetic drugs. To convert total or high-density lipoprotein (HDL) serum cholesterol to mmol/l, multiply by 0.0259; to convert blood glucose to mmol/l, multiply by 0.0559. An asterisk indicates a significant between-group difference.

Table S3. Renal function at baseline by quartiles of the distribution of the follow-up-to-baseline blood lead ratio

Characteristic	<1.91	1.91-3.45	3.45-5.66	≥5.66	*P* value
Number in group	62	63	63	63	…
Serum creatinine, mg/dl	0.932 (0.173)	0.955 (0.157)	0.988 (0.186)	0.955 (0.152)	0.28
Serum cystatin C, mg/l	0.671 (0.114)	0.680 (0.103)	0.678 (0.104)	0.675 (0.107)	0.86
eGFRcrt, ml/min/1.73 m^2	106.2 (18.1)	106.4 (17.5)	102.9 (16.2)	106.1 (16.2)	0.69
eGFRcys, ml/min/1.73 m^2	124.6 (15.1)	125.1 (12.5)	125.5 (12.3)	125.2 (13.4)	0.77
eGFRcc, ml/min/1.73 m^2	115.5 (16.7)	115.0 (13.9)	113.2 (12.6)	115.2 (14.2)	0.75
Serum osmolality, mOsm/kg	287.1 (3.4)	286.3 (4.0)	287.0 (4.1)	286.7 (3.6)	0.77
Serum sodium, mmol/l	138.4 (1.7)	138.1 (1.8)	138.4 (1.9)	138.2 (1.7)	0.83
Blood glucose, mg/dl	95.1 (14.4)	92.3 (10.5)	94.9 (22.5)	94.7 (13.5)	0.85
Serum insulin, U/l	6.94 (4.00, 12.9)	7.87 (3.60, 14.6)	6.84 (3.60, 11.0)	7.25 (3.50, 13.2)	0.99
Blood urea nitrogen, mg/dl	14.1 (3.9)	14.0 (3.8)	14.0 (3.3)	13.9 (3.1)	0.70
BUN-to-SCRT ratio	15.5 (4.6)	14.8 (3.9)	14.3 (3.5)	15.0 (4.5)	0.38
Urine specific gravity,	1.017 (0.0080)	1.020 (0.0075)	1.021 (0.0079)	1.019 (0.0073)	0.15
ACR, mg/g	4.54 (2.92, 6.70)	4.42 (3.12, 6.18)	4.37 (2.65, 6.55)	4.63 (2.93, 5.93)	0.90

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both. BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). Values are arithmetic mean (SD) or geometric mean (IQR). *P* values are for trend across increasing categories of the follow-up-to-baseline blood lead concentration ratio. To convert serum creatinine to µmol/l, multiply by 88.42; to convert cystatin C from mg/l to nmol/l, multiply by 74.9; to convert blood glucose to mmol/l, multiply by 0.0559; to convert blood urea nitrogen to mmol/l, multiply by 0.3571.

Table S4. Renal function at last follow-up by quartiles of the distribution of the follow-up-to-baseline blood lead ratio

Characteristic	<1.91	1.91-3.45	3.45-5.66	≥5.66	*P* value
Number in group	62	63	63	63	…
Serum creatinine, mg/dl	0.994 (0.210)	1.038 (0.157)	1.022 (0.178)	1.019 (0.156)	0.56
Serum cystatin C, mg/l	0.693 (0.164)	0.704 (0.148)	0.722 (0.165)	0.720 (0.143)	0.26
eGFRcrt, ml/min/1.73 m^2	98.9 (19.1)	97.0 (15.9)	98.0 (16.3)	98.4 (16.0)	0.97
eGFRcys, ml/min/1.73 m^2	121.5 (20.2)	121.4 (18.1)	120.3 (20.2)	119.2 (18.8)	0.46
eGFRcc, ml/min/1.73 m^2	109.6 (17.9)	108.1 (14.9)	107.7 (14.8)	107.7 (16.1)	0.49
Serum osmolality, mOsm/kg	289.5 (5.9)	288.8 (4.5)	289.0 (3.9)	289.1 (3.4)	0.76
Serum sodium, mmol/l	139.5 (2.8)	139.0 (2.0)	139.3 (1.9)	139.4 (1.7)	0.98
Blood glucose, mg/dl	90.3 (19.4)	87.3 (16.3)	86.5 (16.9)	89.3 (21.2)	0.71
Insulin U/l	9.50 (5.20, 20.7)	8.57 (3.90, 19.2)	8.16 (4.30, 14.7)	9.28 (4.00, 17.2)	0.84
Blood urea nitrogen, mg/dl	15.2 (3.3)	16.4 (4.7)	15.4 (3.0)	15.1 (3.7)	0.52
BUN-to-SCRT ratio	15.8 (4.0)	15.7 (3.8)	15.3 (3.0)	15.1 (3.8)	0.21
Urinary specific gravity,	1.022 (0.0068)	1.023 (0.0077)	1.021 (0.0070)	1.022 (0.0067)	0.89
ACR, mg/g	4.45 (2.82, 7.77)	4.51 (2.63, 6.25)	4.83 (2.89, 7.19)	4.65 (2.86, 6.17)	0.68

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both. BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). Values are arithmetic mean (SD) or geometric mean (IQR). *P* values are for trend across increasing categories of the follow-up-to-baseline blood lead concentration ratio. To convert serum creatinine to µmol/l, multiply by 88.42; to convert cystatin C from mg/l to nmol/l, multiply by 74.9; to convert blood glucose to mmol/l, multiply by 0.0559; to convert blood urea nitrogen to mmol/l, multiply by 0.3571.

Table S5. Association between changes in renal function and in blood lead in 223 workers not on antihypertensive drug treatment

Variable	Unadjusted		Adjusted		Fully adjusted	
	β (95% CI)	*P* value	β (95% CI)	*P* value	β (95% CI)	*P* value
Serum creatinine, $\times 10^{-2}$ mg/dl	-0.07 (-1.82, 1.69)	0.94	0.44 (-1.12, 2.00)	0.58	0.55 (-1.05, 2.16)	0.50
Serum cystatin C, $\times 10^{-2}$ mg/l	0.63 (-0.97, 2.22)	0.44	1.11 (-0.41, 2.64)	0.15	1.17 (-0.41, 2.76)	0.15
eGFRcrt, ml/min/1.73 m^2	-0.11 (-1.88, 1.66)	0.90	-0.64 (-2.20, 0.92)	0.42	-0.82 (-2.43, 0.80)	0.32
eGFRcys, ml/min/1.73 m^2	-0.82 (-2.71, 1.06)	0.39	-1.43 (-3.24, 0.38)	0.12	-1.51 (-3.39, 0.36)	0.11
eGFRcc, ml/min/1.73 m^2	-0.50 (-2.06, 1.06)	0.53	-1.14 (-2.53, 0.25)	0.11	-1.31 (-2.75, 0.13)	0.074
Serum osmolality, mOsm/kg	-0.10 (-0.79, 0.58)	0.77	-0.21 (-0.71, 0.30)	0.42	-0.21 (-0.73, 0.32)	0.43
Serum sodium, mmol/l	0.06 (-0.28, 0.40)	0.73	0.02 (-0.24, 0.29)	0.86	0.03 (-0.24, 0.29)	0.85
Blood glucose, mg/dl	-1.87 (-4.36, 0.63)	0.14	-1.18 (-3.11, 0.75)	0.23	-1.64 (-3.61, 0.32)	0.10
Insulin, %	-4.05 (-15.7, 9.27)	0.53	-5.72 (-16.3, 6.17)	0.33	-8.18 (-17.7, 2.49)	0.13
Blood urea nitrogen, mg/dl	-0.30 (-0.84, 0.24)	0.28	-0.32 (-0.78, 0.14)	0.17	-0.24 (-0.70, 0.22)	0.30
BUN-to-SCRT ratio	-0.20 (-0.73, 0.32)	0.45	-0.44 (-0.90, 0.02)	0.063	-0.39 (-0.86, 0.08)	0.10
Urinary specific gravity, $\times 10^{-2}$	-0.03 (-0.15, 0.09)	0.62	-0.05 (-0.14, 0.04)	0.25	-0.04 (-0.13, 0.05)	0.36
ACR, %	1.14 (-8.87, 12.3)	0.83	-2.06 (-9.88, 6.43)	0.62	-2.32 (-10.2, 6.27)	0.58

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both (reference [8]). BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). Changes in serum insulin and the urinary albumin-to-creatinine ratio are expressed as percentage differences from baseline to follow-up. Association sizes (β), given with 95% confidence interval, express the change in the dependent variable for a 3-fold increase in the blood lead concentration. Adjusted models accounted for sex, age, the change of age from baseline to follow-up (equivalent to between-visit interval), the time of day of blood sampling (nighttime *vs* daytime), and the baseline renal function measure being analysed. Fully adjusted models additionally accounted for baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), the total-to-HDL cholesterol ratio and γ-glutamyltransferase.

Table S6. Associations between renal function and blood lead at baseline and at last follow-up

Variable	Baseline		Last follow-up		*Pslope*
	β (95% CI)	*P* value	β (95% CI)	*P* value	
Serum creatinine, $\times 10^{-2}$ mg/dl	-1.15 (-3.80, 1.49)	0.39	0.38 (-2.81, 3.58)	0.81	0.47
Serum cystatin C, $\times 10^{-2}$ mg/l	0.26 (-1.43, 1.95)	0.76	2.96 (-0.06, 5.97)	0.056	0.13
eGFRcrt, ml/min/1.73 m^2	1.31 (-1.23, 3.85)	0.31	-0.80 (-3.78, 2.18)	0.60	0.29
eGFRcys, ml/min/1.73 m^2	-0.19 (-2.03, 1.65)	0.84	-3.36 (-6.65, -0.07)	0.047	0.099
eGFRcc, ml/min/1.73 m^2	0.63 (-1.43, 2.69)	0.55	-2.33 (-4.94, 0.29)	0.083	0.082
Serum osmolality, mOsm/kg	-0.42 (-1.06, 0.23)	0.21	0.23 (-0.69, 1.14)	0.62	0.26
Serum sodium, mmol/l	-0.23 (-0.53, 0.07)	0.14	0.21 (-0.22, 0.64)	0.35	0.10
Blood glucose, mg/dl	-0.44 (-3.10, 2.21)	0.74	-2.78 (-6.36, 0.79)	0.13	0.30
Insulin, %	3.25 (-2.64, 9.51)	0.29	-5.62 (-12.8, 2.17)	0.15	0.074
Blood urea nitrogen, mg/dl	0.19 (-0.40, 0.78)	0.53	-0.09 (-0.82, 0.64)	0.80	0.56
BUN-to-SCRT ratio	0.00 (-0.00, 0.01)	0.21	-0.00 (-0.01, 0.00)	0.50	0.18
Urine specific gravity$\times 10^{-2}$	-0.04 (-0.17, 0.09)	0.57	-0.01 (-0.14, 0.13)	0.89	0.77
ACR, %	-2.49 (-7.67, 2.97)	0.37	0.08 (-6.86, 7.55)	0.98	0.57

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both (reference [8]). BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). Changes in serum insulin and the urinary albumin-to-creatinine ratio are expressed as percentage differences from baseline to follow-up. Association sizes (β), given with 95% confidence interval, express the change in the dependent variable for a 3-fold increase in the blood lead concentration. All models were adjusted for sex, age, the time of day of blood sampling (nighttime *vs* daytime), body mass index, smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), the total-to-HDL cholesterol ratio and γ-glutamyltransferase. *Pslope* derived by a z-statistic expresses the significance of the difference in the βs between baseline and last follow-up.

Table S7. Association between changes in renal function and in blood lead stratified by the median baseline age

Variable	Baseline age <26.6 years (N = 127)		Baseline age ≥26.6 years (N = 124)		*Pint*
	β (95% CI)	*P* value	β (95% CI)	*P* value	
Serum creatinine, $\times 10^{-2}$ mg/dL	0.22 (-2.21, 2.66)	0.86	1.26 (-0.82, 3.34)	0.23	0.45
Serum cystatin C, $\times 10^{-2}$ mg/L	1.20 (-0.90, 3.29)	0.26	1.41 (-0.98, 3.79)	0.24	0.80
eGFRcrt, ml/min/1.73 m^2	-0.32 (-2.75, 2.10)	0.79	-1.15 (-3.14, 0.84)	0.25	0.49
eGFRcys, ml/min/1.73 m^2	-1.51 (-3.97, 0.95)	0.23	-1.90 (-4.66, 0.85)	0.17	0.76
eGFRcc, ml/min/1.73 m^2	-1.24 (-3.33, 0.84)	0.24	-1.43 (-3.33, 0.46)	0.14	0.58
Serum osmolality, mOsm/kg	0.36 (-0.35, 1.06)	0.32	-0.49 (-1.40, 0.42)	0.29	0.057
Serum sodium, mmol/l	0.39 (0.05 , 0.73)	0.026	-0.32 (-0.77, 0.14)	0.17	0.002
Blood glucose, mg/dl	-1.91 (-4.56, 0.74)	0.16	1.75 (-1.31, 4.80)	0.26	0.043
Serum insulin, %	-5.37 (-19.1, 10.6)	0.49	-6.30 (-20.8, 10.9)	0.45	0.66
Blood urea nitrogen, mg/dl	-0.45 (-1.03, 0.13)	0.12	0.04 (-0.64, 0.72)	0.90	0.36
BUN-to-SCRT ratio	-0.44 (-1.05, 0.16)	0.15	-0.34 (-1.03, 0.35)	0.33	0.85
Urine specific gravity, $\times 10^{-2}$	-0.11 (-0.23, -0.00)	0.045	0.11 (-0.03, 0.25)	0.12	0.019
ACR, %	1.33 (-8.88, 12.7)	0.81	-0.34 (-14.2, 15.7)	0.96	0.64

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both (reference [8]). BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). Changes in serum insulin and the urinary albumin-to-creatinine ratio are expressed as percentage differences from baseline to follow-up. Association sizes (β), given with 95% confidence interval, express the change in the dependent variable for a 3-fold increase in the blood lead concentration. All models were adjusted for sex, age, follow-up duration, the time of day of blood sampling (nighttime *vs* daytime), the baseline renal function measure being analysed, and baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), total-to-HDL cholesterol ratio and γ-glutamyltransferase. *Pint* expresses the significance of the between-group interaction.

Table S8. Association between changes in renal function and in blood lead stratified by the median baseline blood lead

Variable	Baseline blood lead <4.32 µg/dl (N = 126)		Baseline blood lead ≥4.32 µg/dl (N = 125)		*Pint*
	β (95% CI)	*P* value	β (95% CI)	*P* value	
Serum creatinine, $\times 10^{-2}$ mg/dl	2.70 (0.38 , 5.01)	0.023	1.47 (-1.79, 4.73)	0.37	0.64
Serum cystatin C, $\times 10^{-2}$ mg/l	1.25 (-1.49, 3.98)	0.37	1.20 (-1.74, 4.14)	0.42	0.79
eGFRcrt, ml/min/1.73 m^2	-2.78 (-5.15, -0.41)	0.022	-1.49 (-4.51, 1.53)	0.33	0.75
eGFRcys, ml/min/1.73 m^2	-1.52 (-4.69, 1.65)	0.34	-1.24 (-4.60, 2.12)	0.47	0.73
eGFRcc, ml/min/1.73 m^2	-2.33 (-4.33, -0.32)	0.024	-2.05 (-4.95, 0.85)	0.16	0.93
Serum osmolality, mOsm/kg	-0.00 (-0.73, 0.72)	>0.99	0.40 (-0.69, 1.49)	0.47	0.91
Serum sodium, mmol/l	-0.02 (-0.41, 0.37)	0.91	0.23 (-0.26, 0.71)	0.36	0.91
Blood glucose, mg/dl	0.56 (-2.60, 3.72)	0.72	-3.74 (-7.71, 0.23)	0.065	0.96
Insulin, %	-3.83 (-18.5, 13.5)	0.64	-7.36 (-25.5, 15.2)	0.49	0.47
Blood urea nitrogen, mg/dl	0.16 (-0.48, 0.81)	0.62	0.39 (-0.46, 1.24)	0.36	0.49
BUN-to-SCRT ratio	-0.30 (-0.94, 0.35)	0.36	0.01 (-0.90, 0.93)	0.98	0.46
Urine specific gravity, $\times 10^{-2}$	0.02 (-0.11, 0.15)	0.77	0.02 (-0.15, 0.19)	0.79	0.94
ACR, %	-3.85 (-15.5, 9.41)	0.55	9.49 (-8.20, 30.6)	0.31	0.45

eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both (reference [8]). BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). Changes in serum insulin and the urinary albumin-to-creatinine ratio are expressed as percentage differences from baseline to follow-up. Association sizes (β), given with 95% confidence interval, express the change in the dependent variable for a 3-fold increase in the blood lead concentration. All models were adjusted for sex, age, follow-up duration, the time of day of blood sampling (nighttime *vs* daytime), the baseline renal function measure being analysed, and baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), total-to-HDL cholesterol ratio and γ-glutamyltransferase. *Pint* expresses the significance of the between-group interaction.

Table S9. Association between changes in renal function and in blood lead stratified by the median cumulative blood lead index

Variable	CBLI < 34.6 µg/dl × year (N = 123)		CBLI < 34.6 µg/dl × year (N = 128)		*Pint*
	β (95% CI)	*P* value	β (95% CI)	*P* value	
Serum creatinine, $\times 10^{-2}$ mg/dl	0.83 (-1.59, 3.24)	0.50	1.89 (-0.42, 4.21)	0.11	0.24
Serum cystatin C, $\times 10^{-2}$ mg/l	0.59 (-1.61, 2.79)	0.59	2.83 (0.53, 5.12)	0.016	0.063
eGFRcrt, ml/min/1.73 m^2	-0.72 (-3.23, 1.79)	0.57	-1.62 (-3.85, 0.61)	0.15	0.39
eGFRcys, ml/min/1.73 m^2	-0.93 (-3.51, 1.65)	0.48	-3.46 (-6.10, -0.82)	0.011	0.054
eGFRcc, ml/min/1.73 m^2	-0.93 (-3.03, 1.17)	0.38	-2.66 (-4.62, -0.69)	0.008	0.068
Serum osmolality, mOsm/kg	0.10 (-0.57, 0.77)	0.77	-0.04 (-0.90, 0.83)	0.94	0.34
Serum sodium, mmol/l	0.21 (-0.16, 0.58)	0.27	-0.04 (-0.44, 0.37)	0.86	0.12
Blood glucose, mg/dl	-1.06 (-4.28, 2.16)	0.52	0.14 (-3.32, 3.59)	0.94	0.41
Insulin, %	-11.6 (-24.3, 3.34)	0.12	4.45 (-11.8, 23.7)	0.61	0.16
Blood urea nitrogen, mg/dl	-0.22 (-0.85, 0.40)	0.48	0.13 (-0.58, 0.83)	0.72	0.42
BUN-to-SCRT ratio	-0.26 (-0.88, 0.36)	0.41	-0.36 (-1.08, 0.35)	0.31	0.95
Urine specific gravity, $\times 10^{-2}$	-0.07 (-0.19, 0.04)	0.21	0.02 (-0.11, 0.15)	0.79	0.58
ACR, %	2.99 (-8.37, 15.7)	0.62	1.95 (-12.4, 18.7)	0.80	0.59

CBLI is the cumulative blood lead index. eGFRcrt, eGFRcys, and eGFRcc refer to the glomerular filtration rate estimated from serum creatinine, serum cystatin C or both (reference [8]). BUN-to-SCRT ratio is the ratio of blood urea nitrogen (mg/dl) to serum creatinine (mg/dl). Changes in serum insulin and the urinary albumin-to-creatinine ratio are expressed as percentage differences from baseline to follow-up. Association sizes (β), given with 95% confidence interval, express the change in the dependent variable for a 3-fold increase in the blood lead concentration. All models were adjusted for sex, age, follow-up duration, the time of day of blood sampling (nighttime *vs* daytime), the baseline renal function measure being analysed, and baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), the total-to-HDL cholesterol ratio and γ-glutamyltransferase. *Pint* expresses the significance of the between-group interaction.

Table S10. Red blood cell biomarkers changes from baseline to last follow-up by quartiles of the distribution of the follow-up-to-baseline blood lead concentration ratio

Characteristic	<1.91	1.91-3.45	3.45-5.66	≥5.66	*P* value
Number in group	62	63	63	63	…
Haemoglobin, g/dl	-0.19 (-0.39, 0.00)	-0.06 (-0.26, 0.14)	-0.07 (-0.26, 0.11)	-0.23 (-0.41, -0.05)	0.77
Haematocrit, %	-0.01 (-0.02, -0.00)	-0.01 (-0.01, -0.00)	-0.01 (-0.01, -0.00)	-0.01 (-0.02, -0.00)	0.88
RBC count, $\times 10^{12}$ /l	-0.14 (-0.21, -0.07)	-0.15 (-0.20, -0.09)	-0.10 (-0.17, -0.03)	-0.15 (-0.21, -0.09)	0.97
RDW, %	15.8 (15.1, 16.4)	15.5 (14.6, 16.4)	15.6 (14.9, 16.3)	16.2 (15.8, 16.7)	0.33
MCV, fl	0.47 (-0.19, 1.14)	0.90 (0.19, 1.61)	0.41 (0.00, 0.82)	0.64 (-0.03, 1.31)	>0.99
MCH, pg	0.66 (0.13, 1.19)	0.85 (0.34, 1.35)	0.69 (0.31, 1.07)	0.37 (0.05, 0.70)	0.31
MCHC, g/dl	0.33 (0.12, 0.54)	0.45 (0.29, 0.62)	0.37 (0.15, 0.58)	0.23 (-0.04, 0.50)	0.43

RBC, RDW, MCV, MCH, MCHC indicate red blood cell, red cell distribution width, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration, respectively. Within-group changes are arithmetic mean (SD) or geometric mean (IQR). P values denote the significance of the between-group differences.

Table S11. Association between changes in red blood cell biomarkers and in blood lead

Variable	Unadjusted		Adjusted		Fully adjusted	
	β (95% CI)	*P* value	β (95% CI)	*P* value	β (95% CI)	*P* value
Haemoglobin, g/dl	0.00 (-0.03, 0.03)	0.87	0.01 (-0.02, 0.04)	0.63	0.01 (-0.02, 0.04)	0.65
Haematocrit, %	0.01 (-0.08, 0.10)	0.90	0.03 (-0.06, 0.12)	0.56	0.03 (-0.07, 0.12)	0.57
RBC count, $\times 10^{12}$ /l	-0.00 (-0.01, 0.00)	0.70	-0.00 (-0.01, 0.01)	0.95	-0.00 (-0.01, 0.01)	0.84
RDW, %	-0.63 (-5.13, 3.86)	0.78	0.04 (-0.23, 0.31)	0.77	0.09 (-0.19, 0.37)	0.52
MCV, fl	0.00 (-0.13, 0.14)	0.97	0.03 (-0.10, 0.17)	0.64	0.02 (-0.12, 0.16)	0.78
MCH, pg	0.00 (-0.01, 0.02)	0.99	0.00 (-0.01, 0.02)	0.83	0.00 (-0.01, 0.02)	0.78
MCHC, g/dl	0.01 (-0.00, 0.02)	0.28	0.01 (-0.01, 0.02)	0.34	0.00 (-0.01, 0.02)	0.36

RBC, RDW, MCV, MCH, MCHC indicate red blood cell, red cell distribution width, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration, respectively. Association sizes (β), given with 95% confidence interval, express the change in the dependent variable for a 3-fold increase in the blood lead concentration. Adjusted models accounted for sex, age, follow-up duration, the time of day of blood sampling (nighttime *vs* daytime), and the baseline red blood cell biomarker being analysed. Fully adjusted models additionally accounted for baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), the total-to-HDL cholesterol ratio and γ-glutamyltransferase.

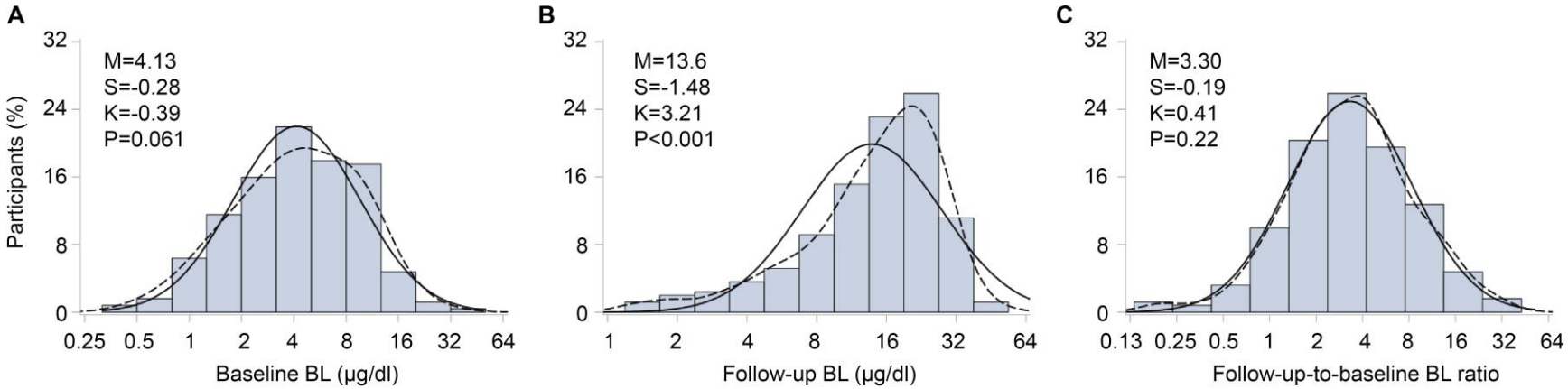


Figure S1. Distributions of blood lead at baseline (A) and last follow-up (B) and of the last-follow-up-to-baseline blood lead ratio (C). The solid and dotted lines represent the normal and kernel density distributions. *P* values are for departure of the actually observed distribution from normality according to the Shapiro-Wilk statistic. M indicates the geometric mean. Skewness (S) and kurtosis (K) were computed as the third and fourth moments about the mean divided by the cube of the standard deviation.

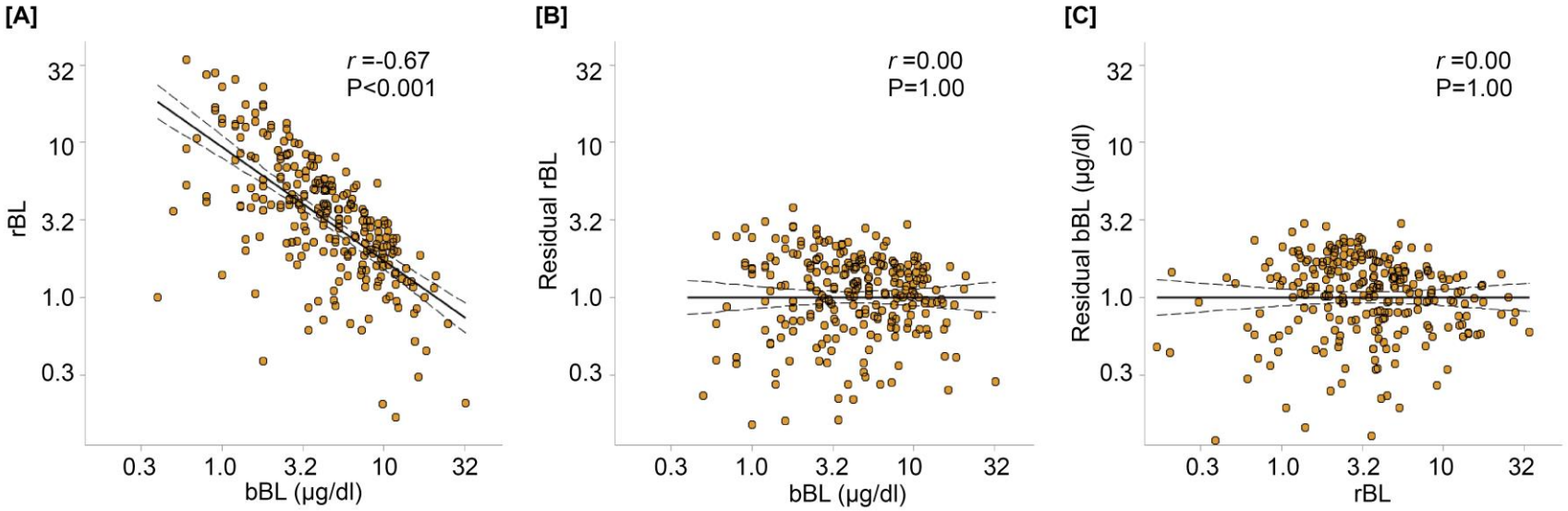


Figure S2. Linear associations between baseline blood lead (bBL) and the last-follow-up-to-baseline BL ratio (rBL; panel A), between bBL and the residual of rBL regressed on bBL (panel B), and rBL and the residual of bBL regressed on rBL (panel C). The regression lines are given with 95% confidence interval for the prediction of the mean of the dependent variable at any level of the explanatory variable. The residuals remove any variance in the dependent variable explained by the explanatory variable, as evidenced by the null correlation coefficients (*r*) and the absence of any significance (*P*) in panels B and C.

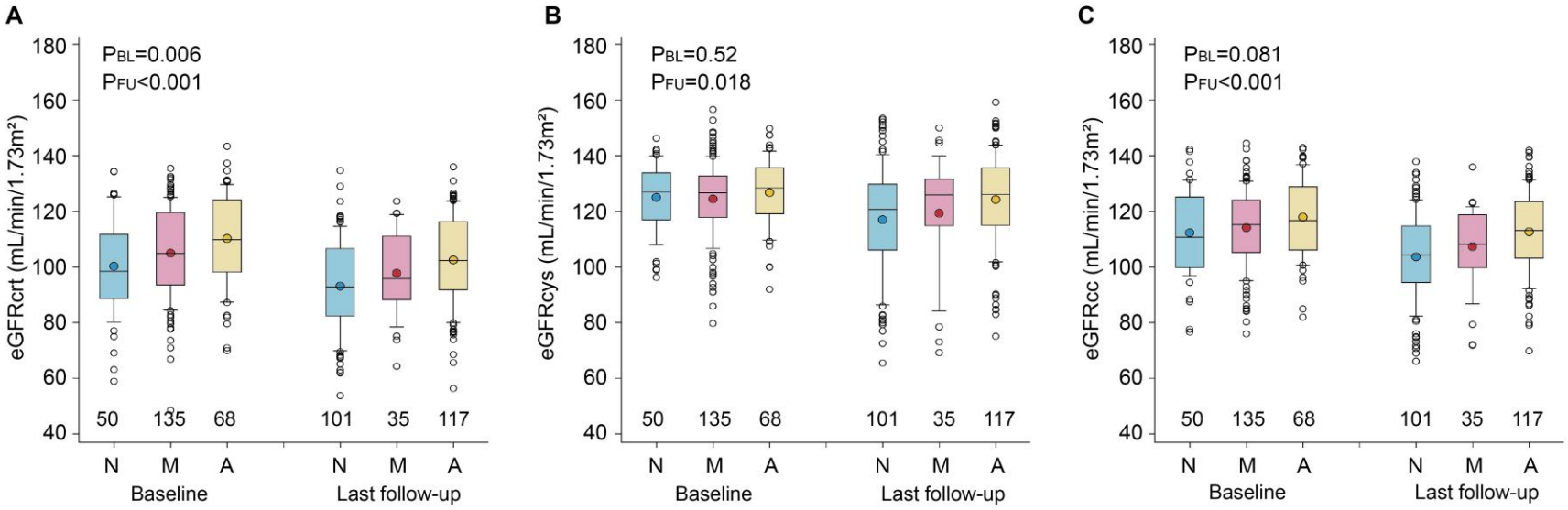


Figure S3. Boxplots showing the distributions of the glomerular filtration rate derived from serum creatinine (eGFRcrt; panel A), serum cystatin C (eGFRcys; panel B), or both serum creatinine and cystatin C (eGFRcc; panel C) by study phase (baseline and last follow-up) and by work shift (night [N], morning [M], and afternoon [A]). The central line, the upper and lower lines, and the upper and lower caps represent the median, interquartile range, and the 10th to 90th percentile interval. The arithmetic means and extreme measurements are represented by circles inside the box and outside the whiskers, respectively. The number of data points contributing to each whisker plot is given along the horizontal axis. *P* values denote the significance of the overall difference between the estimates of the glomerular filtration rate by time of day for baseline (P_{BL}) and follow-up (P_{FU}).

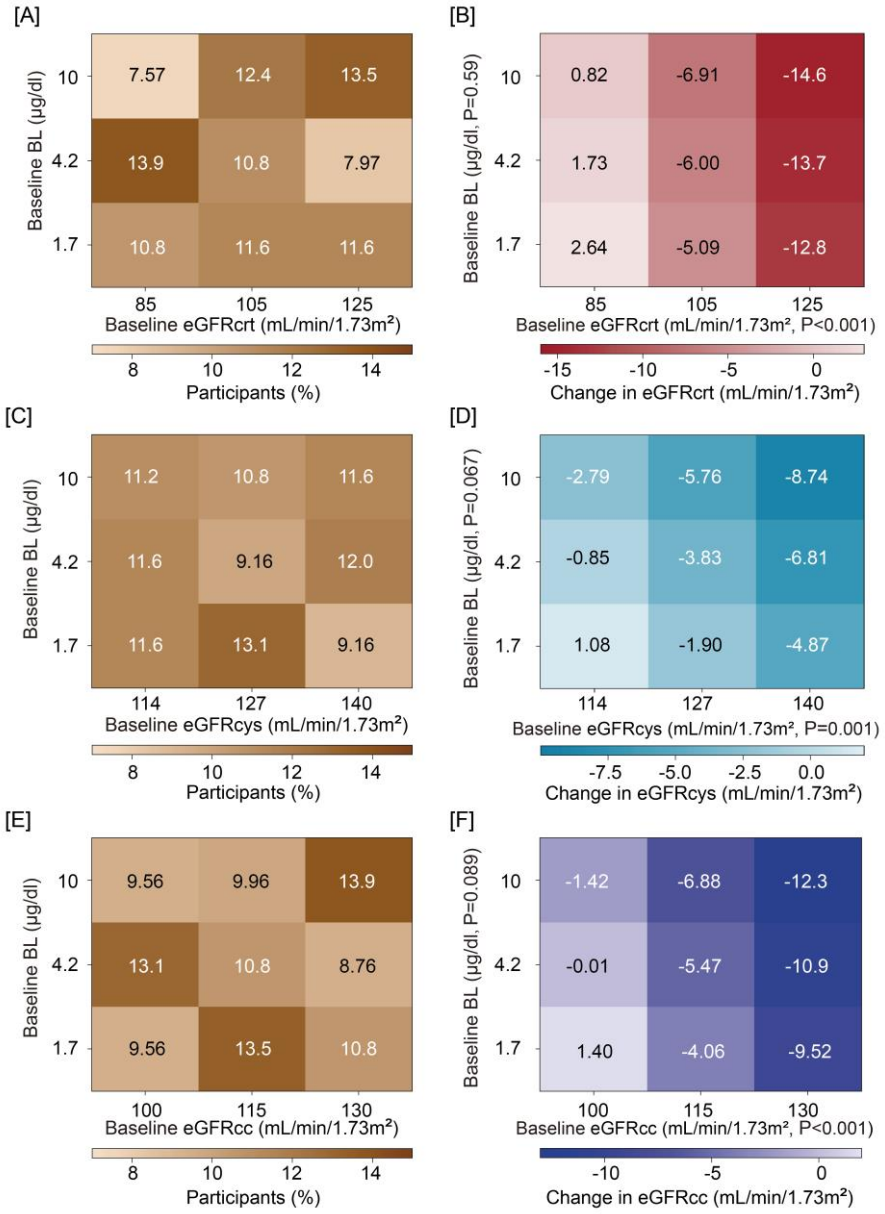


Figure S4. Heat maps relating the changes in glomerular filtration to their baseline values and the baseline blood lead (BL) concentration. Participants were cross-classified by thirds of the distributions of the baseline BL and the baseline glomerular filtration rate derived from serum creatinine (eGFRcrt; panels A and B), serum cystatin C (eGFRcys; panels C and D) or both (eGFRcc; panels E and F). The percentage of participants contributing to each cell of heat maps is given in panels A, C, and E. Associations sizes are point estimates corresponding with the thick marks and were derived from fully adjusted mixed models, which accounted for sex, age, follow-up duration, the time of day of blood sampling (daytime *vs* nighttime), body mass index, change in body weight, the follow-up-to-baseline BL ratio, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), total-to-HDL cholesterol ratio, and γ-glutamyltransferase. The *P*-values of the interaction terms between baseline BL and the baseline eGFR were 0.50, 0.11 and 0.70 for eGFRcrt (B), eGFRcys (E) and eGFRcc (F), respectively.

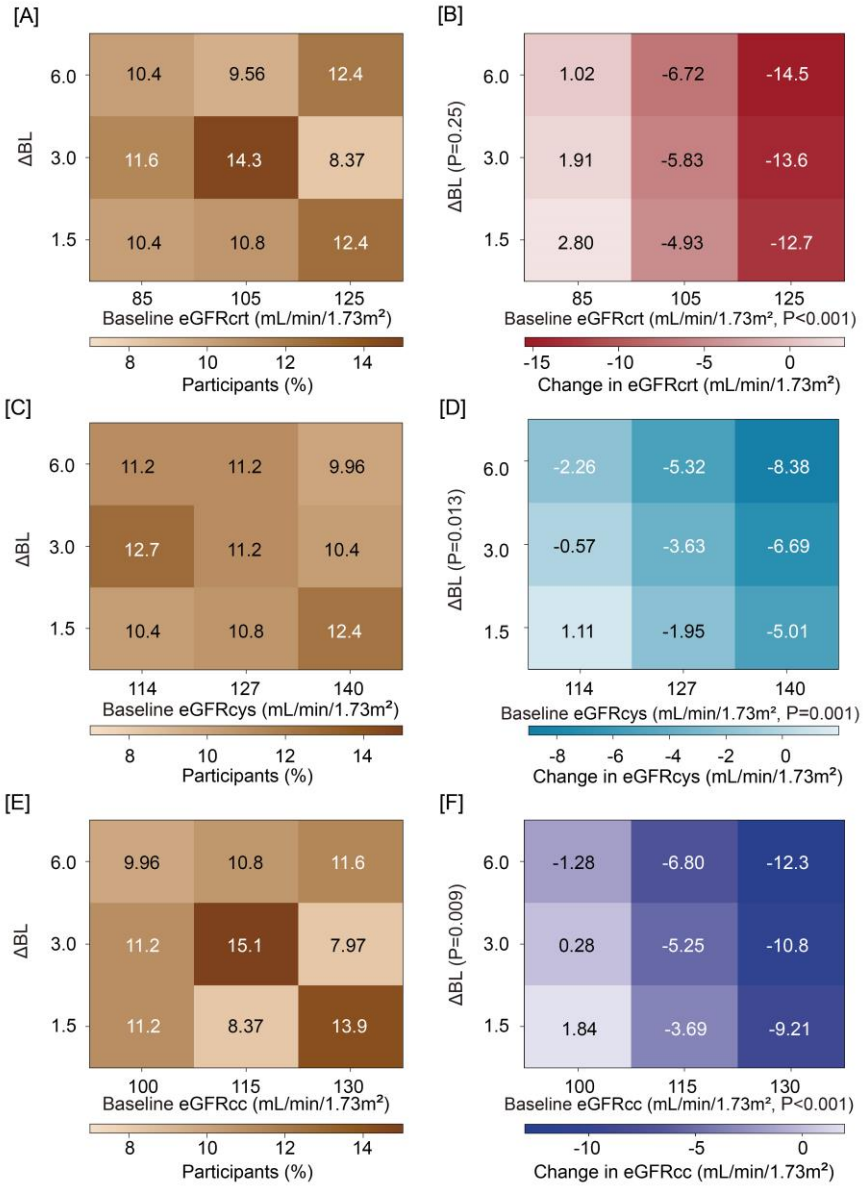


Figure S5. Heat maps relating the changes in glomerular filtration to their baseline values and the follow-up-to-baseline blood lead (BL) concentration ratio. Participants were cross-classified by thirds of the distributions of the follow-up-to-baseline BL ratio and the baseline glomerular filtration rate derived from serum creatinine (eGFRcrt; panels A and B), serum cystatin C (eGFRcys; panels C and D) or both (eGFRcc; panels E and F). The percentage of participants contributing to each cell of heat maps is given in panels A, C, and E. Associations sizes are point estimates corresponding with the thick marks and were derived from fully adjusted mixed models, which accounted for sex, age, follow-up duration, the time of day of blood sampling (daytime *vs* nighttime), body mass index, change in body weight, baseline BL, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure, antihypertensive medication (yes *vs* no), the total-to-HDL cholesterol ratio, and γ-glutamyltransferase. The *P*-values of the interaction terms between the follow-up-to-baseline BL ratio and the baseline eGFR were 0.65, 0.33 and 0.85 for eGFRcrt (B), eGFRcys (E) and eGFRcc (F), respectively.

Chapter 2

Unhealthy ageing in relation to lead exposure

Part 2

Blood pressure and renal function responses in workers exposed to lead for up to six years

Yu-Ling Yu, De-Wei An, MD, Wen-Yi Yang, Peter Verhamme,
Karel Allegaert, Tim S. Nawrot, Jan A. Staessen

Previously published in:
Journal of Clinical Hypertension. 2023; 25: 1086-1095.

Abstract

Background: The Study for Promotion of Health in Recycling Lead (SPHERL) assessed the blood pressure (BP) and renal function (RF) responses for up to six years in the workers without previous occupational lead exposure.

Methods: BP was the average of 5 consecutive readings and the estimated glomerular filtration rate was derived from serum creatinine (eGFRcrt) and cystatin C (eGFRcys). Blood lead (BL) was measured by inductively coupled plasma mass spectrometry (detection limit 0.5 μg/dL). The statistical methods included multivariable-adjusted mixed models and interval-censored Cox regression analysis.

Results: The 234 workers analyzed were on average 28.5 years old and included 91.9% men. The baseline BL concentration was 4.35 μg/dL and increased 3.17-fold over follow-up (median: 2.03 years; range: 0.92-6.45 years). The changes in BP and RF were not significantly correlated with the follow-up-to-baseline BL ratio ($p \geq 0.51$ and $p \geq 0.18$, respectively). The fully-adjusted changes in systolic/diastolic BP associated with a doubling of BL were -0.25/-0.12 mm Hg (CI: -0.94 to 0.44/-0.66 to 0.42 mm Hg). Accordingly, the incidence of stage-1 or -2 hypertension was not associated with the BL change ($p \geq 0.063$). Similarly, the changes in eGFRcrt and eGFRcys associated with a 3-fold BL increment were not significant, amounting to -0.70 mL/min/1.73 m^2 (CI: -1.70 to 0.30 mL/min/1.73 m^2) and -1.06 ml/min/1.73 m^2 (-2.16 to 0.03 mL/min/1.73 m^2).

Conclusions: In conclusion, the BP and RF responses to an over 3-fold BL increment were small and not significant confirming the safety of modern lead-handing facilities operating under current safety rules.

Introduction

Lead is a widespread environmental toxicant. Although previous publications have demonstrated the significant associations of lead exposure with the risks of hypertension [1,2] or renal function (RF) decline [3-6], these studies, either in environmental [2-5] or occupational [1,6] settings, were based on high-level lead exposure, had a cross-sectional design, or gave insufficient consideration to previous exposure or other confounders. The 2017 Global Burden of Disease (GBD) report [7] indicated that lead exposure, via increasing blood pressure (BP), might cause or aggravate a wide variety of cardiovascular diseases and renal dysfunction. However, the GBD investigators listed as potential limitations of their findings, residual confounding, the uncertainty as to the extent to which effect sizes were generalizable, the impossibility to account for temporal changes in the exposure to risk factors.

In the United States, the variation of lead in the environmental setting was assessed in consecutive cycles of the National Health and Nutrition Examination Survey (NHANES). The average blood lead (BL) concentration fell sharply from 13.1 μg/dL (1976-1980) [8] to 1.2-2.76 μg/dL (1988-1994) [8], and further to 1.64 μg/dL (1999-2002) [9,10]. These BL levels (post-1988) were similar to the BL level of 2 μg in the pre-industrial human [7] and much lower than in occupational settings. However, there is currently no consensus whether low-level lead exposure raises BP or induces kidney dysfunction. The Study for Promotion of Health in Recycling Lead (SPHERL; NCT02243904) [11] is a real-world study assessing the health responses prior to and up to six years after starting lead exposure in the occupational setting. In the current manuscript, we aimed to investigate the predefined co-primary endpoints – longitudinal BP and renal function (RF) – to lead exposure over up to three examination cycles.

Methods

Study participants

SPHERL is a longitudinal study of newly hired workers without known previous occupational lead exposure employed at battery manufacturing and lead recycling plants in the United States [11,12]. The US Occupational Safety and Health Administration Standard (www.osha.gov/laws-regs/regulations/standardnumber/1910/1910.1025), including regular health check-ups, proper workplace ventilation, and the obligatory use of personal protective equipment, was implemented at all recruitment sites. SPHERL complies with the Helsinki declaration for investigations in humans. The Ethics Committee of the University Hospitals Leuven (Belgium) approved the study protocol

(B322201421631) [11]. Changes in BP and RF in response to the first occupational lead exposure were the predefined co-primary endpoints [11].

Of 746 newly hired workers invited to participate in the study, 601 (80.6%) provided informed written consent. However, in the interval between consent and the planned baseline examination (median: 19 days; 5th-95th percentile interval: 9-59 days), 95 laborers left the companies or withdrew consent. From 25 January 2015 to 19 September 2017, 506 workers underwent the baseline examination, of whom 289 (57.1%) had at least one follow-up visit and 236 (46.6%) had two or more follow-up visits (**Figure 1**). The first, second and third follow-up visits were conducted from 13 September 2016 to 28 March 2019, from 13 September 2017 to 4 October 2019, and from 2 September 2021 to 23 February 2022, respectively. Of 289 participants with at least one follow-up visit, 55 were excluded because of missing information, leaving 234 workers for statistical analysis (**Figure 1**).

Clinical and biochemical measurements

At the study sites, trained nurses applied current guidelines to measure office BP at the brachial artery. After the workers had rested for 5 minutes in the sitting position, the nurses obtained five consecutive BP readings to the nearest 2 mm Hg by auscultation of the Korotkoff sounds, using standard mercury sphygmomanometers. The five readings were averaged for analysis. Heart rate was counted over 15 seconds. BP was categorized according to the 2017 American College of Cardiology/American Heart Association guideline (**Table S1**) [13,14]. Detailed description of BP measurements is given in the Data Supplement.

The clinical variables and the analytical methods of laboratory tests, including the measurements of RF and BL, the quality control of these measurements, and the supporting references are given in the Data Supplement. In short, BL was determined on whole blood by inductively coupled plasma mass spectrometry at an analytical laboratory certified for BL analysis in compliance with the provisions of the OSHA Lead Standard, 29CFR 1910.1025 (Occupational Safety and Health Administration [www.osha.gov]). The BL detection limit was 0.5 μg/dL.

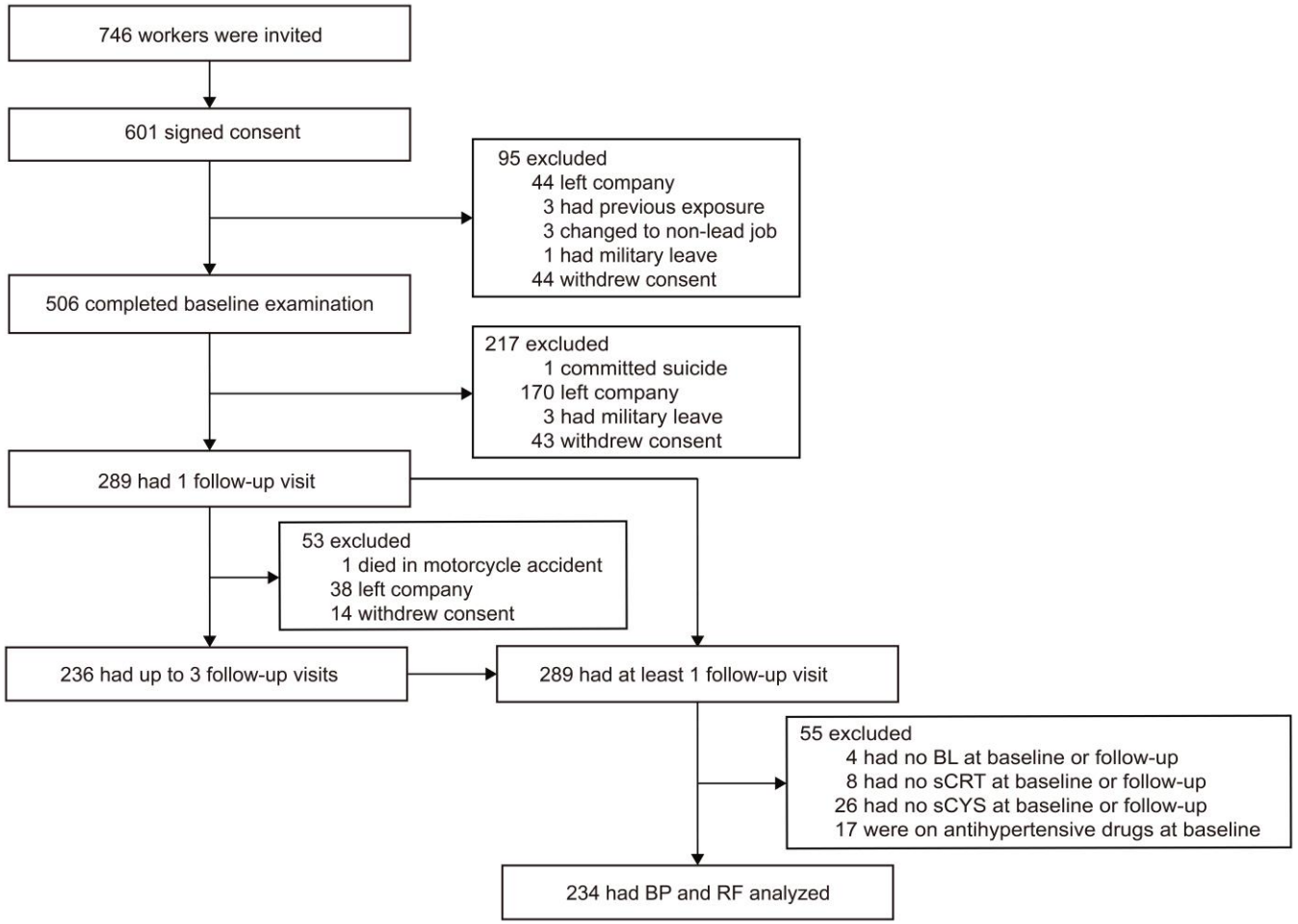


Figure 1. Flow Chart.

Abbreviations: BL, blood lead; BP, blood pressure; RF, renal function; sCRT, serum creatinine; sCYS, serum cystatin C.

The estimated glomerular filtration rate was derived from serum creatinine (eGFRcrt) and serum cystatin C (eGFRcys), using the Chronic Kidney Disease Epidemiology Collaboration equations [15]. To estimate the decline in glomerular filtration over the study period, age at each visit was introduced into the aforementioned equations. Serum osmolality was computed according to the formula: serum osmolality (mOsm/kg) = 2 × (serum Na^+ [mmol/L]) + (blood glucose [mg/dL] / 18) + (blood urea nitrogen [mg/dL] / 2.8) [16]. In addition, serum osmolality was measured by the freezing point depression technique in 37 workers to ascertain the accuracy of the formula against the true measurement of serum osmolality, using the Bland and Altman approach (**Figure S1**).

Statistical analysis

For database management and statistical analysis, we used the SAS software, version 9.4, maintenance level 5 (SAS Institute Inc., Cary, NC). Departure from normality was evaluated by the Shapiro-Wilk statistic. To normalize the distributions, we applied a logarithmic transformation (base 10) to urinary albumin-to-creatinine ratio (ACR), γ-glutamyltransferase, (index of alcohol consumption) and BL. We reported the central tendency and spread of continuously distributed variables as mean and standard deviation (SD) or as geometric mean and the interquartile range (IQR) or the 5th-95th percentile interval (PI) for logarithmically transformed variables. To compare means and proportions, we applied the t statistic or ANOVA for continuous variables, and the Fisher exact test for categorical variables, respectively.

In exploratory analyses, we assessed the BP and RF of workers across fourths of the distribution BL changes from baseline to the last follow-up. The changes in BP and RF responses to the change in BL were expressed for a doubling of the follow-up-to-baseline BL ratio. Estimates were derived from mixed models including the first and repeated follow-up visits, while accounting for clustering within participants using a random effect. The interval-censored proportional hazards regression was applied to estimate the association between incident hypertension and the BL change. The baseline hazard function was modelled using cubic splines with 3 knots. For the analysis of BP and incident hypertension, the covariables were ethnicity (white *vs* others), sex, baseline age, baseline BP, the baseline value of body mass index and change in body weight during follow-up, and the baseline values of and the changes during follow-up in heart rate, smoking status, total-to-HDL serum cholesterol ratio, γ-glutamyltransferase and serum creatinine. For the analysis of RF, the covariables were sex, baseline age, baseline RF, follow-up duration, the time of day of blood sampling (nighttime *vs* daytime), the baseline

value of body mass index and change in body weight during follow-up, and the baseline values of and the changes during follow-up in smoking status, mean arterial pressure (diastolic BP plus one third of the difference between systolic and diastolic BP), the total-to-HDL cholesterol ratio and γ-glutamyltransferase, and changes in antihypertensive medication (yes *vs* no) during follow-up.

Results

Characteristics of workers

Table 1 lists the baseline characteristics of the 234 workers, of whom 215 (91.9%) were men. The cohort included 112 (47.9%) Whites, 112 (47.9) Hispanics, 9 (3.85) Blacks, and 9 (3.85) workers with another self-reported ethnic background. At enrollment, age averaged 28.5 years, total and HDL cholesterol 169.9 and 46.3 mg/dL, and the corresponding total-to-HDL cholesterol ratio 3.9 (**Table 1**). The values of blood pressure and renal function at baseline and the last follow-up visit, and the corresponding changes are shown in Table S2. In the 37 duplicate measurements of serum osmolality (**Figure S1**), the bias determined according to the Bland and Altman approach was glutamyl transferase 1.86 mOsm/kg (95% CI: -0.06 to 3.78, *P*=0.057).

Blood lead

During the median follow-up time of 2.03 years (IQR: 1.96-2.16 years; range: 0.92-6.45 years), the geometric mean BL concentration increased from 4.35 μg/dL (IQR: 2.50-7.90 μg/dL; 5th-95th percentile interval: 0.90-15.2 μg/dL) at baseline, to 16.6 μg/dL (IQR: 10.5-22.2 μg/dL; 5th-95th percentile interval: 3.80-30.8 μg/dL) at the first follow-up visit, and to 15.3 μg/dL (IQR: 8.90-22.1 μg/dL; 5th-95th percentile interval: 3.20-31.4 μg/dL) at the last follow-up visit, respectively. The follow-up-to-baseline BL ratio averaged 3.17 (IQR: 1.88-5.46; 5th-95th percentile interval, 0.62-14.0).

Table 1. Baseline Characteristics of 234 Workers

Characteristic	N (%)[a]	Characteristic	Mean (SD/IQR)[a]
Male	215 (91.9)	Age, years	28.5 (8.59)
Female	19 (8.12)	Body mass index, kg/m^2	28.6 (5.97)
White ethnicity	112 (47.9)	Systolic blood pressure, mm Hg	119.3 (10.0)
Hispanic ethnicity	104 (44.4)	Diastolic blood pressure, mm Hg	79.2 (8.72)
Black ethnicity	9 (3.85)	Heart rate, beats per minute	74.3 (12.1)
Other ethnicities	9 (3.85)	Serum creatinine, mg/dL[c]	0.96 (0.17)
Current smokers	57 (24.4)	Serum cystatin C, mg/L[c]	0.67 (0.10)
Alcohol intake	102 (43.6)	eGFRcrt, mL/min/1.73 m^2 [b]	106.4 (16.6)
Normotension	99 (42.3)	eGFRcys, mL/min/1.73 m^2 [b]	126.0 (12.6)
Elevated blood pressure	27 (11.5)	Serum osmolality, mOsm/kg	286.8 (3.78)
Hypertension stage ≥1	108 (46.2)	Urinary specific gravity	1.02 (0.01)
Hypertension stage ≥2	29 (12.4)	Total-to-HDL cholesterol ratio	3.88 (1.27)
Treated hypertension	0 (0.00)	ACR, mg/g	4.36 (2.83, 6.10)
Diabetes mellitus	7 (2.99)	γ-glutamyltransferase, U/L	22.1 (16.0,31.0)

[a]Values are number of participants (%), arithmetic mean (SD) or geometric mean (IQR). Blood pressure was the average of five readings. Normotension, elevated blood pressure and hypertension were categorized according to the 2017 ACC/AHA guideline, irrespective of treatment status (reference 13). Diabetes mellitus was a self-reported diagnosis, a fasting blood glucose of ≥126 mg/dL, or use of antidiabetic drugs.
[b]eGFRcrt and eGFRcys refer to the glomerular filtration rate estimated from serum creatinine and serum cystatin C (reference 15).
[c]To convert serum creatinine from mg/dL to micromol/L, multiply by 88.42; to convert cystatin C from mg/L to nmol/L, multiply by 74.9.
Abbreviations: ACR, urinary albumin-to-creatinine ratio; HDL, high-density lipoprotein; IQR, interquartile range; N (%), number of participants (percent); SD, standard deviation.

Blood pressure and incident hypertension

Baseline systolic/diastolic BP amounted to 119.3/79.2 mm Hg (**Table 1**). The corresponding mean arterial pressure was 92.6 mm Hg (SD: 8.5 mm Hg) and pulse pressure was 40.1 mm Hg (SD: 7.0 mm Hg). In exploratory analyses, there was no trend ($P \geq 0.51$) in the BP changes across increasing quartiles of the follow-up-to-baseline BL ratio (**Table S3**). In the unadjusted, adjusted and fully adjusted mixed models, accounting for the clustering within participants, the associations were not significant between the change in BP and the changes in BL ($P \geq 0.29$, **Table 2**). In the fully adjusted model (**Table 2**), the association sizes associated with a doubling of follow-up-to-baseline BL ratio were -0.25 mm Hg (CI: -0.94 to 0.44 mm Hg) for systolic BP and -0.12 mm Hg (CI, -0.66 to 0.42 mm Hg) for diastolic BP. Heat maps (**Figure 2**) demonstrated that the changes in BP were mainly determined by baseline BP ($P < 0.0001$), while the change in BL did not reach significance in these analyses ($P \geq 0.47$).

Cross-classification of the baseline and follow-up BP (**Table S4**) demonstrated that of 99 workers, who were normotensive as baseline, 14 moved up to untreated elevated BP, 42 to untreated stage-1 hypertension, 7 to untreated stage-2 hypertension, no worker to untreated severe hypertension, and 1 to treated hypertension, whereas 35 workers stayed normotensive. Taken into account the baseline BP status, overall, 65/126 (51.6%) workers developed ≥stage-1 hypertension, 22/205 (10.0%) stage-2 hypertension, and 97/238 (40.8%) moved up across the blood pressure classification. In the unadjusted, adjusted and fully adjusted analyses, the hazard ratios (HRs) for incident hypertension were not significant ($p \geq 0.063$, **Table 2**). For incident hypertension ≥ stage 1, the fully adjusted HR was 1.21 (CI: 0.99 to 1.48, **Table 2**). For moving up across the BP categories (**Table 2**), the fully adjusted HR was 1.09 (CI: 0.94 to 1.25).

Renal function

At baseline, eGFRcrt averaged 106.4 mL/min/1.73 m^2 and eGFRcys 126.0 mL/min/1.73 m^2 (**Table 1**). In exploratory analyses, there was no trend ($P \geq 0.18$) in the RF changes across increasing quartiles of the follow-up-to-baseline BL ratio (**Table S5**). In addition, 12 (5.13%) workers had a self-reported history of nephrolithiasis at enrollment, but no new or recurrent incidence occurred during follow-up. There was no significant correlation between the changes in BP and changes in RF ($P \geq 0.71$). The age-dependent changes in eGFRcrt and eGFRcys were -1.77 and -1.21 mL/min/1.73 m^2, respectively.

Table 2. Association between Changes in Blood Pressure, Incident Hypertension and Change in Blood Lead

Outcome	Unadjusted		Adjusted		Fully Adjusted	
Blood pressure[a]	**Estimate (95% CI)**	***p*-value**	**Estimate (95% CI)**[b]	***p*-value**	**Estimate (95% CI)**[c]	***p*-value**
Systolic blood pressure, mm Hg	-0.11 (-0.95, 0.73)	0.80	-0.39 (-1.07, 0.29)	0.26	-0.25 (-0.94, 0.44)	0.47
Diastolic blood pressure, mm Hg	0.32 (-0.44, 1.09)	0.40	-0.13 (-0.66, 0.39)	0.62	-0.12 (-0.66, 0.42)	0.66
Mean arterial pressure, mm Hg	0.16 (-0.54, 0.85)	0.66	-0.19 (-0.70, 0.33)	0.47	-0.13 (-0.65, 0.39)	0.63
Pulse pressure, mm Hg	-0.30 (-1.10, 0.50)	0.46	-0.25 (-0.83, 0.34)	0.41	-0.20 (-0.81, 0.41)	0.52
Incident hypertension[d] **(n/N**[e]**)**	**HR (95% CI)**	***p*-value**	**HR (95% CI)**[g]	***p*-value**	**HR (95% CI)**[h]	***p*-value**
Hypertension ≥ stage 1 (65/126)	1.15 (0.96, 1.38)	0.14	1.15 (0.95, 1.39)	0.13	1.21 (0.99, 1.48)	0.063
Hypertension ≥ stage 2 (22/205)	0.94 (0.75, 1.18)	0.58	0.95 (0.75, 1.19)	0.65	1.03 (0.79, 1.34)	0.84
Moving up categories[f] (97/234)	1.11 (0.97, 1.27)	0.12	1.08 (0.95, 1.24)	0.25	1.09 (0.94, 1.25)	0.25

[a]For the analyses of blood pressure (BP), estimates express the difference in BP associated with a doubling of the follow-up-to-baseline blood lead ratio. Estimates were derived from mixed models accounting for clustering within participants using a random effect.
[b]Adjusted models accounted for sex and age, BP and body mass index at baseline.
[c]Fully adjusted models additionally accounted ethnicity (white *vs* other), change in body weight during follow-up, and the baseline values of and the changes during follow-up in heart rate, smoking status, total-to-HDL serum cholesterol ratio, γ-glutamyltransferase, and serum creatinine.
[d]Hypertension was categorized according to the to 2017 ACC/AHA guideline (references 13), irrespective of treatment status. Stage-1 and stage-2 hypertension were systolic or diastolic BP levels of 130-139/80-89 mm Hg and 140-159/90-99 mm Hg, respectively. If systolic and diastolic BP were in different categories, the highest category was applied. For the analysis of incident hypertension, Hazard ratios (HR), given with 95% confidence interval, HRs and 95% CI were obtained from proportional hazard models for interval censored data, using a cubic spline function to model the baseline hazard. HRs express the risk associated with a doubling of the follow-up-to-baseline blood lead level.
[e]n/N refers to the number of incident cases of hypertension and the total number of workers at risk. Workers at risk were those without stage-1 or stage-2 hypertension at baseline or all workers for moving up categories.
[f]Moving up categories indicates an increase in the blood pressure category by one or more steps during follow-up.
[g]Adjusted models accounted for sex, age, baseline body mass index and baseline mean arterial pressure, i.e., diastolic blood BP plus one third of the difference between systolic and diastolic BP.
[h]Fully adjusted models additionally accounted for ethnicity (white *vs* other), change in body weight during follow-up, and the baseline value of and change during follow-up in heart rate, smoking status, total-to-HDL serum cholesterol ratio, γ-glutamyltransferase, and serum creatinine.
Abbreviations: BP, blood pressure; HR, hazard ratio; 95% CI, 95% confidence interval.

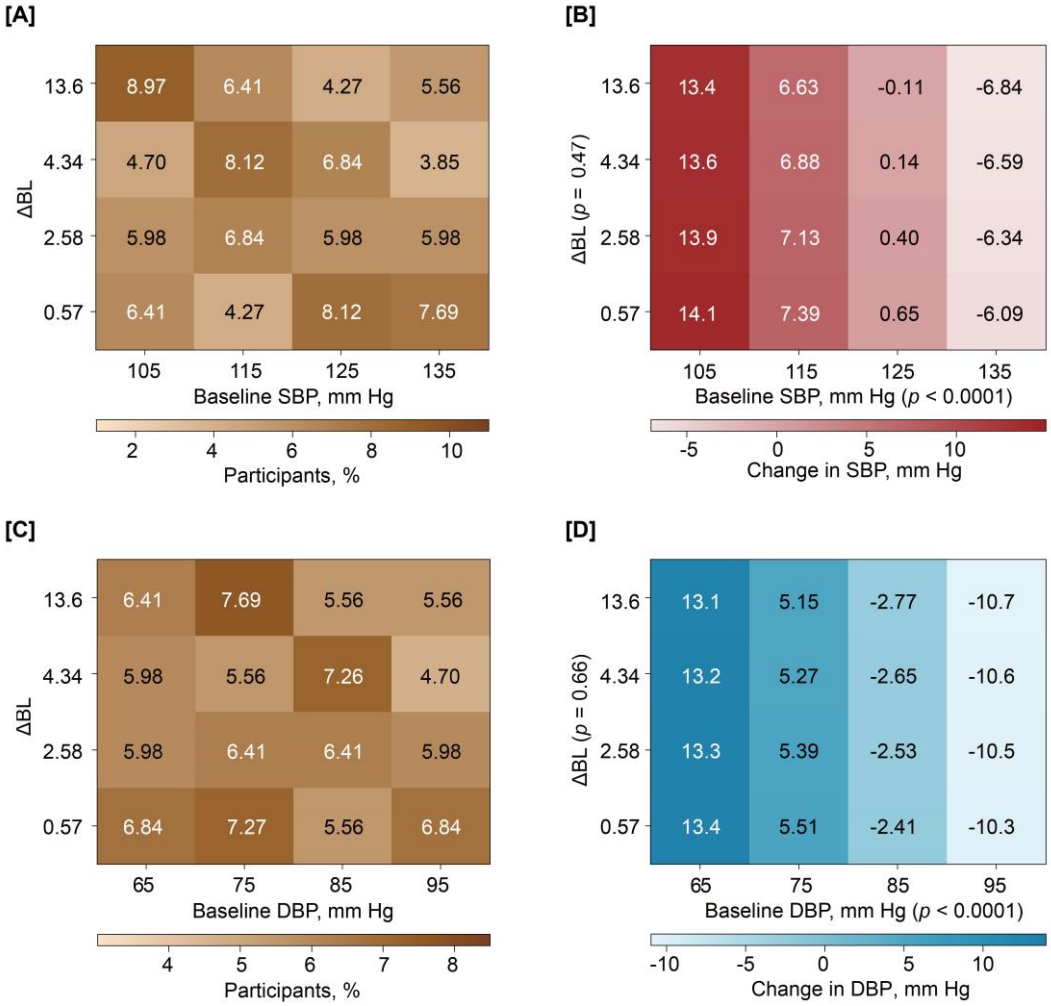


Figure 2. Heat Maps Relating the Change in Systolic and Diastolic Blood Pressure to the Blood Pressure Level at Baseline and the Change in Blood Lead from Baseline to Last Follow-Up. Abbreviations: ΔBL: change in blood lead; SBP: systolic blood pressure; DBP: diastolic blood pressure. For SBP (B) and DBP (D), the associations were derived by mixed models (see table 2). All models were fully adjusted. The percentage of participants (A and C) contributing to the cross-classification between the baseline blood pressure (horizontal axis) and the fold change in BL (vertical axis) are given for each analysis run.

In the unadjusted and adjusted mixed models, a doubling of BL was not significantly associated with the changes in RF ($P \geq 0.073$; **Table 3**). In the fully adjusted models, the association sizes associated with a doubling of follow-up-to-baseline BL ratio were -0.70 mL/min/1.73 m^2 (CI: -1.70 to 0.30 mL/min/1.73 m^2) for eGFRcrt and -1.06 mL/min/1.73 m^2 (CI: -2.16 to 0.03 mL/min/1.73 m^2) for eGFRcys (**Table 3**). Moreover, the changes in serum creatinine, serum cystatin C, serum osmolality, urinary specific gravity and ACR were not significantly associated with the changes in BL ($P \geq 0.081$; **Table 3**). Heat maps (**Figure 3**) indicated that the changes in RF were mainly determined by baseline eGFRcrt and eGFRcys ($P \leq 0.0011$), while the change in BL did not reach significance in these analyses ($P \geq 0.057$).

Discussion

In a real-world occupational setting, the over 3-fold BL increment was not significantly associated with increases in any BP component, higher risk of hypertension, or with a decline in a variety RF measurements in a cohort of workers without known previous occupational lead exposure. These findings confirmed the previously published cross-sectional analyses at baseline [12,17] and the longitudinal analyses at follow-up in the second cycle of the SPHERL project [18,19]. Compared with the previous publications [18,19], the current study went through a longer follow-up period and indicated a stable BL levels after the first year, which could provide a more reliable outcome interpretation after starting the first occupational exposure. The HR and CI of incident hypertension ≥ stage 1 did not reach significant statistically, while this result could not exclude the risk of incident hypertension, as defined by the stricter criteria in 2017 American College of Cardiology/American Heart Association guideline (**Table S1**) [13,14], with certainty. In addition, the Bland and Altman approach indicated reasonable repeatability in the serum osmolarity measurements in 37 workers. The heat maps demonstrated that baseline BP (**Figure 2**) and baseline eGFRcrt and eGFRcys (**Figure 3**) were the principal determinants of their corresponding changes during follow-up, while the over 3-fold increment in BL did not significantly produce adverse health outcomes. Due to regression to mean, as revealed in all clinical measurements [20] the workers with lower BP at baseline were more likely to have an increased BP level at follow-up or to move up across BP categories, while this tendency was inverse for the workers at the top tail of the baseline BP distribution (**Figure 2**). Similarly, the same regression to mean phenomenon affected the findings for eGFRcrt and eGFRcys (**Figure 3**).

Table 3. Association between Changes in Renal Function and Change in Blood Lead

Variable	Unadjusted		Adjusted[d]		Fully adjusted[e]	
	Estimate (95% CI)[c]	*p*-value	Estimate (95% CI)[c]	*p*-value	Estimate (95% CI)[c]	*p*-value
Serum creatinine, $\times 10^{-2}$ mg/dL	-0.09 (-1.18, 1.01)	0.88	0.47 (-0.55, 1.48)	0.37	0.56 (-0.48, 1.59)	0.29
Serum cystatin C, $\times 10^{-2}$ mg/L	-0.37 (-1.33, 0.58)	0.44	0.76 (-0.16, 1.68)	0.10	0.83 (-0.10, 1.77)	0.081
eGFRcrt, mL/min/1.73 m^2 [a]	0.08 (-0.98, 1.13)	0.88	-0.62 (-1.59, 0.36)	0.22	-0.70 (-1.70, 0.30)	0.17
eGFRcys, mL/min/1.73 m^2 [a]	0.40 (-0.73, 1.53)	0.48	-0.98 (-2.06, 0.09)	0.073	-1.06 (-2.16, 0.03)	0.057
Serum osmolality, mOsm/kg	0.16 (-0.25, 0.56)	0.44	-0.05 (-0.36, 0.26)	0.75	-0.06 (-0.38, 0.26)	0.71
Urine specific gravity, $\times 10^{-2}$	-0.02 (-0.09, 0.06)	0.68	-0.03 (-0.08, 0.03)	0.34	-0.01 (-0.06, 0.04)	0.68
ACR, % [b]	-1.45 (-7.06, 4.49)	0.62	-0.71 (-5.93, 4.80)	0.80	-0.81 (-6.05, 4.71)	0.77

[a]eGFRcrt and eGFRcys refer to the glomerular filtration rate estimated from serum creatinine or serum cystatin C (reference 15).
[b]Change in urinary albumin-to-creatinine ratio are expressed as percentage differences from baseline to follow-up.
[c]Estimates express the difference in renal function associated with a doubling of the follow-up-to-baseline blood lead ratio. Estimates were derived from mixed models accounting for clustering within participants as random effect.
[d]Adjusted models accounted for sex, age, follow-up duration, the time of day of blood sampling (nighttime *vs* daytime), and the baseline renal function measure being analyzed.
[e]Fully adjusted models additionally accounted for baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure (diastolic BP plus one third of the difference between systolic and diastolic BP, the total-to-HDL cholesterol ratio and γ-glutamyltransferase, and changes in antihypertensive medication (yes *vs* no) during follow-up.
Abbreviations: ACR, urinary albumin-to-creatinine ratio; 95% CI, 95% confidence interval.

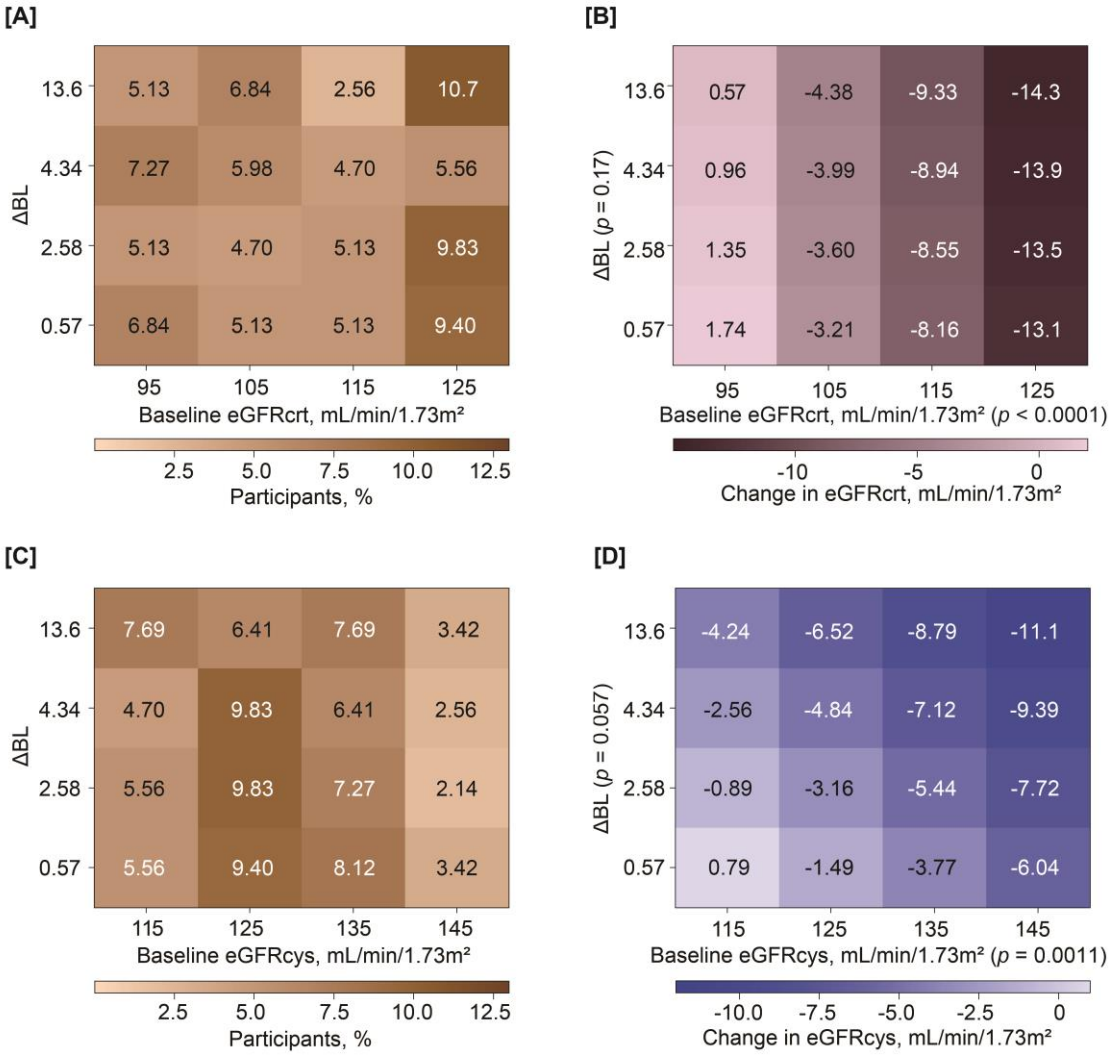


Figure 3. Heat Maps Relating the Change in eGFRcrt and eGFRcys to the eGFR Level at Baseline and the Change in Blood Lead from Baseline to Last Follow-Up. Abbreviations: ΔBL: change in blood lead; eGFRcrt: the glomerular filtration rate estimated from serum creatinine; eGFRcys: the glomerular filtration rate estimated from serum cystatin C. For eGFRcrt (B) and eGFRcys (D), the associations were derived by mixed models (see table 3). All models were fully adjusted. The percentage of participants (A and C) contributing to the cross-classification between the baseline blood pressure (horizontal axis) and the fold change in BL (vertical axis) are given for each analysis run.

The eGFRcys, as derived from serum cystatin C, is a better biomarker reflecting glomerular filtration than eGFRcrt, for its higher reliability in the presence of confounding by ethnicity, sex, age, muscle mass and protein intake [15]. The expected age-related decline in eGFRcrt and eGFRcys (-1.77 and -1.21 mL/min/1.73 m^2, respectively), was of the similar magnitude compared with the corresponding fully adjusted changes in eGFRcrt and eGFRcys in relation to the baseline-to-follow-up BL ratio (**Table 3**).

In addition to regression to the mean, the present study highlights the importance to account in longitudinal studies for concealed albeit not unexpected confounders and to distinguish crudely observed changes in the glomerular filtration rate in the context of the biomarker of exposure of interest, BL in the current study. Both serum creatinine and serum cystatin C, from which glomerular filtration is derived, show a diurnal rhythm with little influence of meals or meat ingestion on serum cystatin C level, whereas these confounders increase serum creatinine level [21]. Along similar lines, during sleep, urine flow decreases and the tubular reabsorption of water increases [22,23]. The newly hired workers recruited into SPHERL transited not only from environmental to occupational exposure but also from a sedentary to a physically demanding lifestyle. On the basis of the published tables [24], the jobs offered to the workers required an energy expenditure of 6 to >8 metabolic equivalents defined as the amount of oxygen consumed while resting in the sitting position. Physical labor induces acute renal changes in healthy adults [25]. In young adults, exercise reduces renal plasma flow and glomerular filtration with smaller effects on eGFRcys than on eGFRcrt [26].

In the current analysis, the changes in BP and in glomerular filtration were not significantly correlated. In contrast, a recent NHANES study (1999-2014) [27] with 20,073 participants (49.01% men, mean age, 47.27 years, mean BL, 1.81 μg/dL), demonstrated a positive association of BP with BL and an inverse association of eGFR with both BL and BP. However, this cross-sectional analysis did not allow excluding the alternative hypothesis that lead accumulation is a consequence instead of a cause of kidney dysfunction. Among 7341 male Korean lead exposed workers (mean age, 31.1 years; mean BL, 5.3 μg/dL), the odds ratio relating the risk of hypertension to BL in the highest quartile of BL distribution (6.87-10.0 μg/dL) was 1.54 (95% CI, 1.26-1.89) [1]. However, this study did not account for the previous occupational lead exposure and could not demonstrate a continuous association between BP and BL over the whole BL range [1]. A previous study by Mujaj and coworkers reported an inverse association between ACR and BL [12]. The present study also showed an inverse association, although it was not statistically significant (**Table 3**). Creatinine is removed from the

circulating blood by glomerular filtration but also by proximal tubular secretion. Little or no tubular reabsorption of creatinine occurs. Small proteins that pass the glomerular sieve undergo tubular reabsorption. The aforementioned publication [12] could not clarify whether the inverse association between ACR and BL was induced by higher tubular reabsorption of small proteins or higher proximal tubular excretion of creatinine. Along similar lines, several studies reported an inverse association between eGFR and BL in response to environmental [4,5,28-31] or occupational [6] exposure. However, as 70% of lead is excreted via kidney [32,33], whether higher BL induces renal impairment or *vice versa* cannot be distinguished.

Lead is a cumulative toxicant, 90-95% of which is stored in bone, from where it is recirculated with a half-life of 20-25 years [34,35]. BL, which for 99% is carried by red blood cells, reflects recent exposure over the past 1-2 months and the amount of lead released and recirculated from bone stores [34] Bone lead correlates with BL [35,36] and explains around 20% of the variance in BL, depending on seasonality [35] and hormonal and other endogenous and environmental stimuli influencing the balance between bone formation and resorption [36]. Therefore, BL in older individuals increasingly represent the pre-existing body burden [34,35] originating from the historical environmental lead exposure. In the United States, leaded paint was effectively prohibited in 1976 and leaded gasoline was completely phased out in 1995 [37]. The SPHERL finding are not vulnerable to this phenomenon, given that young age of most of its participants and the exclusion of workers with previous occupational lead exposure.

The strong points of SPHERL study are the longitudinal study design in young workers without known previous occupational exposure and the rigorous quality control of BL concentrations with a detection limit as low as 0.5 μg/dL. An important concept highlighted by the SPHERL experience is that in longitudinal studies in an occupational setting not accounting for confounders, such as aging, the diurnal variation in body functions, shift work, or the physiological changes normally associated with physically strenuous work might lead to falsely attributing noxious effects to the pollutant under study. On the other hand, the current study must also be interpreted within the context of its limitations. First, the attrition rate among 506 workers originally was 42.9%, predominately because of occupational mobility. Therefore, more than 500 participants were enrolled to meet the sample size for addressing BP and RF as the co-primary endpoints. Second, the healthy worker effect[38] might potentially account for the non-significance of the current findings, given the mean age of 28.5 years in this occupational cohort. Third, although the ethnic distribution of the workers was representative for the

population at the recruitment sites, women were under-represented. Only 11.6% of study participants were female, which precluded analyses stratified by sex. The current observations should not be unthoughtfully generalized and are therefore not applicable to older individuals or patients with comorbidities, such as diabetes, which increases the vulnerability of renal function. Fourth, taking five consecutive measurements is in keeping with all previous epidemiological and other research conducted by our group. Moreover, if a drop of the blood pressure level occurs from the first to the fifth, this decreases the risk of white-coat hypertension and falsely classifying a worker as being hypertensive [39-41]. Finally, the co-exposure of cadmium was not assessed, which is commonly present in lead recycling plants and has adverse effects on renal tubular and glomerular function.

Conclusions

A real-world experiment involving workers without know previous lead exposure did not demonstrate significant associations of BP, the risk of hypertension or RF measurements with an over 3-fold BL increment. Thus, using a state-of-the art longitudinal cohort design, SPHERL confirmed that in modern lead-handing facilities operating under current rules imposed by OSHA, workers over an employment period of 2 years, in some extending to up to 6 years, were well protected with minimal risk for hypertension or renal dysfunction.

Funding

The Alliance for the Promotion of Preventive Medicine is a not-profit research association (URL: www.appremed.org; Belgian registration number, 739849385), which received a non-binding grant from OMRON Healthcare Co. Ltd., Kyoto, Japan.

Conflict of interest

All authors declare no conflict of interests.

References

1. Kim MG, Kim YW, Ahn Y-S. Does low lead exposure affect blood pressure and hypertension? *J Occup Health.* 2019;62:e12107.
2. Nawrot TS, Thijs L, Den Hond EM, Roels HA, Staessen JA. An epidemiological re-appraisal of the association between blood pressure and lead : a meta-analysis. *J Hum Hypertens.* 2002;16:123-131.
3. Park Y, Lee S-J. Association of blood heavy metal levels and renal function in Korean Adults. *Int J Environ Res Public Health.* 2022;19:6646.
4. Staessen JA, Lauwerys RR, Buchet JP, Bulpitt CJ, Rondia D, Vanrenterghem Y, Amery A, the Cadmibel Study Group. Impairment of renal function with increasing blood lead concentrations in the general population. *N Engl J Med.* 1992;327:151-156.
5. Kim R, Rotnitzky A, Sparrow D, Weiss ST, Wagner C, Hu H. A longitudinal study of low-level lead exposure and impairment of renal function. The Normative Aging Study. *J Am Med Assoc.* 1996;275:1177-1181.
6. Chowdhury R, Darrow L, McClellan W, Sarnat S, Steenland K. Incident ESRD among participants in a lead surveillance program. *Am J Kidney Dis.* 2014;64:25-31.
7. GBD 2017 Risk Factor Collaborators. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risk factors or clusters of risks for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* 2018;392:1923-1994.
8. Pirkle JL, Brody DJ, Gunter EW, Kramer RA, Paschal DC, Flegal KM, Matte TD. The decline in blood lead levels in the United States. The National Health and Nutrition Examination Surveys (NHANES). *J Am Med Assoc.* 1994;272:284-291.
9. Muntner P, Menke A, DeSalvo KB, Rabito FA, Batuman V. Continued decline in blood lead levels among adults in the United States : the National Health and Nutrition Examination Surveys. *Arch Intern Med.* 2005;165:2155-2161.
10. Hara A, Thijs L, Asayama K, Gu YM, Jacobs L, Zhang ZY, Liu YP, Nawrot TS, Staessen JA. Blood pressure in relation to environmental lead exposure in the National Health and Nutrition Examination Survey 2003 to 2010 *Hypertension.* 2015;65:62-69.

11. Hara A, Gu YM, Petit T, Liu YP, Jacobs L, Zhang ZY, Jin Y, Thijs L, Wei FF, Nawrot TS, Staessen JA. Study for promotion of health in recycling lead - rationale and design. *Blood Press.* 2015;24:147-157.

12. Mujaj B, Yang WY, Zhang ZY, Wei FF, Thijs L, Verhamme P, Staessen JA. Renal function in relation to low-level environmental lead exposure. *Nephrol Dial Transplant.* 2018;34:941-946.

13. Whelton PK, Carey RM, Aronow WS, Casey DEJr, Collins KJ, Dennison Himmelfarb C, DePalma SM, Gidding S, Jamerson KA, Jones DW, MacLaughlin EJ, Muntner P, Ovbiagele B, Smith SCJr, Spencer CC, Stafford RS, Taler SJ, Thomas RJ, Williams KASr, Williamson JD, Wright JTJr. 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA guideline for prevention, detection, evaluation, and management of high blood pressure in adults: A report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol.* 2018;71:e127-e248.

14. Cheng YB, Thijs L, Zhang ZY, Kikuya M, Yang WY, Melgarejo JD, Boggia J, Wei FF, Hansen TW, Yu CG, Asayama K, Ohkubo T, Dolan E, Stolarz-Skrzypek K, Malyutina S, Casiglia E, Lind L, Filipovský J, Maestre GE, Imai Y, Kawecka-Jaszcz K, Sandoya E, Narkiewicz K, Li Y, O'Brien E, Wang JG, Staessen JA, IDACO Investigators. Outcome-driven thresholds for ambulatory blood pressure based on the new American College of Cardiology/American Heart Association classification of hypertension. *Hypertension.* 2019;74:776-783.

15. Inker LA, Schmid CH, Tighiouart H, Eckfeldt JH, Feldman HI, Greene T, Kusek JW, Manzi J, Van Lente F, Zhang YL, Coresh J, Levey AS, for the CKD-EPI Investigators. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med.* 2012;367:20-29.

16. Rasouli M. Basic concepts and practical equations on osmolality: biochemical approach. *Clin Biochem.* 2016;49:936-941.

17. Yang WY, Efremov L, Mujaj B, Zhang ZY, Wei FF, Huang QF, Thijs L, Vanassche T, Nawrot TS, Staessen JA. Association of office and ambulatory blood pressure with blood lead in workers before occupational exposure. *J Am Soc Hypertens.* 2018;12:14-24.

18. Yu YL, Yang WY, Thijs L, Melgarejo JD, Yu CG, Wei DM, Wei FF, Nawrot TS, Zhang ZY, Staessen JA. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *Hypertension.* 2020;76:1299-1307.

19. Yu YL, Thijs L, Wei DM, Melgarejo JD, Yu CG, Yang WY, Roels HA, Zhang ZY. Two-year responses of renal function to first occupational lead exposure. *KI Reports.* 2022;7:1198-1209.

20. Gasowski J, Li Y, Kuznetsova T, Richart T, Thijs L, Grodzicki T, Clarke R, Staessen JA. Is "usual" blood pressure a proxy for 24-hour ambulatory blood pressure in predicting cardiovascular outcomes ? *Am J Hypertens.* 2008;21:994-1000.

21. Hilderink JM, van der Linden N, Kimenai DM, Litjens EJR, Klinkeberg LJJ, Aref BM, Aziz F, Kooman JP, Rennenberg RJMW, Bekers O, Koopmans RP, Meex SJR. Biological variation of creatinine, cystatin C,, and eGFR over 24 hours. *Clin Chem.* 2018;64:851-860.

22. Sirota JH, Baldwin DS, Villareal H. Diurnal variation of renal function in man. *J Clin Invest.* 1950;29:187-192.

23. Dyer AR, Martin GJ, Burton WN, Levin M, Stamler J. Blood pressure and diurnal variation in sodium, potassium, and water excretion. *J Hum Hypertens.* 1998;12:363-371.

24. Ainsworth BE, Haskel WL, Hermann SD, Meckes N, Bassett DR, Jr., Tudor-Locke C, Greer JL, Vezina J, Whitt-Glover MC, Leon AS. 2011 Compendium of Physical Activities: a second update of codes and MET values. *Med Sci Sports Exerc.* 2011;43:1575-1581.

25. Poortmans JR, Ouchinsky M. Glomerular filtration rate and albumin excretion after maximal exercise in aging sendentary and active men. *J Gerontol A Biol Sci Med Sci.* 2006;61:1181-1185.

26. Mingels A, Jacobs L, Kleijnen V, Wodzig W, van Dieijen-Visser M. Cystatin C a marker for renal function after exercise. *Int J Sports Med.* 2009;30:668-671.

27. Huang Y-Q, He G-D, Feng Y-Q. The association of lead exposure with blood pressure and hypertension: a mediation analyses of estimated glomerular filtration rate. *Environ Sci Pollut Res.* 2023;30:59689-59700.

28. Muntner P, He J, Vupputuri S, Coresh J, Batuman V. Blood lead and chronic kidney disease in the general United States population: results from NHANES III. *Kidney Int.* 2003;63:1044-1050.

29. Åkesson A, Lundh T, Vahter M, Bjellerup P, Lidfeldt J, Nerbrand C, Samsioe G, Stromberg U, Skerfving S. Tubular and glomerular kidney effects in Swedish

women with low environmental cadmium exposure. *Environ Health Perspect.* 2005;113:1627-1631.

30. Lai LH, Chou SY, Wu FY, Chen JJ, Kuo HW. Renal dysfunction and hyperuricemia with low blood lead levels and ethnicity in community-based study. *Sci Tot Environ.* 2008;401:39-43.
31. Harari F, Sallsten G, Christensson A, Petkovic M, Hedblad B, Forsgard N, Melander O, Nilsson PM, Borné Y, Engström G, Barregard L. Blood lead levels and decreased kidney function in a population-based cohort. *Am J Kidney Dis.* 2018;72:381-389.
32. O'Flaherty EJ. Physiologically based methods for bone-seeking elements. IV. Kinetics of lead disposition in humans. *Toxicol Appl Pharmacol.* 1993;118:16-29.
33. Leggett RW. An age-specific kinetic model of lead metabolism in humans. *Environ Health Perspect.* 1993;101:598-616.
34. Rabinowitz MB. Toxicokinetics of bone lead. *Environ Health Perspect.* 1991;91:33-37.
35. Oliveira S, Aro A, Sparrow D, Hu H. Season modifies the relationship between bone and blood lead levels : the Normative Aging Study. *Arch Environ Health.* 2002;57:466-472.
36. Korrick SA, Schwartz J, Tsaih SW, Hunter DJ, Aro A, Rosner B, Speizer FE, Hu H. Correlates of bone and blood lead levels among middle-aged abd elderly women. *Am J Epidemiol.* 2002;156:335-343.
37. Needleman HL. Review. The removal of lead from gasoline : historical perspective and personal reflections. *Environ Res.* 2000;84:20-35.
38. Nuyts GD, Elseviers MM, De Broe ME. Healthy worker effect in a cross-sectional study of lead workers. *J Occup Med.* 1993;35:387-391.
39. Alwan H, Pruijm M, Ponte B, Ackermann D, Guessous I, Ehret G, Staessen JA, Asayama K, Vuistiner P, Younes SE, Paccaud F, Wuerzner G, Pechere-Bertschi A, Mohaupt M, Vogt B, Martin P-Y, Burnier M, Bochud M. Epidemiology of masked and white-coat hypertension: the family-based SKIPOGH study. *PLoS One.* 2014;9:e2522.
40. Conen D, Aeschbacher S, Thijs L, Li Y, Boggia J, Asayama K, Hansen TW, Kikuya M, Björklund-Bodegård K, Ohkubo T, Jeppesen J, Gu YM, Torp-Pedersen C, Dolan E, Kuznetsova T, Stolarz-Skrzypek K, Tikhonoff V, Schoen T, Malyutina S, Casiglia

E, Nikitin Y, Lind L, Sandoya E, Kawecka-Jaszcz K, Mena L, Maestre GE, Filipovský J, Imai Y, O'Brien E, Wang JG, Risch L, Staessen JA. Age-specific differences between conventional and ambulatory daytime blood pressure values. *Hypertension.* 2014;64:1073-1079.

41. Asayama K, Thijs L, Staessen JA. Averaging clinic and home measures associated with reductions in within-patient variability and may give a better indication of blood pressure control. *Ann Intern Med.* 2011;154:781-788.

Data supplement

Expanded methods

Blood pressure measurement

At the study sites, trained nurses measured office blood pressure at the brachial artery. At the enrolment visit, in line with current guidelines [1], they measured upper arm circumference. Standard cuffs had a 12 × 24 cm inflatable portion, but if upper arm girth exceeded 31 cm, larger cuffs with 15 × 35 cm bladders were applied. Blood pressure readings were obtained at the non-dominant arm, unless at the enrollment visit the difference in systolic or diastolic blood pressure between both arms was 10 mmHg or more. In such case, the upper-arm cuffs were applied to the arm giving the highest blood pressure read-out. After the workers had rested for 5 minutes in the sitting position, the nurses measured office blood pressure to the nearest 2 mm Hg by auscultation of the Korotkoff sounds, using standard mercury sphygmomanometers. For analysis, the five readings were averaged. Heart rate was counted over 15 seconds. Terminal digit preference [2,3] and number preference [3] were the criteria applied for quality control of the office blood pressure. Terminal digit preference [2,3] refers to the observer rounding off the blood pressure reading to a digit of her or his choosing, most often five or zero. Number preference refers to the number of identical blood pressure values made by an observer within a series of consecutive blood pressure readings within a participant [3].

Office blood pressure were categorized according to the 2017 American College of Cardiology/American Heart Association (ACC/AHA) guideline [4]. Normotension and elevated blood pressure on office measurement were systolic levels of <120 mm Hg and 120-129 mm Hg and a diastolic level of <80 mm Hg. Stage-1 and stage-2 hypertension and severe hypertension were systolic or diastolic levels of 130-139/80-89 mm Hg, 140-159/90-99 mm Hg and ≥160/≥100 mm Hg, respectively. If systolic and diastolic blood pressure were in different categories, the highest level was used to categorize workers. To compare the blood pressure classifications, the thresholds of 2017 ACC/AHA guideline and 2018 European Society of Cardiology/European Society of Hypertension (ESC/ESH) guideline [5] were given in **Table S1**. Stage-1 and stage-2 hypertension thresholds in 2017 ACC/AHA guideline are comparable to the high normal blood pressure and grade-1 hypertension thresholds in 2018 ESC/ESH guideline [5].

Biochemical measurements

Venous blood samples were obtained after 8 to 12 hours of fasting. Blood lead levels were determined on whole blood by inductively coupled plasma mass spectrometry at an analytical laboratory certified for blood lead analysis in compliance with the provisions of the OSHA Lead Standard, 29CFR 1910.1025 (Occupational Safety and Health Administration [www.osha.gov]). Prior to analysis, the specimens were digested with nitric acid and spiked with an iridium internal standard. The detection limit was 0.5 mg/dL. The accuracy of the lead tests was verified by use of proficiency samples purchased from the College of American Pathologists (CAP) and the Pennsylvania Department of Blood Lead Programs [6]. Proficiency testing was performed in six separate trial runs, including in total 30 test samples annually. All survey materials were handled in the same manner as the study samples and processed with the normal workflow utilizing the same repeat/dilution protocols and calibration and quality control frequency [6]. Compliance with Clinical Laboratory Improvement Amendments (CLIA), CAP and New York State accreditation and regulatory requirements was verified routinely with test level review of the laboratory services by external auditors. Calibrators with certified accuracy (National Institute of Standards and Technology [www.nist.gov]) were included in each batch of study samples and spanned the range of the analytical measurement range. Accuracy was evaluated on Westgard Rules [7] and defined within the total allowable error established with review of the CAP, Centers for Disease Control and Prevention, CLIA 88 [8], and OSHA guidelines. Accuracy, defined as the deviation from known lead standards ran along with the study samples, was within 10% [6]. The bias determined according to the Bland and Altman approach [9] in 30 spilt blood samples with blood lead concentrations (average in duplicate samples) ranging from 0.70 to 27.9 mg/dL, was 0.08 mg/dL (95% confidence interval [CI], -0.01 to 0.18, p=0.078). The repeatability coefficient, defined as twice the SD of the signed differences between duplicate measurements [9] was 0.52. Expressed as a percentage of the mean blood lead concentration or as a percentage of near maximal variation in blood lead (four times the SD of the logarithmically transformed distribution), the repeatability coefficient was 6.7% and 1.9%, respectively. Lower values indicate better repeatability.

Total and high-density (HDL) lipoprotein serum cholesterol, serum creatinine, serum cystatin C, γ-glutamyltransferase (an index of alcohol intake), blood urea nitrogen, and blood glucose were measured by automated enzymatic methods, serum insulin by ELISA, and serum sodium by flame photometry. Serum creatinine was measured, using Jaffe's method with modifications in a single certified laboratory that applied isotope-

dilution mass spectrometry for calibration [10]. eGFR was derived from serum creatinine, serum cystatin C and both, using the Chronic Kidney Disease Epidemiology Collaboration equations [11]. Fresh urine samples were analyzed for urinary specific gravity and the albumin-to-creatinine ratio (ACR) defined as the urinary albumin in milligrams divided by urine creatinine in grams. Over three evaluations, the laboratory obtained a proficiency score of 100% for the BL measurements and 100% for routine biochemistry.

References

1. O'Brien E, Asmar R, Beilin L, Imai Y, Mallion JM, Mancia G, Mengden T, Myers M, Padfield P, Palatini P, Parati G, Pickering T, Redón J, Staessen J, Stergiou G, Verdecchia P, on behalf of the European Society of Hypertension Working Group on Blood Pressure Monitoring. European Society of Hypertension recommendations for conventional, ambulatory and home blood pressure measurement. *J Hypertens.* 2003; 21: 821-848.
2. Wingfield D, Cooke J, Thijs L, Staessen JA, Fletcher AE, Fagard R, Bulpitt CJ, on behalf of the Syst-Eur Investigators. Terminal digit preference in the Syst-Eur trial. *Blood Press Monit.* 2002; 7: 169-178.
3. Kuznetsova T, Staessen JA, Kawecka-Jaszcz K, Babeanu S, Casiglia E, Filipovský J, Nachev C, Nikitin Y, Peleská J, O'Brien E, on behalf of the EPOGH Investigators. Quality control of the blood pressure phenotype in the European Project on Genes in Hypertension. *Blood Press Monit.* 2002; 7: 215-224.
4. Whelton PK, Carey RM, Aronow WS, Casey DEJr, Collins KJ, Dennison Himmelfarb C, DePalma SM, Gidding S, Jamerson KA, Jones DW, MacLaughlin EJ, Muntner P, Ovbiagele B, Smith SCJr, Spencer CC, Stafford RS, Taler SJ, Thomas RJ, Williams KASr, Williamson JD, Wright JTJr. 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA guideline for prevention, detection, evaluation, and management of high blood pressure in adults: A report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol.* 2018; 71: e127-e248.
5. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, Clement DL, Coca A, de Simone G, Dominiczak A, Kahan T, Mahfoud F, Redon J, Ruilope L, Zanchetti A, Kerins M, Kjeldsen SE, Kreutz R, Laurent S, Lip GYH, McManus R, Narkiewicz K, Ruschitzka F, Schmieder R, Shlyakhto E, Tsioufis C, Aboyans V, Desormais I, Task Force Members. 2018 ESC/ESH Guidelines for the management

of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology and the European Society of Hypertension *J Hypertens.* 2018; 36: 1953-2041.

6. Centers for Disease Control and Prevention. Screening young children for lead poisoning: Guidance for state and local public health officials; Appendix C1: The Lead Laboratory (accessed 18 December 2018). 2018.
7. Westgard JO, Barry PL, Hunt MR, Groth T. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem.* 1981; 27: 493-501.
8. Centers for Disease Control and Prevention. CLIA '88 Focus on Clinic and Office Laborarories (accessed 18 December 2018). 1988.
9. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet.* 1986; 1: 307-310.
10. Myers GL, Miller WG, Coresh J, Fleming J, Greenberg N, Greene T, Hostetter T, Levey AS, Panteghini M, Welch M, Eckfeldt JH. Recommendations for improving serum creatinine measurement: a report from the laboratory working group of the National Kidney Disease Education Program. *Clin Chem.* 2006; 52: 5-18.
11. Inker LA, Schmid CH, Tighiouart H, Eckfeldt JH, Feldman HI, Greene T, Kusek JW, Manzi J, Van Lente F, Zhang YL, Coresh J, Levey AS, for the CKD-EPI Investigators. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med.* 2012; 367: 20-29.

Table S1. Classifications of Office Blood Pressure

Hypertension Category	Systolic, mm Hg		Diastolic, mm Hg
2017 ACC/AHA thresholds			
Normal	<120	and	<80
Elevated blood pressure	120-129	and	<80
Stage-1 hypertension	130-139	or	80-89
Stage-2 hypertension	≥140	or	≥90
2018 ESC/ESH thresholds			
Normal	120-129	and/or	80-84
High normal	130-139	and/or	85-89
Grade-1 hypertension	140-159	and/or	90-99
Grade-2 hypertension	160-179	and/or	100-109
Grade-3 hypertension	≥180	and/or	≥110

Abbreviations: ACC/AHA, American College of Cardiology/American Heart Association; ESC/ESH, European Society of Cardiology/ European Society of Hypertension. The ACC/AHA and ESC/ESH thresholds were published in references 4 and 5, respectively.

Table S2. Baseline and Follow-up Blood Pressure and Renal Function in 234 Workers

Characteristic	Baseline	Last follow-up	Δ (95% CI)	*P* value
Blood pressure				
Systolic blood pressure, mm Hg	119.3 (10.0)	124.2 (10.2)	4.91 (3.54, 6.28)	<0.0001
Diastolic blood pressure, mm Hg	79.2 (8.72)	80.6 (7.44)	1.38 (0.19, 2.59)	0.024
Mean arterial pressure, mm Hg	92.6 (8.54)	95.1 (7.35)	2.56 (1.47, 3.65)	<0.0001
Pulse pressure, mm Hg	40.1 (6.99)	43.6 (8.97)	3.52 (2.17, 4.87)	<0.0001
Renal function				
Serum creatinine, mg/dL	0.96 (0.17)	1.03 (0.18)	0.07 (0.05, 0.09)	<0.0001
Serum cystatin C, mg/L	0.67 (0.10)	0.73 (0.16)	0.05 (0.04, 0.07)	<0.0001
eGFRcrt, mL/min/1.73 m^2	106.4 (16.6)	98.0 (17.2)	-8.41 (-10.1, -6.68)	<0.0001
eGFRcys, mL/min/1.73 m^2	126.0 (12.6)	119.3 (19.5)	-6.77 (-8.74, -4.79)	<0.0001
Serum osmolality, mOsm/kg	286.8 (3.78)	288.6 (4.57)	1.77 (1.10, 2.45)	<0.0001
Urine specific gravity	1.02 (0.01)	1.02 (0.01)	0.22 (0.11, 0.33)	0.0001
ACR, mg/g	4.36 (2.83, 6.10)	4.67 (2.86, 6.88)	7.50 (-3.10, 19.3)	0.17

Values are arithmetic mean (SD) or geometric mean (IQR). Δ is the values of changes from baseline to the last follow-up visit. Changes from baseline to last follow-up are given with 95% confidence interval; for logarithmically transformed ACR changes are expressed as percentage. *P*-values denote the significance of the changes from baseline to last follow-up. eGFRcrt and eGFRcys refer to the glomerular filtration rate estimated from serum creatinine and serum cystatin C (reference 11). To convert serum creatinine from mg/dL to micromol/L, multiply by 88.42; to convert cystatin C from mg/L to nmol/L, multiply by 74.9.
Abbreviations: ACR, urinary albumin-to-creatinine ratio; IQR, interquartile range; SD, standard deviation.

Table S3. Blood Pressure Responses by Quartiles of the Follow-up-to-Baseline Blood Lead Ratio

Characteristic	Quartiles of the distribution of the follow-up-to-baseline blood lead ratio				*P* value
Fold change in blood lead	<1.88	1.88-3.17	3.17-5.46	>5.46	
Number in category	58	59	58	59	
Systolic blood pressure, mm Hg	5.54 ± 12.3	4.16 ± 9.17	3.68 ± 9.23	6.25 ± 11.6	0.78
Diastolic blood pressure, mm Hg	0.24 ± 9.19	2.13 ± 8.41	1.49 ± 8.90	1.67 ± 10.7	0.51
Mean arterial pressure, mm Hg	2.00 ± 8.49	2.80 ± 7.42	2.22 ± 7.85	3.20 ± 9.94	0.54
Pulse pressure, mm Hg	5.30 ± 12.5	2.03 ± 9.52	2.19 ± 9.40	4.58 ± 10.1	0.76

Reported values are arithmetic means (±SD) of the change in blood pressure from baseline to the last follow-up by quartiles of the follow-up-to baseline blood lead ratio. Office blood pressure was the average of five consecutive readings taken after workers had rested for 5 minutes in the sitting position. *P*-values are for linear trend across the quartiles of the follow-up-to-baseline blood lead ratio.

Table S4. Cross-Classification of Blood Pressure Status in 234 Workers

		Baseline						
		Normotension	Untreated Elevated Blood Pressure	Untreated Stage-1 Hypertension	Untreated Stage-2 Hypertension	Untreated Severe Hypertension	Treated Hypertension	All
Last Follow-Up	Normotension	35	8	10	2	0	0	55
	Untreated Elevated Blood Pressure	14	4	10	2	1	0	31
	Untreated Stage-1 Hypertension	42	13	47	11	0	0	113
	Untreated Stage-2 Hypertension	7	0	7	6	1	0	21
	Untreated Severe hypertension	0	0	0	0	0	0	0
	Treated hypertension	1	2	5	4	2	0	14
	All	99	27	79	25	4	0	234

Blood pressure thresholds are given in the Expanded Methods.

Table S5. Renal Function Responses by Quartiles of the Follow-up-to-Baseline Blood Lead Ratio

Characteristic	Quartiles of the distribution of the follow-up-to-baseline blood lead ratio				*P* value
Fold change in blood lead	<1.88	1.88-3.17	3.17-5.46	>5.46	
Number in category	58	59	58	59	
Serum creatinine, mg/dL	7.48 ± 12.2	8.71 ± 10.8	2.59 ± 15.1	6.20 ± 11.6	0.18
Serum cystatin C, mg/L	0.06 ± 0.12	0.03 ± 0.12	0.06 ± 0.15	0.07 ± 0.13	0.51
eGFRcrt, mL/min/1.73 m^2	-8.86 ± 11.7	-11.5 ± 12.4	-4.61 ± 15.8	-8.64 ± 13.0	0.34
eGFRcys, mL/min/1.73 m^2	-7.01 ± 13.3	-4.61 ± 14.3	-6.75 ± 17.6	-8.70 ± 15.7	0.42
Serum osmolality, mOsm/kg	1.60 ± 6.80	2.18 ± 4.55	1.83 ± 4.91	1.49 ± 4.60	0.83
Urinary specific gravity	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	0.56
ACR, %	0.01 ± 0.40	0.06 ± 0.36	0.02 ± 0.30	0.04 ± 0.34	0.87

eGFRcrt and eGFRcys refer to the glomerular filtration rate estimated from serum creatinine and serum cystatin C (reference 11). Reported values are arithmetic means (±SD) of the changes in renal function from baseline to the last follow-up by the quartiles of the follow-up-to-baseline blood lead ratio. For logarithmically transformed ACR, changes are expressed as percentage. *P*-values are for linear trend across the quartiles of the follow-up-to baseline blood lead ratio. To convert serum creatinine to micromol/L, multiply by 88.42; to convert cystatin C from mg/L to nmol/L, multiply by 74.9.
Abbreviation: ACR, urinary albumin-to-creatinine ratio.

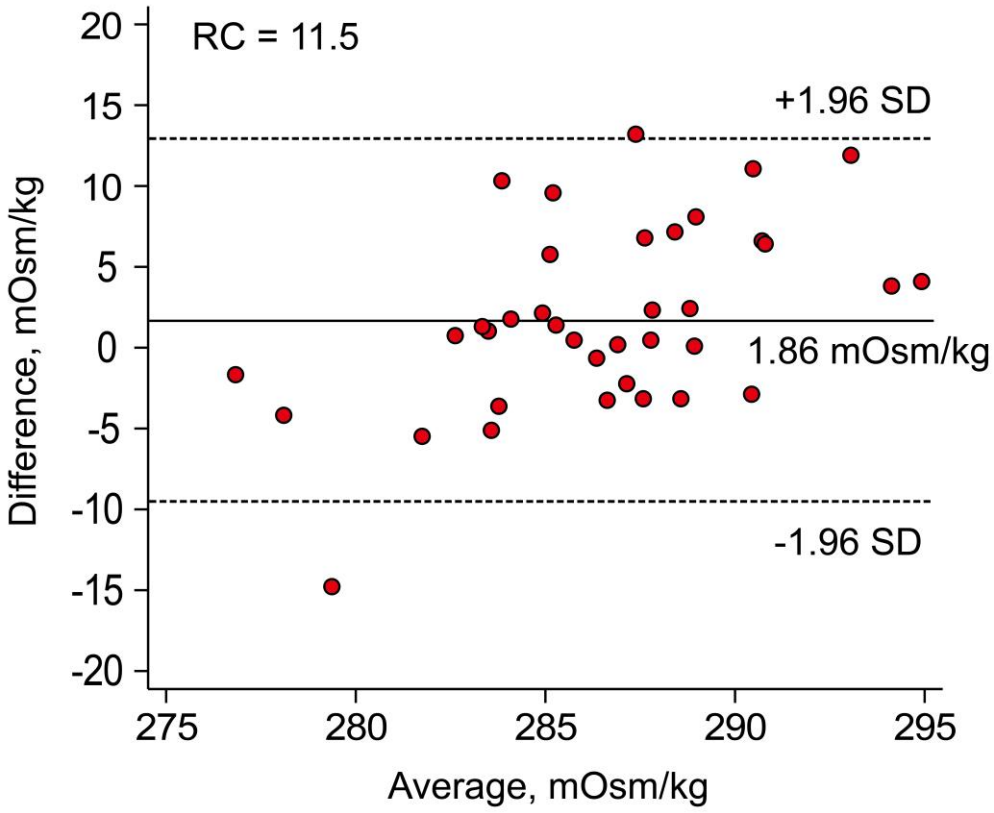


Figure S1. Bland-Altman Plot for Duplicate Serum Osmolality Measurements in 37 Workers. Abbreviations: SD, standard deviation; RC, reproducibility coefficient. The difference between the calculated and measured serum osmolality was plotted against the average of both measurements. The bias (measured minus calculated serum osmolality) was 1.86 mOsm/kg (*P*=0.057). Calculated serum osmolality (mOsm/kg) = 2 × (serum Na+ [mmol/L]) + (blood glucose [mg/dL] / 18) + (blood urea nitrogen [mg/dL] / 2.8). The RC is twice the SD of the signed differences between duplicate measurements.

Chapter 2

Unhealthy ageing in relation to lead exposure

Part 3

Health risks related to environmental and occupational lead exposure

Yu-Ling Yu*, De-Wei An*, Wen-Yi Yang, Dong-Yan Zhang,
Dries S. Martens, Tim S. Nawrot, Jan A. Staessen

* Joint-first authors.

Previously published in:
Kardiologia Polska. 2025. doi: 10.33963/v.phj.104575.
(Online ahead of print)

Abstract

Lead is a ubiquitous toxic metal. This review summarises the toxicokinetics and toxicology of lead, the methods to measure blood lead (BL) and summarises recently published studies, and ends with an appraisal of recent regulations to protect exposed workers. Lead exposure runs via inhalation of lead-contaminated particulate and via gastrointestinal uptake. To assess BL, inductively coupled plasma mass spectrometry and electrothermal atomic absorption spectrometry are commonly applied. BL in the general population declined drastically, approaching pre-industrial levels, at least in developed nations. Using cardiovascular, renal end neurological endpoints, SPHERL, a longitudinal study in a lead-exposed workers demonstrated that current regulations effectively protect against detrimental effects. FLEMENGHO, a Flemish population study, showed how BL declined over the past 20 years and reported on the association of renal and left ventricular function with BL. A re-analysis of NHANES data representative of American adults accounted for the drastic BL fall over time (1999-2020) and produced for cardiovascular mortality estimates of the BL-attributable risk fraction and the number of avoidable deaths that were respectively >3 and >7 times smaller than in earlier analyses, which ignored the BL decline. The worldwide GBD studies published in 2012 and 2021 were inconsistent in considering the BL-associated cardiovascular endpoints and the null-effect BL level that decreased from 2.5 to 0.016 μg/dl. In conclusion, there is large progress in reducing occupational and environmental lead exposure, but designing and reinforcing stricter regulations should rank high on the agenda of policy makers, in particular in low- and middle-income countries.

Introduction

Among toxic metals threatening human health, lead is by far the prime contaminant in this class. Historically, sources of lead exposure comprised pyrolytic zinc ore refinement, indoor and outdoor paints, fuels to which lead was added as antiknock agent, conduits distributing drinking water, and the usage of lead as corrosive-resistant building material. Drafting and reinforcing regulations banning lead in most applications took until the end of the previous century in the developed world, but legislation or its enforcement lag behind in middle- and low-income countries [1]. Lead is still being widely used in manufacturing lead-acid batteries and ammunition. In Western countries, coarse and fine particulate containing lead deposited over the previous centuries remains a prime source of exposure (**Figure 1**) [2], whereas in other countries the gastrointestinal route is an exposure vector due to the environmental contamination of food or water [2,3]. In developed nations, because of the tightening legislation regulating lead exposure, the mean blood lead concentration in adults drawn from the general population currently decreased to approximately 1.5 μg/dl, which is close to the estimated blood lead concentration in pre-industrial humans (2 μg/dl), only exposed to natural sources, as estimated by the Global Burden of Disease (GBD) Consortium [4].

There is currently no consensus whether or not low-level lead exposure raises blood pressure (BP) or induces kidney dysfunction. This review builds on 40 years of research on the health effect of lead exposure, starting with a pilot project in a small Belgian town [5], continuing with multiple cross-sectional [6-8] and longitudinal [9-11] analyses of a random population sample recruited in a defined area in Northern Belgium including municipalities with a high historical exposure to heavy metals due to the emissions of pyrolytic zinc smelters and neighboring municipalities with low exposure, studies in youth [12], systematic literature reviews [13,14], studies in London civil servants [15], analyses focusing on adults representative of the population in the United States [16,17], and culminating in a longitudinal study of newly hired workers without know previous lead exposure employed in battery recycling plants in the United States [18-22]. The latter study was previously extensively reviewed in this *Journal* [23], but for completeness will again be shorty summarised in the section of this review dealing with the potential adverse health effects of lead exposure. From this 40-year research perspective, the aims of the present review were to summarise the toxicokinetics and toxicology of lead in humans, the methods for measuring the lead body burden, the health risks associated with the contemporary low exposure levels as observed in developed countries, and the

regulations to mitigate the health-related effects of environmental and occupational exposure.

Lead toxicokinetics in humans

Depending on urbanization, dietary habits, socio-economic position, employment in lead recycling plants or leisure time activities that involve soldering, exposure occurs via the respiratory or gastrointestinal route. Respirable fine particulate is the main vector of exposure of the general population [24]. Once the finest dust particles reach the lung alveoli, they readily pass the air-blood barrier and are subsequently system-wide distributed via the blood stream. Occupational exposure entails coarser aerosols that deposit in the upper airways and then translocate to the gastro-intestinal tract by mucociliary clearance, where gastrointestinal uptake kinetics prevail (5-10% uptake). The lead in air to BL slope is around 2 for ambient and 0.05 for occupational exposure [24].

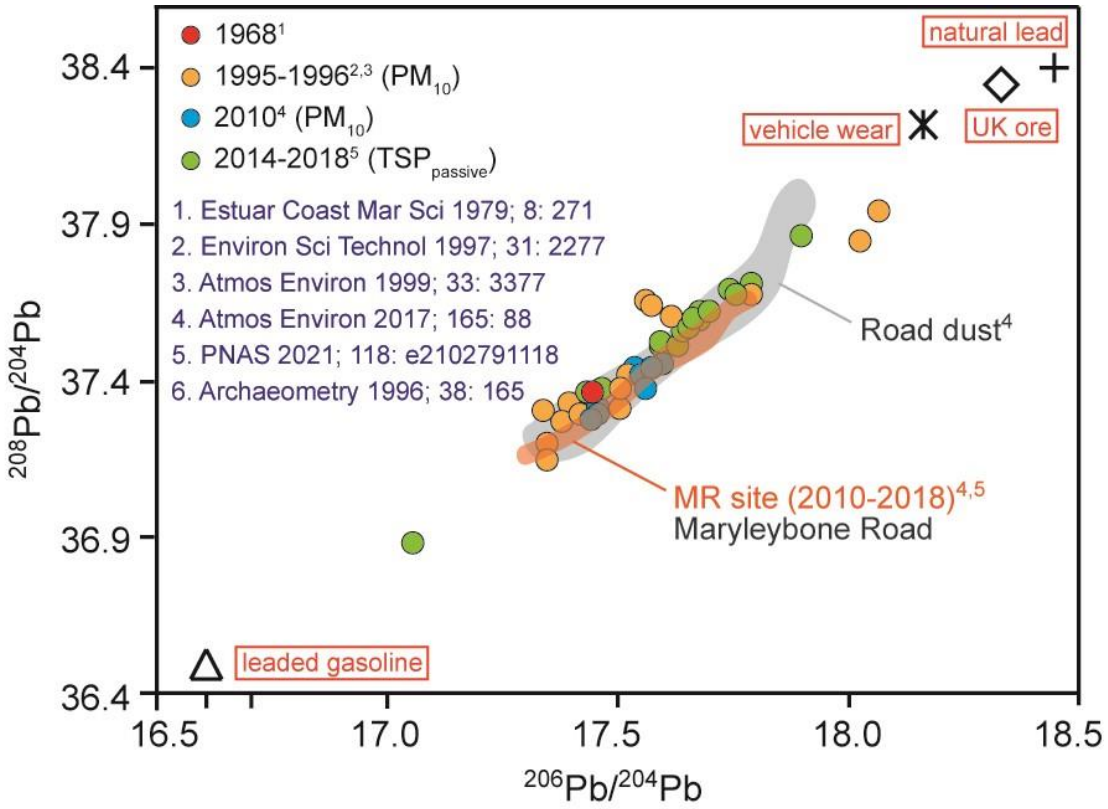


Figure 1. Contribution of 20th Century Lead Deposition to Atmospheric Environment in London. Marleybone Road is a thoroughfare with very heavy traffic in central London within the City of Westminster. The isotopic ratios of lead in dust particles allow to identify the origin of the lead contamination. Reproduced from reference 2.

Lead is a cumulative toxicant, 90-95% of which is stored in bone, from where it is recirculated with a half-life of 20-25 years [25,26]. Blood lead (BL), for 99% carried by red blood cells, reflects recent exposure over the past 1-2 months and the amount of lead released and recirculated from bone stores [25]. Both bone [26,27] and blood [6,26,27] lead increase with advancing age. Bone lead is associated with blood lead [26,27] and explains around 20% of its variance, depending on seasonality [26] and hormonal and other endogenous and environmental stimuli influencing the balance between bone formation and resorption [27]. Recirculation of lead from bone explains why there is a lag time for BL to decline when environmental [9] or occupational [25] lead exposure decreases.

Pathophysiology

The prime pathophysiological mechanism relating adverse health outcomes to lead exposure is by causing hypertension and hypertension-related cardiovascular and renal complications. However, in a meta-analysis of summary statistics extracted from 31 studies and involving 58,518 participants, BL doubling was only associated with a marginally higher systolic/diastolic blood pressure (BP): 1.0/0.6 mmHg (95% confidence interval [CI]: 0.5-1.4/0.4-0.8 mmHg). All studies pooled in this meta-analysis were published before stringent laws regulating lead exposure came into effect (2001).

A multivariable-adjusted analysis of the 2003-2010 data from the National Health and Nutrition Examination Survey (NHANES) showed ethnic inconsistency in the associations of BP with BL, which probably reflected unmeasured confounding and which excluded that environmental lead exposure is a major cause of hypertension in American adults [16]. The 12,725 participants included 21.1% Blacks, 20.5% Hispanics, and 58.4% Whites. Blacks compared to non-Blacks had higher systolic/diastolic BP (126.5/71.9 *vs* 123.9/69.6 mmHg) and higher hypertension prevalence (44.7% *vs* 36.8%). Blood lead was lower in Whites than in non-Whites (1.46 *vs* 1.57 µg/dL). BL doubling was associated with higher systolic/diastolic BP (0.76/0.43 mmHg; CI: 0.38-1.13/0.18-0.68 mmHg), but not with the odds of hypertension (0.95; 0.90-1.01) [16]. Furthermore, a systematic review of five cross-sectional studies demonstrated that systolic BP (0.26 mmHg; CI: 0.02-0.50 mmHg) and the hypertension risk (odds ratio: 1.04; CI: 1.01-1.07) were associated with lead levels in bone (+10 µg/g) [28]. However, according to the same researchers, the evidence relating cardiovascular complications to BL was suggestive at best and insufficient to infer causality [29].

Other mechanisms potentially underpinning the association of cardiovascular disease with lead exposure are speculative and do not carry the weight of the evidence in

humans, not the least because experimental studies in cell or animal models are difficult to extrapolate [30]. Of the multiple mechanisms proposed to play a role are oxidative stress and inflammation, for instance leading to neurotoxicity, endothelial dysfunction or defective DNA repair [31,32]. Lead can substitute or compete with essential divalent cations, preferentially Ca^{2+} [31,32], thereby interfering with signal transmission in the central and peripheral nervous system and the electromechanical coupling in muscles. Finally, individual susceptibility to lead toxicity also depends on several single nucleotide polymorphisms, epigenetic modifications and interference with regulatory RNA molecules [32].

In addition, the kidneys play an important role in the urinary clearance of lead. Lead accumulates in the kidney, resulting at high exposure levels in renal function decline and nephropathy. However, the underlying mechanisms are not well understood and might involve glomerular hypertrophy, infiltration of the renal tissue leukocytes, inflammation, interstitial fibrosis and tubular atrophy [33]. Several studies of populations [34-37] or workers [38] in Europe [6,35], the United States [36-38] and Taiwan [39], reported an inverse association between the glomerular filtration rate estimated from serum creatinine (eGFR) and BL or an increased risk of chronic kidney disease in relation to BL.

Measurement of blood lead

As suggested by World Health Organization [40], the most commonly used laboratory methods for assessing BL are inductively coupled plasma mass spectrometry (ICP-MS) and electrothermal atomic absorption spectrometry (ETAAS). Flame atomic absorption spectrometry (FAAS) can also be used, but FAAS cannot be applied to reliably assess BL in people whose BL is less than 5 µg/dl. Other methods are not frequently used in clinical practice, such as inductively coupled plasma optical emission spectrometry [40].

The Study for the Promotion of Health in Recycling Lead (SPHERL) [41] provides a methodology for the quality control of the BL measurement. SPHERL involved newly hired workers without known previous lead exposure employed at battery manufacturing and lead recycling plants in the United States. At all factories, the medical facilities where blood samples were obtained were located at sites separate from the production lines. The study nurses obtained venous blood samples after participants had fasted for 8 hours. The materials used for blood collection, including test tubes, needles and caps, were certified as being lead free (Becton, Dickinson and Company, Franklin Lakes, NJ). The nurses thoroughly cleansed the brachial venipuncture site and kept the tubes for the measurement of BL closed. BL was determined by ICP-MS at a single laboratory certified for this analysis in compliance with the provisions of the OSHA Lead Standard,

29CFR 1910.1025 (Occupational Safety and Health Administration). This laboratory participated in the US CDC Blood Lead Proficiency Testing Program. Prior to analysis, specimens were digested in nitric acid and spiked with an iridium internal standard. The limit of detection was 0.5 μg/dL. The deviation from known lead standards analyzed along with the samples in each test run was less than 10% [20,41].

Health risks

The health risks associated with lead exposure were reviewed in workers enrolled in SPHERL [22], in adults representative for the population in the United States enrolled in successive cycles of the National Health and Nutrition Examination Survey (NHANES) [42,43], in randomly recruited people in the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) [44], and in publications of the Global Burden of Disease (GBD) publications [45-48]. To our knowledge, SPHERL is the first prospective study that accounted for interindividual variability between people in their vulnerability to the toxic effects of human lead exposure by assessing the participants' health status before and after lead exposure [41] and will therefore be reviewed in greater detail than the other studies.

Environment and Health Study

The goal of the project "*Milieu en Gezondheid*" (Environment and Health) was to explore the feasibility of monitoring exposure to cumulative environmental pollutants by examining youth instead adults [12]. The rational was that at young age cumulative toxins in blood and urine reflect recent exposure, whereas at more advanced age measured levels are predominantly determined by the accrual of the contaminant over the years lived, so that they are no longer indicative of recent exposure. Of the 200 17-year olds, 100 resided near a waste incinerator and a lead processing plant and 100 in a rural area with low exposure levels [12]. Cystatin C in serum and β_2-microglobulin in urine were measured as biomarkers of glomerular and renal tubular function, respectively. BL was higher in the exposed youth (4.2 *vs* 3.5 μg/dl; p=0.04). With adjustments applied for sex, smoking, mean atmospheric ozone concentration, and mean daily temperature in the week before blood samples, the association sizes for a doubling of BL were +3.6% (95% CI: +1.5 to +5.7%; P<0.0001) for cystatin-C and +16.0% (+2.7 to +31.0%; P=0.02) for β_2-microglobulin in urine.

FLEMENGHO

Recruitment for the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) started in 1985 [44]. From August 1985 until November 1990, a random

sample of the households living in a geographically defined area of Northern Belgium was investigated with the goal to recruit an equal number of participants in each of six subgroups by sex and age (20-39, 40-59, and ≥60 years). All household members with a minimum age of 20 years were invited to take part, provided that the quota of their sex-age group had not yet been satisfied. From June 1996 until January 2004 recruitment of families continued, using the former participants (1985-1990) as index persons and also including teenagers.[49] The participants were repeatedly followed-up. In all study phases, the same standardised methods were applied to measure clinical and biochemical variables, to administer questionnaires, and to ascertain the incidence of fatal and nonfatal outcomes.

In an article published in 1992 [6], mean BL was 7.5 µg/dl in 1016 women and 11.4 µg/dl in 965 men. In sex-stratified analyses, a 10-fold higher BL was associated with a 13 and 10 ml/min lower creatinine clearance in women and men, respectively. However, given that approximately 70% of lead excretion occurs via the urine [50,51], observational studies with a cross-sectional design, such as the early FLEMENGH article [6] and other reports [35-39] cannot ascertain the directionality of the association between eGFR and BL, reduced eGFR being associated with less urinary lead excretion and higher BL, or conversely higher BL being associated with a decline in eGFR. Several other FLEMENGHO reports published around the same time could not demonstrate any association of BL with BP or the prevalence or hypertension or cardiovascular disease [7,8]. However, in a prospective FLEMENGHO analysis of 728 individuals [9], BP was measured by auscultation at baseline (1985-1989) and at follow-up (1991-1995) and by 24-h ambulatory BP monitoring at follow-up. Over 5.2 years, BL dropped by 32% from the baseline level of 8.7 µg/dL (range: 1.7-72.5 µg/dL). Changes in the auscultatory BP were unrelated to BL at baseline and the BL changes over follow-up. Along similar lines, the 24-h ambulatory BP was not related to BL at baseline or follow-up.

In 179 FLEMENGHO participants [10], BL and the 24-h urinary cadmium excretion (UVCD) at baseline (1985-2000) averaged urinary 4.14 µg/dl and 0.68 µg, respectively. Systolic and diastolic left ventricular (LV) function was assessed 11.9 years (median) later (2005-2010) by Doppler imaging of the transmitral blood flow and the mitral annular movement and speckle tracking. In multivariable-adjusted linear regression, LV systolic function decreased with BL. For a doubling of exposure, estimates were -0.392% for global longitudinal strain (P=0.034), -0.618% and -0.113 s^{-1} for regional longitudinal strain (P=0.028) and strain rate (P=0.008), and -0.056 s^{-1} for regional radial strain rate (P=0.050). Regional longitudinal strain rate (-0.066 s^{-1}; P=0.009) and regional radial

strain (-2.848%; *P*=0.015) also decreased with UVCD. Models including both exposure indexes did not allow differentiating whether LV dysfunction was predominately related to BL or UVCD. Diastolic LV function was not associated with BL or UVCD (*P*≥0.159). Although effect sizes were small, these FLEMENGHO results suggested that environmental exposure to lead, cadmium, or both might be a forerunner of heart failure, particularly in susceptible patients with comorbidities [10].

In view of the potential role of fine particulate as transmission vector in lead exposure, air pollutants were also investigated in a further FLEMENGHO study [11]. In 671 participants (51.7% women; mean age, 50.4 years), the annual exposures to black carbon, $PM_{2.5}$, PM_{10} and NO_2 averaged 1.19, 13.0, 17.7, and 16.8 mg/m3 (**Figure 2**). Systolic left ventricular function was worse (*P*≤0.027) with higher black carbon, $PM_{2.5}$, PM_{10} and NO_2, and mitral E and a' peak velocities were lower (*P*≤0.021) with higher black carbon, $PM_{2.5}$ and PM_{10}. Moreover, systemic inflammation was identified as a possible mediator of associations with black carbon [11].

NHANES

Analysis of the NHANES III data (1988-1994) [43] illustrates how metrics covering the adverse health effects of lead exposure in the general population are dependent on assumptions [14]. The hazard ratio (HR) relating mortality over 19.3 years of follow-up to a baseline BL increment from the 10th-90th percentile (1.0-6.7 µg/dL) was 1.37 (95% CI: 1.17-1.60). The BL-associated population-attributable risk fraction (PAF) was 18.0% and the number of attributable deaths (NAD) 412,000 per year. This NHANES III analysis inflated estimates by the range for which association sizes were computed and completely ignored the drastic fall in BL among US adults [43]. Surprisingly given the pathophysiology of lead-associated hypertension, the findings were not affected by adjustment for BP or hypertension [43].

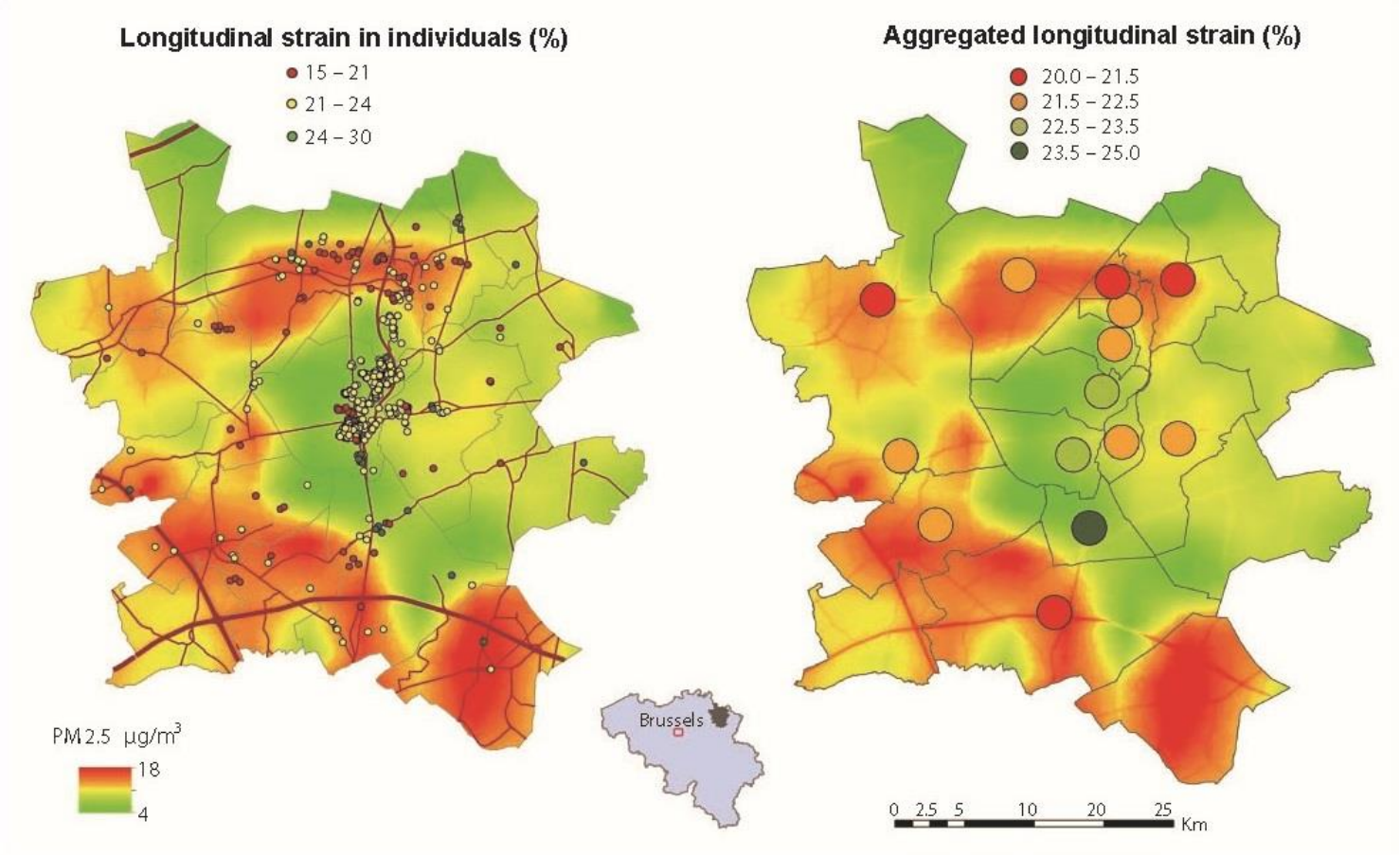


Figure 2. Geographical Association of Multivariable-adjusted Longitudinal LV Strain at the Individual Level or Aggregated per Municipality were Associated with PM2.5 Air Pollution Contours. Grey lines indicate borders of municipalities. Red lines represent the air pollution contours of major roads. The Spearman rank correlation between longitudinal LV strain and exposure to PM2.5 was -0.13 (*P*=0.0005) and -0.90 (*P*<0.0001) in individual and aggregated data, respectively. Abbreviations: LV: left ventricular; PM: particulate matter. Reproduced from reference 10.

A recently published analysis of the 2-year NHANES examination cycles from 1999 until 2020, including 34,806 individuals, accounted for the drastic fall in BL and included a mediation analysis considering the direct pathway relating BL to mortality and the indirect pathways running via BP or socioeconomic status (SES) [42]. In time-stratified analyses, BL decreased from 1.76 μg/dL in 1999-2004 to 0.93 μg/dL in 2017-2020, while the proportion of individuals with BL <1 μg/dL increased from 19.2% to 63.0%. Total mortality was unrelated to BL (HR for a 4-fold BL increment: 1.05 [95% CI: 0.93-1.17]). The HR for cardiovascular death was 1.44 (95%CI: 1.01-2.07) in the 1999-2000 cycle, but lost significance thereafter. BL was directly related to cardiovascular mortality, whereas the indirect BL pathway via BP was not significant. Low SES was directly related to BL and cardiovascular mortality, but the indirect SES pathway via BL lost significance in 2007-2010. From 1999-2004 to 2017-2020, the PAF for cardiovascular mortality decreased from 7.8% (0.17-14.4%) to 2.5% (0.05-4.7%) and NAD from 53,878 to 7539 deaths [42]. The findings of this study may be applicable to developed regions such as the European Union, where repeated BL measurement in the same population showed a declining trend over 20 years (**Figure 3**). However, their generalizability to low-or middle-income countries in Africa or South-East Asia may be limited.

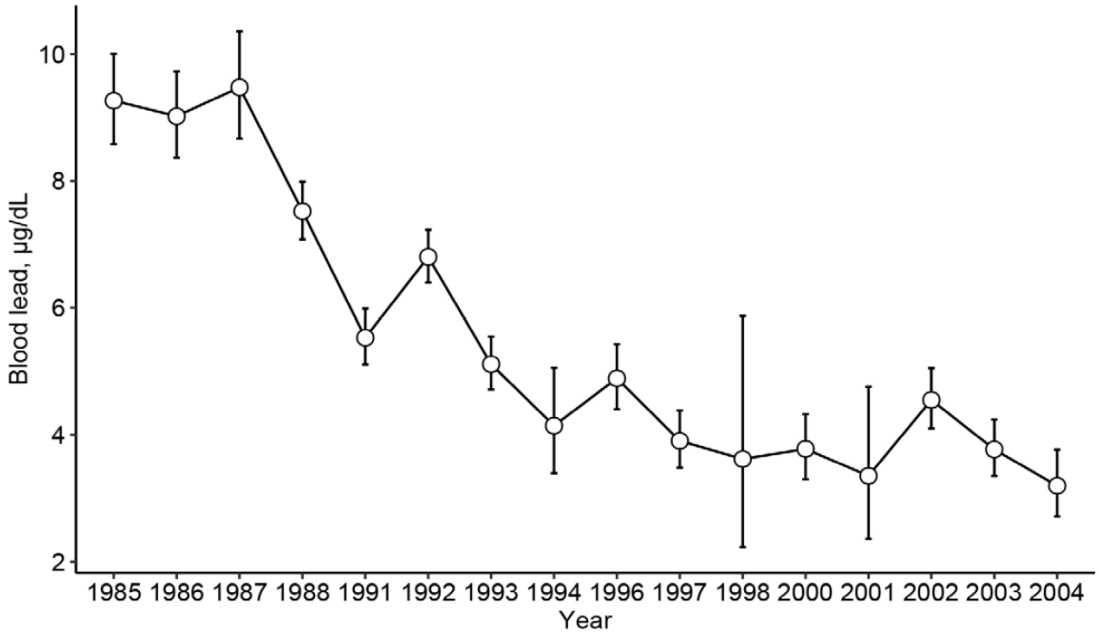


Figure 3. Secular Trend of Blood Lead Level of Participants with Repeated Measures in FLEMENGHO Study. Shown is the geometric mean and standard error of blood lead. Abbreviation: FLEMENGHO: Flemish Study on Environment, Genes and Health Outcomes. Reproduced from reference 42.

GBD Publications

The Global Burden Disease (GBD) study 1999-2010 proposed that from the age of 25 years onwards, there is a causal association between systolic BP and lead exposure [45]. High BP was the leading single risk factor globally, accounting for 9.4 million deaths (95% uncertainty interval [UI]: 8.6-10.1 million) and 7.0% (UI: 6.2-7.7%) of global disability-adjusted life-years (DALYs), which account for both years lived with a disability and the years of life lost [45]. For environmental lead exposure, these estimates were 0.67 million deaths (UI: 0.58-0.78 million) and 0.56% of DALYs lost (UI: 0.47-0.66%) [45]. The GBD investigators listed among possible limitations of their result: (i) residual confounding; (ii) uncertainty as to the extent to which the effect sizes were generalizable; (iii) and the impossibility to account for temporal changes in the exposure to risk factors [45]. Furthermore, the issue of residual confounding requires calculating PAF for clusters of risk factors, rather than for a single risk indicator. Indeed, cardiovascular risk factors and exposures to various environmental pollutants cluster within individuals. Later GBD [46] or GBD-associated [47,48] publications moved the focus of lead-associated adverse health outcomes from hypertension to ischemic heart disease and suggested that the pre-industrial blood lead concentration in man might have been as low as 0.016 μg/dl, based on extrapolation by linear regression of BL on bone lead in data obtained from environmentally or occupationally exposed humans. One wonders why stroke, the complication most closely associated with the BL level was not considered as primary endpoint rather than ischemic heart disease and how blood and bone samples collected in the early 1990's and the linear extrapolation might justify the 0.016 μg/dl BL level, which with the current technology is even not measurable.

SPHERL

The Study for the Promotion of Health in Recycling Lead (SPHERL [NCT02243904]) is the first prospective study, which monitored the health effects related to occupational lead exposure in newly hired workers without known previous exposure [22]. The co-primary endpoints, for which SPHERL was powered [41], were the changes in BP and renal function. The secondary endpoints included the autonomous nervous regulation of the cardiovascular system, as captured by heart rate variability (HRV), neurocognitive function and peripheral nerve conductivity. The workers underwent follow-up visits 1 and 2 years after enrolment. Detailed flow charts describing the selection of workers analyzed for each endpoint have been published [18-22].

For BP and renal function [22], the 234 workers analyzed were on average 28.5 years old and included 91.9% men. The baseline BL concentration was 4.35 μg/dL and

increased 3.2-fold over 2 years of follow-up (range: 0.92-6.45 years). The changes in BP and renal function were not significantly correlated with the follow-up-to-baseline BL ratio. The fully-adjusted changes in systolic/diastolic BP associated with a doubling of BL were -0.25/-0.12 mmHg (CI: -0.94 to 0.44/-0.66 to 0.42 mmHg). Heat maps (**Figure 4**) demonstrated that the changes in BP were mainly determined by the baseline BP ($P<0.0001$), while the change in BL did not reach significance in these analyses ($P\geq0.47$). Accordingly, in the unadjusted, adjusted and fully adjusted analyses, the hazard ratios (HRs) for incident hypertension were not significant ($p\geq0.063$). Similarly, the changes in eGFR derived from serum creatinine or serum cystatin C were not related to the follow-up-to-baseline BL ratio, amounting to -0.70 ml/min/1.73m^2 (CI: -1.70 to +0.30 ml/min/1.73 m^2) and to -1.06 mL/min/1.73 m^2 (-2.16 to +0.03 mL/min/1.73 m^2), respectively. Heat maps (**Figure 5**) indicated that the changes in renal function were mainly determined by the baseline eGFR ($P\leq0.0011$), while the change in BL did not reach significance in these analyses ($P\geq0.057$).

HRV was measured from 5-minute ECG recordings in the supine and standing positions, using the Cardiax software, V.4.14.0 (International Medical Equipment Development, Budapest, Hungary). The analyses of heart rate and HRV [19] included 195 workers (91.3% men; mean age, 27.8 years). Study participants with irregular heart rhythm or taking antihypertensive agents or neuro-active drugs (benzodiazepines, neuroleptics, anti-epileptics, sympathomimetics, amphetamines, and recreational drugs) were excluded from analysis. In analyses stratified by quartiles of blood lead changes, trends in heart rate and in HRV derived by the Fourier or autoregressive approach did not reveal a dose-response curve [19]. In multivariable-adjusted mixed models, heart rate, and HRV were unrelated to BL with no differences between baseline and follow-up. Along similar lines, the orthostatic heart rate responses were not altered by increasing lead exposure. Thus, an over 3-fold BL increment did not affect autonomous neural function, as captured by HRV [52].

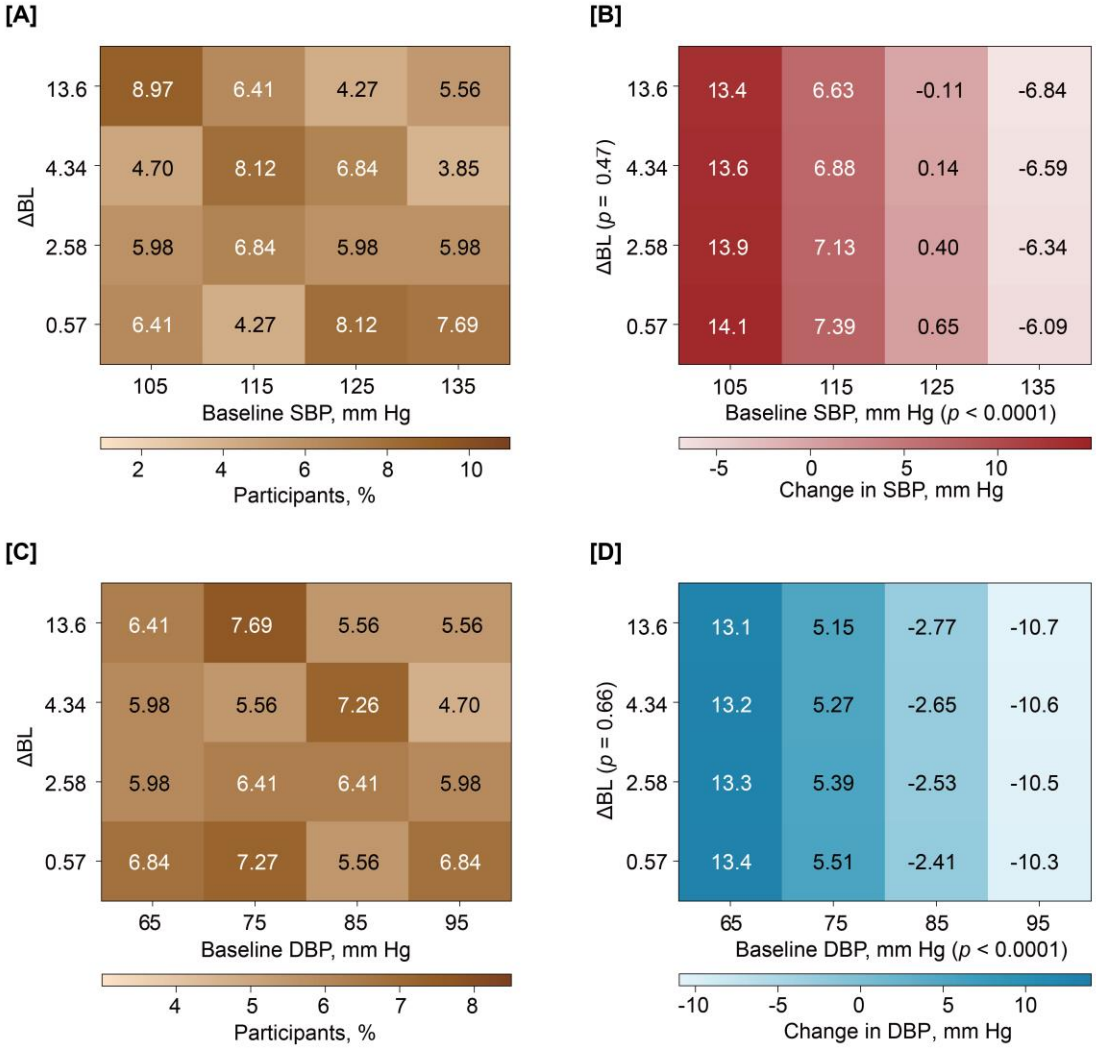


Figure 4. Heat Maps Relating the Change in Systolic and Diastolic Blood Pressure to the Blood Pressure Level at Baseline and the Change in Blood Lead from Baseline to Last Follow-Up. Abbreviations: ΔBL: change in blood lead; SBP: systolic blood pressure; DBP: diastolic blood pressure. For SBP (B) and DBP (D), the associations were derived by mixed models accounted for sex, age, BP and body mass index at baseline, ethnicity (white vs other), change in body weight during follow-up, and the baseline values of and the changes during follow-up in heart rate, smoking status, total-to-HDL serum cholesterol ratio, γ-glutamyltransferase, and serum creatinine. All models were fully adjusted. The percentage of participants (A and C) contributing to the cross-classification between the baseline blood pressure (horizontal axis) and the fold change in BL (vertical axis) are given for each analysis run. Reproduced from reference 22, which was published was an open-access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License.

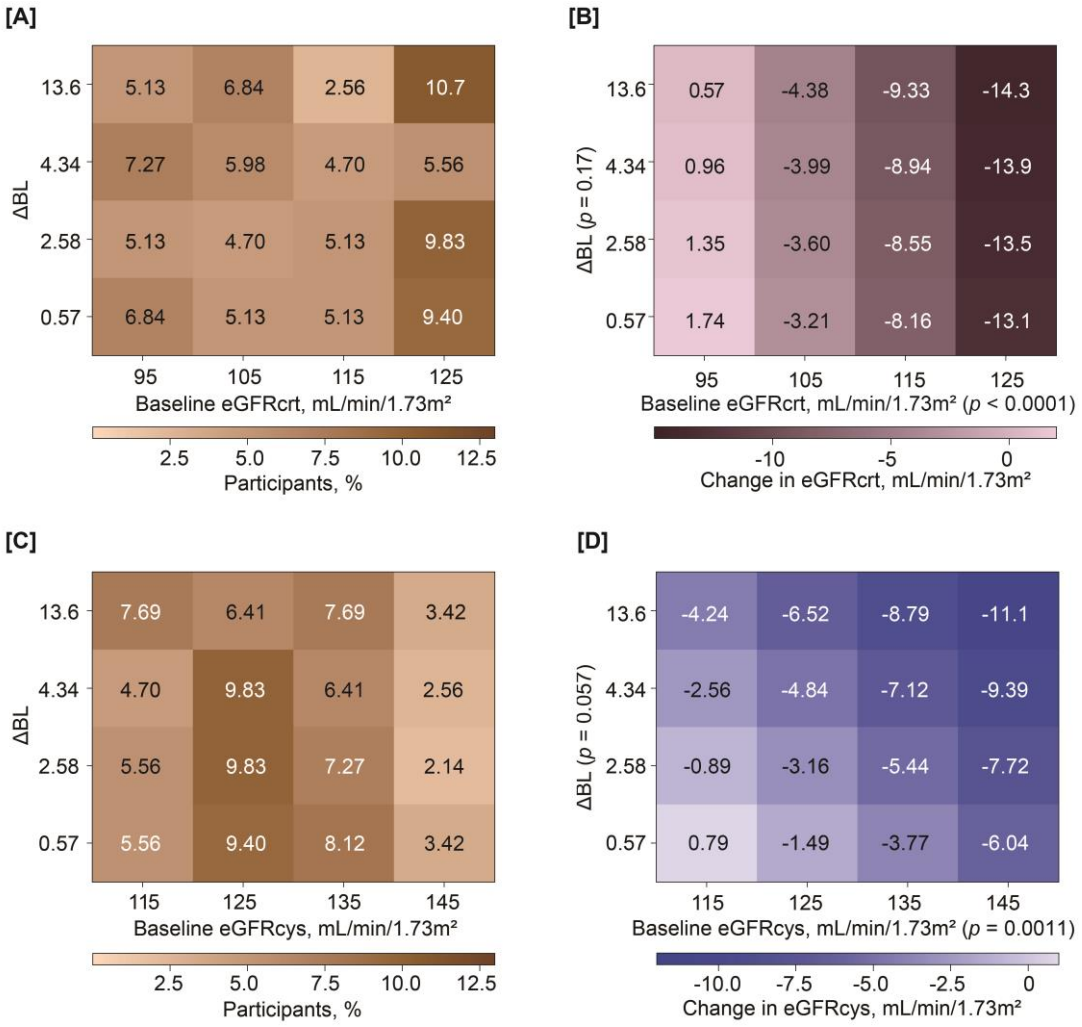


Figure 5. Heat Maps Relating the Change in eGFRcrt and eGFRcys to the eGFR Level at Baseline and the Change in Blood Lead from Baseline to Last Follow-Up. Abbreviations: ΔBL: change in blood lead; eGFRcrt: the glomerular filtration rate estimated from serum creatinine; eGFRcys: the glomerular filtration rate estimated from serum cystatin C. For eGFRcrt (B) and eGFRcys (D), the associations were derived by mixed models, sex, age, follow-up duration, the time of day of blood sampling (nighttime vs daytime), the baseline renal function measure being analyzed, baseline body mass index, change in body weight, and the baseline values of and changes during follow-up in smoking status, mean arterial pressure (diastolic BP plus one third of the difference between systolic and diastolic BP, the total-to-HDL cholesterol ratio and γ-glutamyltransferase, and changes in antihypertensive medication (yes vs no) during follow-up. All models were fully adjusted. The percentage of participants (A and C) contributing to the cross-classification between the baseline blood pressure (horizontal axis) and the fold change in BL (vertical axis) are given for each analysis run. Reproduced from reference 22, which was published was an open-access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License.

Nerve conduction velocity (NCV) is a common test of the function of the peripheral nervous system in lead-exposed workers [53,54]. The study nurses used a handheld device and related software (Brevio Nerve Conduction Monitoring System, NeuMed, West Trenton, NJ) to stimulate the left and right median nerve at a gradually increasing voltage, until the maximum compound motor action potential of the short thumb abductor muscle was reached [52]. Peripheral NCV was assessed in 192 workers. From the lowest to the top quartile of the distribution of the follow-up-to-baseline blood lead concentration ratio, the percent changes in latency time were 2.91% (CI: -0.89 to 6.85%; *P*=0.14), 0.86% (CI: -2.48 to 4.32%; *P*=0.064), 2.14% (CI: -0.86 to 5.23%; *P*=0.057), and 2.57% (CI: -0.93 to 6.19%; *P*=0.67); the *P*-value for linear trend was 0.98. The percent changes in latency time associated with a doubling of blood lead from baseline to follow-up were -0.05% (CI: -0.57 to 0.46%; *P*=0.84) unadjusted and -0.09% (CI: -0.58 to 0.40%; *P*=0.72) fully adjusted for confounders. As a real-world experiment and using a wide range of endpoints [41], SPHERL confirmed the safety of workers in battery-recycling plants in the United States operating under OSHA regulations.

Regulations addressing lead exposure

Tightening environmental regulations led to the banning of lead containing paint (1976), the phasing out of leaded gasoline by the end of the 20th century [55], the elimination of lead as construction material, the replacement of lead pipes in drinking water distribution, the elimination of lead solder in food cans and the compulsory and systematic recycling of lead batteries and other lead waste. Accordingly, BL in American adults dropped from 13.1 μg/dL in NHANES II (1976-1980) to 2.76 μg/dL in NHANES III (1988-1994), to 1.64 μg/dL in NHANES IV (1999-2002), and to 0.93 μg/dL in the 2017-2020 NHANES cycles [16,42].

OSHA provides regulations stipulating how lead exposure at the workplace should be contained. The compulsory measures comprise regular medical monitoring, proper workplace ventilation, and the obligatory use of personal protective equipment. Recently, EU Directive (6417/23) was adopted, amending Council Directives 98/24/EC and 2004/37/EC with regard to the occupational limits for BL and inorganic lead compounds and diisocyanates. The new Directive proposes a reduction of lead in air from 0.15 mg/m^3 to 0.03 mg/m^3 and decreases permissible BL thresholds from 70 µg/dL to 30 µg/dL until 2028 and further to 15 µg/dL thereafter. Workers whose BL is above thresholds due to past exposure must undergo regular medical surveillance, but can continue working if their BLs show a decreasing trend. To mitigate reprotoxic effects, the BL level requiring medical surveillance in female workers of childbearing age was set at

4.5 µg/dL. The Directive, for the first time in Europe, imposes limits for isocyanate functional groups of diisocyanate compounds (NCOs): 6 µg NCO/m^3 for chronic and 12 NCO/m^3 for short-term exposure. However, the Directive recognised that it may be difficult to comply with the proposed NCO limits, because of measurement feasibility and the time needed to implement risk management. Therefore, a transitional value of 10 µg NCO/m^3 with an associated short-term exposure limit of 20 µg NCO/m^3 applies until the end of 2028. A matter of concern is that in middle- and low-income countries, legislation to protect lead-exposed workers is still under revision or only loosely reinforced [1].

Perspectives

Lead is worldwide a ubiquitous metal with high toxicity affecting not only the cardiovascular system in adults, as currently reviewed, but most other organs as well. Even though lead exposure had substantially declined in developed countries, the long-term health effects of lead exposure in low- and middle-income countries still remain a matter of concern [56], because regulations are still being developed or insufficiently reinforced [57,58]. However, the literature on the health effects of lead exposure is skewed by publication bias favoring articles supporting a relation between disease and lead exposure and by selective citation of such studies, as illustrated by a recently published review [59]. This review did not include a stated strategy to search the literature and to select publications for inclusion and in contrast to other articles [29], did not include any reference reaching conflicting interpretations. To be clear on this point, the current review, as explicitly stated in the Introduction, summarised a 40-year research track of a single consortium of investigators.

Although since the turn of the century huge progress has been made in reducing occupational and environmental lead exposure, further lowering exposure to practicable limits should rank high on the agenda of policy makers, especially in low-income countries with outdated industrial infrastructure. Along with eliminating the few remaining point sources of lead, some of which until now escaped detection, lead-exposure containing environmental policies should focus on reducing the exposure to particulate loaded with lead deposited in the previous century [2] and on educating and empowering deprived social groups, particularly in low- and middle-income countries.

From clinical point of view, the 2024 Guidelines for the Management of Elevated Blood Pressure and Hypertension [60], state that the pathogenesis of hypertension involves complex interactions between environmental and behavioral factors, genes, hormonal networks, and multiple organs systems, including the renal cardiovascular and central nervous system. The take home message for the practicing clinicians is therefore that in

the pathogenic work-up of unexplained hypertension or chronic kidney disease, environmental pollutants and their exposure vectors should always be considered. If lead exposure is suspected and given that co-exposure is common, blood or urinary lead and cadmium should be measured, but only at a certified laboratory qualified for the measurement of heavy metals.

Funding

The International Lead Association (www.ila-lead.org) provided an unrestricted grant to the Research Unit Hypertension and Cardiovascular Epidemiology, KU Leuven Department of Cardiovascular Sciences partially supporting data collection and management and statistical analysis of the SPHERL project. The Non-Profit Research Association Alliance for the Promotion of Preventive Medicine, Mechelen, Belgium (URL, www.appremed.org) received a nonbinding grant from OMRON Healthcare Co Ltd, Kyoto, Japan. These funders played no role in the decision to write this invited review and to submit this article for publication.

Conflict of interest

None declared.

References

1. Ericson B, Hu H, Nash E, Ferraro G, Sinitsky J, Taylor MP. Blood lead levels in low-income and middle-income countries: a systematic review. *Lancet Planet Health.* 2021; 5: e145-e153.
2. Resongles E, Dietze V, Green DC, Weiss DJ. Strong evidence for the continued contribution of lead deposited during the 20th century to the atmospheric environment in London today. *Proc Natl Acad Sci.* 2021; 118: e2102791118.
3. Jin T, Amini H, Kosheleva A, Yazdi MD, Wei Y, Castro E, Di Q, Shi L, Schwartz J. Association between long-term exposures to airborne PM2.5 components and mortality in Massachusetts: mixture analysis exploration. *Environ Health.* 2022; 21: 96.
4. GBD 2017 Risk Factor Collaborators. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risk factors or clusters of risks for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* 2018; 392: 1923-1994.
5. Staessen J, Bulpitt CJ, Roels H, Bernard A, Fagard R, Joossens JV, Lauwerys R, Lijnen P, Amery A. Urinary cadmium and lead concentrations and their relation to blood pressure in a population with low exposure. *Brit J Ind Med.* 1984; 41: 241-248.
6. Staessen JA, Lauwerys RR, Buchet JP, Bulpitt CJ, Rondia D, Vanrenterghem Y, Amery A, the Cadmibel Study Group. Impairment of renal function with increasing blood lead concentrations in the general population. *N Engl J Med.* 1992; 327: 151-156.
7. Staessen J, Bruaux P, Claeys-Thoreau F, De Plaen P, Ducoffre G, Lauwerys R, Roels H, Rondia D, Sartor F, Amery A. The relationship between blood pressure and environmental exposure to lead and cadmium in Belgium. *Environ Health Perspect.* 1988; 78: 127-129.
8. Staessen J, Amery A, Bernard A, Bruaux P, Buchet JP, Bulpitt CJ, Claeys F, De Plaen P, Ducoffre G, Fagard R, Lauwerys RR, Lijnen P, Nick L, Saint-Remy A, Roels H, Rondia D, Sartor F, Thijs L. Blood pressure, the prevalence of cardiovascular diseases and exposure to cadmium: a population study. *Am J Epidemiol.* 1991; 134: 257-267.

9. Staessen JA, Roels H, Fagard R, for the PheeCad Investigators. Lead exposure and conventional and ambulatory blood pressure. A prospective population study. *J Am Med Assoc.* 1996; 275: 1563-1570.
10. Yang WY, Zhang ZY, Thijs L, Cauwenberghs N, Wei FF, Jacobs L, Luttun A, Verhamme P, Kuznetsova T, Nawrot TS, Staessen JA. Left ventricular structure and function in relation to environmental exposure to lead and cadmium. *J Am Heart Assoc.* 2017; 6: e004692.
11. Yang WY, Zhang ZY, Thijs L, Bijnens EM, Jansen BG, Vanpoucke C, Lefebvre W, Cauwenberghs N, Wei FF, Luttun A, Verhamme P, Van Hecke E, Kuznetsova T, D'Hooge J, Nawrot T, Staessen JA. Left ventricular function in relation to chronic residential air pollution in a general population. *Eur J Prev Cardiol.* 2017; 24: 1416-1428.
12. Staessen JA, Nawrot T, Den Hond E, Thijs L, Fagard R, Hoppenbrouwers K, Koppen G, Nelen V, Schoeters G, Vanderschueren D, Van Hecke E, Verschaeve L, Vlietinck R, Roels HA, for the Environment and Health Study Group. Renal function, cytogenetic measurements, and sexual development in adolescents in relation to environmental pollutants : a feasibility study. *Lancet.* 2001; 357: 1660-1669.
13. Nawrot TS, Thijs L, Den Hond EM, Roels HA, Staessen JA. An epidemiological re-appraisal of the association between blood pressure and lead : a meta-analysis. *J Hum Hypertens.* 2002; 16: 123-131.
14. Staessen JA, Thijs L, Yang WY, Yu CG, Wei FF, Roels HA, Nawrot TS, Zhang ZY. Interpretation of population health metrics: environmental lead expsoure as an exemplary case. *Hypertension.* 2020; 75: 603-614.
15. Staessen J, Yeoman WB, Fletcher AE, Markowe HLJ, Marmot MG, Rose G, Semmence A, Shipley MJ, Bulpitt CJ. Blood lead concentration, renal function, and blood pressure in London civil servants. *Brit J Ind Med.* 1990; 47: 442-447.
16. Hara A, Thijs L, Asayama K, Gu YM, Jacobs L, Zhang ZY, Liu YP, Nawrot TS, Staessen JA. Blood pressure in relation to environmental lead exposure in the National Health and Nutrition Examination Survey 2003 to 2010. *Hypertension.* 2015; 65: 62-69.
17. Agarwal S, Zaman T, Tuzcu EM, Kapadia SR. Heavy metals and cardiovascular disease: results from the National Health and Nutrition Examination Survey (NHANES) 1999-2006. *Angiology.* 2011; 62: 422-429.

18. Yu YL, Thijs L, Saenen N, Melgarejo JD, Wei DM, Yang WY, Yu CG, Roels HA, Nawrot TS, Maestre GE, Staessen JA, Zhang ZY. Two-year neurocognitive responses to first occupational lead exposure. *Scand J Work Environ Health.* 2020; 45: 298-307.
19. Yu YL, Thijs L, Yu CG, Yang WY, Melgarejo JD, Wei DM, Wei FF, Nawrot TS, Verhamme P, Roels HA, Staessen JA, Zhang ZY. Two-year responses of heart rate and heart rate variability to first occupational lead exposure. *Hypertension.* 2021; 77: 1775-1786.
20. Yu YL, Yang WY, Thijs L, Melgarejo JD, Yu CG, Wei DM, Wei FF, Nawrot TS, Zhang ZY, Staessen JA. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *Hypertension.* 2020; 76: 1299-1307.
21. Yu YL, Thijs L, Wei DM, Melgarejo JD, Yu CG, Yang WY, Roels HA, Zhang ZY. Two-year responses of renal function to first occupational lead exposure. *KI Reports.* 2022; 7: 1198-1209.
22. Yu YL, An DW, Yang WY, Verhamme P, Allegaert K, Nawrot TS, Staessen JA. Blood pressure and renal function responses in workers exposed to lead for up to six years. *J Clin Hypertens.* 2023; 25: 1086-1095.
23. Yu YL, An DW, Chori BS, Nawrot TS, Staessen JA. Blood pressure and hypertension in relation to lead exposure updated according to present-day blood lead levels. *Kardiol Pol.* 2023; 81: 580-588.
24. Saenen ND, Bové H, Steuwe C, Roeffaers MBJ, Provost EB, Lefebvre W, Vanpoucke C, Ameloot M, Nawrot TS. Children's urinary environmental carbon load. A novel marker reflecting residential ambient air pollution exposure ? *Am J Respir Crit Care Med.* 2017; 196: 873-881.
25. Rabinowitz MB. Toxicokinetics of bone lead. *Environ Health Perspect.* 1991; 91: 33-37.
26. Oliveira S, Aro A, Sparrow D, Hu H. Season modifies the relationship between bone and blood lead levels : the Normative Aging Study. *Arch Environ Health.* 2002; 57: 466-472.
27. Korrick SA, Schwartz J, Tsaih SW, Hunter DJ, Aro A, Rosner B, Speizer FE, Hu H. Correlates of bone and blood lead levels among middle-aged abd elderly women. *Am J Epidemiol.* 2002; 156: 335-343.

28. Navas-Acien A, Schwartz BS, Rothenberg SJ, Hu H, Silbergeld EK, Guallar E. Bone lead levels and blood pressure endpoints: a meta-analysis. *Epidemiology.* 2008; 19: 496-504.
29. Navas-Acien A, Guallar E, Silbergeld EK, Rothenberg SJ. Lead exposure and cardiovascular disease — a systematic review. *Environ Health Perspect.* 2007; 115: 472-482.
30. Staessen JA, Lauwerys RR, Bulpitt CJ, Fagard R, Lijnen P, Roels H, Thijs L, Amery A. Is a positive association between lead exposure and blood pressure supported by animal experiments? *Curr Opin Nephrol Hypertens.* 1994; 3: 257-263.
31. Virgolini MB, Aschner M. Molecular mechanisms of lead neurotoxicity. *Adv Neurotoxicol.* 2021; 5: 159-213.
32. Mitra P, Sharma S, Purovit P, Sharma P. Clinical and molecular aspects of lead toxicity: an update. *Crit Rev Clin Lab Sci.* 2017; 54: 506-528.
33. Orr SE, Bridges CC. Chronic kidney disease and exposure to nephrotoxic metals. *Int J Mol Sci.* 2017; 18: 1039.
34. Åkesson A, Lundh T, Vahter M, Bjellerup P, Lidfeldt J, Nerbrand C, Samsioe G, Stromberg U, Skerfving S. Tubular and glomerular kidney effects in Swedish women with low environmental cadmium exposure. *Environ Health Perspect.* 2005; 113: 1627-1631.
35. Harari F, Sallsten G, Christensson A, Petkovic M, Hedblad B, Forsgard N, Melander O, Nilsson PM, Borné Y, Engström G, Barregard L. Blood lead levels and decreased kidney function in a population-based cohort. *Am J Kidney Dis.* 2018; 72: 381-389.
36. Muntner P, He J, Vupputuri S, Coresh J, Batuman V. Blood lead and chronic kidney disease in the general United States population: results from NHANES III. *Kidney Int.* 2003; 63: 1044-1050.
37. Kim R, Rotnitzky A, Sparrow D, Weiss ST, Wagner C, Hu H. A longitudinal study of low-level lead exposure and impairment of renal function. The Normative Aging Study. *J Am Med Assoc.* 1996; 275: 1177-1181.
38. Chowdhury R, Darrow L, McClellan W, Sarnat S, Steenland K. Incident ESRD among participants in a lead surveillance program. *Am J Kidney Dis.* 2014; 64: 25-31.
39. Lai LH, Chou SY, Wu FY, Chen JJ, Kuo HW. Renal dysfunction and hyperuricemia with low blood lead levels and ethnicity in community-based study. *Sci Tot Environ.* 2008; 401: 39-43.

40. World Health Organization. *Brief guide of analytical methods for measuring lead in blood.* Geneva, 2020, pp 1-22.
41. Hara A, Gu YM, Petit T, Liu YP, Jacobs L, Zhang ZY, Jin Y, Thijs L, Wei FF, Nawrot TS, Staessen JA. Study for promotion of health in recycling lead - rationale and design. *Blood Press.* 2015; 24: 147-157.
42. An DW, Yu YL, Hara A, Martens DS, Yang WY, Cheng YB, Huang QF, Asayama K, Stolarz-Skrzypek K, Rajzer M, Verhamme P, Nawrot.T.S., Li Y, Staessen JA. Lead-associated mortality in the US 1999-2020: a time-stratified analysis of a national cohort. *J Hypertens.* 2024; 42: 1322-1330.
43. Lanphear BP, Rauch S, Auinger P, Allen RW, Hornung RW. Low-level lead exposure and mortality in US adults: a population-based cohort study. *Lancet Public Health.* 2018; 3: e177-e184.
44. Staessen J, Bulpitt CJ, Fagard R, Joossens JV, Lijnen P, Amery A. Familial aggregation of blood pressure, anthropometric characteristics and urinary excretion of sodium and potassium - a population study in two Belgian towns. *J Chron Dis.* 1985; 38: 397-407.
45. Global Burden of Disease Collaboration. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* 2012; 380: 2224-2260.
46. GBD 2021 Diseases and Injuries Collaborators. Global incidence, prevalence, years lived with disability (YLDs), disbaility-adjusted life-years (DALYs) and healthy life expectancy in 204 countries and territories and 811 submational locations, 1990-2021: a systematic analysis of the Global Burden of DIsease Study 2021. *Lancet.* 2024; 403: 2133-2161.
47. Safiri S, Karamzad N, Singh K, Carson-Chahhoud K, Adams C, Nejadghaderi SA, Almasi-Hashiani A, Sullman MJM, Mansournia MA, Bragazzi NL, Kaufman JS, Collins GS, Kolahi AA. Burden of ischemic heart disease and its attributable risk factors in 204 countries and territories, 1990-2019. *Eur J Prev Cardiol.* 2022; 29: 420-431.
48. Karimi H, Mahdavi S, Moghaddam SS, Abbasi-Kangevari M, Soleimani Z, Esfahani Z, Masinaei M, Fateh SM, Golestani A, Dilmaghani-Marand A, Kompani F, Rezaei N, Ghasemi E, Larijani B, Farzadfar F. Unveiling the lead exposure attributed burden in

Iran from 1990 to 2019 through the lens of the Global Burden of Disease study 2019. *Sci Rep.* 2024; 14: 8688.

49. Staessen JA, Wang JG, Brand E, Barlassina C, Birkenhäger WH, Herrmann SM, Fagard R, Tizzoni L, Bianchi G. Effects of three candidate genes on prevalence and incidence of hypertension in a Caucasian population. *J Hypertens.* 2001; 19: 1349-1358.
50. O'Flaherty EJ. Physiologically based methods for bone-seeking elements. IV. Kinetics of lead disposition in humans. *Toxicol Appl Pharmacol.* 1993; 118: 16-29.
51. Leggett RW. An age-specific kinetic model of lead metabolism in humans. *Environ Health Perspect.* 1993; 101: 598-616.
52. Yu CG, Wei FF, Yang WY, Zhang ZY, Mujaj B, Thijs L, Feng YM, Staessen JA. Heart rate variability and peripheral nerve conduction velocity in relation to blood lead in newly hired lead workers. *Occup Environ Med.* 2019; 76: 382-388.
53. Chia SE, Chia KS, Chia HP, Ong CN, Jeyaratnam J. Three-year follow-up of serial nerve conduction among lead-exposed workers. *Scand J Work Environ Health.* 1996; 22: 374-380.
54. Chia SE, Chia HP, Ong CN, Jeyaratnam J. Cumulative blood lead level and nerve conduction parameters. *Occup Med.* 1996; 46: 59-64.
55. Needleman HL. Review. The removal of lead from gasoline: historical perspective and personal reflections. *Environ Res.* 2000; 84: 20-35.
56. Hu H, Aro A, Payton M, Korrick S, Sparrow D, Weiss ST, Rotnitzky A. The relationship of bone and blood lead to hypertension. The Normative Aging Study. *J Am Med Assoc.* 1996; 275: 1171-1176.
57. Yan LD, Rouzier V, Pierre JL, Lee MH, Muntner P, Parsons PJ, Apollon A, St-Preux S, Malebranche R, Pierre G, Emmanuel E, Nash D, Kingery J, Walsh KF, Smith CE, Metz M, Tymejczyk O, Deschamps M, Pape JW, Fitzgerald DW, McNairy ML. High lead exposure associated with higher blood pressure in Haiti: a warning sign for low-income country. *Hypertension.* 2022; 79: 283-290.
58. Obadia PM, Kayembe-Kitenge T, Haufroid V, Nkulu CBL, Nemery B. Preeclampsia and blood lead (and other metals) in Lubumbashi, DR Congo. *Environ Res.* 2024; 167: 468-471.
59. Lanphear B, Navas-Acien A, Bellinger DC. Lead poisoning. *N Engl J Med.* 2024; 391: 1621-1631.

60. McEvoy JW, McCarthy CP, Bruno RM, Brouwers S, Canavan MD, Cecon C, Christodorescu MD, Daskalopoulou SS, Ferro CJ, Gerdts E, Hanssen H, Harris J, Lauder L, McManus RJ, Molloy GJ, Rahimi K, Regitz-Zagrosek V, Rossi GP, Sandset EC, Scheenaerts B, Staessen JA, Uchmanowicz II, Volterrani M, Touyz RM, and ESC Scientific Document Group. 2024 ESC Guidelines for the management of elevated blood pressure and hypertension. *Eur Heart J.* 2024; 45: 1-107.

Chapter 3

Unhealthy ageing in relation to urinary proteomic profiling

Part 1

Osteoporosis in relation to a bone-related ageing biomarker derived from the urinary proteomic profile: a population study

Yu-Ling Yu, Dries S. Martens, De-Wei An, Babangida Chori, Agnieszka Latosinska, Justyna Siwy, Augustine N. Odili, Katarzyna Stolarz-Skrzypek, Gladys E. Maestre, Kei Asayama, Yan Li, Peter Verhamme, Karel Allegaert, Harald Mischak, Tim S. Nawrot, Jan A. Staessen

Previously published in:
Aging and Disease. 2024. 16: 633-644.

Abstract

Background: Screening for and prevention of osteoporosis and osteoporotic fractures is imperative, given the high burden on individuals and society. This study constructed and validated an ageing-related biomarker derived from the urinary proteomic profile (UPP) indicative of osteoporosis (UPPost-age).

Methods: In a prospective population study done in northern Belgium (1985-2019), participants were invited for a follow-up examination in 2005-2010 and participants in the 2005-2010 examination again invited in 2009-2013. Participants in both the 2005-2010 and 2009-2013 examinations (*n*=519) constituted the derivation (2005-2016 data) and time-shifted validation (2009-2013 data) datasets; 187 participants with only 2005-2010 data formed the synchronous validation dataset. The UPP was assessed by capillary electrophoresis coupled with mass spectrometry. Analyses focused on 2372 sequenced urinary peptides (101 proteins) with key roles in maintaining the integrity of bone tissue.

Results: In multivariable analyses with correction for multiple testing, chronological age was associated with 99 urinary peptides (16 proteins). Peptides derived from IGF2 and MGP were upregulated in women compared to men, whereas COL1A2, COL3A1, COL5A2, COL10A1 and COL18A1 were down-regulated. Via application of a 1000-fold bootstrapped elastic regression procedure, finally, 29 peptides (10 proteins) constituted the UPPost-age biomarker, replicated across datasets. In cross-sectional analyses of 2009-2013 data (*n*=706), the body-height-to-arm-span ratio, an osteoporosis marker, was negatively associated with UPPost-age ($p<0.0001$). Over 4.89 years (median), the 10-year risk of osteoporosis associated with chronological age and UPPost-age (53 cases including 37 fractures in 706 individuals) increased by 21% and 36% ($p \leq 0.044$). Among 357 women, the corresponding estimates were 55% and 60% for incident osteoporosis (37 cases; $p \leq 0.0003$) and 42% and 44% for osteoporotic fractures (25 cases; $p \leq 0.017$).

Conclusions: In conclusion, an ageing-related UPP signature with focus on peptide fragments derived from bone-related proteins is associated with osteoporosis risk and available for clinical and trial research.

Introduction

Osteoporosis is an age-related skeletal disorder characterised by the loss of bone mass, induced by increased osteoclast-stimulated bone resorption and inhibition of osteoblast-stimulated bone formation, and leading to the fragility of bone tissue and high risk of fractures [1]. Based on National Health and Nutrition Examination Survey 2005-2010, the prevalence of osteoporosis in American adults aged 50 years or older was 10.3%, translating into 10.2 million people at risk [2]. With increasing age the prevalence of osteoporosis raised exponentially in Americans, from 5.1% at age 50-59 years to 26.2% in octogenarians and the very elderly [2]. Moreover, 71% of osteoporotic fracture occurred among women [3], who had a higher prevalence of osteoporosis and osteoporotic fracture risk at any given age compared with men [2,4]. Because osteoporosis and its related fractures worsen quality of life [5] and represent a high socio-economic burden [3,6,7], early screening and prevention are imperative [8].

Urine contains more than 5000 sequenced peptides, which identify the parental proteins and in the case of osteoporosis reveal the dysregulated molecular features in the skeletal system [9,10]. Moreover, biological age, evaluated by standardised physiological measurements, functional tests or sophisticated “omics” technologies [11], allows a better prediction of the functional capacity later in life than chronological age. Previous studies demonstrated that the urinary peptidomic profile (UPP) is an effective predictor ageing-associated disabilities [9]. However, few studies addressed how osteoporosis is related to the ageing-related UPP signature [12]. Thus, the aims of the current study were to construct an ageing-related biomarker indicative of osteoporosis (UPPost-age) and to validate the UPPost-age in the family-based Flemish Study on Environment, Genes, and Health Outcomes (FLEMENGHO) [9].

Material and methods

Study population

FLEMENGHO complies with the Helsinki declaration and is registered with the Belgian Data Protection Authority (III 11/1234/13; Aug 22, 2013). The ethics committee of the University Hospitals Leuven, Belgium, approved the secondary use of FLEMENGHO data (B32220083510). From August 20, 1985, to December 14, 1990, a random sample of the households living in a geographically defined area of northern Belgium was investigated with the goal to recruit an equal number of participants in each of six subgroups stratified by sex and age (20-39 years, 40-59 years, and ≥60 years). All household members aged ≥20 years were invited to take part, provided that the quota of their sex-age group had not yet been met. From April 3, 1996, to May 12, 2007, recruitment of families continued,

including young people aged 10-19 years. Participants younger than 18 years provided informed assent and their parents or custodians gave informed consent. Of 4286 people invited to participate in FLEMENGHO, 3343 consented (participation rate 78.0%).

From May 30, 2005, until May 31, 2010, participants were invited to a follow-up examination if their last known address was within 15 km of the local examination center (Eksel, Belgium) and if they had not withdrawn consent in any of the previous examination cycles (1985-2004). All participants who had a follow-up visit from 2005 to 2010 were invited to an additional follow-up examination from October 28, 2009, until March 19, 2013. Among 828 participants who renewed consent (**Figure 1**), 122 were excluded from analysis, if they had not undergone UPP profiling (n=108), or were aged younger than 20 years (n=14). Therefore, three datasets were constructed including 706 participants (**Figure 1**). The participants, who took part in both 2005-2010 and 2009-2013 follow-up examinations constituted the derivation dataset (n=519), which included the 2005-2010 data, and the time-shifted validation dataset (n=519), which included the 2009-2013 follow-up data. The remaining participants, who only took part in the 2005-2010 examination, constituted the synchronous validation dataset (n=187).

Clinical and biochemical variables

At each examination, study nurses administered a standardised questionnaire about each participant's medical history, smoking and drinking habits, intake of medications and risk factors. Body height was measured with participants barefoot and standing firm against a wall. Arm span, measured by a flexible meter, was the distance from the tip of the middle finger from the left hand to the tip of the middle finger from the right hand with the both arms abducted at 90°. Body height and arm span were determined to the nearest centimeter. Urine samples for UPP analysis were aliquots of a 24-h urine sample collected within one week of the examination and deep frozen at -40 C. Venous blood samples were obtained after 8 hours of fasting. After centrifugation and aliquoting, blood-derived specimens were stored at -80°C. A single certified laboratory assessed the routine biochemistry, using quality controlled automated methods. The estimated glomerular filtration rate (eGFR) was computed from serum creatinine [13], measured by a modification of Jaffe's methods with isotope-dilution calibration [14]. According to the Chronic Kidney Disease Epidemiology Collaboration equation [14], eGFR = 141 × minimum (Scrt/κ, 1)$^{\alpha}$ × maximum (Scrt/κ,1)$^{-1.209}$ × 0.993^{age} × 1.018 (if female), where Scrt is the serum creatinine concentration in μmol/L, κ is 61.9 for women and 79.6 for men, and α is -0.329 for women and -0.411 for men; minimum indicates the minimum of Scrt/κ or 1 and maximum indicates the maximum of Scrt/κ or 1. Desphospho-

uncarboxylated matrix Gla protein (dp-ucMGP), a biomarker reflecting vitamin K status [15], was measured on citrated plasma by ELISA.

Among 357 women, 35 (9.80%) had bone mineral density (BMD) assessed in an earlier examination cycle. Bone mineral density (BMD) was measured at the forearm just above the wrist by single photon absorptiometry (ND1100 bone density scanner; Nuclear Data Inc, Schaumburg, IL, USA). Distal scans of adult forearms traverse a mean of 35% trabecular bone, whereas in proximal scans this proportion declines to nearly 5 % [16]. Low BMD was defined as the distal and proximal bone density corrected for fat and bone width lower than the 40th percentile.

Outcomes

Body-height-to-arm-span-ratio (HASR) is an anthropometric parameter reflecting the osteoporotic compression of the vertebrae [17,18]. Body height and arm span are approximately equal in young adults. With arm span as reference unaffected by ageing, HASR reflects the age-related loss of body height. In 656 FLEMENGHO participants (50.6% women; median age at the first measurement: 40.5 year), examined twice in the interval from 1985 until 2008, height change over a median of 9.6 years (IQR: 6.5-15.5 years, range: 0.74-23.4 years), averaged 0.46 cm (SD: 1.51) [19] and was significantly correlated with HASR (r=-0.17, P<0.0001; **Figure S1**). The sex-specific correlation coefficients were -0.24 in women (red circles) and -0.095 in men (green squares), respectively (p=0.057 for the sex difference). Therefore, HASR was analyzed as a continuous outcome reflecting osteoporosis and the absolute difference of body height minus arm span dichotomised by the ≤ -3 cm threshold [17,18] as binary outcome.

At annual intervals, the vital status of FLEMENGHO participants was ascertained by record linkage with the National Population Registry in Brussels, Belgium. For all participants, data on the incidence of nonfatal outcomes were collected by a standardised questionnaire at follow-up visits, via structured telephone interviews, and by searching participants' medical records at the four regional hospitals and University Hospitals Leuven, all serving the catchment area. The outcomes of interest were osteoporosis (ICD8 code 723.0; nonaccidental fractures, invasive osteosynthesis, spine stabilizing surgery, or initiation of bisphosphonate treatment). The history of fracture was blindly and independently reviewed by two researchers against the source data to exclude the accidental cases not attributable to osteoporosis. For analysis of the incidence of osteoporosis and osteoporotic fracture, the date of urine collection in 2005-2010 served as the baseline. For all outcomes, participants were censored at occurrence of the first event or on June 30, 2019, if no event had occurred.

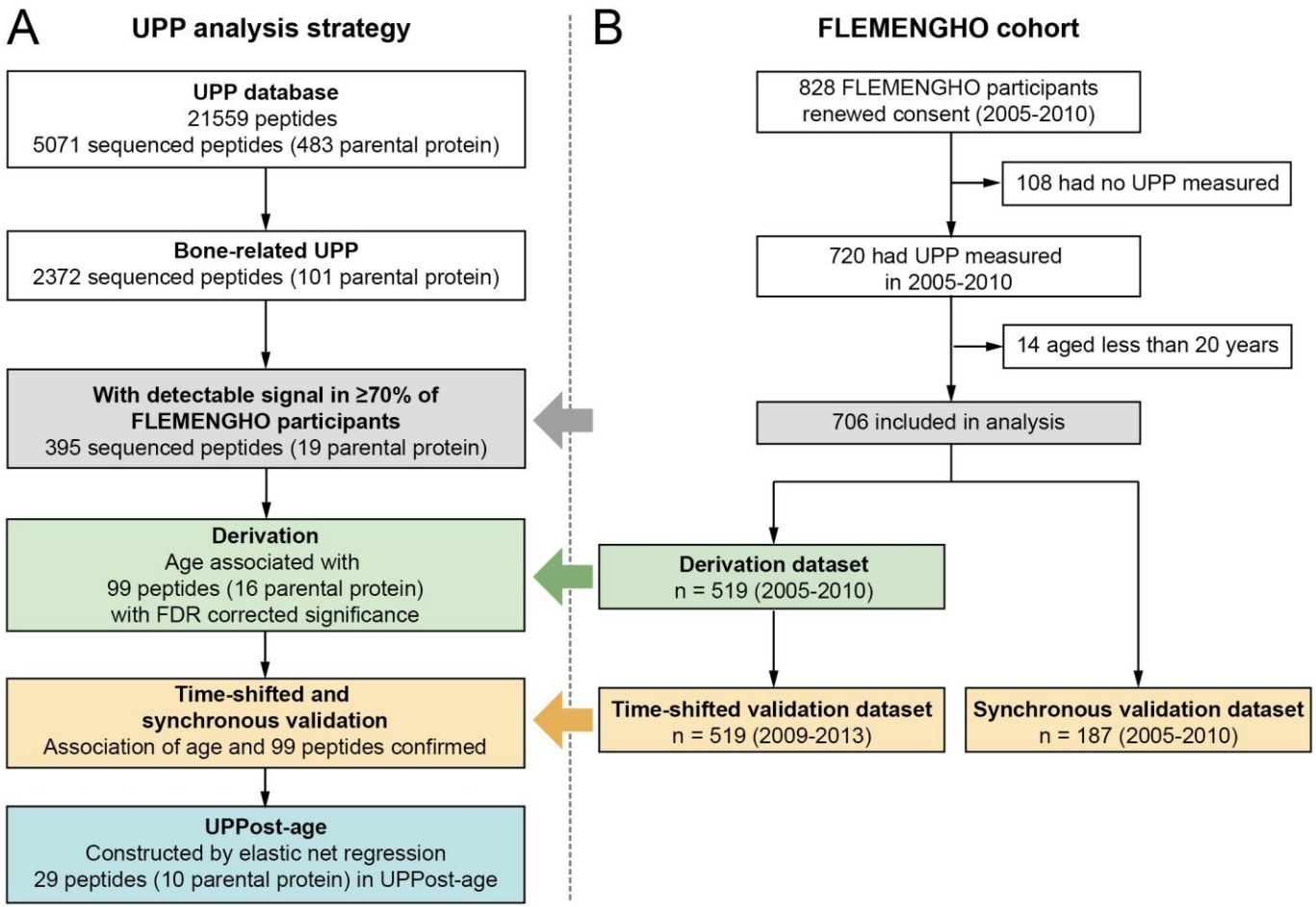


Figure 1. Flow chart depicting the analysis strategy and the selection of FLEMENGHO participants. Bone-related UPP refers to 2372 of 5071 sequenced urinary peptides derived from proteins implicated in skeleton development, bone formation and mineralization, ossification and other bone-related mechanism and metabolic processes (https://www.uniprot.org). Abbreviations: FLEMENGHO: Flemish Study on Environment, Genes, and Health Outcomes; UPP: urinary peptidomic profile; UPPost-age: age as predicted by UPP; FRD: false discovery rate.

Urinary peptidomics

The methods for urine sample preparation, capillary electrophoresis coupled with mass spectrometry (CE-MS), peptide sequencing, calibration, and quality control of the mass spectrometric data have been published [20-22] and are described in the Data Supplement. By identifying the function of the parental protein (**Table S1**) from the UniProt database (https://www.uniprot.org), we analyzed peptides derived from proteins implicated in skeleton development, bone formation and mineralization, ossification and other bone-related mechanism and metabolic processes. Among the bone-related sequenced urinary peptides, those with a detectable signal in ≥70% of study participants (**Figure 1** and **Table S1**), which is a common approach in UPP analysis [23,24], were carried through to further analysis. This selection procedure resulted in a short list of 395 of 5071 sequenced peptides originating from 19 parental proteins of interest, of which the biological function is highlighted in **Table S1**.

Statistical analysis

Database management and statistical analysis were done using SAS, version 9.4 (maintenance level 5). The distributions of UPP were rank normalised, by sorting measurements from the smallest to the highest value and then applying the inverse cumulative normal function [25]. Within-group changes of continuous and categorical variables were assessed by the t statistic and McNemar's test, respectively. Between-group means and proportions were compared using the large-sample z test and Fisher's exact test. Significance was a *p*-value of 0.05, with adjustment for multiple testing if indicated.

The analysis was done according to predefined steps (**Figure 1**). First, the association between chronological age and the selected 395 urinary peptides was investigated in the 2005-2010 derivation dataset (n=519), with cumulative adjustments applied for the clustering within families (random effect), sex, body mass index, eGFR, current smoking, γ-glutamyltransferase (index of alcohol intake), fasting plasma glucose, physical activity, use of diuretics (yes *vs* no) [26], and socioeconomic status (high or middle *vs* low; 1 *vs* 0). Physical activity and socioeconomic status were derived by questionnaire as described previously [27]. After applying Benjamini-Hochberg false discovery rate (FDR) to adjust significance, 99 urinary peptides associated with chronological age were retained in further analyses.

Because the prevalence of osteoporosis differs considerably between women and men, the association between the urinary peptide levels and sex was investigated in the 2005-2010 derivation dataset (n=519) by general linear models, first unadjusted and next

adjusted as described above, but omitting the sex adjustment. Volcano plots were then constructed to evaluate the consistency of sex-specific UPP distributions among the derivation, time-shifted validation and synchronous validation datasets. To develop UPPost-age, the 99 peptides were subjected to elastic net regression. The L1 and L2 regression penalties were determined by 10-fold random cross-validation. This procedure was bootstrapped 1000 times to obtain the final estimates of L1 and L2 and the 95% confidence interval of the regression coefficients, linking each protein fragment with chronological age. Next, to validate the ageing biomarker, the UPPost-age biomarker was evaluated in the time-shifted validation and synchronous validation datasets. Linear regression, logistic regression and Cox regression models were applied to assess the associations of HASR, difference between body height and arm span (HASD) ≤ -3 cm (0, 1), and incident osteoporosis (0, 1) and osteoporotic fracture (0,1), with chronological age and UPPost-age. In a sensitivity analysis, the risk of osteoporosis was assessed by the median of plasma desphospho-uncarboxylated matrix Gla protein (dp-ucMGP) in view of the risk modulation in a previous air pollution study [28].

Results

Characteristics of participants

The derivation dataset and the time-shifted validation dataset (n=519, **Table 1**) included 261 women (50.3%) and 258 men (49.7%). Age averaged 49.8 (SD: 14.4 years) and 54.6 (14.3 years) at the 2005-2010 and 2009-2013 examinations, respectively. At baseline, mean body height and arm span were 169.3 (9.38 cm) and 171.0 (11.0 cm), and their corresponding ratio, HASR, was 0.99 (0.03). Overall, 47 (9.1%) participants were on diuretics in the derivation dataset and 54 of 261 (20.7%) women were taking contraceptive pills and 13 (5.0%) were on hormonal substitution therapy. The characteristics of participants included in the derivation dataset (n=519) and synchronous validation dataset (n=187) were largely similar ($p \geq 0.046$; **Table 1**). From the 2005-2010 to the 2009-2013 examinations, mean body mass index increased by +0.75 kg/m2 (SE: 0.27; P=0.0054), mean eGFR declined by -5.20 mL/min/1.73m^2 (SE: 0.93; P<0.0001), while the prevalence of hypertension (from 43.5% to 51.8%; P=0.012) and the proportion of women reaching menopause (from 43.4% to 56.7%; P=0.056) increased.

Associations with chronological age

With multivariable adjustment and correction for multiple testing applied, 99 sequenced peptides, derived from 16 parental proteins, were significantly associated with chronological age (**Table S2**). For peptides derived from the same parental protein, these associations were directionally similar in most but not all instances (**Table S2**).

Table 1. Characteristics of FLEMENGHO participants (starts)

Characteristic	Derivation dataset (*n*=519)	Time-shifted validation dataset (*n*=519)	*P*-value	Synchronous validation dataset (*n*=187)	*P*-value
Anthropometrics					
Women (%)	261 (50.3)	261 (50.3)	…	96 (51.3)	0.13
Natural menopause (% of women)	121 (43.4)	148 (56.7)	0.056	44 (45.8)	0.95
Surgical menopause (% of women)	13 (5.0)	15 (5.7)	0.70	5 (5.2)	0.90
Men (%)	258 (49.7)	258 (49.7)	…	91 (48.7)	0.81
Age (years)	49.8 ± 14.4	54.6 ± 14.3	<0.0001	51.8 ± 16.4	0.13
Body weight (kg)	76.0 ± 14.7	78.2 ± 15.0	0.019	75.9 ± 15.3	0.91
Body height (cm)	169.3 ± 9.38	169.3 ± 9.48	0.98	168.3 ± 9.19	0.20
Arm span (cm)	171.0 ± 11.0	170.7 ± 10.9	0.67	170.1 ± 11.1	0.37
Body mass index (kg/m^2)	26.5 ± 4.34	27.2 ± 4.34	0.0054	26.7 ± 4.29	0.57
HASR	0.99 ± 0.03	0.99 ± 0.03	0.39	0.99 ± 0.03	0.67
Biochemical measurements					
Serum creatinine (mg/dL)	86.1 ± 15.6	89.5 ± 21.3	0.0041	86.6 ± 13.7	0.69
Total serum calcium (mg/dL)	9.56 ± 0.44	9.72 ± 0.40	<0.0001	9.52 ± 0.44	0.85
Fasting plasma glucose (mg/dL)	88.7 ± 13.7	89.1 ± 14.4	0.71	89.8 ± 15.7	0.36
eGFR (mL/min/1.73m^2)	81.2 ± 14.8	76.0 ± 15.2	<0.0001	79.6 ± 16.0	0.21
dp-ucMGP (μg/L)	4.35 (2.98, 6.01)	…	…	4.89 (3.03, 6.41)	0.20
γ-glutamyltransferase (U/L)	22.0 (15.0, 33.0)	22.0 (16.0, 33.0)	0.47	23.0 (14.0, 33.0)	0.96
FSH in women (U/L)	18.5 (4.00, 52.9)	40.2 (5.70, 63.3)	<0.0001	23.0 (4.75, 53.2)	0.66

Table 1 Characteristics of FLEMENGHO participants (ends)

Characteristic	Derivation dataset (*n*=519)	Time-shifted validation dataset (*n*=519)	*P*-value	Synchronous validation dataset (*n*=187)	*P*-value
Life style					
Physical activity (kcal/day)	1768 (1322, 2218)	1682 (1330, 2163)	0.18	1863 (1389, 2261)	0.45
Current smoking (%)	96 (18.5)	75 (14.5)	0.079	41 (21.9)	0.31
Current alcohol intake (%)	148 (28.5)	156 (30.1)	0.59	68 (36.4)	0.046
Middle or high socioeconomic status (%)	194 (37.4)	192 (37.0)	0.90	56 (29.9)	0.068
Comorbidities					
Diabetes mellitus (%)	20 (3.9)	35 (6.7)	0.38	6 (3.2)	0.69
Hypertension (%)	226 (43.5)	269 (51.8)	0.012	79 (42.2)	0.43
Use of medications					
Diuretics (%)	47 (9.1)	62 (12.0)	0.16	22 (11.8)	0.31
Contraceptive pill intake (% of women)	54 (20.7)	45 (17.2)	0.32	24 (25.0)	0.38
Hormonal substitution (% of women)	13 (5.0)	13 (5.0)	…	6 (6.2)	0.64

Abbreviations: eGFR: glomerular filtration rate derived from serum creatinine, using the Chronic Kidney Disease Epidemiology Collaboration equation; dp-ucMGP: desphospho-uncarboxylated matrix Gla protein; HASR: body-height-to-arm-span ratio; FSH: follicle-stimulating hormone. Data are n (%), mean ± SD, or median (IQR); *p*-values denote the significance of the difference between the derivation dataset and the validation datasets. Diabetes was as fasting plasma glucose of ≥126 mg/dL (≥7.0 mmol/L), a self-reported diagnosis, diabetes documented in practice or hospital records, or use of antidiabetic drugs. Hypertension was a blood pressure of ≥140 mm Hg systolic or ≥90 mm Hg diastolic or use of antihypertensive drugs. To convert serum creatinine from mg/dL to μmol/L, multiply by 88.42; total serum calcium from mg/dL to mmol/L, multiply by 0.25; serum cholesterol from mg/dL to mmol/L, multiply by 0.0259; glucose from mg/dL to mmol/L, multiply by 0.0555, dp-ucMGP from μg/L to pmol/L, multiply by 94.299.

Associations were positive for IGF2 (insulin-like growth factor II; same directionality in 2 of 2 peptides) and MGP (matrix Gla protein; 2 of 2). For peptides derived from collagens the associations were predominantly negative: COL1A1 (33 of 38), COL1A2 (13 of 14), COL2A1 (6 of 10), COL3A1 (17 of 21) and COL6A1 (3 of 3). For peptides with the highest significance derived from the same parental protein in the derivation dataset, the consistency of the associations was confirmed in the time-shifted validation and synchronous validation datasets (**Table S3**), based on the FDR-corrected *p* value in a 1-sided test (given the prior probability established by the significance in the derivation dataset). Of the 16 parental proteins, 9 (56.3%) and 10 (62.5%) were confirmed in the time-shifted validation or the synchronous validation datasets, respectively. Only COL11A1 and COL11A2 remained unconfirmed in either validation dataset (**Table S3** and **Table S4**).

The sex-specific association of the 99 bone-related sequenced urinary peptides with chronological age was further explored, using multivariable-adjusted linear regression analysis. The peptide levels derived from IGF2 and MGP were significantly upregulated in women compared to men, whereas COL1A2, COL3A1, COL5A2, COL10A1 and COL18A1 were significantly down-regulated (**Figure 2** and **Table S5**). Volcano plots demonstrated the consistency of these sex-specific associations with chronological age in the derivation, time-shifted validation and synchronous validation datasets (**Figure 2**).

Associations with UPPost-age

Elastic net regression retained 29 of 99 (29.3%) of the bone-related sequenced urinary peptides in the UPPost-age prediction model (**Table S6**). The selected peptides originated from 10 parental proteins, mainly collagen fragments, IGF2 and MGP (**Table S6**). The UPPost-age was similar to the chronological age in the derivation set (49.8 years [SE: 0.49] *vs* 49.8 years [0.63]; *P*=0.98) and synchronous validation set (50.3 years [0.94] *vs* 51.8 years [1.20]; *P*=0.088), whereas UPPost-age was lower than chronological age in the time-shifted validation dataset (52.1 years [0.50] *vs* 54.6 years [0.63]; *P*<0.0001). Compared to the derivation set, the regression slopes were similar in the synchronous validation dataset (*P*=0.17; **Figure 3**), but slightly smaller in time-shifted validation dataset (*P*=0.012; **Figure 3**). For a 10-years increase in the UPPost-age (**Figure 3**), the chronological age increased by 9.9 years (95% CI: 9.2-10.6 years; *r*=0.77) in the derivation datasets, 8.5 years (95% CI: 7.7-9.4 years; *r*=0.67) in the time-shifted validation dataset, and 8.9 years (95% CI: 7.5-10.2 years; *r*=0.69) in the synchronous validation dataset. Among the 35 women who had both BMD and UPP measured, UPPost-age was significantly higher in those with low BMD (59.6 years [SD: 7.12] *vs* 54.0 years [6.68], *P*=0.047).

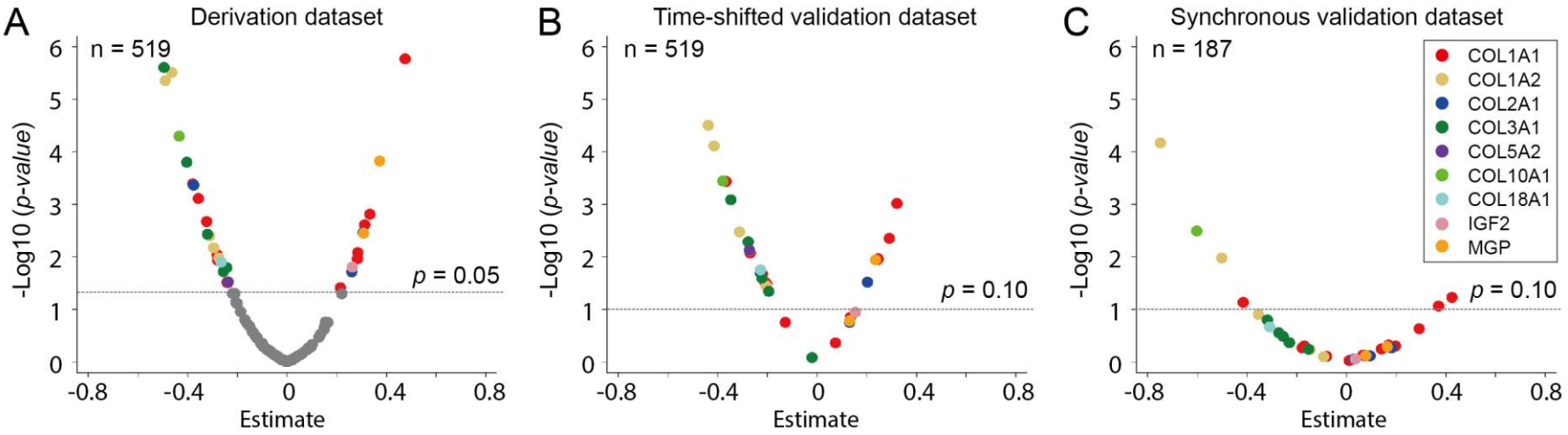


Figure 2. Volcano plots depicting the sex-specific expression of bone-related urinary proteomics in the derivation (A), time-shifted validation (B) and synchronous validation (C) datasets. Estimate was the association size of difference in urinary peptide level in women compared to men (men as reference) derived from the rank-normalised data in the adjusted general linear models. Adjusted models accounted for the clustering within families, age, body mass index, the glomerular filtration rate calculated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration equation, current smoking, γ-glutamyltransferase as index of alcohol intake, fasting plasma glucose, physical activity, use of diuretics (yes *vs* no), and middle or high socioeconomic status (yes *vs* no). The 99 urinary peptides associated with chronological age were included in the analysis in the derivation dataset, and only those reaching significance ($p < 0.05$, colored dots; refer to Table S5) were included in the analysis of the time-shifted and synchronous validation datasets. The insignificant urinary peptides (gray dots in A) were not included in the replication analyses. *p*-values were corrected for the Benjamini-Hochberg false discovery rate ($p < 0.10$ in the validation datasets, given the prior probability established by the significance in the derivation dataset). 32 urinary peptides reached significance and identified 9 parental proteins in the adjusted models.

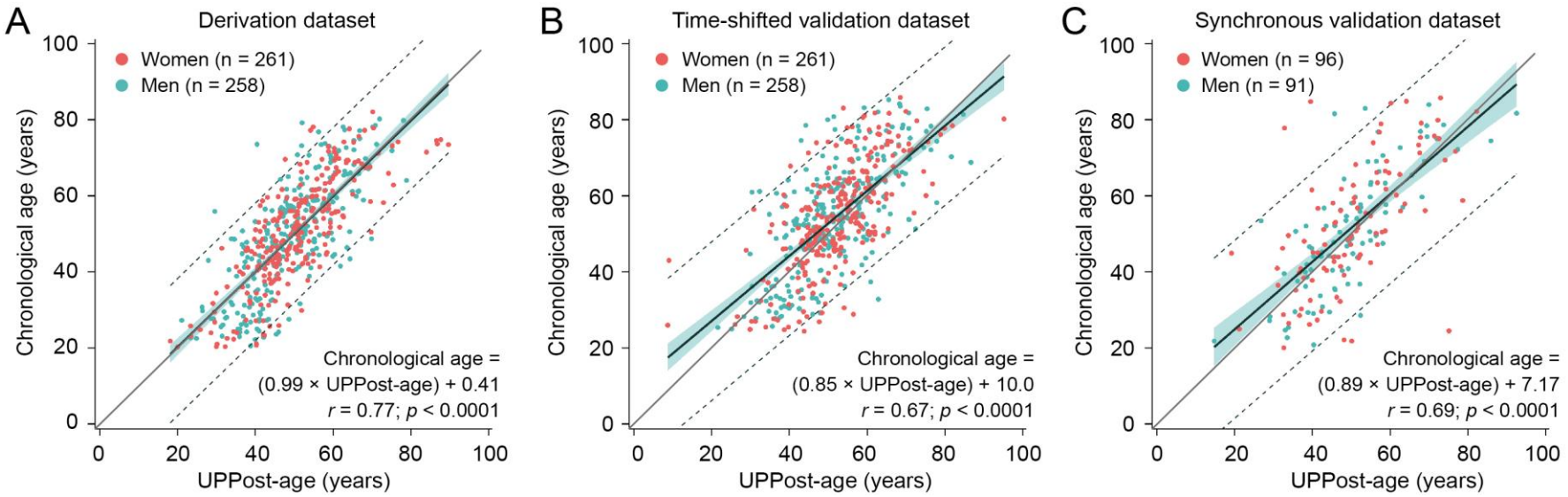


Figure 3. Correlations between chronological age and the UPPost-age predicted by bone-related urinary proteome in FLEMENGHO participants. In the derivation (A), time-shifted validation (B) and synchronous validation (C) datasets, the regression lines (solid black) are given with 95% confidence intervals for the predicted mean of chronological age (green band) and the predicted chronological age of individual participants (dotted lines). The grey line in the correlation plots is the identity line. Red and green dots represent women and men, respectively. The correlation coefficients (*r*), the significance (*p*) and the linear regression equation are given for each dataset. Abbreviations: FLEMENGHO: Flemish Study on Environment, Genes, and Health Outcomes; UPP: urinary peptidomic profile; UPPost-age: age as predicted by UPP.

Association of HASD and HASR with age

In a cross-sectional analysis of the FLEMENGHO baseline data (2005-2010), the risk of having HASD ≤ -3 cm, was related to age and UPPost-age (**Table S7**). The odds ratios expressing the risk of having HASD ≤ -3 cm per 10-year increment were significant for age in all participants, men, women and postmenopausal women ($P \leq 0.025$). For UPPost-age, significance was maintained in all participants and in women with odds ratios amounting to 1.31 (95% CI: 1.12, 1.53; $P < 0.0007$) and 1.43 (1.14, 1.81; $P = 0.0024$), respectively (**Table S7**).

The 706 participants making up the FLEMENGHO study population included 108 unrelated participants and 598 related participants from 16 single-generation families and 58 multigeneration pedigrees. In multivariable-adjusted regression analyses, HASR was significantly associated with chronological age ($P \leq 0.001$) and UPPost-age ($P \leq 0.011$) in all FLEMENGHO participants, men and women, with a similar trend in postmenopausal women. When the population was dichotomised per the -3 cm HASD threshold, the directionality of the associations was maintained, but significance was lost in women and postmenopausal women (**Table 2**).

Incidence of osteoporosis

During a median follow-up interval of 4.89 years (IQR: 4.44-5.19 years), 53 participants developed osteoporosis, of whom 16 had an osteoporosis diagnosis in practice or hospital files and 37 experienced an osteoporotic fracture. With adjustments applied for clustering within families and sex, in all participants, the risk of osteoporosis (53 cases) expressed per 10-year increment in age and UPPost-age, increased by 21% and 36% ($P \leq 0.044$), respectively. In women, the corresponding estimates adjusted for clustering within families were 55% and 60% for incident osteoporosis (37 cases; $P \leq 0.0003$) and 42% and 44% for osteoporotic fractures (25 cases; $P \leq 0.017$). Full adjustment for all risk factors for osteoporosis removed the significance of the associations in **Table 3**.

Independent of chronological age (**Figure S2**), serum dp-ucMGP was correlated with UPPost-age ($r = 0.41$; $P < 0.0001$). Dichotomised by the median of dp-ucMGP (≥4.50 and <4.50 μg/L), the risk of osteoporosis was significantly associated with UPPost-age in the higher dp-ucMGP category (HR: 1.35 [1.01, 1.80]; $P = 0.040$), but not in the lower category (HR: 1.31 [0.84, 2.05]; $P = 0.24$).

Table 2. Body-height-to-arm-span ratio in relation to baseline age in FLEMENGHO participants (2005-2010)

HASR (× -3 cm) Group analyzed	*n*	Chronological age		UPPost-age	
		Estimate (95% CI)	*P*-value	Estimate (95% CI)	*P*-value
All participants	706	-4.84 (-6.32, -3.36)	<0.0001	-4.67 (-6.47, -2.87)	<0.0001
HASD > -3 cm	428	-1.85 (-3.26, -0.43)	0.011	-2.22 (-3.89, -0.55)	0.0093
HASD ≤ -3 cm	278	-1.84 (-3.34, -0.34)	0.016	-2.39 (-4.18, -0.59)	0.0095
Men	349	-5.39 (-7.41, -3.36)	<0.0001	-4.31 (-6.90, -1.73)	0.0011
HASD > -3 cm	161	-2.84 (-5.00, -0.67)	0.010	-2.60 (-5.27, 0.07)	0.056
HASD ≤ -3 cm	188	-1.45 (-3.26, 0.36)	0.12	-2.40 (-4.60, -0.20)	0.032
Women	357	-4.51 (-6.77, -2.25)	0.0001	-4.86 (-7.40, -2.32)	0.0002
HASD > -3 cm	267	-1.01 (-2.95, 0.93)	0.30	-1.79 (-3.95, 0.36)	0.10
HASD ≤ -3 cm	90	-2.40 (-5.46, 0.66)	0.12	-2.01 (-5.39, 1.37)	0.24
Menopausal women	183	-4.91 (-10.1, 0.26)	0.063	-3.67 (-8.22, 0.87)	0.11
HASD > -3 cm	121	-1.07 (-5.35, 3.20)	0.62	-3.24 (-6.94, 0.47)	0.086
HASD ≤ -3 cm	62	0.36 (-5.64, 6.36)	0.90	1.28 (-3.86, 6.43)	0.62

Abbreviations; HASD: the difference of body height minus arm span; HASR: body-height-to-arm-span ratio; *n*: the number of participants in the category; UPPost-age: age as predicted by the urinary proteomic profile; 95% CI: 95% confidence interval. Estimates were derived by linear regression analysis with HASR as the continuous outcome in all participants and in two subgroups dichotomised by the HASD threshold (> -3 *vs* ≤ -3 cm). Estimates, given with 95% CI, express the association size of HASR per 10-year increment in chronological age or UPPost-age. Estimates are partial regression coefficients adjusted for clustering within families, sex (in all participants only), current smoking, γ-glutamyltransferase, fasting plasma glucose, physical activity, use of diuretics (yes *vs* no), and socioeconomic status (high or middle *vs* low). To increase readability, leading zeros were removed by multiplying the estimates by 10^3; the actual values can be obtained by multiplying the estimates by 10^{-3}.

Table 3. Risk of osteoporosis in relation to age in FLEMENGHO participants

Variable	n/N	Chronological age		UPPost-age	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
All participants					
Osteoporosis	53/706	1.21 (1.00, 1.46)	0.044	1.36 (1.09, 1.68)	0.0057
Osteoporotic fracture	37/706	1.05 (0.84, 1.31)	0.66	1.17 (0.90, 1.53)	0.24
Men					
Osteoporosis	16/349	0.72 (0.51, 1.01)	0.058	0.81 (0.52, 1.26)	0.36
Osteoporotic fracture	12/349	0.56 (0.36, 0.86)	0.0083	0.66 (0.39, 1.12)	0.12
Women					
Osteoporosis	37/357	1.55 (1.23, 1.95)	0.0003	1.60 (1.25, 2.03)	0.0001
Osteoporotic fracture	25/357	1.42 (1.07, 1.88)	0.014	1.44 (1.07, 1.94)	0.017
Menopausal women					
Osteoporosis	32/183	1.17 (0.82, 1.67)	0.39	1.31 (0.96, 1.78)	0.087
Osteoporotic fracture	20/183	1.13 (0.72, 1.77)	0.59	1.22 (0.82, 1.81)	0.32

Abbreviations: n/N: number of incident cases/number at risk; UPP: urinary peptidomic profile; UPPost-age: age as predicted by the urinary proteomic profile; HR: hazard ratio; 95% CI: 95% confidence interval. Of 53 incident osteoporosis cases, 16 had the diagnosis documented in practice or hospital records and 37 experienced an osteoporotic fracture. HRs, given with 95% CI, express the relative risk per 10-year increment in chronological age or UPPost-age. The HRs were adjusted for clustering within families and in all participants additionally for sex.

Discussion

We constructed a novel ageing-related biomarker indicative of osteoporosis, UPPost-age, and validated UPPost-age cross-sectionally and longitudinally in the family-based FLEMENGHO study, which is representative for Europeans living in northern Belgium. Four lines of evidence support this statement. First, chronological age was associated with bone-related UPP fragments, including collagens, IGF2 and MGP in the derivation and validation datasets. Second, among the 99 bone-related urinary peptides associated with chronological age, 32 (32.3%) showed a sex-specific distribution probably explaining sexual heterogeneity in the molecular pathogenic mechanisms in osteoporosis. Third, HASR and HASD ≤ -3 cm, anthropometric measurements reflecting osteoporosis, were associated with UPPost-age. Finally, the association between osteoporosis and UPPost-age reached significance in all participants and in women and osteoporotic fracture was associated with UPPost-age in women. In line with the aforementioned results, there was a positive correlation between the plasma levels of dp-ucMGP and UPPost-age and the risk of osteoporosis was higher in participants with high plasma dp-ucMGP, a marker of vitamin K deficiency [15]. Its activation requires a vitamin K-dependent γ-glutamate carboxylation, which is also needed for key proteins maintaining the integrity of bone tissue, including osteocalcin, GAS6, periostin and protein S [29], which play a key role in the differentiation and gene expression in osteoblasts and in the mineralization of the bone extracellular matrix [30].

The current findings are consistent with previous FLEMENGHO publication [9], showing that a general ageing UPP biomarker without focus on bone tissue proteins (UPPost-age) was associated with osteoporosis and fracture risk in all participants and women. However, UPPost-age only had 8 of 29 (27.6%) urinary peptide segment and 3 of 10 (30.0%) parental proteins in common with UPP-age. Furthermore, a case-control study identified a distinctive serum proteomics profile, which differed between healthy controls compared with patients with osteopenia and osteoporosis (Q^2=0.7295, R^2=0.9180). Most of the dysregulated proteins were immunoglobins, complement proteins and cytoskeletal proteins [31]. Focusing on in vitro experiments, a comparative analysis of proteins expressed by three osteoblast-derived extracellular matrix preparations identified more than 1000 proteins with differing levels, including those specifically present in mineralised matrix, such as growth factors and proteins linked to cell migration and angiogenesis [32].

Type-I collagen is the most abundant constituents of the organic extracellular matrix in bones tissue, accounting for 90% of total collagen [33,34]. The main function of

collagens is to provide mechanical support and to act as a scaffold for bone cells. COL1A1 participates in various physiological processes, including bone trabecular formation, ossification, osteoblast differentiation and skeletal system development, while COL1A2 involves in bone mineralization (**Table S1**). In the type-I collagen degradation, small fragments are first released by collagenases, whereas larger fragments are further degraded by gelatinases with the resulting smaller fragments being further cleaved by gelatinases [35]. Only breakdown products of mature collagen appear in urine [35]. Current findings showed an inverse association between chronological age and type-I collagen fragments (**Table S2**), indicating that the level of collagen I fragments decreased in urine with advancing age. Furthermore, in the current study, chronological age was negatively associated with the urinary levels of peptide fragments derived from COL2A1, COL3A1 and COL6A1 (**Table S2**). COL2A1 participate in cartilage development and endochondral bone morphogenesis [36]. COL3A1 serves as the stimulator of cell growth and messenger RNA expression in normal osteoblasts and bone marrow mesenchymal stem cells [37]. COL6A1 deficiency alters the configuration and shape of osteoblasts, which affects the maintenance of bone mass [38,39]. The inverse association of urine peptide fragments derived from these collagens with age might therefore reflect the reduced expression of these functional collagens with age.

The present study demonstrated that the bone-related UPP displayed a sex-specific pattern with women having lower levels than men of urinary peptides derived from collagens, IGF2 and MGP (**Figure 3**). The possible explanation for the up-regulated IGF2 in women might be an upregulation for bone repair and remodeling [40]. Along similar lines, a previous study [41] reported the association of 90 urinary peptides with sex and verified these findings in a general population sample (n=337) and in patients with type-2 diabetes (n=1442). These peptides were derived from 29 parental proteins, including COL1A1, COL1A2, COL3A1, COL10A1, COL18A1 and PIGR [41]. Subgroup analysis in the healthy individuals below 45 years and over 55 years revealed that 51 and 66 urinary peptides were associated with sex, respectively, indicating that these sex-specific differences became more pronounced after menopause [41]. Osteoporosis is an ageing-related skeletal disease with an exceedingly higher prevalence and high incidence of osteoporotic fractures in postmenopausal women, compared to men of similar age [42,43]. Among the urinary peptides retained in the UPPost-age prediction model, 10 (34.5%) displayed the sex-specific difference. Identification of sex-associated UPP differences might help to have a better understanding in pathogenic molecular mechanisms in women compared to men, irrespective of the disease of interest. Our current observations might prompt research into novel circulating or urinary biomarkers

with high diagnostic accuracy over and beyond the levels of sex hormones and UPPost-age.

Strong points and limitations

The strong points of the current study are the replication and validation of UPPost-age prediction model within the FLEMENGHO cohort and the demonstration of its clinical relevance given its association with osteoporosis. Notwithstanding these strong points, the current study must be interpreted within the context of its limitations. First, the validation of UPPost-age prediction model was based on the HASR, HASD ≤ -3 cm and the incidence of osteoporosis. Not all the participants had BMD, the gold standard of osteoporosis diagnosis, which might have led to an underestimation of the incidence of clinically asymptomatic osteoporosis. However, UPPost-age was significantly higher in the women with low BMD in the study population. Furthermore, the incidence rates per 100 persons were 7.51% (95% CI: 5.56, 9.45%) for osteoporosis and 5.24% (3.60, 6.88%) for osteoporotic fracture during the long-term follow-up, which was comparable to previous studies [2,44]. Second, osteoporosis causes high health care expenditures, predominantly due to the treatment and clinical management of severe fractures [45]. A health economic analysis was not within the scope of the current study. Further health economic studies are needed to compare the cost of osteoporosis management with and without the application of UPPost-age as an ageing biomarker indicating the risk of osteoporotic fractures in women and as actionable guide for and early intervention and prevention. Third, the FLEMENGHO cohort was recruited in a high-income country, where residents have ample access to calcium-rich food, such as milk and cheese, and nutrients, such as fermented cheese and green vegetables rich in vitamin K. Therefore, further validation of the UPPost-age prediction model is necessary in other ethnicities, less affluent countries and special populations, in particular among high risk postmenopausal women [46], organ transplantation recipients with immunotherapy [47] and patients with vitamin D deficiency [48]. Finally, the number of events in some strata in Table 3 was too low to allow a full adjustment. However, the cross-sectional analyses in Table 2, support the findings reported in Table 3 with partial adjustment. Independent replication in other cohorts might strengthen our current findings. Moreover, the results in Table 3 are in keeping with the higher osteoporosis risk in women compared with men.

Conclusion

An ageing-related UPP signature focused on peptide fragments derived from bone tissue-related proteins is associated with the risk of osteoporosis and osteoporotic fracture. The

UPPost-age biomarker is registered as an in-vitro diagnostics in Germany and the European Union, providing patients, clinicians and trialists with a novel approach to predict the risk of osteoporosis at an early stage, when prevention of osteoporotic fractures is still possible.

Funding

OMRON Healthcare, Co., Ltd., Kyoto, Japan provided a non-binding grant to the Non-Profit Research Association Alliance for the Promotion of Preventive Medicine (APPREMED), Mechelen, Belgium. Dries S. Martens holds a postdoctoral position granted by the Research Foundation Flanders (FWO grant 12X9623N).

Conflict of interest

JS and AL are employees of Mosaiques-Diagnostics GmbH, Hannover, Germany. HM is the co-founder and co-owner of Mosaiques- Diagnostics GmbH. The other authors declare no conflict of interest.

References

1. Rachner TD, Khosla S, Hofbauer LC. Osteoporosis: now and the future. *Lancet.* 2011; 377: 1276-1287.
2. Veiras LC, Girardi ACC, Curry J *et al*. Sexual dimorphic pattern of renal transporters and electrolyte homeostasis. *J Am Soc Nephrol.* 2017; 28: 3504-3517.
3. Bruge R, Dawson-Hughes B, Solomon DH, John BW, Alison K, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. *J Bone Miner Res.* 2007; 22: 465-475.
4. Hernlund E, Svedbom A, Ivergård M *et al*. Osteoporosis in the European Union: a compendium of country-specific reports. *Arch Osteoporos.* 2013; 8: 136.
5. Gao S, Zhao Y. Quality of life in postmenopausal women with osteoporosis: a systematic review and meta-analysis. *Qual Life Res.* 2023; 32: 1551-1565.
6. Dunnewind T, Dvortsin EP, Smeets HM *et al*. Economic consequences and potentially preventable costs related to osteoporosis in the Netherlands. *Value Health.* 2017; 20: 762-768.
7. Williams SA, Daigle SG, Weiss R, Wang Y, Arora T, Curtis JR. Economic burden of osteoporosis-related fractures in the US medicasre population. *Ann Pharmacother.* 2021; 55: 821-829.
8. GBD 2019 Fracture Collaborators. Global, regional, and national burden of bone fractures in 204 countries and territories, 1990-2019: a systematic analysis from the Global Burden of Disease Study 2019. *Lancet Healthy Longev.* 2021; 2: e580-e592.
9. Martens DS, Thijs L, Latosinska A *et al*. Urinary peptidomics to address age-related disabilities: a prospective population study with replication in patients. *Lancet Healthy Longev.* 2021; 2: e690-e703.
10. Xu J, Cai X, Miao Z *et al*. Proteome-wide profiling reveals dysregulated molecular features and accelerated aging in osteoporosis: a 9.8-year prospective study. *Aging Cell.* 2023; 22: e14035.
11. López-Otín C, Biasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: an expanding universe. *Cell.* 2023; 186: 243-278.
12. Marx D, Anglicheau D, Caillard S *et al*. Urinary collagen peptides: source of markers for bone metabolic processes in kidney transplant recipients. *Proteomics Clin Appl.* 2022; 17: 2200118.

13. Levey AS, Bosch JP, Lewis JB *et al*. A more accurate method to estimate glomerular filtration rate from serum creatinine : a new prediction equation. *Ann Intern Med.* 1999; 130: 461-470.
14. Peake M, Whiting M. Measurement of serum creatinine — Current status and future goals. *Clin Biochem Rev.* 2006; 27: 173-182.
15. Wei FF, Trenson S, Verhamme P, Vermeer C, Staessen J. Vitamin K-dependent matrix Gla protein as multifaceted protector of vascular and tissue integrity. *Hypertension.* 2019; 73: 1160-1169.
16. Schutte R, Nawrot TS, Richart T *et al*. Bone resorption and environmental exposure to cadmium in women: a population study. *Environ Health Perspect.* 2008; 116: 777-783.
17. Verhaar HJJ, Koele JJ, Neijzen T, Dessens JAG, Duursma SA. Are arm span measurements useful in the prediction of osteoporosis in postmenopausal women? *Osteoporos Int.* 1998; 8: 174-176.
18. Ofluoglu D, Unlu F, Akyuz G. Relationship between arm span and height in postmenopausal osteoporotic women. *Rheumatol Int.* 2008; 28: 737-741.
19. Staessen JA, Roels HA, Emelianov D *et al*. Environmental exposure to cadmium, forearm bone-density, and risk of fractures : prospective population study. *Lancet.* 1999; 353: 1140-1144.
20. Jantos-Siwy J, Schiffer E, Brand K *et al*. Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res.* 2009; 8: 268-281.
21. Klein J, Papadopoulos T, Mischak H, Mullen W. Comparison of CE-MS/MS and LC-MS/MS sequencing demonstrates significant complementarity in natural peptide identification in human urine. *Electrophoresis.* 2014; 35: 1060-1064.
22. Mischak H, Vlahou A, Ioannidis JPA. Technical aspects and inter-laboratory variability in native peptide profiling : the CE-MS experience. *Clin Biochem.* 2013; 46: 432-443.
23. Zhang ZY, Ravassa S, Nkuipou-Kenfack E *et al*. Novel urinary peptidomic classifier predicts incident heart failure. *J Am Heart Assoc.* 2017; 6: e005432.
24. An DW, Yu YL, Martens DS *et al*. Statistical approaches applicable in managing OMICS data: urinary proteomics as exemplary case. *Mass Spec Rev.* 2023; 42: 1-18.
25. Blom G. Statistical estimates and transformed beta-variables. *Biom J.* 1961; 3: 285.

26. Zhang R, Yin H, Yang M, Lei X, Zhen D, Zhang Z. Advanced progress of the relationship between antihypertensive drugs and bone metabolism. *Hypertension.* 2023; 80: 2255-2264.

27. Staessen JA, Fagard R, Amery A. Life style as a determinant of blood pressure in the general population. *Am J Hypertens.* 1994; 7: 685-694.

28. Martens DS, An DW, Yu YL *et al*. Association of air pollution with a urinary biomarker of biological aging and effect modifiers in a prospective population study: protective role of antioxidant vitamin K. *Environ Health Perspect.* 2023; 131: 127011.

29. Elshaikh AO, Shah L, Mathew CJ, Lee R, Jose MT, Cancerevic I. Influence of vitamin K on bone mineral density and osteoporosis. *Cureus.* 2020; 12: e10816.

30. Akbari S, Rasouli-Ghahroudi AA. Vitamin K and bone metabolism: a review of the latest evidence in preclinical studies. *Biomed Res Int.* 2018; 4629383: 1-5.

31. Al-Ansari M, Aleidi SM, Masood A *et al*. Protemics profiling of osteoporosis and osteopenia patients and associated network analysis. *Int J Mol Sci.* 2022; 23: 10200.

32. Baroncelli M, van der Eerden B, Kan YY *et al*. Comparative proteomic profiling of human osteoblast-derived extracellular matrices identifies proteins involved in mesenchymal stromal cell osteogenic differentiation and mineralization. *J Cell Physiol.* 2017; 233: 387-395.

33. Lin X, Patil S, Gao YG, Qian A. The bone extracellular matrix in bone formation and regeneration. *Front Phrmacol.* 2020; 11: 757.

34. Licini C, Vitale-Brovarone C, Mattioloni-Belmonte M. Collagen and non-collagenous proteins molecular crosstalk in the pathophysiology of osteoporosis. *Cytokine Growth Factor Rev.* 2019; 49: 59-69.

35. López B, González A, Ravassa S *et al*. Circulating biomarkers of myocardial fibrosis: the need for a reappraisal. *J Am Coll Cardiol.* 2015; 65: 2449-2456.

36. Richards AJ, Laidlaw M, Whittaker J *et al*. High efficiency of mutation detection in type 1 stickler syndrome using a two-stage approach: vitreoretinal assessment coupled with exon sequencing for screening COL2A1. *Hum Mut.* 2006; 27: 696-704.

37. Xia XD, Zhou Z, Yu XH, Zheng XL, Tang CK. Myocardin: a novel player in atherosclerosis. *Atherosclerosis.* 2017; 257: 266-278.

38. Huang J, Yu Z, Wu Y *et al*. Prognostic significance of blood pressure at rest and after performing the six-minite walk test in patinets with acute heart failure. *Am J Hypertens.* 2023; 37: 199-206.
39. Izu Y, Ezura Y, Mizoguchi F *et al*. Type VI collagen deficiency induces osteopenia with distortion of osteoblastic cell morphology. *Tissue Cell.* 2012; 44: 1-6.
40. Min SK, Kim M, Park J-B. Insulin-like growth factor 2-enhanced osteogenic differentiation of stem cell spheroids by regulation of Runx2 and Col1 expression. *Exp Ther Med.* 2021; 383: 1-6.
41. Laurent S, Chatellier G, Azizi M *et al*. SPARTE Study: normalization of arterial stiffness and cardiovascular events in patients with hypertension at medium to very high risk. *Hypertension.* 2021; 78: 983-995.
42. Committee on Clinical Practice Guideline-Gynecology. Osteoporosis prevention, screening, and disgnosis: ACOG clinical practice guideline No. 1. *Obstet Gynecol.* 2012; 138: 494-506.
43. Walker MD, Shane E. Postmenopausal osteoporosis. *N Engl J Med.* 2023; 389: 1979-1991.
44. US Preventive Services Task Force. Screening for osteoporosis to prevent fracture: US preventive services task force recommendation statement. *J Am Med Ass.* 2018; 319: 2521-2531.
45. Dehanne F, Gourdin M, Devleesschauwer B *et al*. Cost-DALY comparison of hip replacement care in 12 Belgian hospitals. *BMJ Open Quality.* 2021; 10: e001263.
46. Huang D, Wang Y, Lv J *et al*. Proteomic profiling analysis of postmenopausal osteoporosis and osteopenia identifies potential proteins associated with low bone mineral density. *PeerJ.* 2020; 8: e9009.
47. Anastasilakis AD, Tsourdi E, Makras P *et al*. Bone disease following solid organ transplatation: a narrative review and recommendations for management from the European Calcified Tissue Society. *Bone.* 2019; 127: 401-418.
48. Holick MF. Vitamin D deficiency. *N Engl J Med.* 2007; 357: 266-281.

Data Supplement

Urinary Proteomics

Sample preparation and CE-MS analysis

As described in detail elsewhere [1], urine aliquots were thawed and 700 µl mixed with 700 µL of 2 M urea, 10 mM NH_4OH containing 0.02 % SDS. Subsequently, samples were ultrafiltered using a Centristat 20 kDa cut-off centrifugal filter device (Satorius, Göttingen, Germany) to eliminate high molecular weight proteins. The obtained filtrate was desalted using a PD 10 gel filtration column (GE Healthcare Bio Sciences, Uppsala, Sweden) to remove urea, electrolytes and salts as well as to enrich the polypeptides. The samples were lyophilised and stored at 4°C before usage. Shortly before CE-MS analysis, the samples were re-suspended in 10 µL HPLC-grade H_2O. Samples were injected into CE-MS with 2 psi for 99 sec, resulting in injection volumes of ~280 nL.

A P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) was coupled with a Micro-TOF MS (Bruker Daltronic, Bremen, Germany). A solution of 20% acetonitrile (Sigma-Aldrich, Taufkirchen, Germany) in HPLC-grade H_2O (Roth, Karlsruhe, Germany) supplemented with 0.94% formic acid (Sigma-Aldrich) was used as running buffer. For CE-MS analysis, the electrospray ionization interface from Agilent Technologies (Palo Alto, CA) was set to a potential of -4.0 to -4.5 kV. Spectra were recorded over an *m*/*z* range of 350-3000 and accumulated every 3 s.

CE-MS data processing

After the CE-MS analysis, mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaFinder software [2]. Only signals with z>1 observed in a minimum of 3 consecutive spectra with a signal-to-noise ratio of at least 4 were considered. The resulting peak list characterises each polypeptide by its mass and migration time. Data were calibrated utilizing 3151 internal standards as reference data points for mass and migration time by applying global and local linear regression, respectively as described previously. Reference signals of 29 abundant peptides were used as internal standards for calibration of signal intensity using local linear regression [3]. This procedure was shown to be an easy and reliable method to address both analytical and dilution variances and differences in glomerular filtration in a single calibration step. This procedure was shown to be highly reproducible and to address both analytical and dilution variances in a single calibration step [3]. The obtained peak list characterises each polypeptide by its calibrated molecular mass (Da), calibrated CE migration time (min) and normalised signal intensity. All detected peptides

were deposited, matched, and annotated in a Microsoft SQL database allowing further statistical analysis.

Sequencing of peptides

Candidate biomarkers were sequenced using CE-MS/MS or LC-MS/MS analysis, as described in detail [4]. MS/MS experiments were using an Ultimate 3000 nano-flow system (Dionex/LC Packings, Thermo Fisher Scientific Inc., Waltham, MA) or a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA), both connected to an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) equipped with a nano-electrospray ion source. The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from *m*/*z* 300–2,000) were acquired in the Orbitrap. Ions were sequentially isolated for fragmentation. Data files were searched against the UniProt human nonredundant database, using Proteome Discoverer 2.4 and the SEQUEST search engine. Relevant settings were: no fixed modifications, oxidation of methionine and proline as variable modifications. The minimum precursor mass was set to 790 Da, maximum precursor mass to 6000 Da with a minimum peak count of 10. The high-confidence peptides were defined by cross-correlation (Xcorr) >1.9 and rank = 1. Precursor mass tolerance and fragment mass tolerance were 5 ppm and 0.05 Da, respectively. For further validation of obtained peptide sequences, the correlation between peptide charge at the working pH of 2 and CE-migration time was utilised to minimise incorrect sequence assignment [5]. Calculated CE-migration time of the sequence candidate based on its peptide sequence (number of basic amino acids) was compared to the experimental migration time.

References

1. Mischak H, Vlahou A, Ioannidis JPA. Technical aspects and inter-laboratory variability in native peptide profiling : the CE-MS experience. *Clin Biochem.* 2013; 46: 432-443.
2. Latosinska A, Siwy J, Mischak H, Frantzi M. Peptidomics and proteomics based on CE-MS as a robust tool in clinical application: the past, the present, and the future. *Electrophoresis.* 2019; 40: 2294-2308.
3. Jantos-Siwy J, Schiffer E, Brand K *et al*. Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res.* 2009; 8: 268-281.

4. Klein J, Papadopoulos T, Mischak H, Mullen W. Comparison of CE-MS/MS and LC-MS/MS sequencing demonstrates significant complementarity in natural peptide identification in human urine. *Electrophoresis.* 2014; 35: 1060-1064.
5. Zürbig P, Renfrow MB, Schiffer E *et al*. Biomarker discovery by CE-MS enables sequence analysis via MS/MS with platform-independent separation. *Electrophoresis.* 2006; 27: 2111-2125.

Table S1. Bone-related function of 19 parental protein identified by 395 sequenced urinary peptides

Parental proteins identified from the amino-acid sequence			Number of peptides in analysis	Bone-related function
Accession number	**Symbol**	**Name**		
P02452	COL1A1	Collagen alpha-1(I) chain	199	Bone trabecula formation, cartilage development involved in endochondral bone morphogenesis, embryonic skeletal system development, endochondral ossification, extracellular matrix structural constituent and organization, ossification, osteoblast differentiation, skeletal system development
P08123	COL1A2	Collagen alpha-2(I) chain	37	Bone mineralization, extracellular matrix structural constituent and organization, skeletal system development
P02458	COL2A1	Collagen alpha-1(II) chain	21	Extracellular matrix structural constituent, cartilage condensation, cartilage development involved in endochondral bone morphogenesis, chondrocyte differentiation, embryonic skeletal joint morphogenesis, endochondral ossification, skeletal system development
P02461	COL3A1	Collagen alpha-1(III) chain	94	Extracellular matrix structural constituent and organization, limb joint morphogenesis
P53420	COL4A4	Collagen alpha-4(IV) chain	3	Extracellular matrix structural constituent, high expression in calvaria
P05997	COL5A2	Collagen alpha-2(V) chain	5	Extracellular matrix organization, ossification, skeletal system development
P12109	COL6A1	Collagen alpha-1(VI) chain	5	Extracellular matrix structural constituent, osteoblast differentiation
Q14055	COL9A2	Collagen alpha-2(IX) chain	2	Extracellular matrix structural constituent and organization, skeletal system development
Q14050	COL9A3	Collagen alpha-3(IX) chain	2	Extracellular matrix structural constituent and organization, involvement in multiple epiphyseal dysplasia
Q03692	COL10A1	Collagen alpha-1(X) chain	1	Extracellular matrix structural constituent and organization, skeletal system development
P12107	COL11A1	Collagen alpha-1(XI) chain	7	Extracellular matrix structural constituent and organization, cartilage condensation, embryonic skeletal system morphogenesis, ossification
P13942	COL11A2	Collagen alpha-2(XI) chain	4	Extracellular matrix structural constituent and organization, cartilage development, skeletal system development
Q5TAT6	COL13A1	Collagen alpha-1(XIII) chain	4	Extracellular matrix structural constituent and organization, endochondral ossification, skeletal system development
P39060	COL18A1	Collagen alpha-1(XVIII) chain	2	Extracellular matrix structural constituent and organization, collagen fibril organization, skeletal system development
Q14993	COL19A1	Collagen alpha-1(XIX) chain	2	Extracellular matrix structural constituent and organization, skeletal system development
Q96P44	COL21A1	Collagen alpha-1(XXI) chain	1	Collagen-containing extracellular matrix, expression in bone marrow
P01344	IGF2	Insulin-like growth factor II	3	Growth factor and hormone activity, osteoblast differentiation
P08493	MGP	Matrix Gla protein	2	Structural constituent of bone, cartilage condensation, ossification, regulation of bone mineralization
Q86Y38	XYLT1	Xylosyltransferase 1	1	Embryonic skeletal system development, ossification involved in bone maturation

For analysis, 395 sequenced urinary peptides derived from 19 parental proteins with a detectable signal in ≥70% FLEMENGHO participants were selected (see Figure 1). Accession numbers refer to the Uniprot database (http://www.uniprot.org/uniprot), which substantiated the bone-related function of the parental proteins.

Table S2. Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (starts)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	*p*-value
		Accession number	Symbol	Name		
e99907132	EpGSpGENGAPGQmGPR	P02452	COL1A1	Collagen alpha-1(I) chain	0.281 (0.203, 0.359)	9.21E-11
e99910072	GLpGTGGPpGENGKpGEpGPKG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.232 (-0.309, -0.155)	3.29E-08
e99901132	cDDYRLcE	P08493	MGP	Matrix Gla protein	0.230 (0.154, 0.305)	3.85E-08
e99906708	pGpAGEKGSpGADGPAGAP	P02452	COL1A1	Collagen alpha-1(I) chain	0.250 (0.169, 0.332)	5.19E-08
e99904196	PSGDQGASGpAGPSGP	P02458	COL2A1	Collagen alpha-1(II) chain	0.237 (0.156, 0.318)	7.19E-08
e99920065	ARGNDGARGSDGQpGppGPPGTAGFPGSpGAKGEVGpAGSpGSNGApG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.241 (-0.323, -0.159)	7.27E-08
e99912851	LDGAKGDAGPAGPKGEpGSpGENGApG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.227 (-0.304, -0.151)	1.52E-07
e99917856	ARGNDGARGSDGQPGPpGppGTAGFpGSpGAKGEVGP	P02461	COL3A1	Collagen alpha-1(III) chain	-0.235 (-0.317, -0.153)	1.78E-07
e99919107	DQGPVGRTGEVGAVGPpGFAGEKGpSGEAGTAGPPGTpGPQG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.218 (-0.297, -0.139)	3.00E-07
e99917922	NTGApGSPGVSGPKGDAGQpGEKGSpGAQGpPGAPGPLG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.234 (-0.318, -0.151)	3.04E-07
e99916966	PpGESGREGApGAEGSpGRDGSPGAKGDRGETGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.215 (-0.295, -0.135)	3.23E-06
e99906441	TGSpGSpGPDGKTGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.204 (-0.283, -0.126)	7.75E-06
e99912986	ADGQpGAKGEpGDAGAKGDAGPPGPAGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.203 (-0.282, -0.125)	9.27E-06
e99901661	DGpSGAEGpPGp	P02458	COL2A1	Collagen alpha-1(II) chain	0.187 (0.107, 0.267)	2.49E-05
e99918573	DQGPVGRTGEVGAVGpPGFAGEKGPSGEAGTAGPpGTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	-0.183 (-0.263, -0.102)	3.26E-05
e99919745	SKGESGNKGEpGSAGPQGPpGpSGEEGKRGPNGEAGSAGPPGPpG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.187 (-0.270, -0.105)	3.43E-05
e99905560	GSpGSpGPDGKTGPPGp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.191 (-0.269, -0.113)	3.61E-05
e99900340	cDDYRLc	P08493	MGP	Matrix Gla protein	0.189 (0.108, 0.270)	4.30E-05
e99909891	SEGSPGHpGQPGpPGpPGApGP	P02461	COL3A1	Collagen alpha-1(III) chain	-0.189 (-0.271, -0.106)	4.88E-05
e99906709	GSpGSpGPDGKTGPpGPAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.186 (-0.264, -0.108)	5.84E-05
e99906154	EDGHpGKPGRpGERG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.180 (-0.261, -0.098)	6.07E-05
e99912949	LDGAKGDAGPAGpKGEpGSpGENGApG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.191 (-0.271, -0.111)	6.40E-05

Table S2. Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (continued)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	*p*-value
		Accession number	Symbol	Name		
e99917311	NTGApGSpGVSGpKGDAGQpGEKGSPGAQGPPG APGp	P02461	COL3A1	Collagen alpha-1(III) chain	-0.186 (-0.270, -0.102)	9.67E-05
e99915778	ESGREGAPGAEGSPGRDGSpGAKGDRGETGp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.186 (-0.266, -0.106)	0.00011
e99911415	IGPpGPAGApGDKGESGPSGPAGPTG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.182 (-0.261, -0.104)	0.00012
e99903016	ApGDRGEPGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.187 (-0.268, -0.105)	0.00016
e99903372	DGQPGAKGEpGDAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.184 (-0.266, -0.103)	0.00017
e99911777	ANGApGNDGAKGDAGApGApGSQGAPG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.188 (-0.271, -0.104)	0.00019
e99903120	EGSpGRDGSpGAK	P02452	COL1A1	Collagen alpha-1(I) chain	0.188 (0.105, 0.272)	0.00021
e99904945	SpGSPGPDGKTGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.186 (-0.270, -0.102)	0.00027
e99906617	MpGSpGGpGSDGKpGpPG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.172 (-0.255, -0.089)	0.00029
e99917288	GPpGESGREGAPGAEGSpGRDGSpGAKGDRGET Gp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.184 (-0.267, -0.100)	0.00031
e99919885	ERGEQGPAGSpGFQGLpGpAGppGEAGKpGEQG VPGDLGAPGPSG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.179 (-0.261, -0.098)	0.00034
e99918864	GpAGPPGPPGPPGTSGHPGSPGSPGYQGPPGEP GQAGPSGPPG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.164 (-0.246, -0.082)	0.00052
e99904847	GGDkGEDGDPGQPGp	P12107	COL11A1	Collagen alpha-1(XI) chain	0.157 (0.074, 0.241)	0.00063
e99917866	NTGApGSpGVSGPKGDAGQPGEKGSpGAQGPPG APGPLG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.166 (-0.250, -0.081)	0.00070
e99912508	KGNSGEpGAPGSKGDTGAKGEpGpVG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.176 (-0.260, -0.092)	0.00073
e99920509	GEHGpPGPPGPIGPVGQPGAAGADGEPGARGPQ GHFGAKGDEGTRGFNGP	P13942	COL11A2	Collagen alpha-2(XI) chain	-0.153 (-0.236, -0.070)	0.00078
e99915442	DVStPPTVLPDNFPRYP	P01344	IGF2	Insulin-like growth factor II	0.148 (0.066, 0.231)	0.00088
e99919425	pGApGVRGFQGQKGSmGDpGLPGPQGLRGDVG DRGPGGAAGP	Q14050	COL9A3	Collagen alpha-3(IX) chain	0.150 (0.066, 0.235)	0.00089
e99917787	GARGLpGpPGSNGNPGPPGPSGSPGKDGPPGPA GNTGAP	P02461	COL3A1	Collagen alpha-1(III) chain	-0.160 (-0.243, -0.076)	0.0011
e99917280	AAGEpGKAGERGVpGPpGAVGPAGKDGEAGAQG PPGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.169 (-0.253, -0.086)	0.0012
e99913779	DDILASPPRLPEPQPYPGAPHHSS	P39060	COL18A1	Collagen alpha-1(XVIII) chain	0.140 (0.059, 0.222)	0.0012

Table S2. Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (continued)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	*p*-value
		Accession number	Symbol	Name		
e99917212	RTGEVGAVGPpGFAGEKGPSGEAGTAGpPGTpGPQG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.149 (-0.231, -0.067)	0.0012
e99909408	GDDGEAGKpGRPGERGPPGp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.166 (-0.247, -0.084)	0.0013
e99906961	GLpGTGGPpGENGKPGEp	P02461	COL3A1	Collagen alpha-1(III) chain	-0.161 (-0.246, -0.075)	0.0013
e99914645	DVStPPTVLPDNFPRYPVG	P01344	IGF2	Insulin-like growth factor II	0.144 (0.061, 0.226)	0.0014
e99905423	VGpPGPPGpPGPPGPPS	P02452	COL1A1	Collagen alpha-1(I) chain	-0.167 (-0.250, -0.084)	0.0014
e99905074	DGHpGKpGRPGERG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.149 (-0.232, -0.066)	0.0014
e99916419	EAGRDGNpGNDGppGRDGQPGHKGERGYpG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.146 (-0.227, -0.064)	0.0016
e99904531	PGTpGSPGPAGASGNPG	P02458	COL2A1	Collagen alpha-1(II) chain	0.147 (0.065, 0.228)	0.0018
e99916785	DPGLMGERGEDGpAGNGTEGFpGFpGYPGNR	P12109	COL6A1	Collagen alpha-1(VI) chain	-0.137 (-0.221, -0.054)	0.0020
e99907513	TGSpGSPGPDGKTGPpGPAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.164 (-0.247, -0.080)	0.0022
e99914815	pGYPGQpGQDGKPGYQGIAGTpGVpGSPG	Q96P44	COL21A1	Collagen alpha-1(XXI) chain	-0.130 (-0.211, -0.049)	0.0023
e99917447	GPpGDpGLMGERGEDGpAGNGTEGFPGFPGYPGN	P12109	COL6A1	Collagen alpha-1(VI) chain	-0.134 (-0.219, -0.049)	0.0030
e99911753	GADGQPGAKGEPGDAGAKGDAGppGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.162 (-0.247, -0.077)	0.0031
e99918044	AGGPGFPGApGAKGEAGpTGARGpEGAQGPRGEPGTPGS	P02458	COL2A1	Collagen alpha-1(II) chain	-0.143 (-0.227, -0.058)	0.0039
e99905705	DGQPGAKGEpGDAGAKG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.153 (-0.235, -0.071)	0.0042
e99906176	VGPpGPpGPPGPpGPPSA	P02452	COL1A1	Collagen alpha-1(I) chain	-0.157 (-0.241, -0.073)	0.0042
e99915411	PQGPpGPTGpGGDKGDTGPpGPQGLQGLpGT	P02461	COL3A1	Collagen alpha-1(III) chain	0.144 (0.060, 0.228)	0.0043
e99918867	QGFQGPAGEPGEPGQTGPAGARGPAGppGKAGEDGHpGKP	P08123	COL1A2	Collagen alpha-2(I) chain	-0.136 (-0.218, -0.053)	0.0043
e99914015	PAGERGEQGAPGPSGFQGLPGPpGPPGE	P02458	COL2A1	Collagen alpha-1(II) chain	-0.140 (-0.224, -0.056)	0.0048
e99911879	EPGPAGSKGESGNKGEPGSAGpQGp	P08123	COL1A2	Collagen alpha-2(I) chain	-0.138 (-0.222, -0.053)	0.0049
e99917184	GPAGFAGPPGADGQPGAKGEQGEAGQKGDAGAPGPQG	P02458	COL2A1	Collagen alpha-1(II) chain	-0.136 (-0.219, -0.054)	0.0050
e99906686	SpGNIGPAGKEGPVGLpG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.132 (-0.215, -0.049)	0.0065
e99911537	GKNGDDGEAGKPGRpGERGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.153 (-0.238, -0.069)	0.0067

Table S2. Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (continued)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	*p*-value
		Accession number	Symbol	Name		
e99910953	DGQpGAKGEpGDAGAKGDAGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	0.148 (0.066, 0.229)	0.0068
e99901648	GpEGGKGAAGPpG	P02461	COL3A1	Collagen alpha-1(III) chain	0.140 (0.054, 0.225)	0.0078
e99918881	HDGEKGPRGKPGDMGPPGPQGPPGKDGPpGVKGENGHp	Q5TAT6	COL13A1	Collagen alpha-1(XIII) chain	-0.118 (-0.203, -0.033)	0.0085
e99912556	KNGDDGEAGKpGRPGERGpPGPQG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.147 (-0.230, -0.064)	0.0094
e99902032	GGpGSDGKpGPpG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.134 (-0.217, -0.050)	0.0095
e99915467	GRDGNpGNDGPpGRDGQpGHKGERGYpG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.126 (-0.210, -0.043)	0.0098
e99919780	GAPGERGETGPpGpAGFAGpPGADGQPGAKGEQGEAGQKGDAGAPG	P02458	COL2A1	Collagen alpha-1(II) chain	-0.128 (-0.212, -0.045)	0.011
e99915129	ERGEAGIpGVpGAKGEDGKDGSPGEpGANG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.129 (-0.210, -0.047)	0.011
e99906024	SpGSPGPDGKTGpPGPAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.144 (-0.228, -0.061)	0.012
e99913707	GApGQNGEPGGKGERGApGEKGEGGppG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.129 (-0.212, -0.046)	0.013
e99908442	AGEKGPSGEAGTAGPpGTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	0.124 (0.039, 0.208)	0.014
e99916417	RTGEVGAVGPpGFAGEKGPSGEAGTAGPpGTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	-0.123 (-0.208, -0.038)	0.015
e99911008	pPGEEGKRGPRGDpGTVGPpGP	P05997	COL5A2	Collagen alpha-2(V) chain	-0.105 (-0.187, -0.022)	0.016
e99914315	NRGERGSEGSPGHpGQpGPPGpPGApGP	P02461	COL3A1	Collagen alpha-1(III) chain	-0.127 (-0.210, -0.043)	0.016
e99913816	ERGEAGIpGVpGAKGEDGKDGSpGEpG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.128 (-0.212, -0.044)	0.016
e99901785	DGESGRPGRpG	P02461	COL3A1	Collagen alpha-1(III) chain	0.126 (0.043, 0.210)	0.017
e99905346	GPpGKpGDDGEAGKpG	P02458	COL2A1	Collagen alpha-1(II) chain	0.123 (0.039, 0.207)	0.017
e99908598	NSGEPGApGSKGDTGAKGEp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.138 (-0.220, -0.055)	0.018
e99902286	PpGEAGKpGEQG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.139 (-0.224, -0.055)	0.020
e99903284	DGSpGAKGDRGET	P02452	COL1A1	Collagen alpha-1(I) chain	0.138 (0.055, 0.222)	0.021
e99915811	GHPGPSGPPGKpGYGSpGLQGEpGLPGPPGPS	Q03692	COL10A1	Collagen alpha-1(X) chain	-0.099 (-0.183, -0.016)	0.021
e99912156	GPAGpPGEKGEPGDDGPSGAEGPPGp	P02458	COL2A1	Collagen alpha-1(II) chain	-0.117 (-0.200, -0.034)	0.025
e99902189	GpPGPpGPpGPpG	P53420	COL4A4	Collagen alpha-4(IV) chain	-0.096 (-0.180, -0.012)	0.025

Table S2. Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (ends)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	*p*-value
		Accession number	Symbol	Name		
e99906650	EGSpGRDGSpGAKGDRG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.129 (-0.209, -0.049)	0.028
e99913065	ADGQpGAKGEpGDAGAKGDAGPpGPAGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.128 (-0.208, -0.048)	0.029
e99917672	GPpGpAGFAGpPGADGQPGAKGEQGEAGQKGDAGAPGP	P02458	COL2A1	Collagen alpha-1(II) chain	-0.116 (-0.201, -0.032)	0.029
e99904330	pPGpPGPpGpPGPPS	P02452	COL1A1	Collagen alpha-1(I) chain	-0.132 (-0.216, -0.048)	0.034
e99909989	DGESGRpGRpGERGLpGPpG	P02461	COL3A1	Collagen alpha-1(III) chain	0.118 (0.034, 0.203)	0.034
e99905551	VGPpGPPGPpGPpGPPS	P02452	COL1A1	Collagen alpha-1(I) chain	-0.133 (-0.218, -0.049)	0.035
e99905559	GpAGpRGERGPPGESG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.110 (-0.196, -0.025)	0.0371
e99920326	GSEGARGAPGPAGPPGDPGLMGERGEDGPAGNGTEGFpGFpGYpGNR	P12109	COL6A1	Collagen alpha-1(VI) chain	-0.094 (-0.179, -0.010)	0.043
e99918041	NTGAPGSpGVSGpKGDAGQpGEKGSpGAQGppGAPGPLG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.115 (-0.200, -0.030)	0.044
e99906697	VGPpGpPGPpGPPGPPSAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.129 (-0.213, -0.044)	0.046

FLEMENGHO: Flemish Study on Environment, Genes, and Health Outcomes. The lower-case letters in the amino-acid sequence identify post-translational modifications: c, disulphide bridges; m, oxidised methionine; n, deaminated asparagine; p, hydroxylated proline; k, hydroxylated lysine; q, deaminated glutamine. Accession numbers refer to the Uniprot database (http://www.uniprot.org/uniprot). Association sizes (β) between urinary peptides and chronological age were expressed per 10-year increment and were adjusted for the clustering within families, sex, body mass index, the glomerular filtration rate calculated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration equation, current smoking, γ-glutamyltransferase, fasting plasma glucose, physical activity, use of diuretics (yes *vs* no), and socioeconomic status (High or middle *vs* low). *p*-values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate for 395 tested associations.

Table S3. Association of chronological age with 16 bone-related urinary peptides

Peptide ID number	Parental protein	Derivation dataset (*n* = 519)		Time-shifted validation dataset (*n* = 519)		Synchronous validation dataset (*n* = 187)	
		β (95% CI)	*p*-value	β (95% CI)	*p*-value	β (95% CI)	*p*-value
e99907132	COL1A1	0.281 (0.203, 0.359)	9.21E-11	0.293 (0.205, 0.381)	1.51E-09	0.151 (0.028, 0.274)	0.093
e99910072	COL3A1	-0.232 (-0.309, -0.155)	3.29E-08	-0.171 (-0.260, -0.082)	0.00059	-0.201 (-0.329, -0.073)	0.026
e99901132	MGP	0.230 (0.154, 0.305)	3.85E-08	0.232 (0.146, 0.318)	8.67E-07	0.238 (0.113, 0.363)	0.0007
e99904196	COL2A1	0.237 (0.156, 0.318)	7.19E-08	0.134 (0.042, 0.226)	0.0098	NS	…
e99919107	COL1A2	-0.218 (-0.297, -0.139)	3.00E-07	-0.106 (-0.198, -0.015)	0.034	-0.218 (-0.343, -0.093)	0.0028
e99904847	COL11A1	0.157 (0.074, 0.241)	0.00063	NS	…	NS	…
e99920509	COL11A2	-0.153 (-0.236, -0.070)	0.00078	NS	…	NS	…
e99915442	IGF2	0.148 (0.066, 0.231)	0.00088	0.100 (0.005, 0.194)	0.04	0.160 (0.026, 0.294)	0.028
e99919425	COL9A3	0.150 (0.066, 0.235)	0.00089	0.129 (0.037, 0.221)	0.011	NS	…
e99913779	COL18A1	0.140 (0.059, 0.222)	0.0012	0.113 (0.023, 0.203)	0.018	NS	…
e99916785	COL6A1	-0.137 (-0.221, -0.054)	0.0020	NS		-0.140 (-0.276, -0.004)	0.05
e99914815	COL21A1	-0.130 (-0.211, -0.049)	0.0023	-0.100 (-0.189, -0.010)	0.033	NS	…
e99918881	COL13A1	-0.118 (-0.203, -0.033)	0.0085	NS	…	-0.134 (-0.268, -0.001)	0.049
e99911008	COL5A2	-0.105 (-0.187, -0.022)	0.016	NS	…	-0.178 (-0.316, -0.040)	0.019
e99915811	COL10A1	-0.099 (-0.183, -0.016)	0.021	NS	…	-0.179 (-0.312, -0.047)	0.016
e99902189	COL4A4	-0.096 (-0.180, -0.012)	0.025	NS	…	-0.145 (-0.278, -0.012)	0.041

Only urinary peptides with the highest significance per identified protein were selected for this analysis to demonstrate consistency of the associations in 519 participants included in the derivation and the time-shifted validation datasets and in the 187 participants included in the synchronous validation dataset. Association sizes (β) of chronological age with the urinary peptides were expressed per 10-year increment and were adjusted for the clustering within families, sex, body mass index, the glomerular filtration rate calculated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration equation, current smoking, γ-glutamyltransferase, fasting plasma glucose, physical activity, use of diuretics (yes *vs* no), and socioeconomic status (high or middle *vs* low). The *p*-values indicate the validation status ($p < 0.10$, given the prior probability established by the significance in the derivation dataset). NS indicates not significant.

Table S4. Number of replicated bone-related urinary peptides identifying parental proteins

Peptide ID number	Parental protein	Number of peptide fragments (Percentage replicated compared with derivation dataset)		
		Derivation dataset (*n* = 519)	Time-shifted validation dataset (*n* = 519)	Synchronous validation dataset (*n* = 187)
e99907132	COL1A1	38	16 (42.1%)	19 (50%)
e99910072	COL3A1	21	10 (47.6%)	6 (28.6%)
e99901132	MGP	2	1 (50%)	2 (100%)
e99904196	COL2A1	10	4 (40%)	5 (50%)
e99919107	COL1A2	14	3 (21.4%)	8 (57.1%)
e99904847	COL11A1	1	0 (0%)	0 (0%)
e99920509	COL11A2	1	0 (0%)	0 (0%)
e99915442	IGF2	2	2 (100%)	1 (50%)
e99919425	COL9A3	1	1 (100%)	0 (0%)
e99913779	COL18A1	1	1 (100%)	0 (0%)
e99916785	COL6A1	3	0 (0%)	1 (33.3%)
e99914815	COL21A1	1	1 (100%)	1 (100%)
e99918881	COL13A1	1	0 (0%)	0 (0%)
e99911008	COL5A2	1	0 (0%)	1 (100%)
e99915811	COL10A1	1	0 (0%)	1 (100%)
e99902189	COL4A4	1	0 (0%)	1 (100%)

Replication indicates similar directionality in the association with chronological age with a *p-value* of <0.10 (given the prior probability established by the significance in the derivation dataset).

Table S5. Sex difference of bone-related urinary peptides in the derivation dataset (starts)

Peptide ID number	Parental protein	Unadjusted		Adjusted	
		Estimate (95% CI)	*p-value*	Estimate (95% CI)	*p-value*
e99918573	COL1A2	-0.551 (-0.716, -0.386)	1.39E-08	-0.490 (-0.663, -0.317)	4.42E-06
e99915129	COL3A1	-0.544 (-0.709, -0.379)	1.25E-08	-0.496 (-0.671, -0.320)	2.49E-06
e99919107	COL1A2	-0.483 (-0.650, -0.316)	7.38E-07	-0.464 (-0.634, -0.295)	3.10E-06
e99915778	COL1A1	-0.440 (-0.608, -0.273)	7.26E-06	-0.324 (-0.497, -0.151)	0.0021
e99909408	COL1A1	-0.426 (-0.594, -0.258)	1.47E-05	-0.357 (-0.533, -0.181)	0.00077
e99917184	COL2A1	-0.424 (-0.592, -0.256)	1.42E-05	-0.375 (-0.553, -0.198)	0.00043
e99913707	COL3A1	-0.419 (-0.587, -0.251)	1.63E-05	-0.403 (-0.582, -0.225)	0.00016
e99915811	COL10A1	-0.418 (-0.586, -0.250)	1.59E-05	-0.435 (-0.614, -0.256)	5.03E-05
e99908598	COL1A1	-0.394 (-0.562, -0.226)	5.59E-05	-0.379 (-0.557, -0.202)	0.00041
e99915411	COL3A1	-0.373 (-0.542, -0.204)	0.00016	-0.321 (-0.501, -0.140)	0.0037
e99903016	COL1A1	-0.355 (-0.524, -0.186)	0.00037	-0.281 (-0.458, -0.105)	0.0091
e99905074	COL1A2	-0.329 (-0.499, -0.160)	0.001	-0.295 (-0.473, -0.117)	0.0068
e99910072	COL3A1	-0.316 (-0.485, -0.146)	0.0017	-0.256 (-0.436, -0.077)	0.019
e99914315	COL3A1	-0.314 (-0.484, -0.145)	0.0017	-0.243 (-0.409, -0.077)	0.016
e99911008	COL5A2	-0.311 (-0.480, -0.141)	0.0019	-0.238 (-0.416, -0.060)	0.03
e99906686	COL1A2	-0.307 (-0.477, -0.138)	0.002	-0.313 (-0.492, -0.133)	0.004
e99913779	COL18A1	-0.291 (-0.461, -0.121)	0.0036	-0.266 (-0.441, -0.091)	0.013
e99912508	COL1A1	-0.287 (-0.457, -0.117)	0.0041	-0.278 (-0.458, -0.097)	0.012
e99916419	COL1A2	-0.284 (-0.454, -0.114)	0.0045	-0.274 (-0.450, -0.099)	0.01
e99906650	COL1A1	-0.271 (-0.441, -0.101)	0.0072	NS	…

Table S5. Sex difference of bone-related urinary peptides in the derivation dataset (ends)

Peptide ID number	Parental protein	Unadjusted		Adjusted	
		Estimate (95% CI)	*p-value*	Estimate (95% CI)	*p-value*
e99904945	COL1A1	-0.254 (-0.424, -0.084)	0.013	NS	…
e99911777	COL1A1	-0.245 (-0.415, -0.074)	0.016	-0.239 (-0.418, -0.060)	0.031
e99913816	COL3A1	-0.240 (-0.411, -0.069)	0.017	NS	…
e99911753	COL1A1	-0.240 (-0.411, -0.070)	0.018	-0.242 (-0.424, -0.060)	0.03
e99917866	COL3A1	-0.232 (-0.402, -0.061)	0.023	NS	…
e99918041	COL3A1	0.207 (0.036, 0.378)	0.048	NS	…
e99915442	IGF2	0.209 (0.038, 0.380)	0.046	0.261 (0.085, 0.438)	0.016
e99906709	COL1A1	0.243 (0.072, 0.413)	0.017	0.215 (0.047, 0.382)	0.039
e99901661	COL2A1	0.248 (0.078, 0.419)	0.015	0.306 (0.133, 0.478)	0.0034
e99903120	COL1A1	0.250 (0.079, 0.420)	0.014	0.282 (0.102, 0.463)	0.011
e99900340	MGP	0.260 (0.090, 0.430)	0.011	0.308 (0.135, 0.481)	0.0036
e99918044	COL2A1	0.305 (0.135, 0.474)	0.0021	0.260 (0.078, 0.441)	0.019
e99913065	COL1A1	0.311 (0.141, 0.480)	0.0018	0.332 (0.160, 0.505)	0.0015
e99906441	COL1A1	0.323 (0.154, 0.493)	0.0013	0.313 (0.143, 0.482)	0.0024
e99901132	MGP	0.340 (0.170, 0.509)	0.00068	0.372 (0.210, 0.535)	0.00015
e99910953	COL1A1	0.342 (0.173, 0.512)	0.00063	0.285 (0.109, 0.460)	0.0083
e99905560	COL1A1	0.465 (0.298, 0.632)	1.81E-06	0.475 (0.306, 0.643)	1.7E-06

NS: not significant. Estimate was the association size of difference in urinary peptide level in women compared to men (men as reference) derived from the rank-normalised data in the unadjusted and adjusted general linear models. Adjusted models accounted for the clustering within families, age, body mass index, the glomerular filtration rate calculated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration equation, current smoking, γ-glutamyltransferase as index of alcohol intake, blood glucose, physical activity, use of diuretics (yes *vs.* no), and the middle or high socioeconomic status (yes vs. no). The 99 urinary peptides associated with chronological age were included in the analysis in the derivation dataset. *P-values* were corrected for the Benjamini-Hochberg false discovery rate for 99 tested association. 37 (9 parental proteins) was identified in the unadjusted models and 32 urinary peptides (9 parental proteins) in the adjusted models, respectively.

Table S6. Weights assigned by elastic net regression to the multidimensional UPP biomarker reflecting ageing (starts)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			Weight (95% confidence interval)
		Accession number	Symbol	Name	
…	…	…	…	…	49.577405 (intercept)
e99911879	EPGPAGSKGESGNKGEPGSAGpQGp	P08123	COL1A2	Collagen alpha-2(I) chain	-0.006570 (-0.010338, -0.002746)
e99919107	DQGPVGRTGEVGAVGPpGFAGEKGpSGEAGTAGPPGTpGPQG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.005577 (-0.008878, -0.002536)
e99905705	DGQPGAKGEpGDAGAKG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.005512 (-0.013123, 0.001801)
e99902032	GGpGSDGKpGPpG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.005088 (-0.010774, 0.000741)
e99902189	GpPGPpGPpGPpG	P53420	COL4A4	Collagen alpha-4(IV) chain	-0.005073 (-0.008518, -0.001569)
e99916966	PpGESGREGApGAEGSpGRDGSPGAKGDRGETGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.004863 (-0.006168, -0.003606)
e99903372	DGQPGAKGEpGDAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.004531 (-0.010864, 0.001061)
e99910072	GLpGTGGPpGENGKpGEpGPKG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.004197 (-0.007435, -0.001032)
e99906650	EGSpGRDGSpGAKGDRG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.003875 (-0.009837, 0.002081)
e99906709	GSpGSpGPDGKTGPpGPAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.003650 (-0.004616, -0.002698)
e99912851	LDGAKGDAGPAGPKGEpGSpGENGApG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.003648 (-0.006470, -0.000989)
e99912949	LDGAKGDAGPAGpKGEpGSpGENGApG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.003329 (-0.008020, 0.001176)
e99920509	GEHGpPGPPGPIGPVGQPGAAGADGEPGARGPQGHFGAKGDEGTRGFNGP	P13942	COL11A2	Collagen alpha-2(XI) chain	-0.001102 (-0.001807, -0.000415)
e99906441	TGSpGSpGPDGKTGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.000776 (-0.002173, 0.000571)
e99905560	GSpGSpGPDGKTGPPGp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.000259 (-0.000961, 0.000439)
e99914645	DVStPPTVLPDNFPRYPVG	P01344	IGF2	Insulin-like growth factor II	0.000179 (0.000003, 0.000347)
e99915442	DVStPPTVLPDNFPRYP	P01344	IGF2	Insulin-like growth factor II	0.000350 (-0.000203, 0.000945)

Table S6. Weights assigned by elastic net regression to the multidimensional UPP biomarker reflecting ageing (ends).

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			Weight (95% confidence interval)
		Accession number	Symbol	Name	
e99901132	cDDYRLcE	P08493	MGP	Matrix Gla protein	0.000822 (0.000486, 0.001196)
e99909989	DGESGRpGRpGERGLpGPpG	P02461	COL3A1	Collagen alpha-1(III) chain	0.001280 (0.000333, 0.002376)
e99907132	EpGSpGENGAPGQmGPR	P02452	COL1A1	Collagen alpha-1(I) chain	0.001361 (0.000879, 0.001887)
e99913779	DDILASPPRLPEPQPYPGAPHHSS	P39060	COL18A1	Collagen alpha-1(XVIII) chain	0.002118 (-0.002625, 0.007012)
e99904196	PSGDQGASGpAGPSGP	P02458	COL2A1	Collagen alpha-1(II) chain	0.002342 (-0.001725, 0.006138)
e99910953	DGQpGAKGEpGDAGAKGDAGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	0.002584 (0.001634, 0.003548)
e99903284	DGSpGAKGDRGET	P02452	COL1A1	Collagen alpha-1(I) chain	0.002860 (-0.000424, 0.005988)
e99908442	AGEKGPSGEAGTAGPpGTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	0.002947 (0.000610, 0.005217)
e99905346	GPpGKpGDDGEAGKpG	P02458	COL2A1	Collagen alpha-1(II) chain	0.004666 (0.002066, 0.007324)
e99917280	AAGEpGKAGERGVpGPpGAVGPAGKDGEAGAQGPPGP	P02452	COL1A1	Collagen alpha-1(I) chain	0.005564 (0.003320, 0.007863)
e99916417	RTGEVGAVGPpGFAGEKGPSGEAGTAGPpGTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	0.005706 (0.003379, 0.008284)
e99919425	pGApGVRGFQGQKGSmGDpGLPGPQGLRGDVGDRGP GGAAGP	Q14050	COL9A3	Collagen alpha-3(IX) chain	0.010065 (0.004812, 0.014979)

The derivation dataset included the baseline data of 519 participants in FLEMENGHO. Accession numbers refer to the Uniprot database (http://www.uniprot.org/uniprot). Association sizes (β) between chronological age and the levels of the urinary peptides were derived by elastic net regression, which was bootstrapped 1000 times. The regression slopes (βs) reflect the weight of each of the 29 peptide fragments in the construction of the multidimensional UPPost-age biomarker. Of 99 peptides entering the data reduction analysis, 29 entered in at least 60% of models and identified 10 proteins. The 95% confidence intervals of the βs were derived from the bootstrap distribution.

Table S7. Risk of having HASD ≤ -3 cm in FLEMENGHO participants at baseline (2005-2010).

Variable	n/N	Chronological age		UPPost-age	
		OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
All participants	278/706	1.43 (1.25, 1.63)	<0.0001	1.31 (1.12, 1.53)	0.0007
Men	188/349	1.40 (1.17, 1.67)	0.0002	1.19 (0.96, 1.48)	0.11
Women	90/357	1.51 (1.21, 1.88)	0.0003	1.43 (1.14, 1.81)	0.0024
Postmenopausal women	62/183	1.57 (1.06, 2.34)	0.025	1.22 (0.88, 1.70)	0.24

Abbreviations: HASD: the difference of body height minus arm span; n/N: number of participants with HASD ≤ -3 cm/total number of participants; UPPost-age: age as predicted by the urinary proteomic profile; OR: odds ratio; 95% CI: 95% confidence interval. ORs, given with 95% CI, express the relative risk per 10-year increment in chronological age or UPPost-age with adjustment for clustering within families, sex (in all participants only), current smoking, γ-glutamyltransferase, fasting plasma glucose, physical activity, use of diuretics (yes *vs* no) and socioeconomic status (high and middle *vs* low).

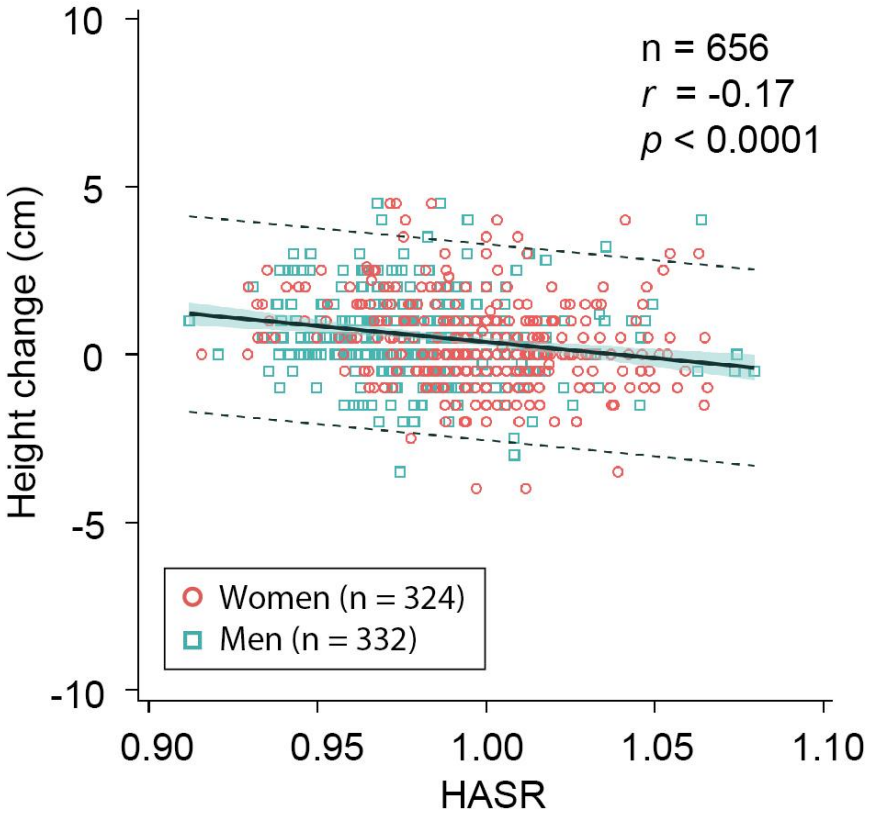


Figure S1. Correlation between height loss and body-height-to-arm-span ratio in 656 FLEMENGHO participants. HASR: body-height-to-arm-span ratio. *r* is the Pearson correlation coefficient. Height change was the differences between two body height measurement examined in the interval from 1985 until 2008. During the median time interval of 9.56 years (interquartile range: 6.54, 15.5 years), the mean height change was 0.46 cm (SD: 1.51 cm) in 656 participants (50.6% women; median age at the first measurement: 40.5 year). Height change was significantly correlated with HASR (r = -0.17, p < 0.0001). The sex-specific correlation coefficients were -0.24 in women (red circles) and -0.095 in men (green squares), respectively (p = 0.057 for the sex difference).

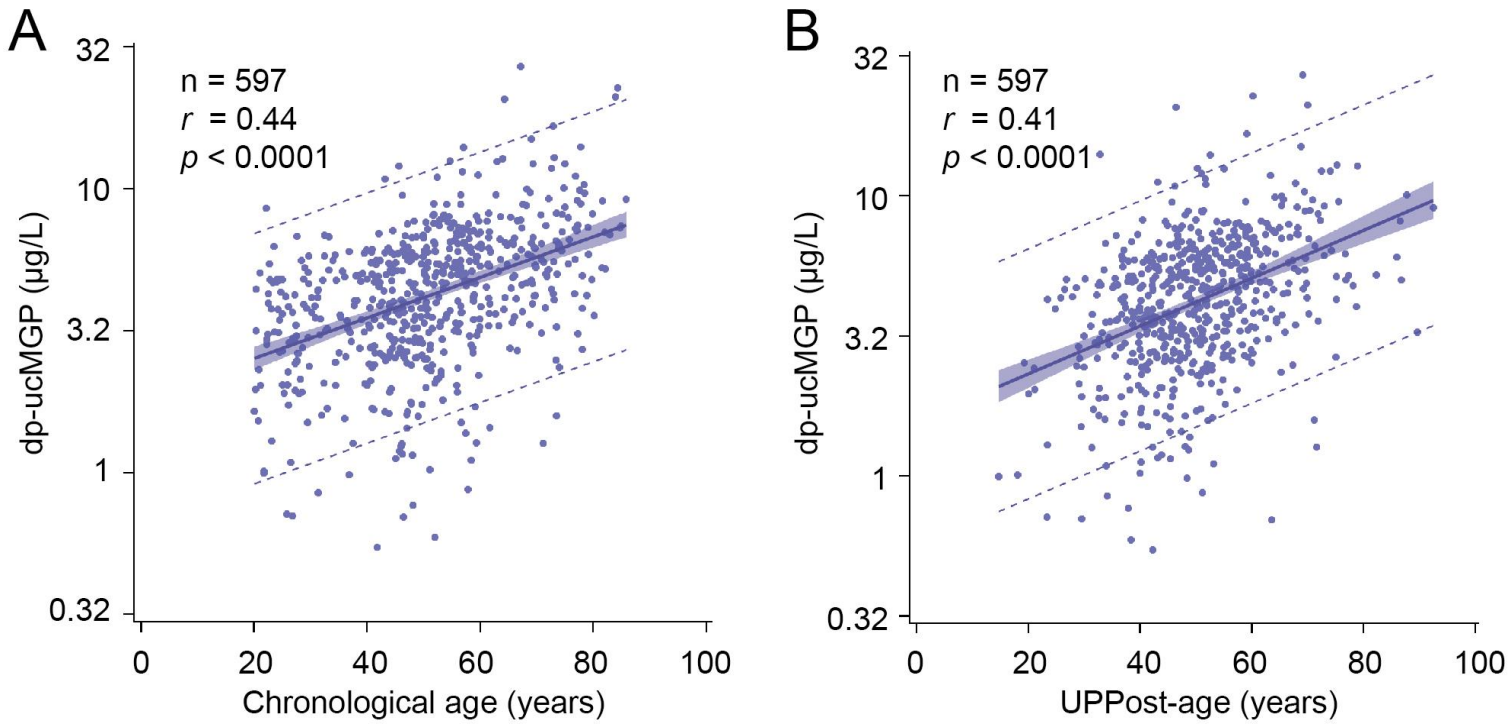


Figure S2. Regression plot of dp-ucMGP with chronological age (A) and UPPost-age (B) in 597 FLEMENGHO participants.
r is the Pearson correlation coefficient. dp-ucMGP denotes desphospho-uncarboxylated matrix Gla protein. UPPost-age is age as predicted by the urinary proteomic profile.

Chapter 3

Unhealthy ageing in relation to urinary proteomic profiling

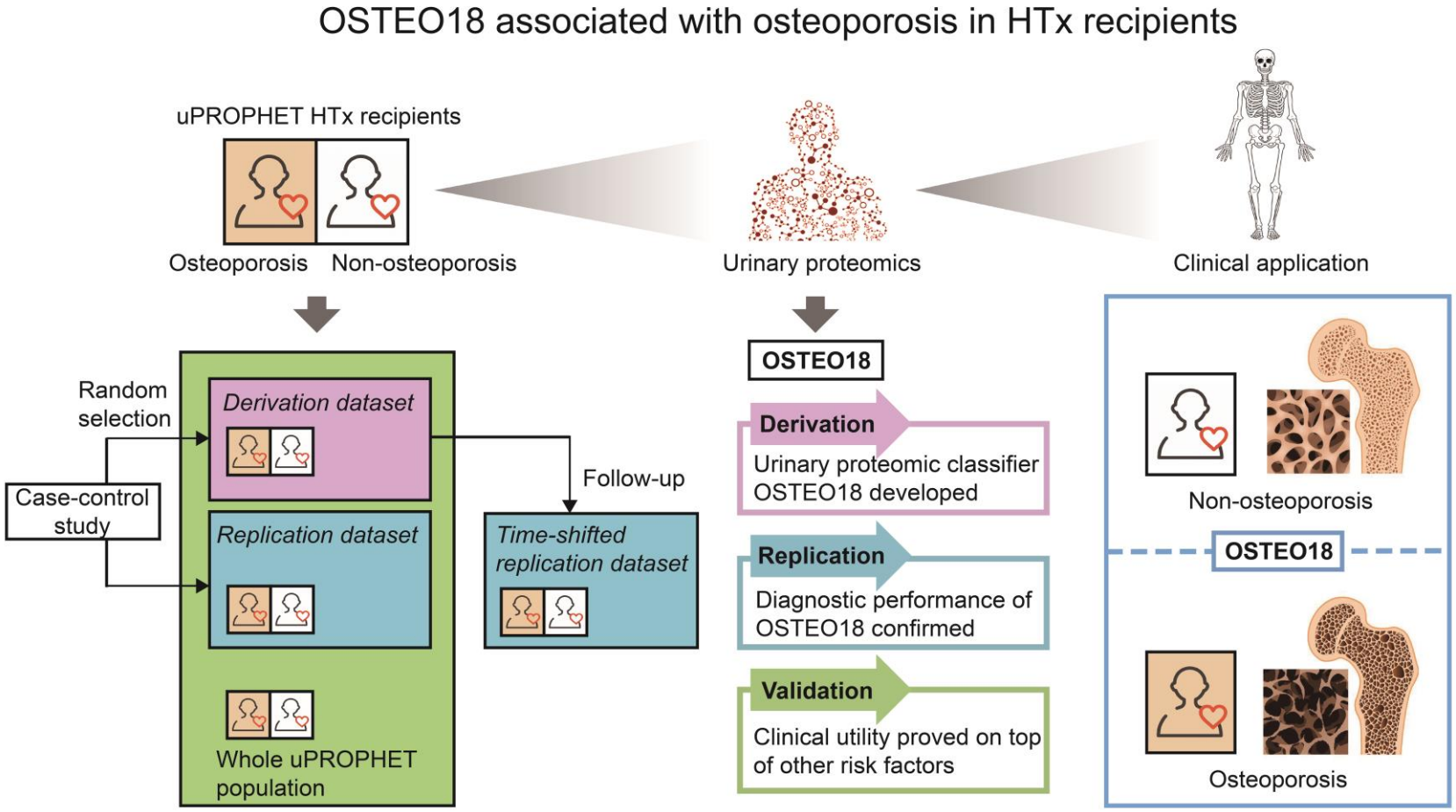
Part 2

OSTEO18, a novel urinary proteomic signature, associated with osteoporosis in heart transplant recipients

Yu-Ling Yu, Qi-Fang Huang, De-Wei An, Julia Raad, Dries S. Martens, Agnieszka Latosinska, Katarzyna Stolarz-Skrzypek, Johan Van Cleemput, Ying-Qing Feng, Harald Mischak, Karel Allegaert, Peter Verhamme, Stefan Janssens, Tim S. Nawrot, Jan A. Staessen

Previously published in:
Heylion. 2024; 10:e24867.

Graphical abstract



Abstract

Background: Immunosuppressive treatment in heart transplant (HTx) recipient causes osteoporosis. The urinary proteomic profile (UPP) includes peptide fragments derived from the bone extracellular matrix. Study aims were to develop and validate a multidimensional UPP biomarker for osteoporosis in HTx patients from single sequenced urinary peptides identifying the parent proteins.

Methods: A single-center HTx cohort was analyzed. Urine samples were measured by capillary electrophoresis coupled with mass spectrometry. Cases with osteoporosis and matching controls were randomly selected from all available 389 patients. In derivation case-control dataset, 1576 sequenced peptides detectable in ≥30% of patients. Applying statistical analysis on these, an 18-peptide multidimensional osteoporosis UPP biomarker (OSTEO18) was generated by support vector modeling. The 2 replication datasets included 118 and 94 patients. For further validation, the whole cohort was analyzed. Statistical methods included logistic regression and receiver operating characteristic curve (ROC) analysis.

Results: In derivation dataset, the AUC, sensitivity and specificity of OSTEO18 were 0.83 (95% CI: 0.76-0.90), 74.3% and 87.1%, respectively. In replication datasets, results were confirmatory. In the whole cohort (154 osteoporotic patients [39.6%]), the ORs for osteoporosis increased (P<0.0001) across OSTEO18 quartiles from 0.39 (95% CI: 0.25-0.61) to 3.14 (2.08-4.75). With full adjustment for known osteoporosis risk factors, OSTEO18 improved AUC from 0.708 to 0.784 (P=0.0004) for OSTEO18 categorised (optimised threshold: 0.095) and to 0.786 (P=0.0003) for OSTEO18 as continuously distributed classifier.

Conclusion: OSTEO18 is a clinically meaningful novel biomarker indicative of osteoporosis in HTx recipients and is being certified as in-vitro diagnostic.

Introduction

Osteoporosis is a common age-related disease caused by imbalance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption, resulting in a decline of bone mineral density (BMD) and an enhanced risk of osteoporotic fractures [1,2]. In recipients of a heart transplant (HTx), immunosuppressants and glucocorticosteroids are routinely prescribed to prevent allograft rejection, thereby increasing the risk of post-transplant osteoporosis due to trabecular bone loss, reduced bone formation and increased bone destruction [3].

Urine contains more than 20,000 peptides, mainly collagen fragments, which are generated in nephron or pass from circulation to tubular fluid through the glomerular barrier [4,5]. Single sequenced urinary peptides allow identifying the parental proteins [6], thereby providing bodywide information on the molecular mechanisms underlying pathophysiological processes. Collagen type I is the most abundant constituent of the organic extracellular matrix in bones [7]. Accelerated biological aging, as captured by urinary proteomic profiling (UPP), is associated with osteoporosis and osteoporotic fractures in the general population [8]. Disease-specific multidimensional urinary classifiers have diagnostic and prognostic value in HTx patients at risk of graft failure [9] or renal dysfunction [10]. However, no previous study investigated whether there is a specific UPP signature indicative of osteoporosis in HTx patients. Using the database of the urinary PROteomics in Predicting HEart Transplantation outcomes study (uPROPHET) [11], the objective of this study was therefore to develop and validate a multidimensional UPP marker from single sequenced urinary peptides with different levels in HTx recipients with and without osteoporosis.

Material and methods

Study population and study endpoint

uPROPHET (registration number, NCT03152422) is a single-center study with as objective to identify and validate UPP signatures for clinical use in HTx patients [11]. uPROPHET complies with the Declaration of Helsinki for research in humans [12]. The Ethics Committee of the University Hospitals Leuven [numbers B322201421186 (S56384) and B322201421045 (S56472) and the European Research Council Executive Agency approved the protocol. HTx recipients provided written informed consents. Recruitment of HTx patients took place at the University Hospital Gasthuisberg in Leuven in collaboration with the transplantation team. All HTx recipients in regular follow-up at the University Hospitals Leuven were invited to provide a 5-mL mid-morning urine sample for UPP analysis.

Of 392 HTx patients enrolled in uPROPHET, 3 were excluded because they had no serum creatinine measurement at baseline (n=1) or because they were younger than 20 years, an age group for which BMD reference values are unavailable (n=2). Therefore, 389 uPROPHET participants were statistically analyzed (**Figure 1**). The osteoporotic endpoint included: (i) a T-score of ≤-2.5 in the hip or lumbar BMD measurements (Data Supplement) as determined by dual energy X-ray absorptiometry (XRA) [13,14] (ii) a history of osteoporotic fracture or the necessity of spine stabilizing surgery; and (iii) clinical, biochemical, hormonal or radiographic signs to start anti-osteoporotic treatment with calcium supplements, vitamin D and/or bisphosphonates. Measurements monitored at the HTx day hospital include loss of body height, complaints compatible with spinal nerve root compression, the development of thoracal kyphosis, lumbar lordosis or scoliosis, serum parathyroid hormone [15] and osteocalcin [16], alkaline phosphatase and bone-specific alkaline phosphatase [17], sex hormone levels (follicle stimulating hormone, estrogens progestogens and testosterone), 25-hydroxy-vitamin D (calcidiol) or 1,25-dihydroxy-vitamin D (calcitriol) [18], 24-h urinary calcium excretion balanced against estimates of dietary calcium intake, and radiographic evidence of vertebral compression fractures [13,14,19].

To generate a multidimensional urinary classifier indicative of osteoporosis, analyses started with a case-control study, in which 129 patients with incident osteoporosis were matched with 129 controls for sex, age (±10 years), eGFR (±15 ml/min/1.73 m^2) and intake of methylprednisolone (**Figure 1**). Cases and controls were randomly subdivided into a derivation dataset (70 cases *vs* 70 controls) and a replication dataset (59 cases *vs* 59 controls). Of the patients in the derivation dataset, 67 cases and 67 controls underwent a second UPP assessment after a median interval of 0.75 years (range: 0.25-6.26 years). These patients constituted the time-shifted replication dataset. For final validation, the urinary classifier was tested in the whole cohort, which included 154 patients with osteoporosis (**Figure 1**).

Assessment of clinical variables

A detailed description of the construction and contents of uPROPHET database is available in the published protocol [11] and the Data Supplement. All potentially relevant clinical information, including anthropometrics, medical antecedents, biochemical measurements and use of immunosuppressive, antihypertensive, lipid-lowering and antidiabetic drugs, was retrieved from the computerised information system of the University Hospitals Leuven. The urine samples was collected within 6 months of the study osteoporotic outcome. The treatments of calcium supplement, vitamin D

supplement, bisphosphonates and immunosuppression drugs were administered at the time of urine sampling.

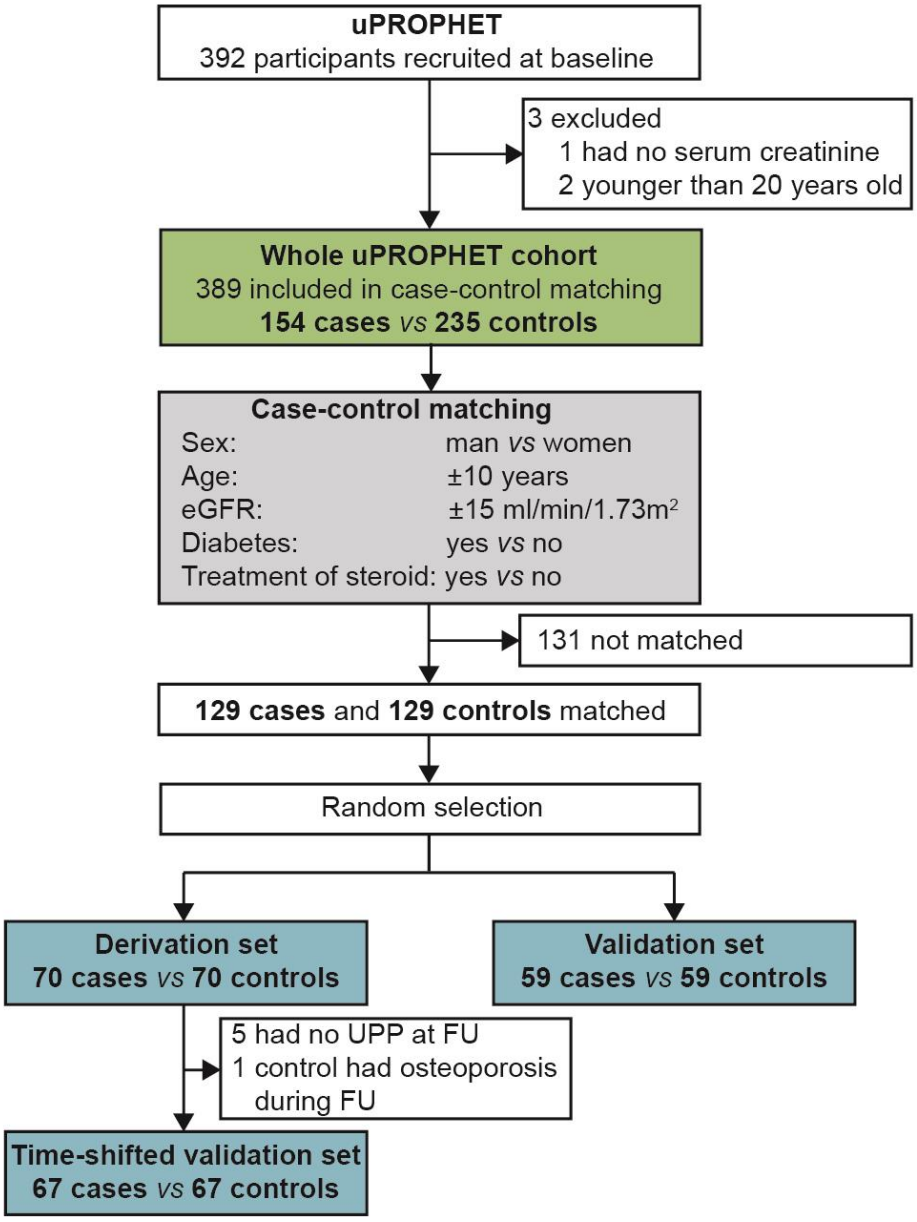


Figure 1 Flow chart.

HTx indicates heart transplantation and FU follow up.

Urinary proteomic profiling

The methods for urine sample preparation, capillary electrophoresis coupled with mass spectrometry (CE-MS), peptide sequencing, calibration, and quality control of the mass spectrometric data have been published [6,20,21] and are described in the Data Supplement. The 1576 sequenced urinary peptides with a detectable signal in ≥30% of study participants (**Figure S1**), a common approach in UPP analysis [22,23], were selected to generate the classifier differentiating cases from controls.

Statistical analysis

For database management and statistical analysis, we used the SAS system, version 9.4 (SAS Institute Inc., Cary, NC). Means were compared using the large-sample z-test or ANOVA and proportions by Fisher's exact test. The 95% confidence intervals (CIs) of rates were computed as $R \pm 1.96 \times \sqrt{(R \times (100 - R) /T)}$, where R is the rate and T is the total number of patients. Derivation, replication and validation of the osteoporosis classifier were done according to predefined steps (**Figure S1**). First, the 1576 selected urinary peptides were compared between cases and controls in the derivation dataset, using the Mann-Whitney U test (**Figure S2**). The significance of the between-group differences was adjusted for multiple comparisons by the Benjamini-Hochberg approach [24]. Next, searching a balance between computational complexity and the minimum number of peptides to construct a UPP classifier, the urinary peptides in the top tail of significance distribution were combined to generate the classifier [25]. The 30 most significant peptides with higher urinary levels in cases and the 30 most significant peptides with higher levels in controls (**Figure S2**) were selected and combined using support vector modeling (SVM) with 94 support vectors and the radial basis kernel function. Models were optimised using a take-one-out procedure until no further optimization was possible by reducing the numbers of support vectors. The so-obtained classifier, OSTEO18 (**Table S1**), included 18 of the original 60 urinary peptides. Reference signals of 29 abundant peptides were used as internal standards for calibration using local linear regression [20], therefore OSTEO18 scores are the dimensionless ratio with the calibration signal in the denominator.

The risk of osteoporosis in the derivation and replication case-control studies and in the whole uPROHPET cohort (**Figure S1**) was related to the OSTEO18 level by logistic regression models, first unadjusted, next partially adjusted for sex, age, the glomerular filtration rate estimated from serum creatinine (eGFR) [26], diabetes (yes *vs* no) and treatment with methylprednisolone (yes *vs* no), and finally additionally adjusted for the time interval since HTx, ionized calcium, treatment with thiazides (yes *vs* no) [27],

tacrolimus [28] (yes *vs* no) and cyclosporine (yes *vs* no) [29]. Ionized calcium was calculated from serum total calcium and serum albumin, using the following formula: ionized calcium (mmol/l)=serum total calcium (mmol/l) + 0.02 × (40 - serum albumin (g/l)) [30]. These covariables were selected because their clinical relevance to osteoporosis in HTx patients [27-29,31-33]. In these models, the OSTEO18 score was introduced as categorized variable approximating the quartiles of the OSTEO18 distribution (<-1, -1-0, 0-1 and ≥1) and as a continuously distributed variable. For the analysis of OSTEO18 as categorised variable the deviation from mean coding was applied, a method which does not require the definition of a reference group, provides 95% confidence intervals for all categories and expresses the risk relative to the average risk in the whole study population [34]. The performance of the OSTEO18 was further assessed by the receiver operating curve (ROC) and the area under curve (AUC) approach. The OSTEO18 threshold optimised by the Youden index was 0.095. The 95% CI of the AUC was calculated by the DeLong method [35]. Significance was a 2-sided *p*-value of ≤0.05.

Results

Case-control studies

Table 1 lists the baseline characteristics of the patients included in the case-control studies. In the derivation dataset, 80.0% of the participants were men (n=112) and 34.3% were on treatment with methylprednisolone (n=48), 67.7% tacrolimus (n=94) and 29.3% cyclosporine (n=41). In the derivation (70 cases *vs* 70 controls), replication (59 *vs* 59) and time-shifted replication (67 *vs* 67) datasets, cases and controls (**Table 1**) were well matched. **Table S2** shows that patients included in the 3 case-control studies had largely similar characteristics with some differences in the treatment with immunosuppressant drugs associated with the time interval since HTx and over-time changing treatment policies.

The OSTEO18 scores were significantly and consistently higher in patients with osteoporosis compared with controls in the derivation (*P*<0.0001, **Figure 2A**), replication (*P*<0.0001, **Figure 2B**) and time-shifted (*P*=0.014, **Figure 2C**) case-control studies. In the derivation dataset, the AUC was 0.83 (95% CI: 0.76-0.90; *P*<0.0001) and sensitivity and specificity were 74.3% and 87.1%, respectively (**Table S3**). Analysis of the replication and time-shifted replication datasets confirmed the discriminatory performance of OSTEO18 (**Table S3**). In the replication case-control study, the AUC, sensitivity and specificity were 0.71 (95% CI: 0.62-0.80; *P*<0.0001), 69.5% and 62.7%, and in the time-shifted replication dataset 0.62 (0.53-0.72; *P*<0.011), 73.1% and 53.7%, respectively.

Table 1. Patient characteristics in the derivation, replication and time-shifted case-control studies (starts)

Characteristic	Derivation dataset			Replication dataset			Time-shifted replication dataset		
	Controls (*n*=70)	Cases (*n*=70)	*p*-value	Controls (*n*=59)	Cases (*n*=59)	*p*-value	Controls (*n*=67)	Cases (*n*=67)	*p*-value
Time after HTx (years)	9.91 (4.53, 14.3)	11.1 (5.28, 18.3)	0.17	3.28 (0.41, 9.29)	5.75 (0.76, 12.9)	0.20	11.3 (5.26, 15.0)	12.0 (5.74, 19.8)	0.18
Age (years)	61.3 (12.1)	60.2 (13.5)	0.61	56.6 (12.6)	59.4 (13.0)	0.23	62.2 (12.4)	60.9 (13.2)	0.58
BMI (kg/m^2)	25.4 (3.36)	25.3 (4.26)	0.99	24.8 (4.28)	24.8 (4.53)	0.98	27.0 (11.5)	25.5 (4.53)	0.31
SBP (mm Hg)	145.6 (19.1)	143.5 (22.3)	0.55	140.0 (19.9)	143.1 (21.3)	0.43	143.8 (19.3)	143.0 (21.4)	0.83
DBP (mm Hg)	86.2 (12.6)	86.0 (13.1)	0.94	87.2 (11.2)	88.0 (10.6)	0.71	85.0 (11.1)	85.0 (13.3)	0.99
Serum creatinine (µmol/L)	136.5 (45.1)	130.2 (40.6)	0.38	124.75 (41.6)	129.81 (42.0)	0.51	140.3 (48.0)	133.8 (40.4)	0.40
eGFR (ml/min/1.73m^2)	52.3 (21.1)	54.1 (19.6)	0.59	58.99 (21.4)	56.00 (22.2)	0.46	51.2 (21.8)	52.2 (19.6)	0.77
Serum albumin (g/L)	44.1 (2.97)	43.9 (3.48)	0.71	43.3 (3.78)	44.1 (2.90)	0.17	44.7 (2.77)	43.9 (3.24)	0.14
Total serum calcium (mmol/L)	2.39 (0.09)	2.40 (0.12)	0.58	2.40 (0.13)	2.40 (0.12)	0.78	2.40 (0.10)	2.39 (0.13)	0.65
Ionized calcium (mmol/L)	2.47 (0.14)	2.48 (0.16)	0.82	2.46 (0.19)	2.48 (0.16)	0.46	2.49 (0.14)	2.47 (0.17)	0.37
Sex									
Men, n (%)	57 (81.4%)	55 (78.6%)	0.67	46 (78.0%)	48 (81.4%)	0.65	56 (83.6%)	53 (79.1%)	0.51
Women, n (%)	13 (18.6%)	15 (21.4%)		13 (22.0%)	11 (18.6%)		12 (16.7%)	14 (20.9%)	
Indication for HTx									
Ischemic CMP, n (%)	33 (47.1%)	25 (35.7%)	0.17	19 (32.2%)	29 (49.2%)	0.061	31 (46.3%)	24 (35.8%)	0.22
Dilated CMP, n (%)	27 (38.6%)	27 (38.6%)	…	29 (49.2%)	21 (35.6%)	0.14	26 (38.8%)	26 (38.8%)	…
Other, n (%)	10 (14.3%)	18 (25.7%)	0.091	8 (13.6%)	6 (10.2%)	0.57	10 (14.9%)	17 (25.4%)	0.13

Table 1. Patient characteristics in the derivation, replication and time-shifted case-control studies (ends)

Characteristic	Derivation dataset			Replication dataset			Time-shifted replication dataset		
	Controls (*n*=70)	Cases (*n*=70)	*p*-value	Controls (*n*=59)	Cases (*n*=59)	*p*-value	Controls (*n*=67)	Cases (*n*=67)	*p*-value
Past smoker, n (%)	47 (67.1%)	43 (61.4%)	0.48	37 (62.7%)	36 (62.1%)	0.94	44 (65.7%)	43 (64.2%)	0.86
Hypertension, n (%)	66 (94.3%)	65 (92.9%)	0.73	50 (84.7%)	55 (93.2%)	0.14	65 (97.0%)	60 (89.6%)	0.084
Diabetes, n (%)	15 (21.4%)	10 (14.3%)	0.27	6 (10.2%)	11 (18.6%)	0.19	15 (22.4%)	9 (13.4%)	0.18
Osteoporotic fracture, n (%)	…	7 (10.0%)	…	…	4 (6.8%)	…	…	6 (9.0 %)	…
BMD T-score ≤-2.5, n (%)	…	23 (32.9%)	…	…	20 (33.9%)	…	…	22 (32.8%)	…
Calcium supplements, n (%)	…	53 (75.7%)	…	…	47 (79.7%)	…	…	51 (76.1%)	…
Vitamin D, n (%)	…	52 (74.3%)	…	…	47 (79.7%)	…	…	50 (74.6%)	…
Bisphosphonates, n (%)	…	14 (20.0%)	…	…	5 (8.5%)	…	…	13 (19.4%)	…
Thiazides, n (%)	15 (21.4%)	5 (7.1%)	0.016	4 (11.8%)	3 (9.1%)	0.72	11 (16.4%)	4 (6.0%)	0.055
Immunosuppression									
Tacrolimus, n (%)	53 (75.7%)	41 (58.6%)	0.031	36 (62.1%)	27 (47.4%)	0.15	49 (73.1%)	37 (55.2%)	0.031
Cyclosporine, n (%)	14 (20.0%)	27 (38.6%)	0.016	5 (8.5%)	12 (20.3%)	0.067	14 (20.9%)	26 (38.8%)	0.024
Everolimus, n (%)	4 (5.7%)	3 (4.3%)	0.70	4 (9.3%)	3 (6.0%)	0.56	0 (0%)	1 (1.5%)	0.32
Mycophenolic acid, n (%)	55 (78.6%)	51 (72.9%)	0.43	32 (57.1%)	33 (61.1%)	0.42	49 (73.1%)	48 (71.6%)	0.85
Azathioprine, n (%)	6 (8.6%)	6 (8.6%)	…	3 (7.0%)	1 (2.1%)	0.26	0 (0%)	0 (0%)	…
Methylprednisolone, n (%)	25 (35.7%)	23 (32.9%)	0.72	12 (20.3%)	13 (22.0%)	0.82	0 (0%)	0 (0%)	…

Values are mean (SD), median (IQR) or number of patients (%). Abbreviations: HTx, heart transplantation; BMI, body mass index; SBP/DBP, systolic/diastolic blood pressure; eGFR, estimated glomerular filtration rate; CMP, cardiomyopathy; BMD, bone mineral density. To convert serum creatinine from µmol/L to mg/dL, multiply by 0.0113. To convert serum albumin from g/L to g/dL, multiply by 0.1. To convert serum total and ionized calcium from mmol/L to mg/dL, multiply by 4. *p*-values refer to the difference between cases and controls in the derivation, replication and time-shifted case-control studies. An ellipsis indicates that the variable was not measured or that the *p*-value was not calculated.

Performance of OSTEO18 in all uPROPHET patients

Table 2 and **Table S4** list the characteristics of all 389 uPROPHET patients as well as the characteristics of 154 patients (39.6%) with osteoporosis and 235 patients (60.4%) without any sign of osteoporosis. Of 389 patients, 78 (20.1%) were examined within the first year after HTx. Of 154 patients with osteoporosis (**Table S4**), only 8 (5.19%) were diagnosed before HTx. As shown in Figure S3, the distribution of OSTEO18 was close to normally distributed (*P*=0.034).

In exploratory analyses without any adjustment, the 389 uPROPHET patients were stratified by their OSTEO18 score: low (<-1), medium-low (-1 to 0), medium-high (0 to 1) and high (≥1). Across increasing OSREO18 categories (**Table 2**), age (low *vs* high: 54.0 *vs* 62.5 years; trend *p*-value: <0.0001), the time interval since HTx (4.33 *vs* 10.2 years; *P*=0.0024), serum creatinine (118.4 *vs* 135.0 μmol/L; *P*=0.0062) and the use of cyclosporine (12.9% vs 34.8%; *P*<0.0001) increased, whereas eGFR (63.7 *vs* 51.6 mL/min/1.73 m^2; *P*=0.002) and tacrolimus treatment (82.8% *vs* 54.3%; *P*=0.0001) decreased. The components of the osteoporotic endpoint were positively associated with higher OSTEO18 strata (**Table 2**). The osteoporosis rates expressed in cases per 100 patients were 17/93 (18.3% [95% CI: 10.4-26.1%]) in the low OSTEO18 category, 27/107 (25.2% [17.0-33.5]) and 48/97 (49.5% [39.5-59.4]) in the medium-low and medium-high categories, and 62/92 (67.4% [57.8-77.0%]) in the high category (trend *p*-value: 0.019).

Using unadjusted, partially adjusted and fully adjusted logistic regression, there was a significantly positive association between the risk of osteoporosis and the OSTEO18 level, irrespective of adjustment (**Table 3**) and irrespective of whether OSTEO18 was analyzed as a categorical or continuously distributed variable with an optimised threshold of 0.095 (**Table 4**). From the low to high OSTEO18 category (**Table 3**), the ORs increased from 0.39 (95% CI: 0.25-0.61) to 3.14 (2.08-4.75), resulting in a trend p-value of <0.0001. With full adjustments applied (model 2), OSTEO18 significantly improved the AUC from 0.708 to 0.786 (*P*=0.0003) for the continuously distributed classifier (**Figure 3A and 3B**) and to 0.784 (*P*=0.0004) for the categorised classifier (**Figure 3A and 3C**). To place the discriminatory performance into context, **Table S5** lists the ORs associated with single risk factors for osteoporosis for which the model 2 (**Figure 3A, 3B and 3C**) was adjusted. The AUC of OSTEO18 was 0.74 (95%CI: 0.69-0.79), which achieved good discriminative performance compared with other single risk factors (**Table S5**).

Table 2. Characteristics of 389 uPROPHET patients by OSTEO18 categories (starts)

Characteristic	OSTEO18 score				*p*-value
	< -1	-1-0	0-1	≥1	
Number in group, n (%)	93 (23.9)	107 (27.5)	97 (24.9)	92 (23.7)	
Time after HTx (years)	4.33 (1.26, 13.2)	6.26 (1.52, 11.2)	7.53 (1.74, 15.6)	10.2 (3.87, 16.1)	0.0024
Age (years)	54.0 (13.5)	55.3 (15.4)	57.1 (14.2)	62.5 (12.2)	<0.0001
BMI (kg/m^2)	25.7 (4.12)	25.3 (4.50)	24.4 (4.24)	25.8 (4.04)	0.78
SBP (mm Hg)	142.3 (18.8)	141.2 (20.7)	141.7 (21.8)	144.3 (21.6)	0.50
DBP (mm Hg)	86.4 (11.2)	85.0 (12.1)	84.4 (11.5)	86.6 (10.9)	>0.99
Serum creatinine (µmol/L)	118.4 (35.9)	121.0 (45.8)	126.7 (45.0)	135.0 (47.6)	0.0062
eGFR (ml/min/1.73m^2)	63.7 (24.2)	63.6 (26.2)	58.3 (25.3)	51.6 (20.1)	0.0002
Serum albumin (g/L)	43.9 (3.06)	44.2 (3.36)	44.0 (3.03)	43.0 (3.47)	0.084
Total serum calcium (mmol/L)	2.38 (0.11)	2.38 (0.12)	2.40 (0.11)	2.40 (0.12)	0.16
Ionized serum calcium (mmol/L)	2.46 (0.15)	2.46 (0.17)	2.48 (0.14)	2.46 (0.17)	0.76
Sex					
Men, n (%)	73 (78.5)	81 (75.7)	70 (72.2)	70 (76.1)	0.58
Women, n (%)	20 (21.5)	26 (24.3)	27 (27.8)	22 (23.9)	
Indication for HTx					
Ischemic cardiomyopathy, n (%)	34 (36.6)	38 (35.5)	34 (35.1)	44 (47.8)	0.14
Dilated cardiomyopathy, n (%)	38 (40.9)	45 (42.1)	40 (41.2)	34 (37.0)	0.58
Other, n (%)	21 (22.6)	23 (21.5)	23 (23.7)	14 (15.2)	0.30

Table 2. Characteristics of 389 uPROPHET patients by OSTEO18 categories (ends)

Characteristic	OSTEO18 score				*p*-value
	< -1	-1-0	0-1	≥1	
Past smoker, n (%)	58 (63.0)	61 (57.0)	61 (63.5)	59 (64.8)	0.58
Hypertension, n (%)	79 (84.9)	95 (88.8)	88 (90.7)	86 (93.5)	0.056
Diabetes, n (%)	22 (23.7)	23 (21.5)	22 (22.7)	23 (25.0)	0.78
Osteoporotic fracture, n (%)	1 (1.1)	2 (1.9)	2 (2.1)	7 (7.6)	0.021
BMD T-score ≤-2.5, n (%)	10 (10.8)	11 (10.3)	17 (17.5)	18 (19.6)	0.037
Anti-osteoporotic treatment, n (%)	11 (11.8)	21 (19.6)	39 (40.2)	52 (56.5)	<0.0001
Thiazides, n (%)	10 (10.8)	6 (5.6)	14 (14.4)	12 (13.0)	0.25
Immunosuppression					
Tacrolimus, n (%)	77 (82.8)	86 (80.4)	66 (68.0)	50 (54.3)	<0.0001
Cyclosporine, n (%)	12 (12.9)	10 (9.3)	22 (22.7)	32 (34.8)	<0.0001
Everolimus, n (%)	5 (5.4)	8 (7.5)	4 (4.1)	4 (4.3)	0.52
Mycophenolic acid, n (%)	65 (69.9)	82 (76.6)	70 (72.2)	64 (69.6)	0.77
Azathioprine, n (%)	9 (9.7)	4 (3.7)	9 (9.3)	4 (4.3)	0.40
Methylprednisolone, n (%)	28 (30.1)	36 (33.6)	33 (34.0)	37 (40.2)	0.17

Values are mean (SD), median (IQR) or number of patients (%). Abbreviations: HTx, heart transplantation; BMI, body mass index; SBP/DBP, systolic/diastolic blood pressure; eGFR, estimated glomerular filtration rate; BMD, bone mineral density. Anti-osteoporotic treatment refers to calcium supplementation, vitamin D supplements and/or bisphosphonates. To convert serum creatinine from µmol/L to mg/dL, multiply by 0.0113. To convert serum albumin from g/L to g/dL, multiply by 0.1. To convert serum total and ionized calcium from mmol/L to mg/dL, multiply by 4. *p*-values refer to the difference between cases and controls in the derivation, replication and time-shifted replication datasets.

Table 3. Association of osteoporosis with OSTEO18 in 389 uPROPHET patients

Variable	n/N (%)	Unadjusted		Adjusted		Fully adjusted	
		OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
OSTEO18 score (categorised)							
<-1	17/93 (18.3)	0.36 (0.23, 0.55)	<0.0001	0.37 (0.24, 0.58)	<0.0001	0.39 (0.25, 0.61)	<0.0001
-1-0	27/107 (25.2)	0.54 (0.37, 0.79)	0.0016	0.54 (0.36, 0.79)	0.0017	0.53 (0.35, 0.79)	0.0017
0-1	48/97 (49.5)	1.57 (1.09, 2.24)	0.015	1.57 (1.09, 2.27)	0.016	1.57 (1.07, 2.29)	0.020
≥1	62/92 (67.4)	3.31 (2.26, 4.84)	<0.0001	3.18 (2.14, 4.73)	<0.0001	3.14 (2.08, 4.75)	<0.0001
p for linear trend			<0.0001		<0.0001		<0.0001
OSTEO18 score (continuous, +1)	154/389 (39.6)	2.07 (1.70, 2.50)	<0.0001	2.02 (1.67, 2.46)	<0.0001	2.03 (1.65, 2.49)	<0.0001

Abbreviations: OR, odds ratio; CI, confidence interval. n/N indicates the number of participants with osteoporosis, the number of participants at risk in each category, and the corresponding percentages. In logistic regression, OSTEO18 was introduced as categorical and as continuously distributed variable. The categorised OSTEO18 score (<-1, -1-0, 0-1 and ≥1) was modeled by the deviation from mean coding, so that ORs express the relative risk compared with the average risk in the whole study population. Adjusted logistic models accounted for sex, age, eGFR, diabetes (yes *vs* no) and treatment with methylprednisolone (yes *vs* no). Fully adjusted logistic models additionally accounted for the time interval since HTx, ionized calcium, treatment with thiazides (yes *vs* no), tacrolimus (yes *vs* no) and cyclosporine (yes *vs* no).

Table 4. Discriminative performance of OSTEO18 in 389 uPROPHET patients

Performance	Estimate (95%CI)
n/N	154/389
Continuously distributed OSTEO18	
AUC	0.74 (0.69, 0.79)
Categorised OSTEO18	
Youden cut-off threshold	0.095
Sensitivity, %	71.4 (63.6, 78.4)
Specificity, %	69.8 (63.5, 75.6)
PLR	2.36 (1.90, 2.94)
NLR	0.41 (0.31, 0.53)
Accuracy	70.4 (65.6, 74.9)

Abbreviations: CI, confidence interval; AUC, area under curve; PLR, positive likelihood ratio (true positive rate/false positive rate); NLR, negative likelihood ratio (false negative rate/true negative rate). n/N indicates the number of participants with osteoporosis and the number of participants at risk. Accuracy is the overall probability that a patient is correctly classified. All estimates in this table were unadjusted for other osteoporosis risk factors.

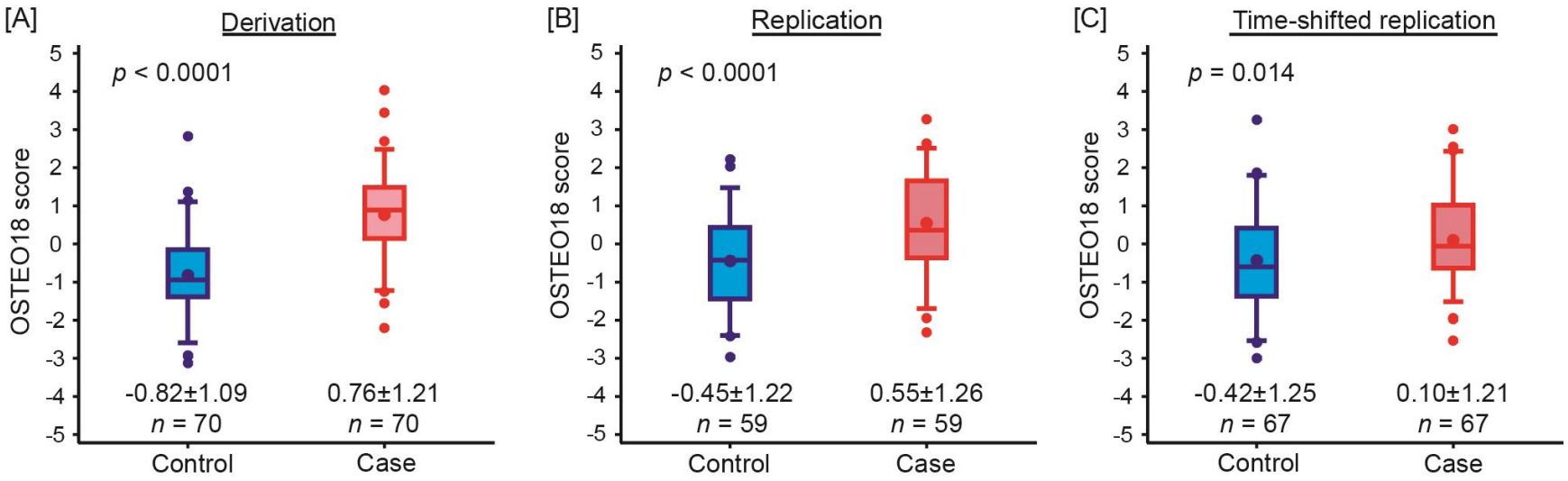
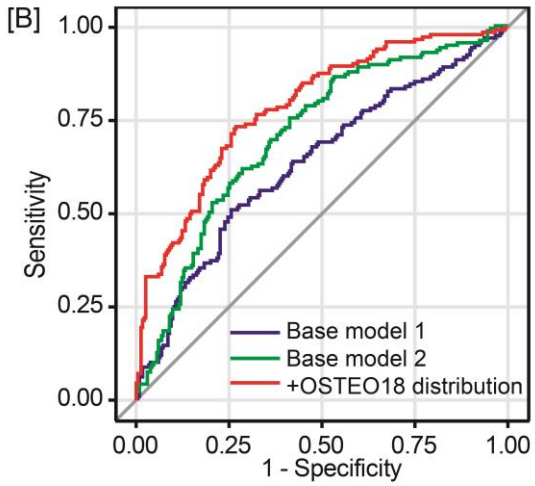


Figure 2. Distribution of the multidimensional classifier OSTEO18 in cases (red) and controls (blue) in the derivation (A), replication (B) and time-shifted (C) case-control studies. OSTEO18 was developed by support vector modelling and optimised by take-one-out cross validation procedure. The central line, the upper and lower lines, and the upper and lower whiskers represent the median, interquartile range, and the 10th to 90th percentile interval, respectively. The arithmetic means and extreme measurements are represented by circles inside the box and outside the whiskers, respectively. Arithmetic means and standard deviations are presented along the horizontal axis. *n* denotes the number of patients in each group. *p* values denote the significance in the OSTEO18 score between cases and controls in each dataset.

[A]

Osteoporosis (n/N = 154/389)

	AUC (95% CI)	*p*-value
Base model 1	0.632 (0.574, 0.689)	
Base model 2	0.708 (0.655, 0.760)	0.0026
+OSTEO18		
Continuous	0.786 (0.740, 0.832)	0.0003
Categorized threshold, 0.095	0.784 (0.737, 0.831)	0.0004



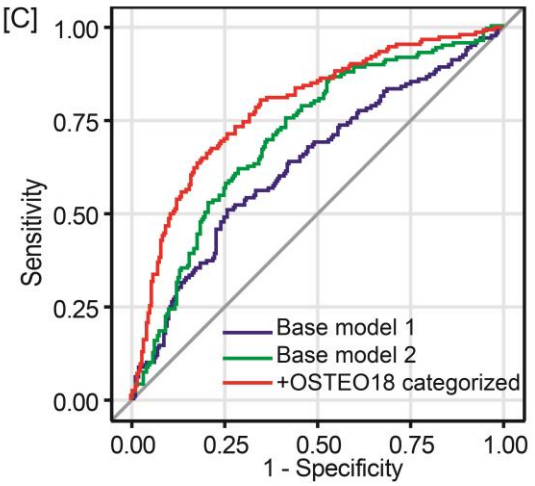


Figure 3. Performance of the OSTEO18 classifier on top of other osteoporosis risk factors in 389 uPROPHET patients. n/N indicates the number of patients with osteoporosis and the number of participants at risk (panel A). Base model 1 includes sex, age, the estimated glomerular filtration rate, diabetes (yes *vs* no) and treatment with methylprednisolone (yes *vs* no). Base model 2 additionally accounted for the time interval since HTx, ionized calcium, treatment with thiazides (yes *vs* no), tacrolimus (yes *vs* no) and cyclosporine (yes *vs* no). In subsequent steps, OSTEO18 was added to the previous model as a continuously distributed variable (panel B) or as a categorised variable (panel C) based on the 0.095 optimized threshold. At each step, *p*-values are for the comparison with the preceding model. AUC is the area under curve given with 95% confidence interval.

Discussion

The key finding of the current study was the development, replication and validation of a novel 18-peptide multidimensional urinary biomarker indicative of osteoporosis in HTx patients. On top of relevant osteoporosis risk factors, OSTEO18 improved the AUC as categorised variable with optimised threshold of 0.095 as well as continuously distributed biomarker. Collagens type I, III, and V are the most abundant constituents of the organic extracellular matrix in bones [36,37]. The main function of collagens is mechanical support and to act as a scaffold for bone cells. Type-I collagen accounts for 90% of total collagen in bone tissue [36]. Only breakdown products of mature collagen appear in urine [38]. In the first step of type-I collagen degradation small fragments are released by collagenases, while larger fragments are further degraded by gelatinases with the resulting smaller fragments being further cleaved by gelatinases [38]. The OSTEO18 urinary biomarker includes 8 fragments (44.4%) derived from type-I collagen and 2 (11.1%) from type-III collagen and plausibly reflects the turnover of the extracellular bone matrix. Furthermore, calcium is absorbed in the mammalian small intestine by two general mechanisms: a transcellular active transport process, located largely in the duodenum and upper jejunum; and a paracellular, passive process that functions throughout the length of the intestine [39]. The polymeric immunoglobulin receptor (PIGR) has the dual role of transporting locally produced dimeric IgA across mucosal epithelia, and serving as the precursor of secretory component and plays a key role in mucosal immunity [40]. The presence of a PIGR-derived urinary peptide in OSTEO18 might be explained by dysregulation of its role by immunosuppression.

In addition to aging, menopause and renal dysfunction compromising calcium homeostasis and the activation of calcidiol to active calcitriol, the root causes of osteoporosis in HTx patients are the use of glucocorticosteroids and immunosuppressive drugs, such as cyclosporin and tacrolimus, to prevent allograft rejection. Glucocorticosteroids reduce bone formation mainly at the trabecular core of the vertebrae [41]. The calcineurin blockers cyclosporine and tacrolimus stimulate the secretion of parathyroid hormone [3] and are nephrotoxic [42,43]. Cyclosporine induces tubular renal cell apoptosis and increases the calcium levels of renal tubular cells [29]. Furthermore, cyclosporine stimulates both osteoblasts and osteoclasts. However, the osteoclastic activity dominates and induces elevated bone turnover and delayed repair of renal osteodystrophy [3,42]. In experimental rat studies [28], tacrolimus decreased bone mineral density in the tibia and femur without influence on bone formation. Keeping in mind that over half of the peptide fragments included in OSTEO18 are derived from type-I

and type-III collagens, the predominant collagens in bone tissue, the novel biomarker might be applied to monitor the osteotoxic effects of glucocorticosteroids, cyclosporine and tacrolimus.

Osteoporosis is a silent disease until it is complicated by fractures—fractures that occur following minimal trauma or, in some cases, with no trauma. Therefore, osteoporosis represents a major public health issue [44], in terms of the quality of life of the patients and social health cost. Several risk factors and circulating biomarkers reflecting osteoporosis in HTx patients can be monitored, although on their own they have an AUC not exceeding 60% (**Table S5**), whereas a 1-unit increment in OSTEO18 was associated with a doubling of the osteoporosis risk with an AUC of 0.74 (95% CI: 0.69-0.79). More importantly, on top of sex, age, the time interval since HTx, eGFR, diabetes, and treatment with methylprednisolone, tacrolimus, cyclosporin and thiazide diuretics, OSTEO18 maintained its discriminatory performance, irrespective of whether the optimised 0.095 threshold was applied or OSTEO18 was analyzed as continuously distributed variable. BMD testing by dual energy XRA is consensually the gold standard in the diagnosis and management of osteoporosis, because the so-determined BMD at the lumbar spine and hip closely correlates with bone strength and is an excellent predictor of future fracture risk [13,14]. Nevertheless, XRA also has limitation due to the confounding effects on the BMD measurement of surrounding soft tissue, bone artefacts caused by osteoarthritis, degenerated discs, aortic calcification and vertebral compression fractures [14]. Other limitations in the administration of XRA scans are imposed by the applicable health insurance. In Belgium (nomenclatuurart17ter _20230401_01.pdf available at www.riziv.be), a dual energy XRA (code: N72) is reimbursed at a rate of €40.25. In the context of HTx, it should be noted that the examination is only reimbursed if the patient has a low-impact vertebral fracture or has been on therapy with glucocorticosteroids for ≥3 months at a daily dose equivalent to 7.5 mg prednisolone. However, in 2012, legislation changed making dual energy XRA only reimbursable at 5-year instead of annual intervals. These rules do not support efficient prevention of osteoporotic complications in HTx patients, because a vertebral fracture already indicates the presence of the disease and because the nomenclature disregards the osteotoxic side-effects of cyclosporine and tacrolimus. Given reimbursement rules, which differ from country to country, the low diagnostic and prognostic value of single osteoporosis risk factors and some pitfalls potentially associated with dual energy XRA, OSTEO18 might be added testing armamentarium provided that cost-effectiveness can be demonstrated, as discussed in the next paragraph.

Cost-effectiveness balances health-care costs against non-monetary units, such as quality-adjusted life-years (QALYs) [45]. The QALY-based value proposition is well established in the UK, Sweden, Belgium, the Netherlands, Luxembourg, and some eastern European countries, but health-care insurers in Germany and France prefer assessing changes in clinical outcomes instead [45]. Bone loss progresses rapidly after HTx. Osteoporosis causes a high economic and social burden, predominantly due to severe fractures. In the United States, among 885 676 Medicare beneficiaries, the per-patient annual health insurance costs from January 2010 until September 2014 were substantially higher in patients (mean age: 80.5 years; 94% female) with an osteoporosis-related fracture than in the matched nonfracture population ($47163.25 *vs* $16034.61) [46]. In 12 Belgian hospitals, the in-patient costs Belgium associated with total hip replacement, which is the feasible treatment for severe fracture, averaged €8013 [47]. In addition, the estimated total costs were €465 million for anti-osteoporotic medication, preventive supplement and the treatment of osteoporosis-related fracture, according the Achmea Health Database (108,013 individuals) in 2010 in the Nederland [48]. Along similar lines, in France, the annual per-person osteoporosis-related costs amounted to €18,040 per person, of which €17,905 was directly related to the fracture and €135 to the management of osteoporosis [49]. The timely institution of anti-osteoporotic is therefore of paramount clinical importance to avoid bone fractures and associated debilitating complications and risky and costly orthopedic surgery [38]. OSTEO18 is being certified as an in-vitro diagnostic and is therefore ready to enter the clinical management of HTx patients. However, the aforementioned costs have to be balanced against those of an OSTEO18 test (€850), which requires further clinical validation in HTx patients.

Strong points and limitations

Strong points of current study are the derivation and replication of the multidimensional urinary classifier OSTEO18 in the case-control studies, as well as the validation in the whole uPROPHET cohort. The UPP is unbiased in the sense that it does not depend on predefined markers, but is only determined by peptides passing the glomerular barrier or generated along the nephron, and because it is platform independent [6]. Notwithstanding the consistency of the findings, the current study must also be interpreted within the context of its limitations. First, the current study had a cross-sectional design, albeit that the time-shifted case-control study added some longitudinal dimension in the replication of the results from the discovery dataset. Second, cases and controls in the discovery and two replication datasets were well matched for the use of methylprednisolone, but it was impossible to achieve complete matching for

immunosuppressive drugs, because treatment strategies changed over time. Third, there was a preponderance of short amino-acid chains among the sequenced peptides, which may be due to the reduced success of sequencing longer-chain peptides. Unknown posttranslational modifications are the most likely cause for the failure to identify a peptide sequence with confidence. Peptide abundance also impacts on the quality of the MS/MS spectra. However, while only 17% of the detected urinary peptides were sequenced, those currently sequenced represented 63% of the total peptide mass [50]. Fourth, the current results were generated in a single-center study of HTx recipients, with specific immunosuppressant treatments and vulnerability inducing higher risks of osteoporosis [3] and other aging-related diseases [51]. The OSTEO18 findings cannot be extrapolated to other patients, such as for instance postmenopausal women. Finally, because our proof-of-concept study is retrospective, osteoporotic biomarkers other than bone specific alkaline phosphatase were not measured.

Conclusion

OSTEO18 is a clinically meaningful novel biomarker indicative of osteoporosis in HTx recipients. Given that OSTEO18 is being certified as in-vitro diagnostic, the test is ready to enter the clinical management of HTx patients. However, to be endorsed by HTx transplantation guidelines, OSTEO18 required further evidence from prospective studies and demonstration of its cost-effectiveness.

Funding

uPROPHET was supported by the European Research Council (Advanced Researcher Grant-2011-294713-EPLORE and Proof-of-Concept Grant-713601-uPROPHET awarded to JAS). OMRON Healthcare, Co., Ltd., Kyoto, Japan provided a non-binding grant to the Non-Profit Research Association Alliance for the Promotion of Preventive Medicine (APPREMED), Mechelen, Belgium. Dries S. Martens holds a postdoctoral grant by the Research Foundations Flanders (FWO grants 12X9623N).

Conflict of interest

Julia Raad and Agnieszka Latosinszka are employees of Mosaiques-Diagnostics GmbH, Hannover, Germany. Harald Mischak is the co-founder and co-owner of Mosaiques-Diagnostics GmbH. The other authors declare no conflict of interest.

References

1. Committee on Clinical Practice Guideline-Gynecology. Osteoporosis prevention, screening, and disgnosis: ACOG clinical practice guideline No. 1. *Obstet Gynecol.* 2012; 138: 494-506.
2. Löfdahl E, Rådegran G. Osteoporosis following heart transplantation and immunosuppressive therapy. *Transplant Rev.* 2017;
3. Kovvuru K, Kanduri SR, Vaitla P *et al*. Risk factors and management of osteoporosis post-transplant. *Medicina (Kaunas).* 2020; 56: 302.
4. Mischak H, Kolch W, Aivaliotis M *et al*. Comprehensive human urine standards for comparability and standardization in clinical proteome analysis. *Proteomics Clin Appl.* 2010; 4: 464-478.
5. Mavrogeorgis E, Mischak H, Latosinska A, Siwy J, Jankowski V, Jankowski J. Reproducibility evaluation of urinary peptide detection using CE-MS. *Molecules.* 2021; 26: 7260.
6. Klein J, Papadopoulos T, Mischak H, Mullen W. Comparison of CE-MS/MS and LC-MS/MS sequencing demonstrates significant complementarity in natural peptide identification in human urine. *Electrophoresis.* 2014; 35: 1060-1064.
7. Saito M, Marumo K. Effects of collagen crosslinking on bone material properties in health and disease. *Calcif Tissue Int.* 2015; 97: 242-261.
8. Martens DS, Thijs L, Latosinska A *et al*. Urinary peptidomics to address age-related disabilities: a prospective population study with replication in patients. *Lancet Healthy Longev.* 2021; 2: e690-e703.
9. Huang QF, Trenson S, Zhang ZY *et al*. Biomarkers to assess right heart pressures in recipients of a heart transplant: a proof-of-concept study. *Transplant Direct.* 2018; 4: e346.
10. Huang QF, Zhang ZY, Van Keer J *et al*. Urinary peptidomic biomarkers of renal function in heart transplant recipients. *Nephrol Dial Transplant.* 2019; 34: 1336-1343.
11. Huang QF, Trenson S, Zhang ZY *et al*. Urinary Proteomics in Predicting Heart Transplantation Outcomes (uPROPHET) - Rationale and database description. *PLoS One.* 2017; 12: e0184443.
12. World Medical Association. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. *J Am Med Assoc.* 2013; 310: 2191-2194.

13. Cosman F, de Beur SJ, LeBoff MS *et al*. Clinician's guide to prevention and treatment of osteoporosis. *Osteoporos Int.* 2014; 25: 2539-2381.

14. Lorentzon M, Cummings SR. Osteoporosis: the evolution of a diagnosis. *J Intern Med.* 2015; 277: 650-661.

15. Braverman ER, Chen TJH, Chen ALC *et al*. AGe-related increases in parathyroid hormaone may be antecedent to both osteoporosis and dementia. *BMC Endocr Disord.* 2023; 9: 21.

16. Singh S, Kumar D, Lal AK. Diagnostic biomarker for primary osteoporosis in women. *J Clin Diagn Res.* 2015; 9: RC04-RC07.

17. Hata K, Tokuhiro H, Nakatsuka K *et al*. Measurement of bone-specific alkaline phosphatase by an immunoselective enzyme assay method. *Ann Clin Biochem.* 1996; 33: 127-131.

18. Richart T, Li Y, Staessen JA. Renal versus extrarenal activation of vitamin D in relation to atherosclerosis, arterial stiffening, and hypertension. *Am J Hypertens.* 2007; 20: 1007-1015.

19. Bouquegneau A, Salam S, Delanaye P, Eastell R, Khwaja A. Bone disease after kidney transplantation. *Clin J Am Soc Nephrol.* 2016; 11: 1282-1296.

20. Jantos-Siwy J, Schiffer E, Brand K *et al*. Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res.* 2009; 8: 268-281.

21. Mischak H, Vlahou A, Ioannidis JPA. Technical aspects and inter-laboratory variability in native peptide profiling : the CE-MS experience. *Clin Biochem.* 2013; 46: 432-443.

22. Zhang ZY, Ravassa S, Nkuipou-Kenfack E *et al*. Novel urinary peptidomic classifier predicts incident heart failure. *J Am Heart Assoc.* 2017; 6: e005432.

23. An DW, Yu YL, Martens DS *et al*. Statistical approaches applicable in managing OMICS data: urinary proteomics as exemplary case. *Mass Spec Rev.* 2023; 42: 1-18.

24. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc B.* 1995; 57: 289-300.

25. Wendt R, Thijs L, Kalbitz S *et al*. A specific urinary peptidomic profile predicts outcome in SARS-CoV-2-infected patients. *eClinicalMedicine.* 2021; 35: 100883.

26. Levey AS, Stevens LA, Schmid CH *et al*. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009; 150: 604-612.

27. Cheng L, Zhang K, Zhang Z. Effectiveness of thiazides on serum and urinary calcium levels and bone mineral density in patients with osteoporosis: a systematic review and meta-analysis. *Drug Des Devel Ther.* 2018; 12: 3929-3935.

28. Kanda J, Izumo N, Furukawa M *et al*. Effects of the calcineurin inhibitors cyclosporine and tacrolimus on bone metabolism in rats. *Biomed Res.* 2018; 39: 131-139.

29. Sambrook PN. Cyclosporine and bone mass. *Clin Exp Rheumatol.* 2000; 18: S93-S96.

30. Karbhari DS, Karbhari NS, Patel S. Significance of measurement of corrected calcium inpatients with normoalbuminemia. *Int J Sci Public Health.* 2017; 6: 1069-1071.

31. Pouresmaeili F, Kamalidehghan B, Kamareher M, Goh TM. A comprehensive overview on osteoporosis and its risk factors. *Ther Clin Risk Manag.* 2018; 14: 2029-2049.

32. Anastasilakis AD, Tsourdi E, Makras P *et al*. Bone disease following solid organ transplatation: a narrative review and recommendations for management from the European Calcified Tissue Society. *Bone.* 2019; 127: 401-418.

33. Hsu CY, Chen L-R, Chen K-H. Osteoporosis in patients with chronic kidney disease: a systemic review. *Int J Mol Sci.* 2020; 21: 6846.

34. Hosmer DW, Jr., Lemeshow S. Polytomous independent variable. *Applied logistic regression.* John Wiley & Sons, New York: 1989; 47-56

35. DeLong ER, DeLong DM, Clarke-Peterson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics.* 1988; 44: 837-845.

36. Lin X, Patil S, Gao YG, Qian A. The bone extracellular matrix in bone formation and regeneration. *Front Phrmacol.* 2020; 11: 757.

37. Licini C, Vitale-Brovarone C, Mattioloni-Belmonte M. Collagen and non-collagenous proteins molecular crosstalk in the pathophysiology of osteoporosis. *Cytokine Growth Factor Rev.* 2019; 49: 59-69.

38. López B, González A, Ravassa S *et al*. Circulating biomarkers of myocardial fibrosis: the need for a reappraisal. *JACC.* 2015; 65: 2449-2456.

39. Bronner F. Mechanisms of intestinal calcium absorption. *J Cell Biochem.* 2003; 88: 387-393.

40. Johansen FE, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. *Mucosal Immunol.* 2011; 4: 598-602.

41. Reid IR, Billington EO. Drug therapy for osteoporosis in older adults. *Lancet.* 2022; 399: 1080-1092.

42. Sikma MA, van Maarseveen EM, van de Graaf EA *et al*. Pharmacokinetics and toxicity of tacrolimus early after heart and lung transplantation. *Am J Transplant.* 2015; 15: 2301-2313.

43. Wu Q, Wang X, Nepovimova E, Wang Y, Yang H, Kuca K. Mechanism of cyclosporine A nephrotoxicity: Oxidative stress, autophay, and signaling. *Food Chem Toxicol.* 2018; 118: 889-907.

44. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. *J Am Med Assoc.* 2009; 301: 513-521.

45. Johannesson M. The relationship between cost-effectiveness analysis and cost-benefit analysis. *Soc Sci Med.* 1995; 41: 483-489.

46. Williams SA, Daigle SG, Weiss R, Wang Y, Arora T, Curtis JR. Economic burden of osteoporosis-related fractures in the US medicasre population. *Ann Pharmacother.* 2021; 55: 821-829.

47. Dehanne F, Gourdin M, Devleesschauwer B *et al*. Cost-DALY comparison of hip replacement care in 12 Belgian hospitals. *BMJ Open Quality.* 2021; 10: e001263.

48. Dunnewind T, Dvortsin EP, Smeets HM *et al*. Economic consequences and potentially preventable costs related to osteoporosis in the Netherlands. *Value Health.* 2017; 20: 762-768.

49. Thomas T, Tubach F, Bizouard G *et al*. The economic burden of severe osteoporotic fractures in the French healthcare database: the FRACTOS study. *JBMR.* 2022; 37: 1811-1822.

50. Latosinska A, Siwy J, Mischak H, Frantzi M. Peptidomics and proteomics based on CE-MS as a robust tool in clinical application: the past, the present, and the future. *Electrophoresis.* 2019; 40: 2294-2308.

51. Kobashigawa J, Shah P, Joseph S *et al*. Frailty in heart transplatation: report from the heart workgroup of a consensus conference on frailty. *Am J Transplant.* 2021; 21: 636-644.

Data Supplement

Expanded methods

Bone mineral content

Bone mineral density (BMD) was measured by dual energy X-ray absorptiometry, both in the hip and in the lumbar vertebrae [1,2]. The T-score was calculated by comparing an individual's BMD measurements at the hip or spine with the peak mean BMD in a healthy, young-adult reference population and was expressed as the number of standard deviations from the mean BMD. For the hip, the T-score was calculated for the neck, trochanter and intertrochanter regions combined. For the lumbar vertebrae, the T-score was calculated for vertebrae L2-L4 combined. If measurements were missing in one of the vertebrae, the T-score was not calculated. The T-score was also not calculated in patients younger than 20 years, because normal values for this age group are unavailable. The osteoporotic threshold was a T-score of ≤-2.5 either in hip or in lumbar vertebrae [1,2].

Clinical and biochemical variables

Body mass index was body weight (kg) divided by height squared (m^2). Venous blood samples were drawn within 1 week of urine sampling after at least 8 h of fasting. The concentration of creatinine in serum was determined, using Jaffe's method with modifications and isotope-dilution mass spectrometry for calibration [3]. The glomerular filtration rate was estimated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [4]. Ionized calcium was calculated from serum total calcium and serum albumin, using the following formula: ionized calcium (mmol/l) = serum total calcium (mmol/l) + 0.02 × (40 - serum albumin (g/l)) [5]. Hypertension was an office blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic or use of antihypertensive drugs [6]. Diabetes mellitus was a hospital diagnosis, a fasting plasma glucose of 126 mg/dl or higher, or the use of antidiabetic agents [7].

Urinary proteomics

Sample preparation and CE-MS analysis

As described in detail elsewhere [8], urine aliquots were thawed and 700 µl mixed with 700 µL of 2 M urea, 10 mM NH_4OH containing 0.02 % SDS. Subsequently, samples were ultrafiltered using a Centristat 20 kDa cut-off centrifugal filter device (Satorius, Göttingen, Germany) to eliminate high molecular weight proteins. The obtained filtrate was desalted using a PD 10 gel filtration column (GE Healthcare Bio Sciences, Uppsala, Sweden) to remove urea, electrolytes and salts as well as to enrich the polypeptides.

The samples were lyophilized and stored at 4°C before usage. Shortly before CE-MS analysis, the samples were re-suspended in 10 μL HPLC-grade H_2O. Samples were injected into CE-MS with 2 psi for 99 sec, resulting in injection volumes of ~280 nL.

A P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) was coupled with a Micro-TOF MS (Bruker Daltronic, Bremen, Germany). A solution of 20% acetonitrile (Sigma-Aldrich, Taufkirchen, Germany) in HPLC-grade H_2O (Roth, Karlsruhe, Germany) supplemented with 0.94% formic acid (Sigma-Aldrich) was used as running buffer. For CE-MS analysis, the electrospray ionization interface from Agilent Technologies (Palo Alto, CA) was set to a potential of -4.0 to -4.5 kV. Spectra were recorded over an *m*/*z* range of 350-3000 and accumulated every 3 s.

CE-MS data processing

After the CE-MS analysis, mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaFinder software [9]. Only signals with z>1 observed in a minimum of 3 consecutive spectra with a signal-to-noise ratio of at least 4 were considered. The resulting peak list characterises each polypeptide by its mass and migration time. Data were calibrated utilizing 3151 internal standards as reference data points for mass and migration time by applying global and local linear regression, respectively as described previously. Reference signals of 29 abundant peptides were used as internal standards for calibration of signal intensity using local linear regression [10]. This procedure was shown to be an easy and reliable method to address both analytical and dilution variances and differences in glomerular filtration in a single calibration step. This procedure was shown to be highly reproducible and to address both analytical and dilution variances in a single calibration step [10]. The obtained peak list characterises each polypeptide by its calibrated molecular mass (Da), calibrated CE migration time (min) and normalised signal intensity. All detected peptides were deposited, matched, and annotated in a Microsoft SQL database allowing further statistical analysis.

Sequencing of peptides

Candidate biomarkers were sequenced using CE-MS/MS or LC-MS/MS analysis, as described in detail [11]. MS/MS experiments were using an Ultimate 3000 nano-flow system (Dionex/LC Packings, Thermo Fisher Scientific Inc., Waltham, MA) or a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA), both connected to an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) equipped with a nano-electrospray ion source. The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS

acquisition. Survey full-scan MS spectra (from *m*/*z* 300–2,000) were acquired in the Orbitrap. Ions were sequentially isolated for fragmentation. Data files were searched against the UniProt human nonredundant database, using Proteome Discoverer 2.4 and the SEQUEST search engine. Relevant settings were: no fixed modifications, oxidation of methionine and proline as variable modifications. The minimum precursor mass was set to 790 Da, maximum precursor mass to 6000 Da with a minimum peak count of 10. The high-confidence peptides were defined by cross-correlation (Xcorr) >1.9 and rank = 1. Precursor mass tolerance and fragment mass tolerance were 5 ppm and 0.05 Da, respectively. For further validation of obtained peptide sequences, the correlation between peptide charge at the working pH of 2 and CE-migration time was utilised to minimise incorrect sequence assignment [12]. Calculated CE-migration time of the sequence candidate based on its peptide sequence (number of basic amino acids) was compared to the experimental migration time.

References

1. Cosman F, de Beur SJ, LeBoff MS *et al*. Clinician's guide to prevention and treatment of osteoporosis. *Osteoporos Int.* 2014; 25: 2539-2381.
2. Lorentzon M, Cummings SR. Osteoporosis: the evolution of a diagnosis. *J Intern Med.* 2015; 277: 650-661.
3. Myers GL, Miller WG, Coresh J *et al*. Recommendations for improving serum creatinine measurement: a report from the laboratory working group of the National Kidney Disease Education Program. *Clin Chem.* 2006; 52: 5-18.
4. Levey AS, Stevens LA, Schmid CH *et al*. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009; 150: 604-612.
5. Karbhari DS, Karbhari NS, Patel S. Significance of measurement of corrected calcium inpatients with normoalbuminemia. *Int J Sci Public Health.* 2017; 6: 1069-1071.
6. Williams B, Mancia G, Spiering W *et al*. 2018 ESC/ESH guidelines for the management of arterial hypertension. *Eur Heart J.* 2018; 39: 3021-3104.
7. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabet Care.* 2018; 26: S5-S20.

8. Mischak H, Vlahou A, Ioannidis JPA. Technical aspects and inter-laboratory variability in native peptide profiling : the CE-MS experience. *Clin Biochem.* 2013; 46: 432-443.
9. Latosinska A, Siwy J, Mischak H, Frantzi M. Peptidomics and proteomics based on CE-MS as a robust tool in clinical application: the past, the present, and the future. *Electrophoresis.* 2019; 40: 2294-2308.
10. Jantos-Siwy J, Schiffer E, Brand K *et al*. Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res.* 2009; 8: 268-281.
11. Klein J, Papadopoulos T, Mischak H, Mullen W. Comparison of CE-MS/MS and LC-MS/MS sequencing demonstrates significant complementarity in natural peptide identification in human urine. *Electrophoresis.* 2014; 35: 1060-1064.
12. Zürbig P, Renfrow MB, Schiffer E *et al*. Biomarker discovery by CE-MS enables sequence analysis via MS/MS with platform-independent separation. *Electrophoresis.* 2006; 27: 2111-2125.

Table S1. Eighteen sequenced urinary peptides included in the multidimensional urinary classifier OSTEO18

Urinary peptide					Frequency		Mean amplitude		Fold change
ID	Symbol	Name of parental protein	Accession number	Amino-acid sequence	Control	Case	Control	Case	
e01155	COL1A1	Collagen alpha-1(I) chain	P02452	ApGDKGESGPS	0.93	0.90	2153	1350	0.65
e02344	COL3A1	Collagen alpha-1(III) chain	P02461	SpGPAGPRGPVGP	0.20	0.31	11	22	2.03
e02535	COL11A1	Collagen alpha-1(XI) chain	P12107	PpGQQGNpGpQG	0.89	0.61	770	457	0.59
e03544	S100A8	Protein S100-A8	P05109	KELDINTDGAVN	0.44	0.26	83	37	0.45
e04144	COL1A2	Collagen alpha-2(I) chain	P08123	DGPpGRDGQpGHK	0.47	0.23	33	21	0.63
e04281	COL1A1	Collagen alpha-1(I) chain	P02452	ApGDRGEPGPpGPAG	0.23	0.39	24	43	1.77
e04657	COL11A1	Collagen alpha-1(XI) chain	P12107	EPGppGQQGNpGPQ	0.89	0.66	377	248	0.66
e05380	COL4A4	Collagen alpha-4(IV) chain	P53420	PGDQGPPGpDGPRGAP	0.89	0.73	297	168	0.57
e05918	COL3A1	Collagen alpha-1(III) chain	P02461	GTGGPpGENGKpGEpGP	0.93	0.80	352	240	0.68
e06468	COL1A1	Collagen alpha-1(I) chain	P02452	ApGSKGDTGAKGEpGPVG	0.54	0.36	96	47	0.49
e07807	COL1A1	Collagen alpha-1(I) chain	P02452	GpPGpPGKNGDDGEAGKpG	0.96	0.79	1736	854	0.49
e11559	COL1A1	Collagen alpha-1(I) chain	P02452	GRTGDAGPVGPPGPpGppGpPGPPS	0.83	0.70	1159	619	0.53
e11865	COL1A1	Collagen alpha-1(I) chain	P02452	GADGQpGAKGEpGDAGAKGDAGPpGP	0.71	0.83	538	922	1.71
e13918	COL1A1	Collagen alpha-1(I) chain	P02452	AGpPGApGApGAPGpVGPAGKSGDRGETGP	0.60	0.74	171	499	2.91
e16278	COL9A3	Collagen alpha-3(IX) chain	Q14050	mPGFKGpTGYKGEQGEVGKDGEKGDpGpPG	0.73	0.49	387	195	0.50
e16942	COL9A3	Collagen alpha-3(IX) chain	Q14050	GELGEAGpSGEPGVPGDAGMPGERGEAGHRGSAGA	0.44	0.64	60	88	1.46
e17599	PIGR	Polymeric immunoglobulin receptor	P01833	EEKAVADTRDQADGSRASVDSGSSEEQGGSSRA	0.79	0.84	1301	2327	1.79
e18542	FGA	Fibrinogen alpha chain	P02671	DEAGsEADHEGTHSTKRGHAKSRPV	0.73	0.74	247	347	1.40

Fold change was the ratio of the amplitudes of each peptide in cases *vs* controls. Abbreviation: ID, identification number of urinary peptides.

Table S2. Patient characteristics in the derivation, replication and time-shifted case-control studies (starts)

Characteristic	Derivation dataset (*n* = 140)	Replication dataset (*n* = 118)	*p*-value	Time-shifted replication dataset (*n* = 134)	*p*-value
Time after HTx (years)	10.8 (4.78, 15.6)	5.10 (0.48, 10.3)	<0.0001	11.6 (5.27, 16.6)	0.27
Age (years)	60.8 (12.8)	58.0 (12.9)	0.087	61.5 (12.8)	0.62
BMI (kg/m^2)	25.4 (3.82)	24.8 (4.39)	0.30	26.2 (8.75)	0.27
SBP (mm Hg)	144.6 (20.7)	141.7 (22.2)	0.32	143.4 (20.3)	0.64
DBP (mm Hg)	86.1 (12.8)	86.0 (10.9)	0.96	85.0 (12.2)	0.48
Serum creatinine (µmol/L)	133.3 (42.8)	127.3 (41.7)	0.25	137.0 (44.3)	0.49
eGFR (mL/min/1.73m^2)	53.2 (20.3)	57.5 (21.8)	0.10	51.7 (20.6)	0.54
Serum albumin (g/L)	44.0 (3.22)	43.7 (3.39)	0.46	44.3 (3.03)	0.47
Serum total calcium (mmol/L)	2.40 (0.11)	2.40 (0.12)	0.93	2.40 (0.12)	0.92
Ionized calcium (mmol/L)	2.48 (0.15)	2.47 (0.17)	0.81	2.48 (0.16)	0.83
Sex					
Men, n (%)	112 (80.0)	94 (79.7)	0.95	109 (81.3)	0.78
Women, n (%)	28 (20.0)	20 (20.3)		25 (18.7)	
Indication for HTx					
Ischemic cardiomyopathy, n (%)	58 (41.4)	48 (40.7)	0.90	55 (41.0)	0.95
Dilated cardiomyopathy, n (%)	54 (38.6)	50 (42.4)	0.54	52 (38.8)	0.97
Other, n (%)	28 (20.0)	14 (11.9)	0.078	27 (20.1)	0.98

Table S2. Patient characteristics in the derivation, replication and time-shifted case-control studies (ends)

Characteristic	Derivation dataset (*n* = 140)	Replication dataset (*n* = 118)	*p*-value	Time-shifted replication dataset (*n* = 134)	*p*-value
Past smoker, n (%)	90 (64.3)	73 (62.4)	0.75	87 (64.9)	0.91
Hypertension, n (%)	131 (93.6)	105 (89.0)	0.19	125 (93.3)	0.92
Diabetes, n (%)	25 (17.9)	17 (14.4)	0.45	24 (17.9)	0.99
Osteoporotic fracture, n (%)	7 (5.0)	4 (3.4)	0.52	6 (4.5)	0.84
BMD T-score ≤-2.5, n (%)	23 (16.4)	20 (16.9)	0.91	22 (16.4)	>0.99
Anti-osteoporotic treatment, n (%)	57 (40.7)	48 (40.7)	0.99	55 (41.0)	0.96
Thiazides, n (%)	8 (7.8)	7 (10.4)	0.44	15 (11.2)	0.021
Immunosuppression					
Tacrolimus	94 (67.1)	63 (54.8)	0.15	86 (64.2)	0.61
Cyclosporine	41 (29.3)	17 (14.4)	0.0043	40 (29.9)	0.92
Everolimus	7 (5.0)	7 (7.5)	0.29	1 (0.7)	0.037
Mycophenolic acid	106 (75.7)	65 (59.1)	0.11	97 (72.4)	0.53
Azathioprine	12 (8.6)	4 (4.4)	0.22	0 (0)	0.0005
Methylprednisolone	48 (34.3)	25 (21.2)	0.020	0 (0)	<0.0001

Values are mean (SD), median (IQR) or number or patients (%). Abbreviations: HTx, heart transplantation; BMI, body mass index; SBP/DBP, systolic/diastolic blood pressure; eGFR, estimated glomerular filtration rate; BMD, bone mineral density. Anti-osteoporotic treatment refers to calcium supplementation, vitamin D supplements and/or bisphosphonate therapy. To convert serum creatinine from μmol/L to mg/dL, multiply by 0.0113. To convert serum albumin from g/L to g/dL, multiply by 0.1. To convert serum total and ionized calcium from mmol/L to mg/dL, multiply by 4. *p*-values refer to significance of the between-group differences with the derivation dataset as reference.

Table S3. Discriminatory performance of OSTEO18 in the derivation, replication and time-shifted case-control studies

Performance	Derivation dataset		Replication dataset		Time-shifted replication dataset	
	Performance	*p*-value	Performance	*p*-value	Performance	*p*-value
Number of cases *vs* controls	70 *vs* 70	…	59 *vs* 59	…	67 *vs* 67	…
OSTEO18 (score)	-0.027 (1.39)	…	0.053 (1.33)	…	-0.16 (1.25)	…
Controls (score)	-0.82 (1.09)	<0.0001	-0.45 (1.22)	<0.0001	-0.42 (1.25)	0.014
Cases (score)	0.76 (1.21)		0.55 (1.26)		0.10 (1.21)	
Youden index	0.61	…	0.32	…	0.27	…
Sensitivity (%)	74.3	…	69.5	…	73.1	…
Specificity (%)	87.1	…	62.7	…	53.7	…
AUC	0.83 (0.76, 0.90)	<0.0001	0.71 (0.62, 0.80)	<0.0001	0.62 (0.53, 0.72)	0.011

AUC indicates area under the receiver operating curve. OSTEO18 is presented as mean (SD) and sensitivity, specificity, AUC as point estimates (95% confidence interval).

Table S4. Baseline characteristics of 389 uPROPHET patients (starts)

Characteristic	Overall (*n* = 389)	Osteoporosis-free patients (*n* = 235)	Patients with osteoporosis (n = 154)	*p*-value
Time after HTx (years)	6.56 (1.79, 13.7)	5.52 (1.18, 12.5)	8.25 (3.49, 15.6)	0.0017
Age (years)	57.2 (14.2)	55.7 (14.7)	59.5 (13.3)	0.0092
BMI (kg/m^2)	25.3 (4.26)	25.5 (4.23)	25.0 (4.29)	0.19
SBP (mm Hg)	142.3 (20.7)	61.8 (25.3)	55.9 (23.0)	0.020
DBP (mm Hg)	85.6 (11.5)	141.4 (20.0)	143.6 (21.8)	0.30
Serum creatinine (µmol/L)	125.1 (44.2)	85.0 (11.4)	86.5 (11.6)	0.22
eGFR (mL/min/1.73m^2)	59.5 (24.6)	123.6 (45.5)	127.4 (42.1)	0.41
Serum albumin (g/L)	43.8 (3.26)	43.76 (3.29)	43.9 (3.21)	0.70
Serum total calcium (mmol/L)	2.39 (0.11)	2.38 (0.11)	2.40 (0.12)	0.086
Ionized calcium (mmol/L)	2.47 (0.16)	2.46 (0.16)	2.48 (0.16)	0.16
Sex				
Men, n (%)	294 (75.6)	183 (77.9)	111 (72.1)	0.19
Women, n (%)	95 (24.4)	82 (22.1)	43 (27.9)	
Indication for HTx				
Ischemic cardiomyopathy, n (%)	150 (38.6)	91 (38.7)	59 (38.3)	0.94
Dilated cardiomyopathy, n (%)	157 (40.4)	95 (40.4)	62 (40.3)	0.97
Other, n (%)	81 (20.8)	49 (20.9)	32 (20.8)	0.99

Table S4. Baseline characteristics of 389 uPROPHET patients (ends)

Characteristic	Overall (*n* = 389)	Osteoporosis-free patients (*n* = 235)	Patients with osteoporosis (n = 154)	*p*-value
Past smoker, n (%)	239 (61.9)	151 (64.8)	88 (57.5)	0.15
Hypertension, n (%)	81 (20.8)	205 (87.2)	143 (92.9)	0.077
Diabetes, n (%)	90 (23.1)	62 (26.4)	28 (18.2)	0.061
Osteoporotic fracture, n (%)	12 (7.8)	…	12 (7.8)	…
BMD T-score ≤-2.5, n (%)	56 (14.4)	…	56 (36.4)	…
Anti-osteoporotic treatment, n (%)	123 (31.6)	…	123 (79.9)	…
Thiazides, n (%)	42 (10.8)	31 (13.2)	11 (7.1)	0.060
Immunosuppression	365 (93.8)	226 (96.2)	139 (90.3)	0.018
Tacrolimus, n (%)	279 (71.7)	189 (80.4)	90 (58.4)	<0.0001
Cyclosporine, n (%)	76 (19.5)	31 (13.2)	45 (29.2)	0.0001
Everolimus, n (%)	21 (5.4)	11 (4.7)	10 (6.5)	0.44
Mycophenolic acid, n (%)	281 (72.2)	183 (77.9)	98 (63.6)	0.0022
Azathioprine, n (%)	26 (6.7)	15 (6.4)	11 (7.1)	0.77
Methylprednisolone, n (%)	134 (34.5)	73 (31.1)	61 (39.6)	0.083

Values are mean (SD), median (IQR) or number or patients (%). Abbreviations: HTx, heart transplantation; BMI, body mass index; SBP/DBP, systolic/diastolic blood pressure; eGFR, estimated glomerular filtration rate; BMD, bone mineral density. Anti-osteoporotic treatment refers to calcium supplementation, vitamin D supplements and/or bisphosphonate therapy. To convert eGFR from mL/min to mL/s per 1.73m^2, multiply by 0.0167. To convert serum creatinine from µmol/L to mg/dL, multiply by 0.0113. To convert serum albumin from g/L to g/dL, multiply by 0.1. To convert serum total calcium and ionized calcium from mmol/L to mg/dL, multiply by 4, respectively. *p*-values refer to the difference between patients with/without osteoporosis. An ellipsis indicates that the variable was not measured or the *p*-value was not calculated.

Table S5. Discriminatory performance associated with single risk factors for osteoporosis in 389 uPROPHET patients

Variables	OR (95% CI)	*p*-value	AUC (95% CI)	*p*-value
Female sex (women *vs* men)	1.36 (0.85, 2.18)	0.19	…	…
Age, +10 years	1.22 (1.05, 1.41)	0.010	0.57 (0.51, 0.62)	0.027
eGFR, +15 mL/min/1.73m^2	0.86 (0.76, 0.98)	0.021	0.57 (0.51, 0.63)	0.019
Diabetes (1 *vs* 0)	0.62 (0.38, 1.02)	0.062	…	…
Methylprednisolone (1 *vs* 0)	1.45 (0.95, 2.21)	0.085	…	…
Interval since HTx, +5 years	1.25 (1.08, 1.45)	0.0027	0.59 (0.54, 0.65)	0.0013
Ionized calcium, +0.5 mmol/L	1.66 (0.86, 3.19)	0.13	…	…
Thiazide diuretics (1 *vs* 0)	0.51 (0.25, 1.04)	0.064	…	…
Tacrolimus (1 *vs* 0)	0.34 (0.22, 0.54)	<0.0001	…	…
Cyclosporine (1 *vs* 0)	2.72 (1.63, 4.54)	0.0001	…	…
OSTEO18 score, +1	2.07 (1.71, 2.50)	<0.0001	0.74 (0.69, 0.79)	<0.0001

Abbreviations: OR, odds ratio; CI, confidence interval; AUC, area under curve; eGFR, estimated glomerular filtration rate; HTx, heart transplantation. The ORs and AUCs are presented as point estimates with 95% confidence interval. The AUC was not computed for non-significant ORs and for 2-level categorised variables.

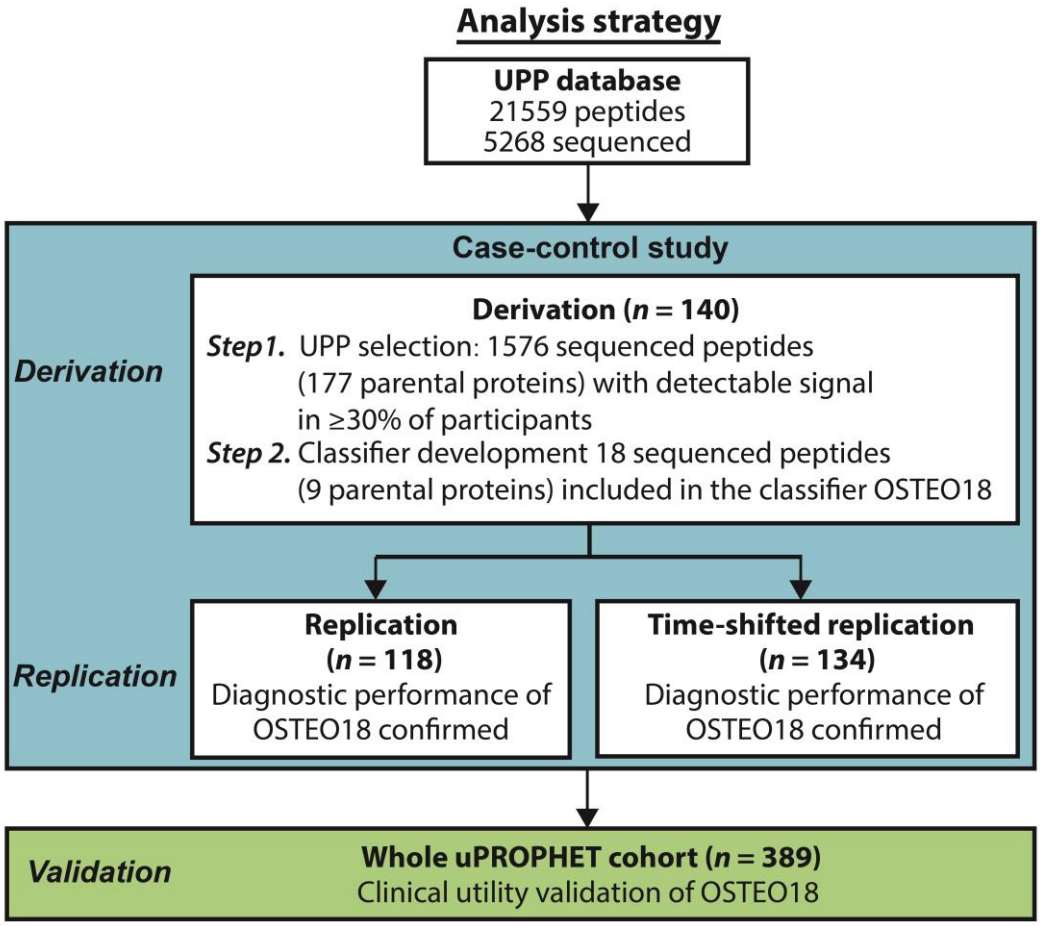


Figure S1. Analysis strategy.

UPP indicates urinary proteomic profiling. *n* denotes the number of patients in each dataset.

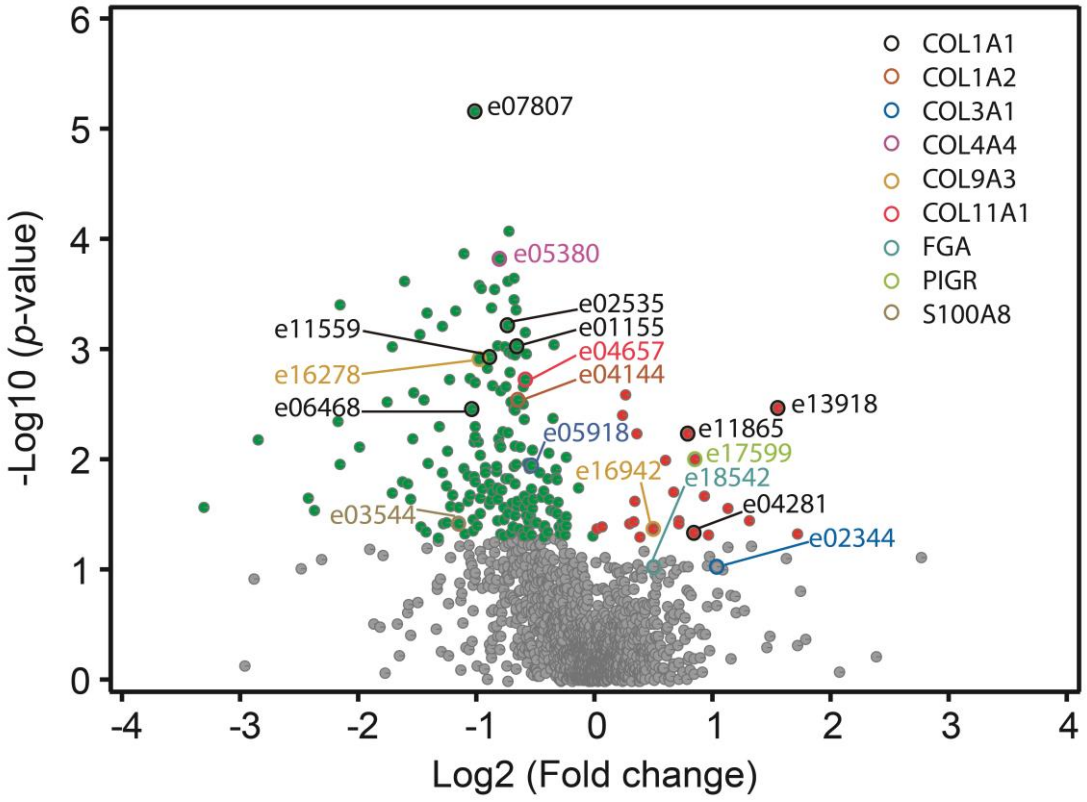


Figure S2. Volcano plot depicting the distribution of the 1576 urinary peptides detected in ≥30% of patients in the derivation case-control study. Fold change is the ratio of the amplitudes of each peptide in cases *vs* controls. The vertical axis denotes the significance of the difference in the urinary peptide levels between cases and controls. The red, green and gray dots respectively indicate the urinary peptides with significantly higher urinary levels in osteoporosis cases, significantly higher levels in controls, and similar levels in cases and controls. Colored circles and the identification numbers denote the 18 urinary peptides included in the classifier OSTEO18 (Table S1). The colors of the circles identify fragments derived from collagen alpha-1(I) chain (COL1A1, black), collagen alpha-2(I) chain (COL1A2, brown), collagen alpha-1(III) chain (COL3A1, blue), collagen alpha-4(IV) chain (COL4A4, purple), collagen alpha-3(IX) chain (COL9A3, orange), collagen alpha-1(XI) chain (COL11A1, red), fibrinogen alpha chain (FGA, dark green), polymeric immunoglobulin receptor (PIGR, light green), and protein S100-A8 (S100A8, beige).

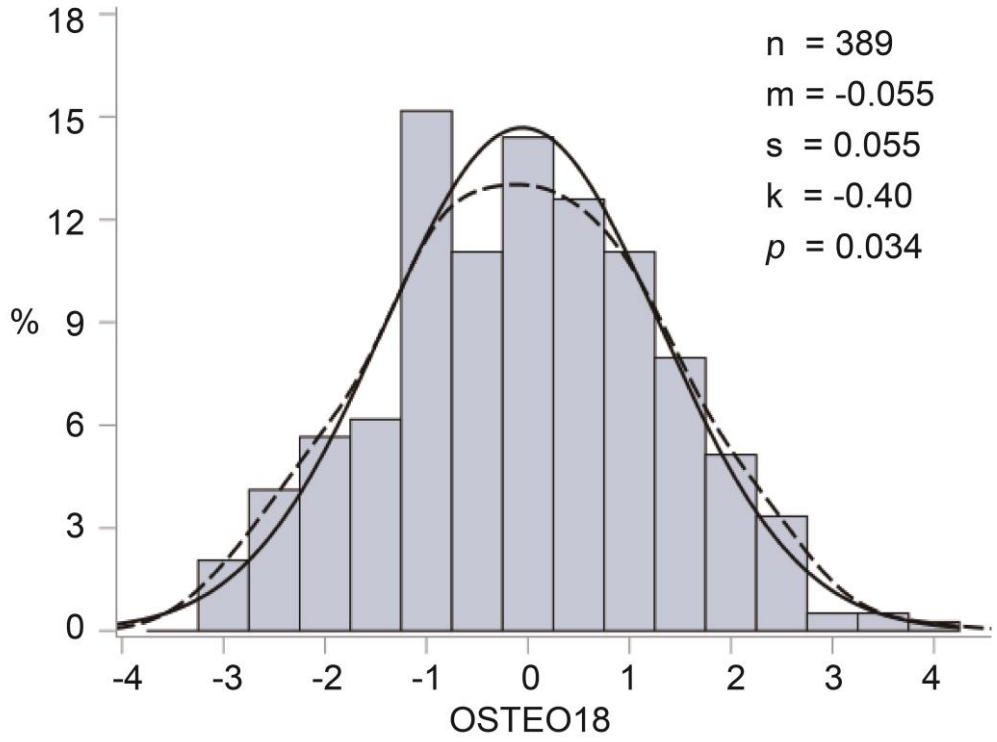


Figure S3. Distribution of OSTEO18 in 389 uPROPHET patients. n, m, s and k indicate the number of patients, the arithmetic mean and the coefficients of skewness and kurtosis. The solid and dotted lines represent the normal and kernel density distributions. The *p*-value is for departure of the actually observed distribution from normality according to the Kolmogorov-Smirnov test.

Chapter 3

Unhealthy ageing in relation to urinary proteomic profiling

Part 3

Urinary proteomic signature of mineralocorticoid receptor antagonism by spironolactone: evidence from the HOMAGE trial

Yu-Ling Yu, Justyna Siwy, De Wei An, Arantxa González, Tine Hansen, Agnieszka Latosinska, Pierpaolo Pellicori, Susana Ravassa, Beatrice Mariottoni, Job A.J. Verdonschot, Fozia Ahmed, Johannes Petutschnigg, Patrick Rossignol, Stephane Heymans, Joe J. Cuthbert, Nicolas Girerd , Andrew L. Clark, Peter Verhamme, Tim S. Nawrot, Stefan Janssens, John G. Cleland, Faiez Zannad, Javier Díez, Harald Mischak, João Pedro Ferreira†, Jan A. Staessen†, on behalf of the HOMAGE investigators‡

† Joint-last authors.

‡ The HOMAGE Investigators are listed in the supplement.

Previously published in:

Heart. 2024. 110: 1180-1187.

Abstract

Objective: Heart failure (HF) is characterised by collagen deposition. Urinary proteomic profiling (UPP) followed by peptide sequencing identifies parental proteins, for over 70% derived from collagens. This study aimed to refine understanding of the antifibrotic action of spironolactone.

Methods: In this substudy (n=290) to the HOMAGE trial, patients were randomised to usual therapy combined or not with spironolactone 25-50 mg/d and followed for 9 months. The analysis included 1498 sequenced urinary peptides detectable in ≥30% of patients and PICP and PICP/CITP as serum biomarkers of COL1A1 synthesis. After rank normalisation of biomarker distributions, between-group differences in their changes were assessed by multivariable-adjusted mixed model ANOVA. Correlations between the changes in urinary peptides and in serum PICP and PICP/CITP were compared between groups using Fisher Z transform.

Results: Multivariable-adjusted between-group differences in the urinary peptides with error 1 rate correction were limited to 27 collagen fragments, of which 16 were upregulated (7 COL1A1 fragments) on spironolactone and 11 downregulated (4 COL1A1 fragments). Over 9 months of follow-up, spironolactone decreased serum PICP from 81 (IQR: 66-95) to 75 (61-90) μg/L and PICP/CITP from 22 (17-28) to 18 (13-26), whereas no changes occurred in the control group, resulting in a difference (spironolactone minus control) expressed in standardised units of -0.321 (95% confidence interval: 0.0007). Spironolactone did not affect the correlations between changes in urinary COL1A1 fragments and in PICP or the PICP/CITP ratio.

Conclusions: Spironolactone decreased serum markers of collagen synthesis and predominantly downregulated urinary collagen-derived peptides, but upregulated others. The interpretation of these opposite UPP trends might be due to shrinking the body-wide pool of collagens, explaining downregulation, while some degree of collagen synthesis must be maintained to sustain vital organ functions, explaining upregulation. Combining urinary and serum fibrosis markers opens new avenues for the understanding of the action of antifibrotic drugs.

Introduction

Fibrosis is the common pathological response to inflammation and chronic tissue injury, such as occur with ageing, hypertension, diabetes mellitus, or ischaemia. Activation of the mineralocorticoid receptor (MR) initiates a cascade of molecular events leading to cell growth and inappropriate expansion and disorganisation of the extracellular matrix (ECM) in the myocardium [1], which is a hallmark of heart failure (HF), irrespective of its cause [2]. The Heart "Omics" in Ageing Study (HOMAGE) was an open-label randomised clinical trial in 527 patients at high risk of developing HF [3]. Patients were randomised to spironolactone 25-50 mg/d on top of usual therapy or usual therapy alone and were followed up for 9 months [3]. Spironolactone caused a fall in serum carboxyterminal propeptide of procollagen I (PICP), a rise in serum carboxyterminal telopeptide of collagen I (CITP) and a fall in the PICP/CITP ratio. PICP and CITP are circulating biomarkers of COL1A1 synthesis and degradation, respectively (**Figure S1**) [3, 4].

Urine contains >20,000 endogenous peptides, of which many have been sequenced, thereby identifying the parental proteins [5]. The urinary proteomic profile (UPP) consists for over 70% of collagen fragments [6]. In a subgroup of patients randomised in the HOMAGE trial, we analysed the between-group differences in the UPP and the associations of urinary with serum fibrosis markers.

Methods

Study participants

HOMAGE was a multicentre open-label trial with blinded endpoint evaluation (registration number: NCT02556450) [3]. Each centre had its own recruitment strategies. The protocol was approved by the Greater Manchester Central Research Ethics Committee (reference: 16/NW/0012; EudraCT number: 2015-000413-48) as well as by each centre's local Ethics Committee (Data Supplement). Patients of either sex, aged ≥60 years, were eligible provided that they were at increased risk of developing HF, because they already had or were likely to develop coronary heart disease. Additionally, eligible patients had to have a serum NT-proBNP of 125-1000 ng/L or a serum BNP of 35-280 ng/L. These ranges excluded patients at low HF risk as well as those with advanced disease requiring further investigation and intensive treatment. Of 877 screened patients, 527 were randomised to spironolactone 25-50 mg per day (n=265) on top of usual treatment or usual treatment alone (n=262). The UPP was analysed in urine samples at baseline and at months 1 and 9, if sequenced peptides had a detectable signal in ≥30% of patients. A flow chart (**Figure S2**) shows the derivation of the HOMAGE trial subgroup dataset currently analysed.

Urinary and circulating biomarkers

Mosaiques-Diagnostics GmbH, Hannover, Germany (MOS) did the UPP profiling for all patients. The methods for sample preparation, capillary electrophoresis coupled with mass spectrometry (CE-MS), peptide sequencing, and for the evaluation, calibration and quality control of the mass spectrometric data have been published [7] and are described in detail in the Data Supplement. In the CE-MS step, 29 abundant endogenous urinary peptides were run along with the samples as internal standards for calibration of the signal intensity. This procedure is highly reproducible and addresses in a single calibration step both analytical and dilution variances, such as the variability in renal function [8]. A total of 1498 sequenced urinary peptides with a detectable signal in ≥30% of participants were analysed. Undetectable peptides were set at the distribution minimum [9]. Glomerular filtration rate was estimated (eGFR) from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [10]. Using methods described in the Data Supplement, serum was analysed for PICP, a marker of COL1A1 synthesis and CITP a marker of COL1A1 degradation (**Figure S1**) [11]. All intra-assay coefficients of variations were <10% [3].

Statistical analysis

For database management and statistical analysis, SAS software, version 9.4, maintenance level 5, was used (SAS Institute Inc., Cary, NC). Deviation from the normal distribution was assessed by the Shapiro-Wilk statistic. The distributions of the serum biomarkers (**Figure S3**) and urinary peptides (**Figure S4**) were rank normalised, by sorting measurements from the smallest to the highest value and then applying the inverse cumulative normal function [12]. Rank normalised variables have mean 0 and SD 1. For non-transformed data, the central tendency (spread) of the data is given as the arithmetic mean (SD) and for rank normalised variables as median (IQR). In unadjusted analyses, means were compared using the large-sample Z-test and proportions by the χ^2 statistic or the Fisher exact test, as appropriate based on cell frequencies. Statistical tests were two-sided.

The within-group changes (time point in the trial) and between-group differences (randomisation group) in the urinary peptides and serum biomarkers were tested, using mixed model ANOVA with the patient modelled as random effect as implement in the PROC GLM procedure in the SAS package. The general linear models were adjusted for the baseline levels of the urinary or serum biomarkers if follow-up data were tested and additionally for sex, age, body mass index (BMI), eGFR, smoking and drinking, history of ischaemic heart disease, and treatment at baseline and subsequent treatment changes

during follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. The antihypertensive drugs applied for adjustment were diuretics (thiazides, thiazide-like agents, and loop diuretics), β-blockers, vasodilators (calcium-channel blockers and α-blockers), and inhibitors of the renin-angiotensin system (angiotensin-converting enzyme inhibitors and angiotensin-II receptor blockers). Lipid-lowering drugs included statins, fibrates and ezetimibe, and antiplatelet drugs aspirin and adenosine diphosphate receptor inhibitors. The antidiabetic drugs coded included insulin, metformin, sulfonylurea, dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 receptor antagonists, thiazolidinediones, and sodium-glucose cotransporter 2 inhibitors.

Urinary peptides with different levels at last follow-up between control and spironolactone-treated patients with a 2-sided significance of 0.01 were selected, and only those keeping Benjamini-Hochberg-adjusted significance [13] of <0.05 were carried through to further analyses. Correlation coefficients of the changes from baseline to follow-up in the urinary peptides regressed on the serum biomarkers were compared between treatment groups (control vs spironolactone), using Fisher Z-transformation [14]. Between-group comparisons of the slopes of these associations were tested by linear regression models, including covariables, randomisation group, the changes in the serum biomarker, and the interaction term between randomisation group and the changes in the serum biomarker [15].

Results

Patient characteristics

Descriptive data for the 290 patients in HOMAGE are shown in **Table 1**. The HOMAGE patients were intensively treated with antihypertensive agents (n=280, 96.6%) and lipid-lowering drugs (n=257, 88.6%), mainly statins (n=251, 86.6%), antiplatelet drugs (n=218, 75.2%), and antidiabetic agents (n=105, 36.2%), insulin in 10 (3.4%) cases. None of the patients randomised in the HOMAGE trial and included in the current substudy was on treatment with sodium-glucose co-transporter-2 inhibitors. The analytical dataset of HOMAGE patients, randomised to control (n=144) or spironolactone (n=146), was well balanced with regard to risk factors, clinical characteristics, routine biochemistry, and serum PICP, CITP and the PICP/CITP ratio (**Table 1**). Compared with the 215 HOMAGE patients not included in the present analyses, the 290 patients analysed had a higher risk profile (**Table S1**).

Table 1. Baseline characteristics of patients

Characteristic	Control	Spironolactone
Number in group (n [%])	144	146
Women	41 (28.5)	28 (19.2)
Past smoking	77 (53.5)	94 (64.4)
Current smoking	11 (7.6)	8 (5.5)
Drinking alcohol	35 (24.3)	34 (23.3)
Hypertension	106 (73.6)	111 (76.0)
Diabetes	33 (22.9)	31 (21.2)
Antihypertensive treatment	140 (97.2)	140 (95.9)
Lipid-lowering treatment	125 (86.8)	132 (90.4)
Antidiabetic agents	54 (37.5)	51 (34.9)
Antiplatelet drugs	106 (73.6)	112 (76.7)
History of ischaemic heart disease	116 (80.6)	119 (81.5)
Clinical characteristics		
Age (y)	73.7±5.9	73.8±6.1
Body mass index (kg/m^2)	28.2±4.7	29.3±5.5
Systolic blood pressure (mm Hg)	139.4±19.0	137.2±17.5
Diastolic blood pressure (mm Hg)	78.1±10.7	77.2±10.0
Heart rate (bpm)	60.9±8.6	62.0±8.8
Biochemistry		
Serum sodium (mmol/L)	139.6±2.7	139.2±3.0
Serum potassium (mmol/L)	4.35±0.37	4.37±0.37
eGFR (mL/min/1.73 m^2)	67.4±13.8	66.7±16.7
Total cholesterol (mg/dL)	151.9±44.4	147.7±40.3
HbA1C (%)	6.20±1.40	6.03±1.21
Markers of collagen turnover		
Serum PICP (μg/L)	81.8 (69.5-94.8)	77.9 (64.2-93.6)
Serum CITP (μg/L)	3.73 (3.15-4.89)	3.78 (3.05-4.89)
PICP/CITP ratio	20.8 (15.5-29.3)	21.9 (16.6-28.1)

Data are presented as arithmetic mean ± standard deviation, *n (%)*, or median (IQR) for variables deviating from the normal distribution. Abbreviations: eGFR, glomerular filtration rate estimated from serum creatinine according to the Chronic Kidney Disease Epidemiology equation; HbA1C, glycated haemoglobin; PICP, procollagen type I C-terminal propeptide; CITP, collagen type I C-terminal telopeptide.

Urinary peptides

The 1498 sequenced urinary peptides retained in the analysis were derived from 212 proteins and included 1109 (74.0%) collagen fragments and 389 (26.0%) peptides derived from other proteins. Peptide fragments were excluded from analysis if they were derived from albumin, β_2-microglobulin and the fibrinogen α-chain (given their high concentration in blood); uromodulin because of its renal origin; and osteopontin as prominent component of mineralised extracellular matrices.

At baseline, the levels of the selected urinary peptides were similar in both randomisation groups (**Table S2**). Given the balanced baseline characteristics of patients randomised to control or spironolactone, the between-group differences in the urinary and serum biomarkers and associated significance levels were not materially affected by adjustment. **Table 2** shows the 27 peptides that differed at follow-up between spironolactone and control. All peptide fragments were derived from collagen. With correction of significance for multiple testing, 11 peptides were derived from COL1A1, four from COL3A1, two from COL1A2, and one peptide from each of 10 other collagens (**Table 2**). **Table S3** lists the amino-acid sequence of the peptides retained in analyses and the protein from which they were derived.

With control patients as reference, the effect of spironolactone on urinary peptides is shown in **Table 3**. Of the 11 peptides derived from COL1A1, seven had higher and four lower levels on spironolactone. Of four peptides derived from COL3A1, three had lower levels on spironolactone and one a higher level. Of the 2 peptides derived from COL1A2, spironolactone decreased both. Of the 27 peptide fragments, spironolactone reduced 16, while the remaining 11 had higher urinary levels on spironolactone compared to spironolactone.

Serum biomarkers

In unadjusted analyses (**Figure 1**), spironolactone shifted the whole distribution of serum PICP and the serum PICP/CITP ratio downwards. Fully adjusted analyses (**Figure S5**), in which the 9-month data were plotted against baseline values confirmed this downward shift. Analyses adjusted for sex, age, BMI, eGFR, smoking and drinking, history of ischaemic heart disease, the baseline value of the biomarker, and treatment at baseline and subsequent treatment changes during follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs (**Table 4**) revealed no between-group differences in CITP at months 1 and 9 (**Table 4**). However, at months 1 and 9, serum PICP and the serum PICP/CITP ratio were lower on spironolactone than control.

Table 2. Urinary peptides with different levels on spironolactone versus control retained in the analyses (starts)

Urinary peptides			*r*	p	p_{BH}
ID	**Symbol**	**Parental protein**			
e04960	COL1A1	collagen alpha-1 (I) chain	0.93	0.00425	0.01392
e08916	COL1A1	collagen alpha-1 (I) chain	1.26	0.00052	0.00010
e09408	COL1A1	collagen alpha-1 (I) chain	1.34	0.00007	0.00252
e10266	COL1A1	collagen alpha-1 (I) chain	1.28	0.00234	0.00649
e10395	COL1A1	collagen alpha-1 (I) chain	1.08	0.00669	0.04818
e10437	COL1A1	collagen alpha-1 (I) chain	1.42	0.00406	0.01217
e10657	COL1A1	collagen alpha-1 (I) chain	1.52	0.00183	0.00470
e10863	COL1A1	collagen alpha-1 (I) chain	1.13	0.00533	0.02132
e11972	COL1A1	collagen alpha-1 (I) chain	0.81	0.00664	0.03985
e17425	COL1A1	collagen alpha-1 (I) chain	0.81	0.00114	0.00273
e18740	COL1A1	collagen alpha-1 (I) chain	0.92	0.00052	0.00111
e10563	COL2A1	collagen alpha-1 (II) chain	1.49	0.01430	0.01716
e03506	COL3A1	collagen alpha-1 (III) chain	0.77	0.00617	0.00723
e05700	COL3A1	collagen alpha-1 (III) chain	0.90	0.00546	0.02457
e07668	COL3A1	collagen alpha-1 (III) chain	2.79	0.00032	0.00033
e18839	COL3A1	collagen alpha-1 (III) chain	0.55	0.00109	0.00245
e05473	COL4A1	collagen alpha-1 (IV) chain	0.90	0.014689	0.01823

Table 2. Urinary peptides with different levels on spironolactone versus control retained in the analyses (continued)

Urinary peptides			*r*	p	p_{BH}
ID	**Symbol**	**Parental protein**			
e17131	COL6A1	collagen alpha-1 (VI) chain	2.03	0.00080	0.00085
e01100	COL7A1	collagen alpha-1 (VII) chain	0.96	0.01862	0.02578
e02933	COL1A2	collagen alpha-2 (I) chain	0.86	0.00346	0.00389
e04987	COL1A2	collagen alpha2 (I) chain	0.74	0.00444	0.01599
e16610	COL4A2	collagen alpha-2 (IV) chain	0.93	0.01586	0.02039
e09267	COL5A2	collagen alpha-2 (V) chain	0.95	0.02508	0.03311
e20509	COL11A2	collagen alpha-2 (XI) chain	0.91	0.01593	0.02124
e15360	COL4A3	collagen alpha-3 (IV) chain	1.43	0.00511	0.00593
e16874	COL5A3	collagen alpha-3 (V) chain	0.90	0.03106	0.04659
e06373	COL4A6	collagen alpha-6 (IV) chain	0.87	0.00323	0.00352

ID is the laboratory identification number of the peptide fragment. Using untransformed data, for each individual and for each peptide in the control and spironolactone group the ratio of the 9-month-to-baseline ratio was computed. From the resulting distributions, the medians were computed. *r* is the ratio of the medians of these distributions (spironolactone/control). A ratio greater than unity indicates an increase in the level of the urinary peptide on spironolactone and *vice versa*. p and p_{BH} refer to the significance of the difference between control and spironolactone, respectively, without and with correction of the significance for multiple testing. Models were adjusted for sex, age, BMI, eGFR, the baseline value of the urinary marker, current smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. An ellipsis indicates that p_{BH} was not computed, because p was not significant.

Table 3. Between-group differences in the urinary peptide levels at last follow-up in HOMAGE trial (starts)

ID	Symbol	Control (n=144)	Spironolactone (n=146)	Δ (95% CI)
e04960	COL1A1	3.9 (3.7-45.1)	3.9 (3.9-27.9)	-0.264 (-0.443, -0.085)†
e08916	COL1A1	1946 (1432-2727)	2695 (1761-3011)	0.391 (0.173, 0.609)‡
e09408	COL1A1	685 (399-1168)	874 (627-1326)	0.461 (0.237, 0.684)†
e10266	COL1A1	14,619 (9745-19,329)	17,047 (12,212-22,490)	0.352 (0.128, 0.577)†
e10395	COL1A1	5997 (4727-7358)	6585 (5204-8017)	0.322 (0.091, 0.553)†
e10437	COL1A1	359 (151-554)	459 (230-757)	0.341 (0.110, 0.571)†
e10657	COL1A1	542 (207-1046)	784 (344-1369)	0.368 (0.139, 0.597)†
e10863	COL1A1	2869 (2004-3984)	3301 (2606-4404)	0.299 (0.090, 0.507)†
e11972	COL1A1	3216 (1617-5187)	2724 (1469-4216)	-0.275 (-0.473, -0.078)†
e17425	COL1A1	58.4 (11.6-145)	19.7 (11.6-106)	-0.322 (-0.514, -0.130)†
e18740	COL1A1	6.6 (2.1-60.9)	2.1 (2.1-31.5)	-0.350 (-0.546, -0.155)‡
e10563	COL2A1	55.3 (3.4-215)	132.5 (3.4-365)	0.292 (0.060, 0.523)*
e03506	COL3A1	234 (55-415)	179.3 (16.4-332)	-0.316 (-0.540, -0.092)‡
e05700	COL3A1	25.8 (18.3-321)	18.8 (18.7-231)	-0.268 (-0.456, -0.081)†
e07668	COL3A1	3.1 (3.0-158)	109 (3.1-292)	0.387 (0.179, 0.595)†
e18839	COL3A1	185 (28.9-454)	113 (3.5-392)	-0.334 (-0.532, -0.136)†
e05473	COL4A1	5.8 (5.7-435)	5.8 (5.7-21.4)	-0.245 (-0.441, -0.050)*
e17131	COL6A1	150 (44-287)	211 (90.9-400)	0.381 (0.161, 0.601)*

Table 3. Between-group differences in the urinary peptide levels at last follow-up in HOMAGE trial (continued)

ID	Symbol	Control (n=144)	Spironolactone (n=146)	Δ (95% CI)
e01100	COL7A1	91.1 (2.5-308)	29.1 (2.5-168)	-0.234 (-0.428, -0.040)*
e02933	COL1A2	481 (333-620)	398 (261-549)	-0.341 (-0.567, -0.114)†
e04987	COL1A2	59.1 (6.1-281)	6.1 (6.1-161)	-0.279 (-0.470, -0.088)†
e16610	COL4A2	5.8 (5.7-31.1)	5.8 (5.7-5.8)	-0.208 (-0.375, -0.040)*
e09267	COL5A2	4.1 (4.0-14.0)	4.1 (4.1-6.8)	-0.211 (-0.394, -0.027)*
e20509	COL11A2	953 (481-2149)	822 (248-1741)	-0.270 (-0.488, -0.052)*
e15360	COL4A3	379 (186-589)	464 (193-779)	0.319 (0.098, 0.541)*
e16874	COL5A3	4.9 (4.9-17.3)	4.9 (4.8-4.9)	-0.200 (-0.380, -0.019)*
e06373	COL4A6	257 (94.7-433)	216 (75.2-371)	-0.278 (-0.462, -0.945)*

ID is the laboratory identification number of the sequenced peptide. Values given for the sequenced peptides are medians of the non-normalised levels (interquartile range). Δ (95% CI) refers to the between-group difference (spironolactone minus control) with 95% confidence interval. Δ (95% CI) provides multivariable-adjusted estimates of the between-group differences in standardised units, as required by the general linear model. Models were adjusted for sex, age, BMI, eGFR, the baseline value of the urinary marker, current smoking and drinking, history of ischaemic heart disease and treatment at baseline and change in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. Significance of the between-group differences was adjusted for multiple comparisons: * $p \leq 0.05$; † $p \leq 0.01$; ‡ $p \leq 0.001$ (see Table 2 for p-values).

Table 4. Serum fibrosis biomarkers at baseline and during follow-up in HOMAGE trial

Circulating biomarker	Control (n=144)	Spironolactone (n=146)	Δ (95% CI)	p
PICP, μg/L				
Baseline	80 (67-96)	81 (66-95)	-0.043 (-0.274, 0.188)	0.72
Month 1	81 (69-97)	73 (60.1-92)	-0.253 (-0.413, -0.093)	0.0025
Month 9	79 (66-100)	75 (61-90)	-0.321 (-0.501, -0.142)	0.0007
CITP, μg/L				
Baseline	3.72 (2.89-5.09)	3.74 (2.90-4.67)	-0.058 (-0.283, 0.168)	0.62
Month 1	3.80 (3.10-5.08)	3.93 (3.15-5.17)	0.105 (-0.048,0.257)	0.18
Month 9	3.82 (3.04-4.99)	4.00 (3.06-5.42)	0.079 (-0.101, 0.259)	0.40
PICP/CITP				
Baseline	21 (16-29)	22 (17-28)	0.001 (-0.225, 0.228)	0.99
Month 1	22 (15-28)	18 (14-25)	-0.240 (-0.406, -0.074)	0.0056
Month 9	21 (16-29)	18 (13-26)	-0.256 (-0.451, -0.061)	0.013

Values given for the circulating biomarkers are medians of the non-normalised non-adjusted levels (interquartile range). Δ (95% CI) refers to the between-group difference (spironolactone minus control) with 95% confidence interval. Δ (95% CI) provides multivariable-adjusted estimates of the between-group differences in standardised units, as required by the general linear model. *p* denotes the significance of the between-group differences. All models were adjusted for sex, age, BMI, eGFR, smoking and drinking, history of ischaemic heart disease, and treatment at baseline antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. The 1- and 9-month models were additionally adjusted for the baseline value of the biomarker and the changes in the treatment status with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. Abbreviations: PICP, procollagen type I C-terminal propeptide; CITP, collagen type I C-terminal telopeptide; PICP/CITP, ratio of PICP to CITP.

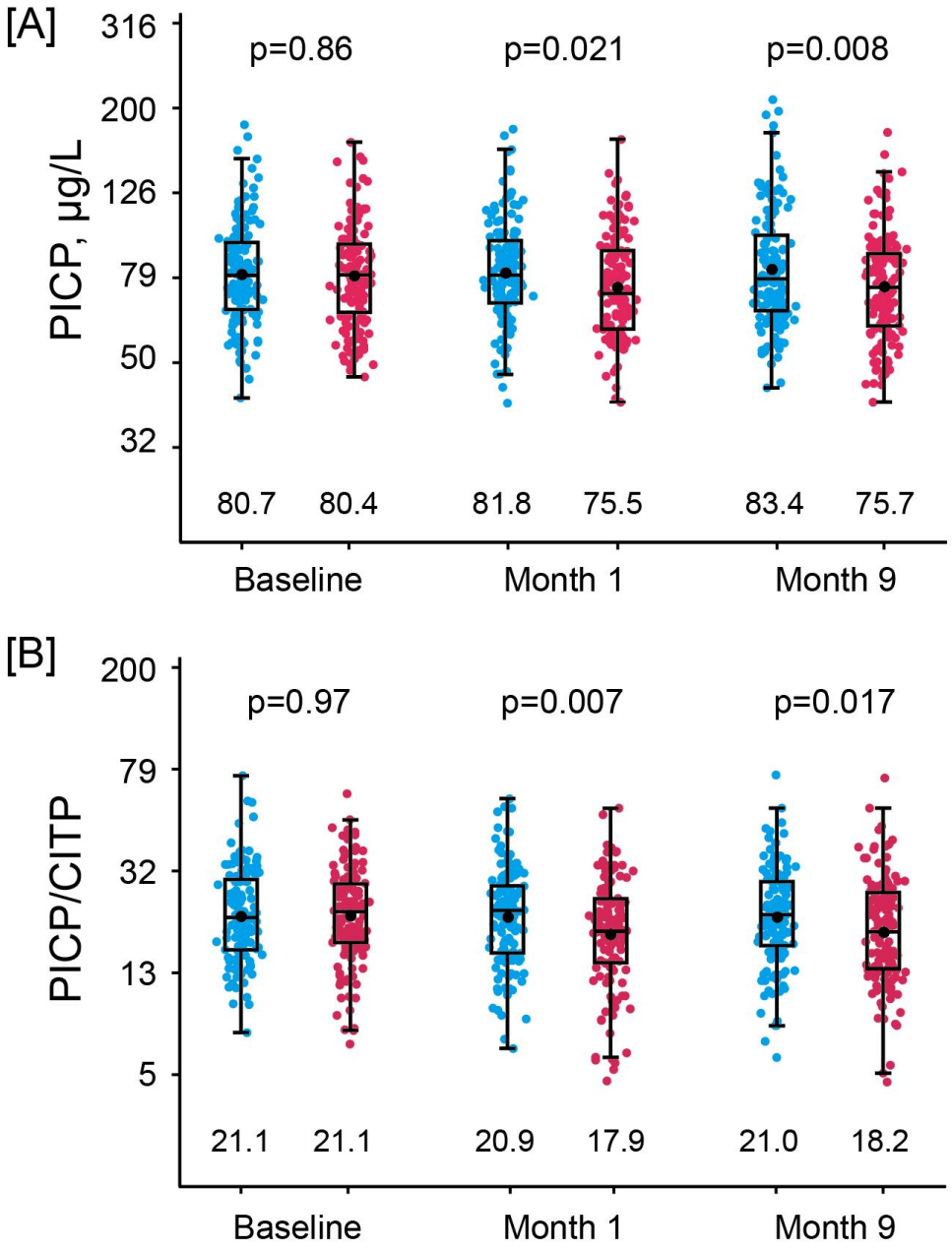


Figure 1. Distribution of serum PICP (A) and the serum PICP/CITP ratio (B) at baseline and during follow-up by treatment group. PICP (carboxyterminal propeptide of procollagen I) is a marker of COL1A1 synthesis and CITP (carboxyterminal telopeptide of collagen I) of COL1A1 (see **Figure S1**). For each box plot, the central line, the upper and lower lines, and the upper and lower caps represent the median, interquartile range, and the 5th to 95th percentile interval. Dots represent individual levels with extremes falling outside the box and whiskers. The geometric means are given along the horizontal axes. p-values are for the between-group differences at each time point.

In fully adjusted analyses (**Figure 2** and **Table S4**), the correlations between the changes from baseline to follow-up in the urinary peptides and the corresponding changes in CITP, the biomarker reflecting degradation of mature COL1A1, were similar in patients randomised to control and spironolactone. None of the correlations with CITP was significant if the COL1A1 fragments decreased from baseline to follow-up (n=4), whereas the correlations with CITP were significant if the COL1A1 fragments increased during follow-up (n=7). Compared with the patients in control group (**Table S5**), serum sodium decreased by 0.90 mmol/L (95% confidence interval: 0.44 to 1.36 mmol/L), whereas serum potassium increased by 0.14 mmol/L (0.06 to 0.22 mmol/L) in the spironolactone group at the last follow-up visit. Moreover, compared to the control, eGFR (**Table S5**) decreased by 2.49 mL/min/1.73 m^2 (-4.94 to -0.47 mL/min/1.73 m^2) on spironolactone.

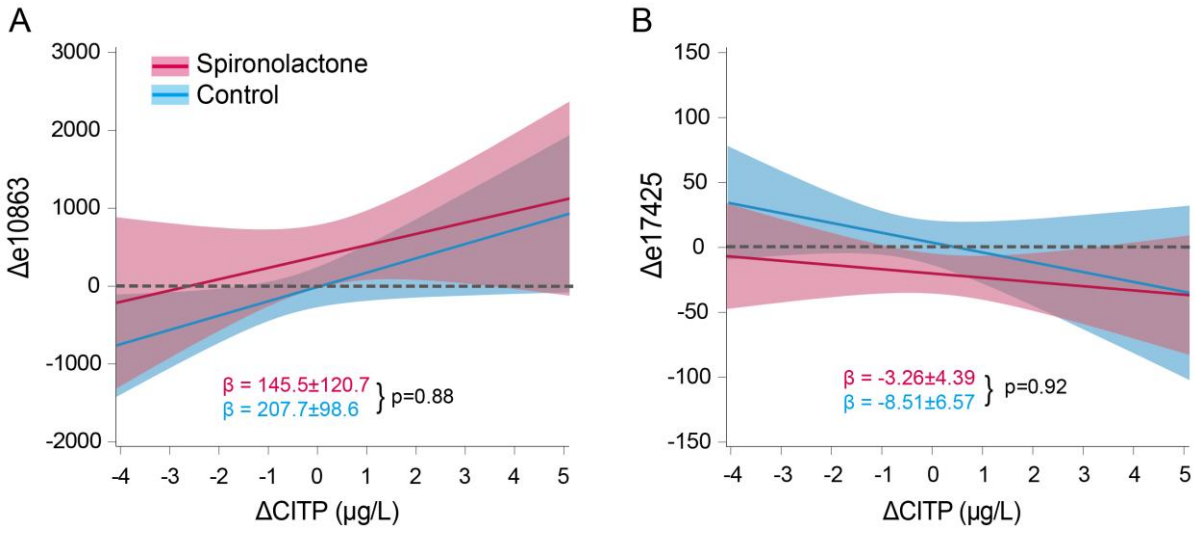


Figure 2. Linear associations between the changes over follow-up (Δ) in the COL1A1-derived peptides e10863 (A) and e17425 (B) regressed on the change (Δ) in carboxyterminal telopeptide of collagen I (CITP). e10863 increased during follow-up, whereas e17425 decreased. CITP is a serum biomarker of the degradation of mature COL1A1. The regression lines are presented with 95% confidence interval. The regression slopes (β) are given with standard error. The dotted lines indicate β=0. The p-values refer to the between-group (control *vs* spironolactone) in the regression slopes. Models are adjusted for sex, age, BMI, eGFR, smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs.

Discussion

This preplanned substudy to the randomised-controlled HOMAGE-RCT trial, to our knowledge for the first time assessed UPP changes in response to spironolactone in patients at high HF risk because of coronary heart disease. The following key observations summarise the results. First, the UPP differences between control and spironolactone were exclusively confined to 27 collagen fragments in line with the observation that collagen fragments constitute >70% of the UPP [6]. Of these 27 fragments, 16 were downregulated on spironolactone and 11 upregulated (**Table 3**). Second, compared to control, serum PICP and the serum PICP/CITP ratio decreased on spironolactone (**Table 4**). Finally, the correlations and regression slopes between the changes from baseline to follow-up in the urinary peptides and the corresponding changes in CITP were similar in patients randomised to control and spironolactone, but these correlations only reached significance, if the COL1A1 fragments were upregulated during follow-up (**Table S4**).

The current study shows associations, of which the interpretation remains speculative although based on literature data. The downward shift of serum PICP and the serum PICP/CITP ratio on spironolactone replicates previous HOMAGE-RCT publications [3]. Given the stochiometric ratios relating PICP to collagen synthesis and CITP to the degradation of mature collagen-1 (**Figure S1**), the downregulation of urinary collagen fragments probably represent reduced synthesis of COL1A1 and by extension a lower body-wide pool of collagens available for degradation. Moreover, the decrease in serum PICP and the PICP/CITP ratio, respectively biomarkers of collagen synthesis and the ratio of collagen synthesis-to-degradation, might reflect the smaller collagen pool available for degradation [16,17]. Two mechanisms might explain why 11 collagen fragments (seven COL1A1 fragments) were upregulated. First, the correlations between the changes from baseline to follow-up in the urinary peptides and the corresponding changes in CITP, the serum marker reflecting degradation of mature COL1A1, were similar in patients randomised to control and spironolactone (**Table S4**), because in all conditions a certain degree of collagen turnover remains necessary to maintain physiological collagen scaffoldings. Another mechanism that might contribute to the discordant trends in the levels of the urinary peptides is a build-up of shorter collagen fragments due to the degradation of longer fragments by proteases along the nephron and the lower urinary tract.

The literature supports our interpretation of the current findings. In a random-effect meta-analysis [18] of 1038 patients randomised in HOMAGE (47.0%) [3], ALDO-DHF

(37.2%) [19] and TOPCAT (15.7%) [20], treatment with spironolactone for 9 to 12 months compared to placebo or usual care reduced PICP by 7.4 mg/L (95% confidence interval: 0.9 to 13.9 mg/L). This association between spironolactone and serum PICP was not mediated by blood pressure [18]. This meta-analysis was consistent with the concept that spironolactone reduces COL1A1 in patients with stages 3-4 of HF [18]. In a post-hoc analysis of 1411 patients receiving spironolactone as add-on therapy in the ASCOT-BPLA trial, the serum concentrations of PIIINP and PICP rose in controls but fell on spironolactone treatment. The adjusted mean changes were +0.52 (95% confidence interval: -0.05 to 1.09) *vs* -0.41 (-0.97 to 0.16) μg/L for PIIINP and +4.54 (-1.77 to 10.9) *vs* -6.36 (-12.5 to -0.21) μg/L for PICP [21]. An aptamer-based proteomic analysis utilised 5284 modified aptamers to 4928 unique proteins in 164 TOPCAT patients with paired plasma samples at baseline and 1 year [22]. The top 4 canonical pathways were enriched for multiple collagens that increased in the placebo group, but decreased on spironolactone [22]. In a previous HOMAGE-RCT report [23], higher serum PICP was associated with left ventricular hypertrophy, left atrial enlargement, and inversely with e' as index of left ventricular stiffness (all $p<0.05$). Moreover, the decrease in serum PICP in response to spironolactone was associated with a decline in E/e' ($p=0.022$).

Injury activates resident fibroblasts or mobilises bone-marrow-derived circulating fibrocytes and epithelial or endothelial cells, and their transdifferentiate into α-smooth muscle actin-expressing myofibroblasts that secrete the ECM components. This process is required for wound repair in acute injury, but produce excessive ECM deposition in response to persistent injury [24]. Antifibrotic drugs remain a critically important unmet medical need, as nearly 45% of all natural deaths in the Western world are attributable to the complications of chronic fibroproliferative disorders [25]. Overall, the current findings might provide new perspectives in the search for refurbished or novel antifibrotic drugs and is therefore relevant for clinical practice [26]. Furthermore, nonsteroidal MR antagonists and sodium-glucose cotransporter-2 inhibitors have potent anti-inflammatory and antifibrotic properties [27]. Given the present findings, UPP analysis combined with measurement of circulating fibrosis biomarkers offers novel perspectives in documenting the antifibrotic properties of novel drug classes. Of note, the serum PICP decrease produced by empagliflozin in the EMPEROR trials was of the same order of magnitude as in the current study: 5% at 12 weeks and 8% at 52 weeks [27].

Study strengths and limitations

The randomised design of the current analysis, the first-time use of UPP data in the assessment of MR antagonism and the exploration of the changes in the urinary and

serum fibrosis markers in response to spironolactone are among the strong points of the current study. However, the present study also has limitations. First, changes in CITP were not significant, because of the smaller sample size compared with the full trial [3], albeit that trends were similar. Second, one possible drawback of the CE-MS approach is the application of the ultrafiltration with the threshold set at 20 kDa, so that larger proteins escape analysis. Finally, proteases active along the nephron and distal urinary tract might affect the urinary peptide fragments detected by UPP analysis. However, in a placebo-controlled study of a dipeptidyl peptidase-4 inhibitor [28], the UPP included pairs of peptide chains, i.e., the substrate for the protease activity (e.g., PPGPPGKNGDDGEAGKPG) and the resulting breakdown product (e.g., GPPGKNGDDGEAGKPG). In the current study, the UPP did not contain such peptide pairs, so that the assumption that spironolactone influenced the UPP by changing protease activity along the urinary tract could not be confirmed for the peptides retained in the analyses. However, this does not exclude degradation of peptides along the urinary tract, which were not retained in the analysis.

Conclusions

In patients prone to heart failure because of coronary heart disease, spironolactone compared to control reduced 16 urinary collagen fragments and increased 11 with no other differential changes in the urinary proteome. Spironolactone did not affect the relation between urinary and serum fibrosis markers. The interpretation of these factual observations is that MR antagonism predominantly downregulated urinary collagen-derived peptides, most likely by shrinking the body-wide pool of collagens. Why some urinary collagen fragments increased might be attributed to the maintenance of some degree of collagen synthesis and scaffolding to sustain vital organ functions or to the activity of proteases along the nephron and lower urinary tract. Combining urinary and serum fibrosis markers opens new avenues for discovery of antifibrotic drugs and refines insight in the action of antifibrotic drugs.

Funding

HOMAGE was funded by the European Union Seventh Framework Program. OMRON Healthcare, Co., Ltd., Kyoto, Japan provided a non-binding grant to the Non-Profit Research Association Alliance for the Promotion of Preventive Medicine (APPREMED), Mechelen, Belgium.

Conflict of interest

Justyna Siwy and Agnieszka Latosinska are employees of Mosaiques-Diagnostics GmbH, Hannover, Germany. Harald Mischak is the co-founder and co-owner of Mosaiques-Diagnostics GmbH. The other authors declare no conflict of interest.

References

1. Epstein M. A podcast discussing aldosterone and mineralocorticoid antagonists in 2021: a paradigm shift. *Diabetes Ther.* 2022;13:583-8.
2. Díez J. Mechanisms of cardiac fibrosis in hypertension. *J Clin Hypertens.* 2007;9:546-50.
3. Cleland JGF, Ferreira JP, Mariottoni B, *et al*. The effect of spironolactone on cardiovascular function and markers of fibrosis in people at increased risk of developing heart failure: the heart 'OMics' in AGEing (HOMAGE) randomized clinical trial. *Eur Heart J.* 2021;42:684-96.
4. Ravassa S, Kuznetsova T, Varo N, *et al*. Biomarkers of cardiomyocyte injury and stress identify left atria and left ventricular remodelling and dysfunction: a population-based study. *Int J Cardiol.* 2015;185:177-85.
5. Latosinska A, Siwy J, Mischak H, *et al*. Peptidomics and proteomics based on CE-MS as a robust tool in clinical application: the past, the present, and the future. *Electrophoresis.* 2019;40:2294-308.
6. Martens DS, Thijs L, Latosinska A, *et al*. Urinary peptidomics to address age-related disabilities: a prospective population study with replication in patients. *Lancet Healthy Longevity.* 2021;2:e690-e703.
7. Mavrogeorgis E, Mischak H, Latosinska A, *et al*. Reproducibility evaluation of urinary peptide detection using CE-MS. *Molecules.* 2021;26:7260.
8. Jantos-Siwy J, Schiffer E, Brand K, *et al*. Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res.* 2009;8:268-81.
9. Lazar C, Gatto L, Ferro M, *et al*. Accounting for the multiple natures of missing values in label-free quantitative proteomics data sets to compare imputation strategies. *J Proteome Res.* 2016;1116-25.
10. Levey AS, Stevens LA, Schmid CH, *et al*. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009;150:604-12.
11. Ravassa S, López B, Ferreira JP, *et al*. Biomarker-based assessment of collagen cross-linking identifies patients at risk of heart failure more likely to benefit from spirolacttone effects on left atrial remodelling. Insights from the HOMAGE clinical trial. *Eur J Heart Fail.* 2022;24:321-31.
12. Blom G. Statistical estimates and transformed beta-variables. *Biom J.* 1961;3:285.

13. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc B.* 1995;57:289-300.

14. Kleinbaum DG, Kupper LL, Nizam A, *et al*. The Correlation Coefficient and Straight-Line Regression Analysis. *Applied Regression Analysis and Other Multivariate Methods*. 4th ed. Andover, Hampshire, UK: BOOKS/COLE CENGAGE Learning, 2010. p. 91-106.

15. Kleinbaum DG, Kupper LL, Nizam A, *et al*. Straight-Line Regression Analysis. *Applied Regression Analysis and Other Multivariate Methods*. 4th ed. Andover, Hampshire, UK: BOOKS/COLE CENGAGE Learning, 2010. p. 41-90.

16. Magalhães P, Pejchinovski M, Markoska K, *et al*. Association of kidney fibrosis with urinary peptides: a path towards non-invasive liquid biopsies? *Sci Rep.* 2017;7:16915.

17. Mavrogeorgis E, Mischak H, Latosinska A, *et al*. Collagen-derived peptides in CKD: a link to fibrosis. *Toxins.* 2022;14:10.

18. Ferreira JP, Cleland JG, Girerd N, *et al*. Spironolactone effect on circulating procollagen type I carboxy-terminal propeptide: pooled analysis of three randomized trials. *Int J Cardiol.* 2023;377:86-8.

19. Edelmann F, Wachter R, Schmidt AG, *et al*. Effect of spironolactone on diastolic function and exercise capacity in patients with heart failure with preserved ejection fraction. The Aldo-HF randomized controlled clinical trial. *J Am Med Assoc.* 2013;309:781-91.

20. Pitt B, Pfeffer MA, Assmann SF, *et al*. Spironolactone for heart failure with preserved ejection fraction. *N Engl J Med.* 2014;370:1383-92.

21. Ferreira JP, Rossignol P, Pizard A, *et al*. Potential spironolactone effects on collagen metabolism biomarkers in patients with uncontrolled blood pressure. *Heart.* 2019;105:307-14.

22. Javaheri A, Diab A, Zhao L, *et al*. Proteomic analysis of effects of spironolactone in heart failure with preserved ejection fraction. *Circ Heart Fail.* 2022;15:e009693.

23. Kobayashi M, Girerd N, Ferreira JP, *et al*. The association between markers of type I collagen synthesis and echocardiographic response to spironolactone in patients at risk of heart failure: findings from the HOMAGE trial. *Eur J Heart Fail.* 2022 Jun 15;24:1559-68.

24. Fan D, Takawale A, Lee J, *et al*. Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. *Fibrogenesis Tissue Repair.* 2012;5:15.

25. Bollong MJ, Yang B, Vergani N, *et al*. Small molecule-mediated inhibition of myofibroblast transdifferentiation for the treatment of fibrosis. *Proc Natl Acad Sci.* 2017;114:4679-84.

26. Li X, Zhu L, Wang B, *et al*. Drugs and targets in fibrosis. *Front Pharmacol.* 2017;8:855.

27. Ferreira JP, Butler J, Anker SD, *et al*. Effects of empagliflozin on collagen biomarkers in patients with heart failure: findings from the EMPEROR trials. *Eur J Heart Fail.* 2024;26: 274-84.

28. Siwy J, Klein T, Rosler M, *et al*. Urinary proteomics as a tool to identify kidney responders to dipeptidyl peptidase-4 inhibition: a hypothesis-generating analysis of the MERLINA-T2D trial. *Proteomics.* 2019;13:1800144.

Data Supplement

HOMAGE investigators

Robertson Centre for Biostatistics, Institute of Health and Wellbeing, University of Glasgow, Glasgow, Scotland, UK — John GF Cleland, MD; Pierpaolo Pellicori, MD; Javed Khan, MD.

Université de Lorraine, Inserm, Centre d'Investigation Clinique Plurithématique, CHRU de Nancy, Nancy, France — João P Ferreira, MD; Franco Cosmi, MD; Anne Pizard, PhD; Nicolas Girerd, MD; Patrick Rossignol, MD; Erwan Bozec, MD; María U Moreno, MD; Faiez Zannad, MD.

Department of Cardiology, Cortona Hospital, Arezzo, Italy — Beatrice Mariottoni, MD.

Department of Cardiology, University of Hull, Castle Hill Hospital, Cottingham, East Riding of Yorkshire, UK — Joe Cuthbert, MD; Andrew L Clark, MD.

Department of Cardiology, Maastricht University Medical Center, Maastricht, the Netherlands — Job AJ Verdonschot PhD; Hans P Brunner La Rocca, MD; Mark Hazebroek, PhD; Stephane Heymans, MD.

Department of Internal Medicine and Cardiology, Campus Virchow Klinikum, Charité, University Medicine Berlin, Berlin Institute of Health (BIH), and German Centre for Cardiovascular research (DZHK), Partner Site Berlin, Germany — Johannes Petutschnigg, MD; Frank Edelmann, MD; Burkert Pieske, MD.

Division of Cardiovascular Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK — Fozia Z Ahmed, MD; Mamas A Mamas, MD.

Centre for Prognosis Research, Institute for Primary Care and Health Sciences, Keele University, Newcastle, UK — Mamas A Mamas, MD.

German Heart Center Berlin, Berlin, Germany — Burkert Pieske, MD.

St. Vincent's University Healthcare Group, and School of Medicine, University College Dublin, Dublin, Ireland — Ken McDonald, MD.

Equipe obésité et insuffisance cardiaque, Université Paul Sabatier, Inserm I2MC, Toulouse, France — Philippe Rouet, MD.

Studies Coordinating Centre, Research Unit Hypertension and Cardiovascular Epidemiology, Department of Cardiovascular Sciences, University of Leuven, Leuven, Belgium — L Thijs, MSc.

Non-Profit Research Association Alliance for the Promotion of Preventive Medicine, Mechelen, Belgium — Jan A Staessen, MD; Kei Asayama, MD; Tine W Hansen, MD; Gladys E Maestre, MD.

Program of Cardiovascular Diseases, CIMA. Universidad de Navarra and IdiSNA, Pamplona, Spain CIBERCV, Carlos III Institute of Health, Madrid, Spain — Arantxa González, PhD; Suzanna Ravassa, PhD; Begoñia López, PhD; Javier Díez, MD.

Departments of Nephrology and Cardiology, Clínica Universidad de Navarra, Pamplona, Spain — Javier Díez, MD.

Department of Cardiovascular Medicine, Istituto di Ricerche Farmacologiche Mario Negri – IRCCS, Milan, Italy —Roberto Latini, MD.

Fondation Force, Research and Consulting Department, EDDH, Centre de Médecine Préventive, Vandoeuvre les Nancy, France — Stephanie Grojean, PhD.

Department of Medical Statistics, London School of Hygiene and Tropical Medicine, London, UK — Tim Collier, PhD.

Urinary proteomics

All steps of the CE-MS analysis and the performance of the analytical platform have recently been described in detail [1].

Sample preparation and CE-MS analysis

Urine aliquots were thawed and 700 µL mixed with 700 µl of 2 M urea, 10 mM NH_4OH containing 0·02 % SDS. Subsequently, samples were ultrafiltered using a Centristat 20 kDa cut-off centrifugal filter device (Satorius, Göttingen, Germany) to eliminate high molecular weight proteins. The obtained filtrate was desalted using a PD 10 gel filtration column (GE Healthcare Bio Sciences, Uppsala, Sweden) to remove urea, electrolytes and salts as well as to enrich polypeptides. The samples were lyophilised and stored at 4°C before usage. Shortly before CE-MS analysis, the samples were re-suspended in 10 µL HPLC-grade H_2O. Samples were injected into CE-MS with 2 psi for 99 sec, resulting in injection volumes of ~280 nL.

A P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) was coupled with a Micro-TOF MS (Bruker Daltronic, Bremen, Germany). A solution of 20% acetonitrile (Sigma-Aldrich, Taufkirchen, Germany) in HPLC-grade water (Roth, Karlsruhe, Germany) supplemented with 0·94% formic acid (Sigma-Aldrich) was used as running buffer. For CE-MS analysis, the electrospray ionization interface from Agilent Technologies (Palo Alto, CA) was set to a potential of -4·0 to -4·5 kV. Spectra were recorded over an *m*/*z* range of 350-3000 and accumulated every 3 s.

CE-MS data processing

After the CE-MS analysis, mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaFinder software [2]. Only signals with z>1 observed in a minimum of 3 consecutive spectra with a signal-to-noise ratio of at least 4 were considered. The resulting peak list characterises each polypeptide by its mass and migration time. Data were calibrated utilizing 3151 internal standards as reference data points for mass and migration time by applying global and local linear regression, respectively. Reference signals of 29 abundant peptides were used as internal standards for calibration of signal intensity using linear regression. This procedure is highly reproducible and addresses both analytical and dilution variances in a single calibration step [3]. The obtained peak list characterises each polypeptide by its calibrated molecular mass [Da], calibrated CE migration time [min] and normalised signal intensity. All detected peptides are deposited, matched, and annotated in a Microsoft SQL database allowing further statistical analysis.

Sequencing of peptides

Candidate biomarkers were sequenced using CE-MS/MS or LC-MS/MS analysis, as described in detail [4]. MS/MS experiments were using an Ultimate 3000 nano-flow system (Dionex/LC Packings, USA) or a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA), both connected to an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Germany) equipped with a nano-electrospray ion source. The mass spectrometer is operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from *m*/*z* 300–2,000) were acquired in the Orbitrap. Ions were sequentially isolated for fragmentation. Data files were searched against the UniProt human nonredundant database using Proteome Discoverer 2·4 and the SEQUEST search engine. Relevant settings were: no fixed modifications, oxidation of methionine and proline as variable modifications. The minimum precursor mass was set to 790 Da, maximum precursor mass to 6000 Da with a minimum peak count of 10. The high-confidence peptides were defined by cross-correlation (Xcorr) >1.9 and rank = 1. Precursor mass tolerance and fragment mass tolerance were 5 ppm and 0.05 Da, respectively. For further validation of obtained peptide sequences, the correlation between peptide charge at the working pH of 2 and CE-migration time was utilised to minimise incorrect sequence assignment [5]: calculated CE-migration time of the sequence candidate based on its peptide sequence (number of basic amino acids) was compared to the experimental migration time.

References

1. Mavrogeorgis E, Mischak H, Latosinska A, *et al*. Reproducibility evaluation of urinary peptide detection using CE-MS. *Molecules.* 2021;26:7260.
2. Latosinska A, Siwy J, Mischak H, *et al*. Peptidomics and proteomics based on CE-MS as a robust tool in clinical application: the past, the present, and the future. *Electrophoresis.* 2019;40:2294-308.
3. Jantos-Siwy J, Schiffer E, Brand K, *et al*. Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res.* 2009;8:268-81.
4. Klein J, Papadopoulos T, Mischak H, *et al*. Comparison of CE-MS/MS and LC-MS/MS sequencing demonstrates significant complementarity in natural peptide identification in human urine. *Electrophoresis.* 2014;35:1060-4.
5. Zürbig P, Renfrow MB, Schiffer E, *et al*. Biomarker discovery by CE-MS enables sequence analysis via MS/MS with platform-independent separation. *Electrophoresis.* 2006;27:2111-25.

Serum biomarkers

PICP was quantified by an enzyme linked immunosorbent assay (Quidel Corporation, San Diego, CA) and CITP by a quantitative radio-immunoassay (Orion Diagnostica, Espoo, Finland). The detection limits were 0.2 μg/L for PICP and 0.6 μg/L for CITP. All inter- and intra-assay coefficients of variation were <10%.

References

López B, Ravassa S, González A, et *al*. Myocardial collagen cross-linking is associated with heart failure hospitalization in patients with hypertensive heart failure. *J Am Coll Cardiol.* 2016;67:251-60.

Ravassa S, López B, Ferreira JP, et *al*. Biomarker-based assessment of collagen cross-linking identifies patients at risk of heart failure more likely to benefit from spironolactone effects on left atrial remodelling. Insights from the HOMAGE clinical trial. *Eur J Heart Fail.* 2022;24:321-31.

Ethics approval

The HOMAGE trial was conducted in nine centres in the United Kingdom, France, Italy, Ireland, Germany and the Netherlands. The trial was approved by relevant ethics committees and regulatory bodies. The name of the Ethics Committee for each country and the reference numbers of the ethical approvals are listed below.

- Greater Manchester Central Research Ethics Committee (No. 16/NW/0012).
- Comité de Protection des Personnes Est-III, Hôpital de Brabois (No. 15.03.04).
- Comitato Etico Regione Toscana (No. 378/CEAVSE).
- Ethics and Medical Research Committee (No. 16/6/2015).
- Ethik-Kommission des Landes Berlin (No. 7.0.21/07/2016).
- De medisch-ethische toetsingscommissie (No. NL52729.068.15).

Table S1. Comparison of patients analysed and not analysed (starts)

Characteristic	Analysed	Not analysed	*P*
Number in group	290	215	…
Number (%)			
Women	69 (23.8)	57 (26.5)	0.4851
Past smoking	171 (59.0)	117 (54.4)	0.3074
Current smoking	19 (6.5)	23 (10.7)	0.0693
Drinking alcohol	69 (23.8)	20 (9.3)	<0.0001
Hypertension	217 (74.8)	177 (82.3)	0.0442
Diabetes	64 (22.1)	20 (9.3)	0.0001
Antihypertensive treatment	280 (96.6)	92 (42.8)	0.1096
Lipid-lowering treatment	257 (88.6)	159 (74.0)	<0.0001
Antiplatelet drugs	218 (75.5)	142 (66.0)	0.0250
Antidiabetic agents	257 (88.6)	201 (93.5)	0.1337
History of IHD	235 (81.1)	130 (60.5)	<0.0001
Clinical characteristics			
Age (y)	73.8±6.0	73.5±7.3	0.5879
Body mass index (kg/m^2)	28.8±5.1	28.9±5.0	0.7618
Systolic blood pressure (mm Hg)	138.3±18.2	139.5±17.7	0.4758

Table S1. Comparison of patients analysed and not analysed (ends)

Characteristic	Analysed	Not analysed	*P*
Diastolic blood pressure (mm Hg)	77.6±10.3	77.8±9.6	0.8491
Heart rate (bpm)	61.4±8.7	62.1±8.5	0.4201
Routine biochemistry			
Serum sodium (mmol/L)	139.2±2.6	139.1±2.6	0.5734
Serum potassium (mmol/L)	4.36±0.37	4.33±0.33	0.3787
eGFR (mL/min/1.73 m^2)	67.1±15.3	70.8±17.4	0.0122
Total cholesterol (mg/dL)	149.8±42.4	141.4±53.1	0.0479
HbA1C (%)	6.11±1.31	6.13±1.02	0.8979

Data are presented as arithmetic mean ± standard deviation, *n (%)* or median (interquartile range) for variables deviating from the normal distribution. p-values were computed by the large sample Z test (after rank normalization, if appropriate) for continuously distributed variables or by the χ^2 statistic or Fisher's exact test, as appropriate according to the cell frequencies, for categorical variables. Abbreviations: eGFR, glomerular filtration rate estimated from serum creatinine according to the Chronic Kidney Disease Epidemiology equation; HBA1C, glycated haemoglobin.

Table S2. Urinary peptide levels at baseline by randomised groups

ID	Symbol	Control (n=144)	Spironolactone (n=146)
e04960	COL1A1	4.81 (4.31-33.9)	4.81 (4.71-37.8)
e08916	COL1A1	2133 (1644-2984)	1981 (1495-2649)
e09408	COL1A1	725 (415-1297)	679 (455-1034)
e10266	COL1A1	14,824 (10,672-20,817)	15,567 (9956-20,058)
e10395	COL1A1	6150 (4872-7577)	5887 (4909-7096)
e10437	COL1A1	415 (197-649)	374 (170-637)
e10657	COL1A1	691 (309-1245)	488 (211-1084)
e10863	COL1A1	2901 (2127-3916)	2803 (2071-3953)
e11972	COL1A1	3179 (1469-4696)	3267 (1818-4775)
e17425	COL1A1	45.4 (11.0-126)	55.9 (11.1-151)
e18740	COL1A1	6.50 (6.49-47.9)	6.62 (6.50-43.2)
e10563	COL2A1	129 (8.15-312)	109 (8.15-312)
e03506	COL3A1	190 (63.5-351)	196 (66.5-355)
e05700	COL3A1	5.17 (4.36-327)	5.52 (5.36-305)
e07668	COL3A1	60.5 (13.3-274)	17.7 (13.3-259)
e18839	COL3A1	209 (23.8-519)	193 (63.9-486)
e05473	COL4A1	2.62 (2.44-26.7)	2.62 (2.44-50.5)
e17131	COL6A1	156 (61.1-347)	157.1 (3.10-339.1)
e01100	COL7A1	94.7 (4.85-253)	87.7 (4.85-206.3)
e02933	COL1A2	468 (273-619)	441 (295-656)
e04987	COL1A2	66.6 (9.97-279)	43.9 (9.97-290.4)
e16610	COL4A2	5.26 (4.89-23.5)	5.26 (4.88-31.7)
e09267	COL5A2	3.34 (2.96-10.8)	3.34 (3.08-13.3)
e20509	COL11A2	879 (362-1776)	1025 (444-1858)
e15360	COL4A3	358 (175-678)	339 (176-643)
e16874	COL5A3	4.56 (4.07-17.0)	4.56 (3.75-20.8)
e06373	COL4A6	190 (72.8-367)	230 (88.0-409)

ID is the laboratory identification number of the peptide fragment. Symbol refers to the parental protein (see table S3). Tabulated values are median (interquartile range) of the urinary peptides retained in the statistical analysis. There were no between-group differences in the urinary peptide levels ($p \geq 0.29$).

Table S3. Urinary peptide sequences retained in the analyses (starts)

ID	Symbol	Amino-acid sequence	Protein (*Uniprot ID*)
e04960	COL1A1	GppGpPGSpGEQGPSG	collagen alpha-1 (I) chain (*P02452)*
e08916	COL1A1	DDGEAGKPGRpGERGpPGP	collagen alpha-1 (I) chain (*P02452*)
e09408	COL1A1	GDDGEAGKpGRPGERGPPGp	collagen alpha-1 (I) chain (*P02452*)
e10266	COL1A1	NGDDGEAGKpGRpGERGPPGP	collagen alpha-1 (I) chain (*P02452*)
e10395	COL1A1	NGDDGEAGKpGRPGERGpPGp	collagen alpha-1 (I) chain (*P02452*)
e10437	COL1A1	AEGSPGRDGSpGAKGDRGETGP	collagen alpha-1 (I) chain (*P02452*)
e10657	COL1A1	AEGSpGRDGSpGAKGDRGETGp	collagen alpha-1 (I) chain (*P02452*)
e10863	COL1A1	DGQPGAKGEpGDAGAKGDAGPPGp	collagen alpha-1 (I) chain (*P02452*)
e11972	COL1A1	ADGQpGAKGEPGDAGAKGDAGppGPA	collagen alpha-1 (I) chain (*P02452*)
e17425	COL1A1	AGPTGARGAPGDRGEPGPpGpAGFAGpPGADGQPGAK	collagen alpha-1 (I) chain (*P02452*)
e18740	COL1A1	DKGETGEQGDRGIKGHRGFSGLQGppGPPGSPGEQGP	collagen alpha-1 (I) chain (*P02452*)
e10563	COL2A1	NPGEPGEpGVSGPMGpRGPpGP	collagen alpha-1 (II) chain (*P02458*)
e03506	COL3A1	SpGERGETGppGP	collagen alpha-1 (III) chain (*P02461*)
e05700	COL3A1	TGpGGDKGDTGPpGPQG	collagen alpha-1 (III) chain (*P02461*)
e07668	COL3A1	GTGGPpGENGKpGEpGPKG	collagen alpha-1 (III) chain (*P02461*)
e18839	COL3A1	GPPGMPGPRGSPGpQGVKGESGKpGANGLSGERGpPGPQG	collagen alpha-1 (III) chain (*P02461*)
e05473	COL4A1	GPpGFTGPPGPPGPPGP	collagen alpha-1 (IV) chain (*P02462*)

Table S3. Urinary peptide sequences retained in the analyses (ends)

ID	Symbol	Amino-acid sequence	Protein (*UniProt ID*)
e17131	COL6A1	PPGDPGLMGERGEDGpAGNGTEGFpGFPGYPGN	collagen alpha-1 (VI) chain (*P12109*)
e01100	COL7A1	DRGEpGPpGP	collagen alpha-1 (VII) chain (*Q02388*)
e02933	COL1A2	GppGPDGNKGEpG	collagen alpha-2 (I) chain (*P08123*)
e04987	COL1A2	pGpQGVQGGKGEQGp	collagen alpha-2 (I) chain (*P08123*)
e16610	COL4A2	DTGNPGAPGTpGTKGWAGDSGpQGRpGVFGLPG	collagen alpha-2 (IV) chain (*P08572*)
e09267	COL5A2	PGPVGApGDAGQRGDPGSRGP	collagen alpha-2 (V) chain (*P05997*)
e20509	COL11A2	GEHGpPGPPGPIGPVGQPGAAGADGEPGARGPQGHFGAKGDEGTRGFNGP	collagen alpha-2 (XI) chain (*P13942*)
e15360	COL4A3	GpKGDpGIpGLDRSGFpGETGSPGIPGHQ	collagen alpha-3 (IV) chain (*Q01955*)
e16874	COL5A3	DLGPpGDpGVSGIDGSpGEKGDPGDVGGPGPPGASG	collagen alpha-3 (V) chain (*P25940*)
e06373	COL4A6	SGpPGFPGLGTTGEKGE	Collagen alpha-6(IV) chain (*Q14031*)

ID is the laboratory identification number of the peptide fragment. Bolded IDs indicate urinary peptide fragments upregulated on spironolactone compared to control (see table 3). A lower case “p” in the amino-acid sequence indicates hydroxyproline. The protein identification number was obtained from the UniProt database (www.uniprot.org).

Table S4. Correlations between the changes over follow-up in COL1A1-derived urinary peptides and CITP

Biomarkers	Control (n=144)		Spironolactone (n=146)		p_{DIF}
	r (95% CI)	*P*	*r* (95% CI)	*P*	
Increasing peptide levels					
Δ e08916 (*COL1A1*)	0.079 (-0.085, 0.240)	0.34	0.025 (-0.138, 0.187)	0.77	0.12
Δ e09408 (*COL1A1*)	0.122 (-0.042, 0.280)	0.14	0.081 (-0.083, 0.240)	0.33	0.053
Δ e10266 (*COL1A1*)	0.145 (-0.019, 0.302)	0.080	0.051 (-0.112, 0.212)	0.54	0.33
Δ e10395 (*COL1A1*)	0.208 (-0.046, 0.359)	0.012	-0.072 (-0.232, 0.091)	0.39	0.47
Δ e10437 (*COL1A1*)	0.259 (0.100, 0.406)	0.0016	0.165 (-0.003, 0.319)	0.045	0.61
Δ e10657 (*COL1A1*)	0.188 (0.025, 0.341)	0.024	0.149 (-0.013, 0.304)	0.071	0.078
Δ e10863 (*COL1A1*)	0.198 (0.035, 0.350)	0.017	0.221 (0.060, 0.370)	0.0071	0.36
Decreasing peptide levels					
Δ e04960 (*COL1A1*)	-0.003 (-0.167, 0.160)	0.97	0.101 (-0.063, 0.259)	0.23	0.97
Δ e11972 (*COL1A1*)	-0.033 (-0.131, 0.195)	0.69	-0.073 (-0.232, 0.091)	0.38	0.81
Δ e17425 (*COL1A1*)	-0.076 (-0.237, 0.089)	0.36	-0.089 (-0.248, 0.075)	0.28	0.66
Δ e18740 (*COL1A1*)	-0.076 (-0.237, 0.089)	0.36	0.078 (-0.085, 0.238)	0.35	0.38

Δ, change from baseline to month 9; CITP, collagen type I C-terminal telopeptide (marker of degradation of mature collagen-1). Tabulated values are partial correlation coefficients (*r*) given with 95% confidence interval. Models are adjusted for sex, age, BMI, eGFR, smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. Peptide levels increasing or decreasing from baseline to month 9 are listed in Table 3. *p* and p_{DIF} refer to the significance of the correlation coefficients and the significance of the between-group differences in the partial correlation coefficients.

Table S5. Between-group differences in serum electrolytes and glomerular filtration at last follow-up visit

Trial Biomarker	Control (n=144)	Spironolactone (n=146)	Δ (95% CI)	*P*
Serum Na^+ (mmol/L)	139.3±0.2	138.4±0.2	-0.90 (-1.36, -0.44)	0.0001
Serum K^+ (mmol/L)	4.39±0.03	4.53±0.03	+0.14 (+0.06, +0.22)	0.0008
eGFR (mL/min/1.73 m^2)	70.7±0.86	68.18±0.85	-2.49 (-4.94, -0.47)	0.047

Values are means ± SE or median (interquartile range). Δ (95% CI) provides multivariable-adjusted estimates of the between-group differences . Models were adjusted for sex, age, BMI, eGFR, the baseline value of the urinary marker, current smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. eGFR is the glomerular filtration rate estimated from serum creatinine according to the Chronic Kidney Disease Epidemiology equation.

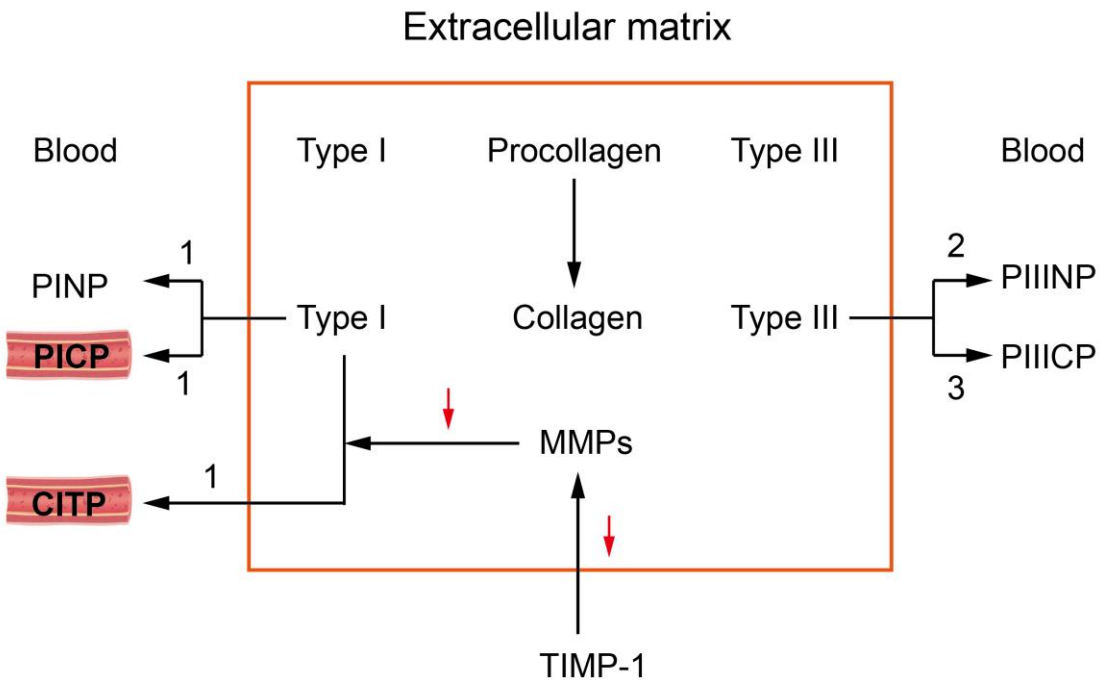


Figure S1. Circulating biomarkers of collagen turnover. Procollagen type-I amino-terminal propeptide (PINP) and procollagen type-I carboxy-terminal propeptide (PICP) are released during conversion of procollagen type-1 to collagen type-1 and carboxyterminal telopeptide of type-I collagen (CITP) during the degradation of collagen type-1 by matrix metalloproteinases (MMPs), which are inhibited by tissue inhibitor of the matrix metalloproteinase type-1 (TIMP1). Procollagen type-III amino-terminal propeptide (PIIINP) and procollagen type-III carboxy-terminal propeptide (PIIICP) are released during conversion of procollagen type-III to collagen type-III. Bracketed numbers indicate the stoichiometric ratio. PICP and CITP, which were analysed in the current HOMAGE Trial dataset, are serum markers of collagen type-1 synthesis and degradation, respectively. PIIINP is an indirect indicator of collagen-III synthesis, because cleavage at the amino-terminus proceeds at a relatively slow rate and, thus, partially processed procollagen molecules remain bound to the surface of collagen type-III fibres (*JACC* 2015;65:2449-2456).

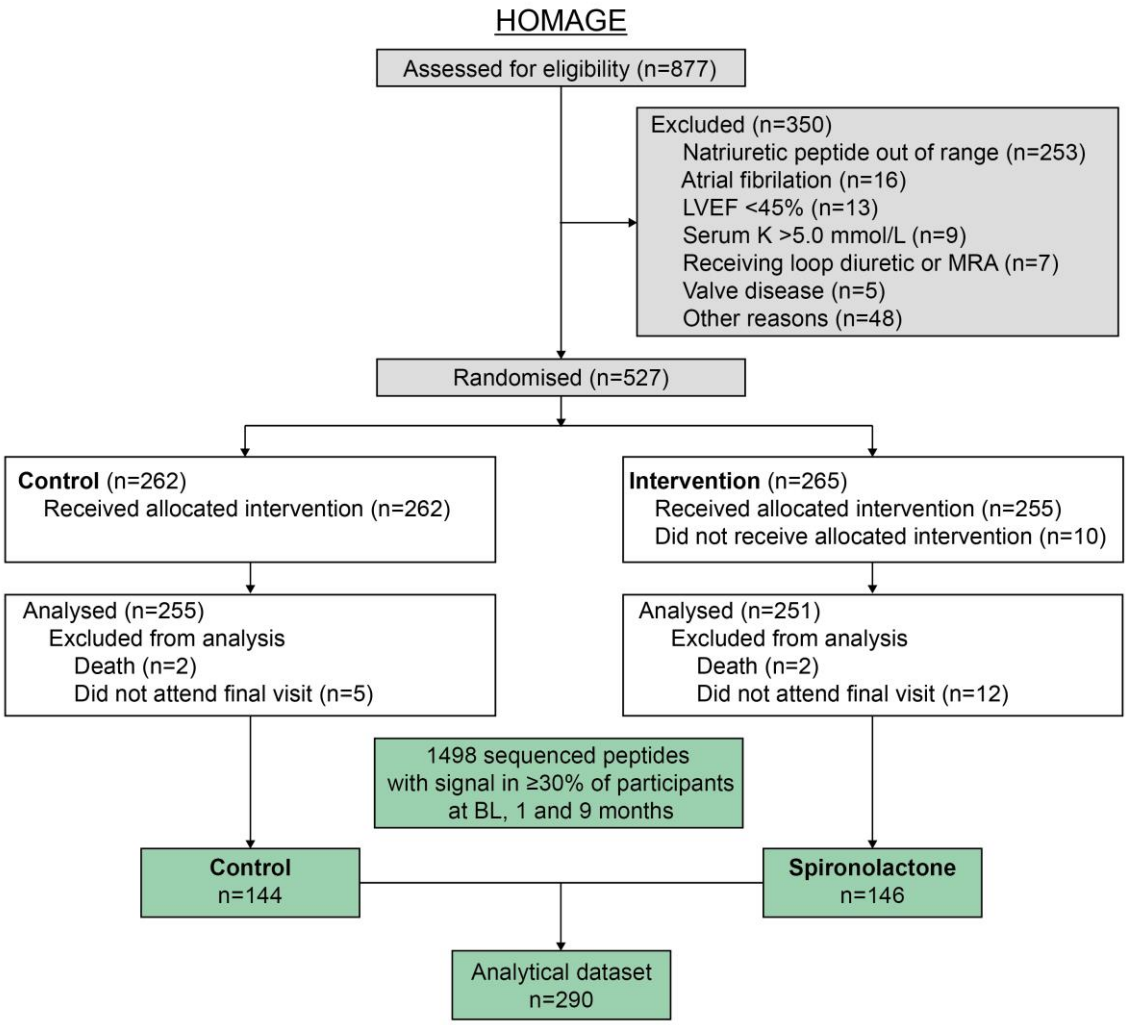


Figure S2. Flow chart of the HOMAGE analytical dataset.

LVEF, left ventricular ejection fraction; BL, baseline.

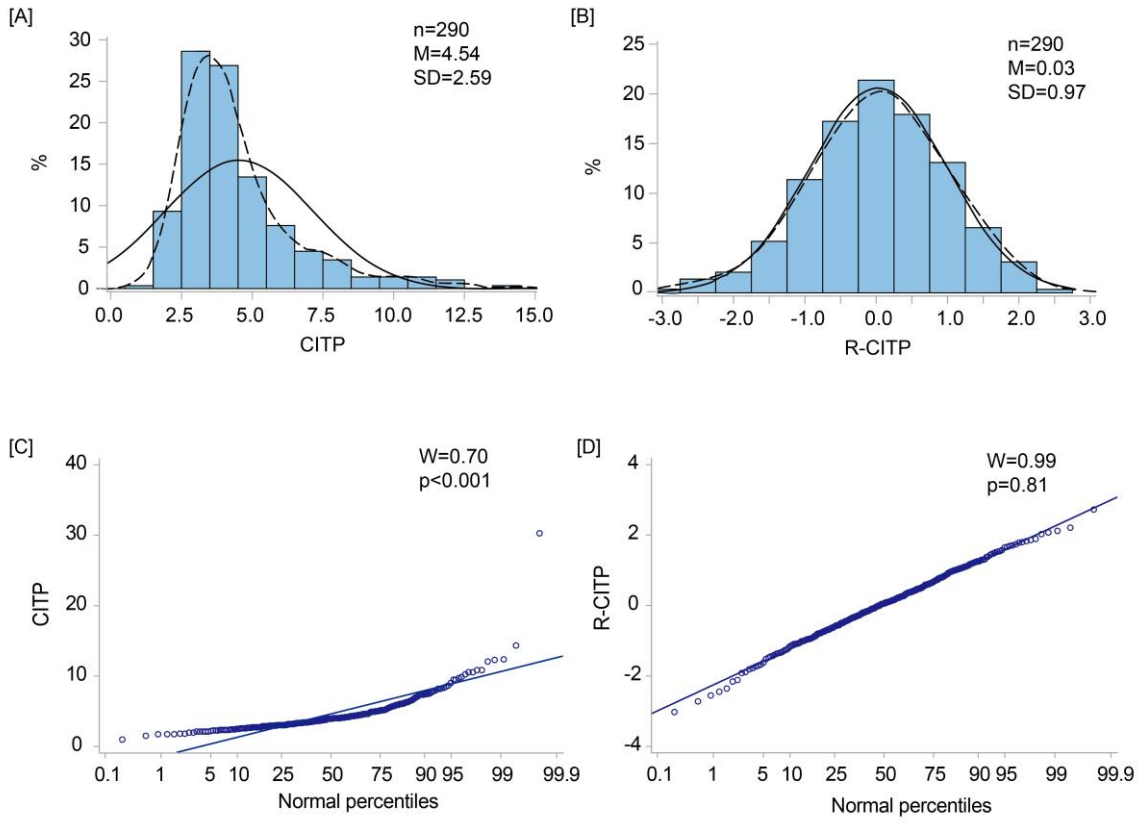


Figure S3. Rank normalisation of a carboxyterminal telopeptide of collagen I (CITP), a serum marker of collagen I degradation. Panels A and B show the distribution plots before (A) and after (B) rank-normalisation; panels C and D show the normal percentile plots before (C) and after (D) rank normalisation. The solid and dotted lines represent the normal and kernel density distributions. *n*, M and SD refer to the number of patients, the arithmetic means and standard deviation. W is the Shapiro-Wilk statistic and p is the associated significance. A significant Shapiro-Wilk test indicates deviation from the normal distribution.

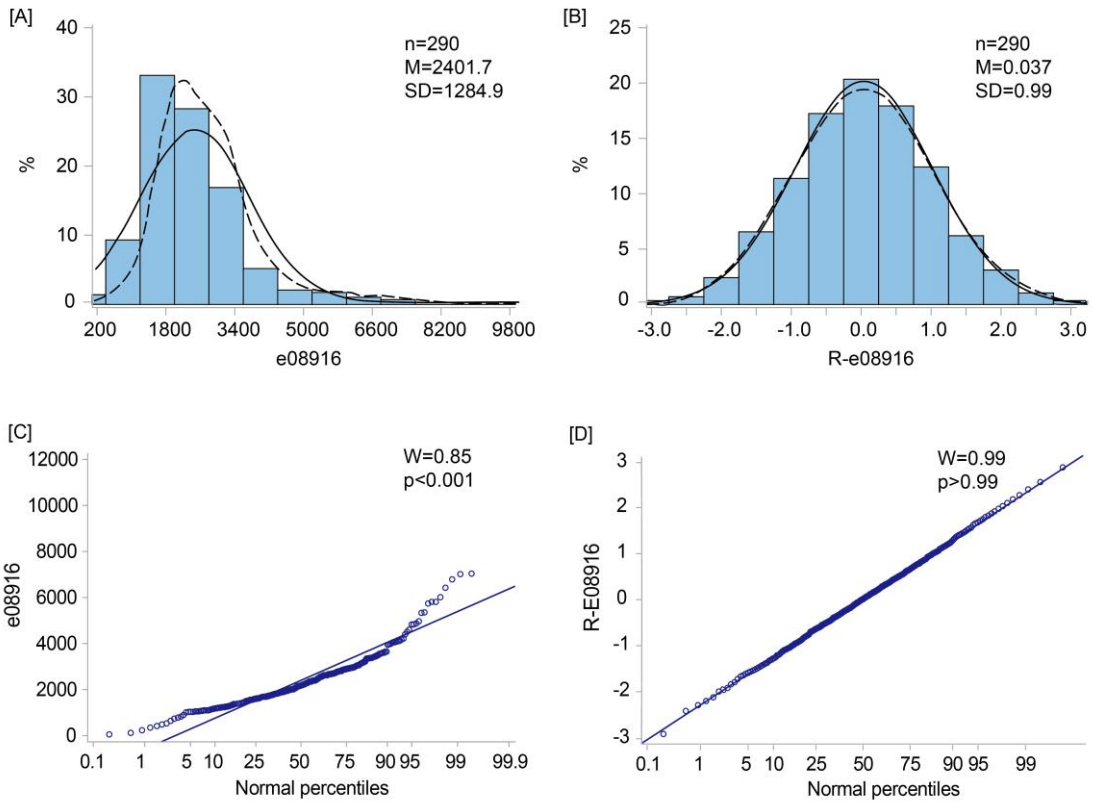


Figure S4. Rank normalisation of urinary peptide fragment e08916 derived from collagen I. Panels A and B show the distribution plots before (A) and after (B) rank-normalisation; panels C and D show the normal percentile plots before (C) and after (D) rank normalisation. The solid and dotted lines represent the normal and kernel density distributions. *n*, M and SD refer to the number of patients, the arithmetic means and standard deviation. W is the Shapiro-Wilk statistic and p is the associated significance. A significant Shapiro-Wilk test indicates deviation from the normal distribution.

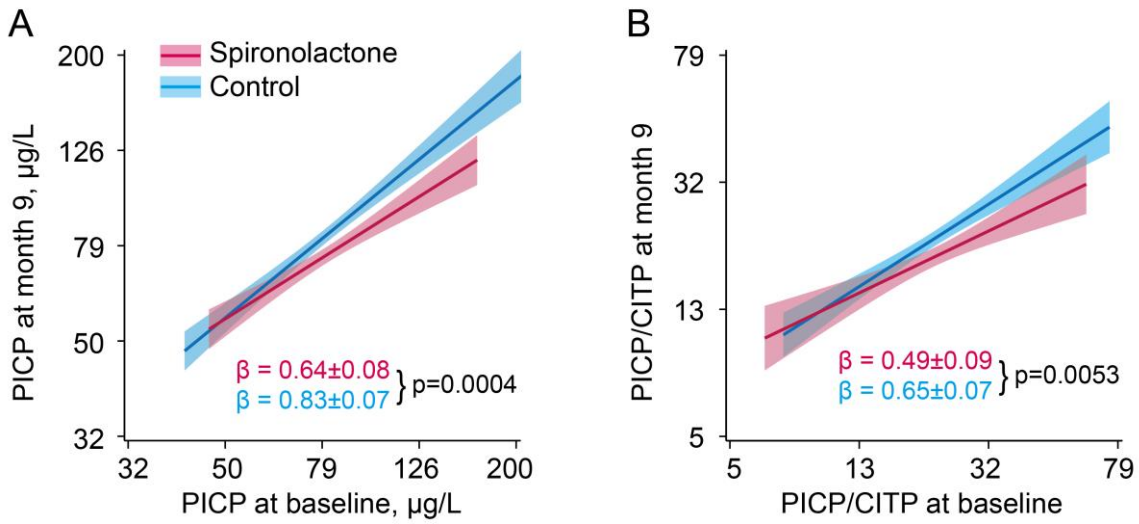


Figure S5. Linear associations between the 9-month serum PICP (A) and the serum PICP/CITP ratio (B) regressed on the baseline levels by treatment group. PICP (carboxyterminal propeptide of procollagen I) is a marker of COL1A1 synthesis and CITP (carboxyterminal telopeptide of collagen I) of COL1A1 degradation (see supplemental figure 1). The regression lines are presented with 95% confidence interval. The regression slopes (β) are given with standard error. p values refer to the between-group (control *vs* spironolactone) in the regression slopes. Models are adjusted for sex, age, BMI, eGFR, smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs.

Chapter 4

Unhealthy ageing in relation to clinical risk factors

Part 1

Blood pressure trajectory from birth to preschool- and school-age: evidence from ENVIRONAGE birth cohort

Yu-Ling Yu, MD, Eleni Renaers, Hanne Sleurs, Anneleen Massot, Daphne Berden, Michelle Plusquin, Rossella Alfano, Dries S. Martens, Dong-Yan Zhang, De-Wei An, Anke Raaijmakers, Karel Allegaert, Peter Verhamme, Jan A. Staessen, Tim S. Nawrot

In preparation.

Abstract

Background: Blood pressure (BP) is a crucial modifiable risk factor strongly associated with cardiovascular disease in adulthood. The modifiable impact of BP might initiates from birth and operates across the whole life span. However, no studies to date tracked BP from birth onwards.

Objective: To explore BP trajectory in childhood and evaluate whether the risk of hypertension later in childhood is related to birth and early childhood BP.

Methods: The ENVIRonmental influence *ON* AGEing in early life (ENVIR*ON*AGE) is a birth cohort, with BP measured at birth, preschool- (4-6 years) and school-age (9-11 years), in 500 children having measurements at multiple time points. Tracker, horse-racer and non-tracker were defined by the change in mean arterial pressure (MAP) percentiles between 2 visits within 0-1 quartile, moving up for 2-3 quartiles, and moving down for 2-3 quartiles, respectively. Elevated BP and hypertension at childhood, standardised for age, sex, and height, were defined following the 2017 American Academy of Pediatrics guidelines. Multivariable adjusted linear, mixed and Cox proportional hazard regression models were applied.

Results: This study included 500 healthy children (53.2% girls). Mean systolic/diastolic BP tracked from 67.3/40.5 mm Hg at birth to 100.2/57.5 mm Hg at preschool-age, and to 107.7/65.1 mm Hg at school-age. Correspondingly, MAP averaged from 51.2 to 74.6 and to 82.1 mm Hg. Each standard-deviation increase in initial BP, BP at the last follow-up increased by 2.66/1.37 mm Hg (1.65 to 3.67/0.28 to 2.46 mm Hg) in systolic/diastolic BP, and 1.97 mm Hg (0.85 to 3.10 mm Hg) in MAP, respectively. The hazard ratios of elevated BP and hypertension were 2.84 (1.50 to 5.38) and 3.75 (1.79 to 7.86), in line with the findings at 4-6 and 9-11 years.

Conclusions: This prospective cohort study demonstrates that BP tracked consistently from birth through childhood. Higher BP levels at birth and in early childhood were strongly associated with an increased risk of elevated BP and hypertension later in childhood. These findings highlight the importance of monitoring and managing BP from birth as a potential strategy to reduce cardiovascular risk later in life.

Introduction

Cardiovascular disease in adulthood is often rooted on the modifiable risk factors present in early childhood. Notably, hypertension is a *"silent"* precursor, because its target organ damages typically occurs only after a long asymptomatic course. In the early 20th century, the pioneering investigators [1,2] reported that the average level of blood pressure (BP) levels in children increases with age. BP tracking across life span plays an important role in predicting whether children will become hypertensive adults and develop its associated complications [3]. Previous epidemiological studies have shown that children with higher BP levels are more likely to develop persistent hypertension [4,5], arterial stiffness [4] and left ventricular hypertrophy [4] in adulthood. Moreover, autopsy [6] and imaging study [7] studies have reported that young patients with hypertension are more likely to experience accelerated vascular ageing and cardiovascular target organ damage [6,7]. To the best of our knowledge, no investigations assessed BP from birth to childhood or evaluated the long-term trajectory of BP development in large population-based study. The ENVIROmental influence *ON* AGEing in early life (ENVIR*ON*AGE) [8] study is a unique birth cohort that assesses BP and other phenotypes at different stages of childhood. To fill the knowledge gap, we explored the BP trajectory in healthy Belgian children in ENVIRONAGE birth cohort, whose BP measurements were taken at birth, preschool- (4-6 years) and school-age (9-11 years), and examined their relation to the incidence of elevated BP and hypertension.

Methods

Study Population

The ongoing ENVIR*ON*AGE study was initiated in 2010 in Limburg, Belgium [8]. Mother-newborn pairs are recruited at birth and are invited for the follow-up visits around the age of 4-6 years and 9-11 years (**Figure 1**). The study complies with the Helsinki Declaration and has been approved by the ethical committees of Hasselt University, Diepenbeek, Belgium (reference no. B371201216090, B371201524537, and B1152021000007). Mothers provided written informed consent. This study was conducted following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

At birth, 1256 newborns and their mothers participated and were visited. Between October 2014 and June 2024, 755 children had the first follow-up visit (FU1) at the age between 4 to 6 years. 501 children were excluded because they passed away (n=2), moved out of the area (n=23), had parental language restriction to fill out questionnaires in Dutch (n=14), withdrew participations (n=402), and had no show at FU1 (n=60).

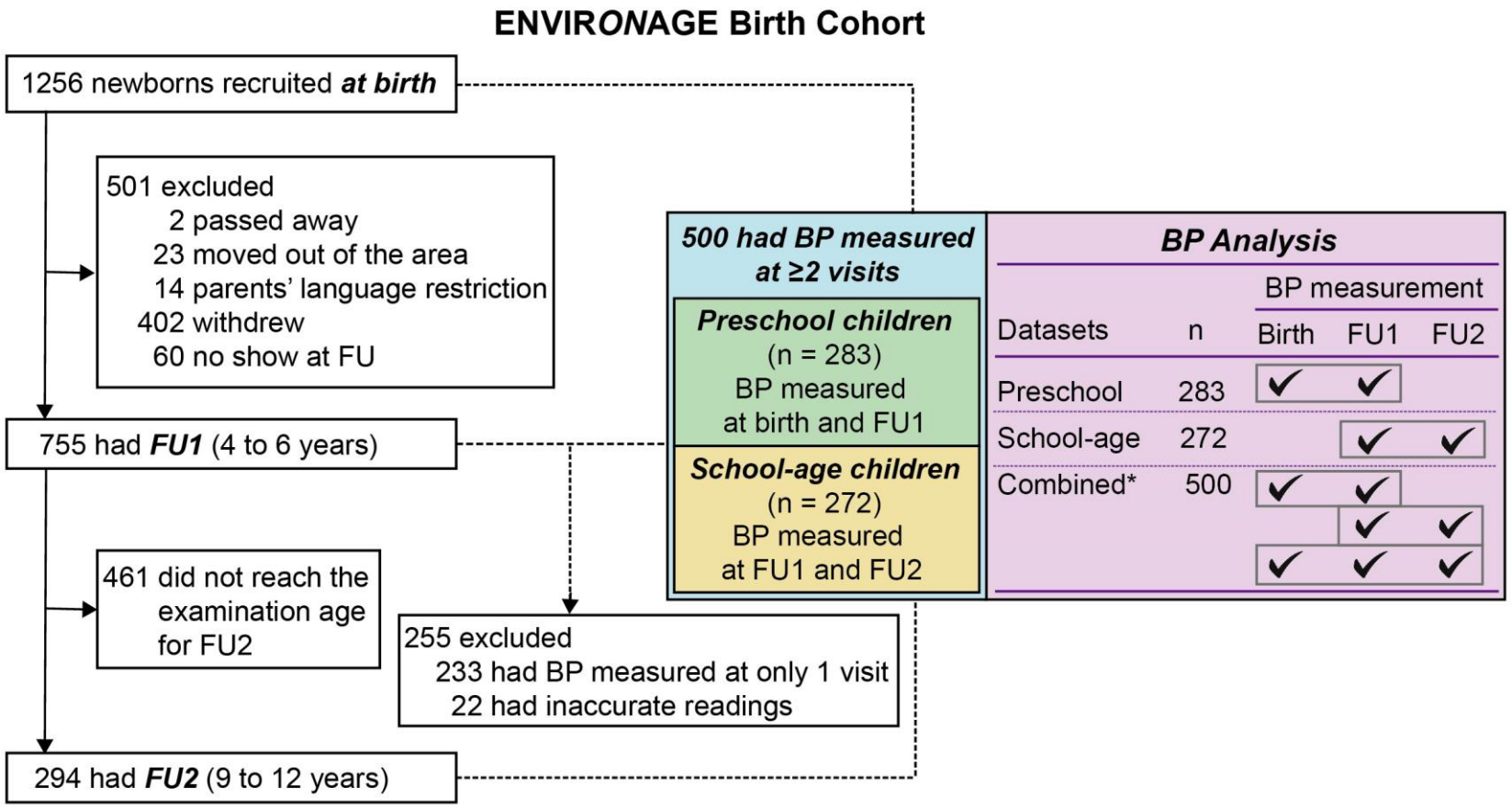


Figure 1. Flow Chart Describing the Recruitment, Disposition and Selection of ENVIRONAGE Children.

BP, blood pressure; ENVIRONAGE, Environmental Influence on Aging in Early Life; FU1, the first follow-up; FU2, the second follow-up; n, number of children. Inaccurate readings refer to the BP readings over the range of ±3 standard deviations. *Among the 500 children in the combined dataset, 228 had BP measured at birth and FU1, 217 had BP measured at FU1 and FU2, and 55 had BP measured at birth, FU1 and FU2.

By 31 October 2024, 294 children had the second follow-up visit (FU2) at the age between 9 to 11 years, except 461 children who did not reach the examination age for FU2.

To explore the BP trajectory though childhood, we only included the children who had 2 or more BP measurements at different visits in the analysis (n=500), of whom 283 had BP measured at birth and FU1 (4-6 years, preschool period), and 272 had BP measured at FU1 and FU2 (9-11 years, school-age period). Therefore, 283 children constructed the preschool dataset, 272 constructed the school-age dataset, and 500 with BP measured at 2 or more visits constructed the combined dataset.

Anthropometrics and Medical Information

At birth, the neonatal information of newborns and pre-pregnancy information of mothers were collected from medical records. Gestational age was recorded in weeks and estimated based on the last menstrual cycle and first fetal ultrasound examination. Birth weight was presented in grams. Maternal pre-pregnancy body mass index (BMI) was calculated as weight in kilograms divided by height in square meter, and was measured at the first antenatal visit. Maternal age, history of gestational hypertension, preeclampsia and diabetes were collected from medical history. Other maternal information was collected via questionnaires filled out by the mothers in the maternity ward, including education attainment categorised by International Standard Classification of Education [9], and smoking habit during pregnancy.

At FU1 and FU2, the body anthropometrics of children were measured, including body height (to the nearest centimeter) and body weight (to the nearest 0.1 kg). Childhood overweight and obesity were defined using age- and sex-specific BMI cut-offs according to the International Obesity Task Force.[10]

Blood Pressure Assessment

At birth, the BP of newborns was measured ±3 days after birth with an advanced oscillometric measurement device (Vital Signs Monitor 6000 Series, Welch Allyn Inc., Skaneateles Falls, NY). To ensure accurate measurements, appropriately sized cuffs were used according to the newborn’s limb circumference [11]. BP was measured between 09:00 a.m. and 11:00 a.m. in between feedings while the newborn was lying in a supine rest position. Newborn BP was taken five times consecutively on the right upper arm with a 2-min interval. Wakefulness during BP measurements was recorded into two categories: asleep and awake.

At follow-up visits (FU1 and FU2), childhood BP was measured with an automated upper-arm BP monitor (Omron 705IT, Omron Corporation) equipped with a cuff adapted

to the arm size of children. Measurements were performed according to the guideline of European Society of Hypertension [12]. After 5 minutes of rest in the supine position, a trained observer obtained 5 consecutive readings at 1-minute intervals. Mean systolic BP (SBP) and diastolic BP (DBP) were based on the mean of the last 3 readings. Mean arterial pressure (MAP) was calculated as DBP plus 40% of pulse pressure (the difference between SBP and DBP).[13] Elevated BP and hypertension were defined according to the 2017 American Academy of Pediatrics (AAP) guidelines [14]. Age-, sex-, and height-specific BP percentiles were calculated for SBP and DBP and referred to as high when above the 90th percentile. The definitions of normal BP, elevated BP, stage 1 hypertension and stage 2 hypertension BP were described in **Table S1**.

Maternal postpartum BP was measured ±3 days after delivery using an automated oscillometric measurement device (Omron 705IT, Omron Corporation, Kyoto, Japan). The measurement was taken on the right upper arm in a sitting position after resting for 5 min. Five consecutive measurements were taken 1 min apart.

Statistical Analysis

All data management and statistical analyses were done using SAS version 9.4 (maintenance level 5; SAS Institute Inc). All statistical tests were 2-sided and a *P* value <0.05 was considered statistically significant. Participants' characteristics were presented as mean with standard deviation (SD) or frequency with percentage. To describe the tracking phenomenon of BP, we defined tracker, horse-racer and non-tracker according to the percentile change in MAP between two visits (**Table S2** and **Figure S1**). Trackers are children with MAP percentiles at the same quartile, or moving up or down for 1 quartile (-50% to +50%) from birth to FU1, or from FU1 to FU2; horse-racers are those moving up for 2 or 3 quartiles (+51% to +100%); and non-trackers are those moving down for 2 or 3 quartiles (-51% to -100%). The BP tracking phenomenon were depicted by Sankey plots, and the absolute changes in BP values were depicted by line graphs, respectively.

Pearson correlations, unadjusted and multivariable-adjusted linear, mixed and proportional hazards Cox regression models were applied. To further explore the blood pressure trajectory and incident hypertension in children, we performed the analysis in continuous outcome as captured by BP and in categorised outcome according to hypertension incidence. For the continuous outcome, the association between follow-up BP (dependent variable) and initial BP (independent variable) were analysed. For the preschool dataset, the initial BP was measured at birth and follow-up BP at FU1. For the school-age dataset, the initial BP was measured FU1 and follow-up BP at FU2. The data

from preschool and school-age datasets were merged as combined dataset. Linear model was applied in the preschool and school-age datasets, and mixed model accounting for clustering of data within participants was applied in the combined dataset. For the categorised outcome, the association between incident elevated BP or hypertension (dependent variable) and initial MAP (independent variable) was analysed. Cox proportional hazard model was applied in the preschool and school-age datasets, and in the combined dataset additionally accounting for clustering of data within participants. The time variable is the time interval between initial BP measurement and the outcome incidence. For both analyses, adjusted models accounted for sex, age, initial body mass index, change in age, change in body height and wakefulness of newborns during BP measurements (when BP at birth involved in analysis). Fully adjusted models additionally accounted for birth weight, gestational age, maternal age at delivery, and maternal pre-pregnancy BMI. The estimates were expressed in per 1-standard-deviation (SD) increase of initial BP.

Additionally, we ran several sensitivity analyses to support the main results. First, a sensitivity analysis including 228 newborns at asleep status were performed in the preschool dataset. Second, we ran a sensitivity analysis in the 240 newborns with normal birth weight. Third, we ran a sensitivity analysis in the nonobese children at 4-6 years (n=477) and 9-11 years (n=206). Fourth, in consideration of mother's characteristics, the fully adjusted models further account for maternal postpartum BP, smoking during pregnancy, and maternal education attainment. Finally, to account for regression to mean, we ran a sensitivity analysis by excluding the 30, 26, and 49 children, who had the initial MAP was lower than 5th percentile or higher than 95th percentile in the preschool, school-age and combined dataset, respectively [15].

Results

Characteristics

The characteristics of the preschool, school-age and combined datasets are shown in **Table 1, Tables S3** and **S4**, respectively. Of the 500 participants included in analysis, 266 (53.2%) were girls. The wakefulness of newborns during BP measurements was recorded, including 228 (80.6%) asleep and 55 (19.4%) awake. Among the 283 newborns, 10 (3.53%), 240 (84.8%) and 33 (11.7%) had a low, normal and high birth weight, respectively (<2500 g, 2500-4000 g, >4000 g). Gestational age averaged 39.2 (SD: 1.57) weeks, and mean birth weight 3384 (515) grams (**Table 1**). The body weight and body height increased, and BMI tracked correspondingly along age (**Table 1, Tables S3** and **S4**).

Table 1. Characteristics of 500 Children in the Combined Dataset

Characteristic	ENVIR*ON*AGE visits		
	Birth (n=283)	FU1 (n=500)	FU2 (n=272)
Age, years	NA	4.70 (0.56)	10.1 (0.82)
Girls, n (%)	147 (51.9)	266 (53.2)	151 (55.5)
Gestational age, weeks	39.2 (1.57)	…	…
Birth weight, grams	3384 (515)	…	…
Body weight, kg	…	19.2 (2.79)	36.4 (8.62)
Body height, cm	50.2 (2.24)	108.8 (5.62)	142.0 (7.64)
BMI, kg/m^2	13.4 (1.26)	16.2 (1.37)	17.9 (3.05)
Overweight, n (%) [b]	…	81 (16.2)	60 (22.1)
Obesity, n (%) [b]	…	13 (2.60)	12 (4.41)
Blood pressure, mm Hg			
SBP	67.3 (8.82)	100.2 (8.49)	107.7 (9.45)
DBP	40.5 (8.34)	57.5 (9.14)	65.1 (6.41)
MAP	51.2 (8.16)	74.6 (7.80)	82.1 (6.74)
Hypertension category, n (%) [a]			
Normal	…	352 (70.4)	183 (67.3)
Elevated	…	58 (11.6)	35 (12.9)
Stage-1 hypertension	…	70 (14.0)	37 (13.6)
Stage-2 hypertension	…	20 (4.00)	17 (6.25)

Abbreviation: FU, follow-up; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.
[a] As defined by 2017 American Academy of Pediatrics guideline using age-, sex-, and height-specific BP percentiles (Table S1 in the Supplement).
[b] As defined by age- and sex-specific BMI cut-offs according to the extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity.

In addition, the mean age of mothers at delivery was 30.9 (3.97) years, and the pre-pregnancy BMI was 24.5 (4.43) kg/m^2. Most of mothers were non-smokers (76.8%) and highly-educated (70.2%). 28 (5.60%) mothers had pregnancy hypertension or preeclampsia and 23 (4.60%) had pregnancy diabetes. The postpartum BP averaged 119.8 (10.9) mm Hg, 75.2 (9.06) mm Hg and 93.0 (9.13) mm Hg in SBP, DBP and MAP, respectively (**Table S5**).

Tracking of Blood Pressure

The BP of the children tracked consistently throughout childhood (**Table 1**). From birth to FU1 (4-6 years) and to FU2 (9-11 years), mean SBP/DBP increased from 67.3/40.5 to 100.2/57.5 and to 107.7/65.1 mm Hg, respectively, and correspondingly mean MAP increased from 51.2 to 74.6 and to 82.1 mm Hg (**Table 1**). In the school-age dataset (**Table S4**), according to the age-, sex-, and height-specific BP percentiles, the proportion of children with normotension significantly decreased by 7.35% (95% confidence interval [CI]: -13.9% to -0.72%) from FU1 to FU2, and stage-2 hypertension increased by 4.04% (0.62% to 7.41%), while the changes in the proportion of elevated BP and stage-1 hypertension did not reach significance.

Of the 283 children in the preschool dataset (birth to FU1), 79.9% were trackers, 10.2% horse-racers and 9.89% non-trackers (**Table S2**). Sankey plot (**Figure 2, panel A**) illustrates the percentile change of MAP by quartiles of the distributions from birth to FU1. The MAP of trackers averaged 51.4 (SD: 7.72) mm Hg at birth and increased to 75.9 (7.45) mm Hg at FU1, but the BP percentiles remained stable (averaged 50.7% and 50.7%). However, the MAP of horse-racers increased substantially from 42.4 (5.35) mm Hg to 83.4 (8.85) mm Hg, and the mean percentiles rose significantly from 19.0% to 82.9%. The MAP of non-trackers changed from 59.0 (4.77) mm Hg to 67.2 (3.20) mm Hg, and the mean percentiles dropped from 79.5% to 13.7% (**Figure 2, panel C** and **Table S2**). A similar pattern in SBP and DBP were observed in **Figures S2** and **S3, panels A** and **B,** respectively. Among the 226 trackers, SBP, DBP and MAP at FU1 were significantly correlated with SBP (r=0.47), DBP (r=0.39) and MAP (r=0.50) at birth, respectively (all $P < 0.0001$; **Figure S4, panel A**).

Similarly, of the 272 children in the school-age dataset (FU1 to FU2), the percentages of trackers, horse-racers and non-trackers were 81.3%, 9.56% and 9.19%, respectively (**Table S2**). From FU1 to FU2 (**Figure 2, panel B**), the MAP of trackers averaged 72.9 (6.09) mm Hg at FU1 and increased to 82.1 (6.38) mm Hg at FU2, but the BP percentiles remained stable (averaged 50.4% and 50.2%). However, the MAP of horse-racers increased substantially from 66.2 (3.32) mm Hg to 89.6 (4.07) mm Hg, and the mean

percentiles increased significantly from 17.7% to 83.2%. The MAP of non-trackers changed from 80.4 (5.57) mm Hg to 75.1 (3.01) mm Hg, and the mean percentiles dropped from 83.9% to 17.5% (**Figure 2, panel D** and **Table S2**). A similar pattern in SBP and DBP were shown in **Figures S2** and **S3, panels A** and **B**, respectively. Among the 221 trackers, SBP, DBP and MAP at FU1 were significantly correlated with SBP (r=0.55), DBP (r=0.35) and MAP (r=0.52) at birth, respectively (all P<0.0001; **Figure S4, panel B**).

Follow-Up and Initial Blood Pressure

In the preschool dataset (**Table 2**), SBP and MAP at 4-6 years was positively associated with the SBP and MAP at birth, respectively, but not in DBP. In the fully adjusted models, with per 1-SD increase in the BP at birth, SBP increased by 1.97 mm Hg (95% CI: 0.70 to 3.24 mm Hg) and MAP increased by 1.83 mm Hg (0.37 to 3.28 mm Hg) at 4-6 years. In the school-age dataset (**Table 2**), with per 1-SD increase in the BP at 4-6 years, SBP increased by 3.94 mm Hg (95% CI: 2.36 to 5.52 mm Hg) and MAP increased by 2.37 mm Hg (0.52 to 4.22 mm Hg) at 9-11 years, but not significant in DBP. With all the participants in the combined dataset (**Table 2**), these association sizes were 2.66 mm Hg (1.65 to 3.67 mm Hg) in SBP, 1.37 mm Hg (0.28 to 2.46 mm Hg) in DBP, and 1.97 mm Hg (0.85 to 3.10 mm Hg) in MAP, respectively.

Incident Hypertension and Initial Blood Pressure

Among the 283 participants in the preschool dataset (**Table 3**), 97 and 61 children developed elevated BP and hypertension, respectively. For each SD increase in MAP at birth, the hazard ration (HR) for developing elevated BP at 4-6 years was 1.39 (1.01 to 1.91) in the fully adjusted model, but the HR for hypertension did not reach significance. Among the 272 participants in the school-age dataset (**Table 3**), 89 and 54 children developed elevated BP and hypertension, and the unadjusted HRs were 2.65 (1.66 to 4.24) and 3.66 (2.04 to 6.56), respectively. The fully adjusted models confirmed these results. In the combined dataset consisting of 500 children, 168 and 105 participants developed elevated BP and hypertension at the last follow-up, respectively (**Table 3**). In the fully adjusted models, 1-SD increase in the initial MAP at birth or at the age of 4-6 years, the HRs of elevated BP and hypertension were 2.84 (1.50 to 5.38) and 3.75 (1.79 to 7.86), respectively.

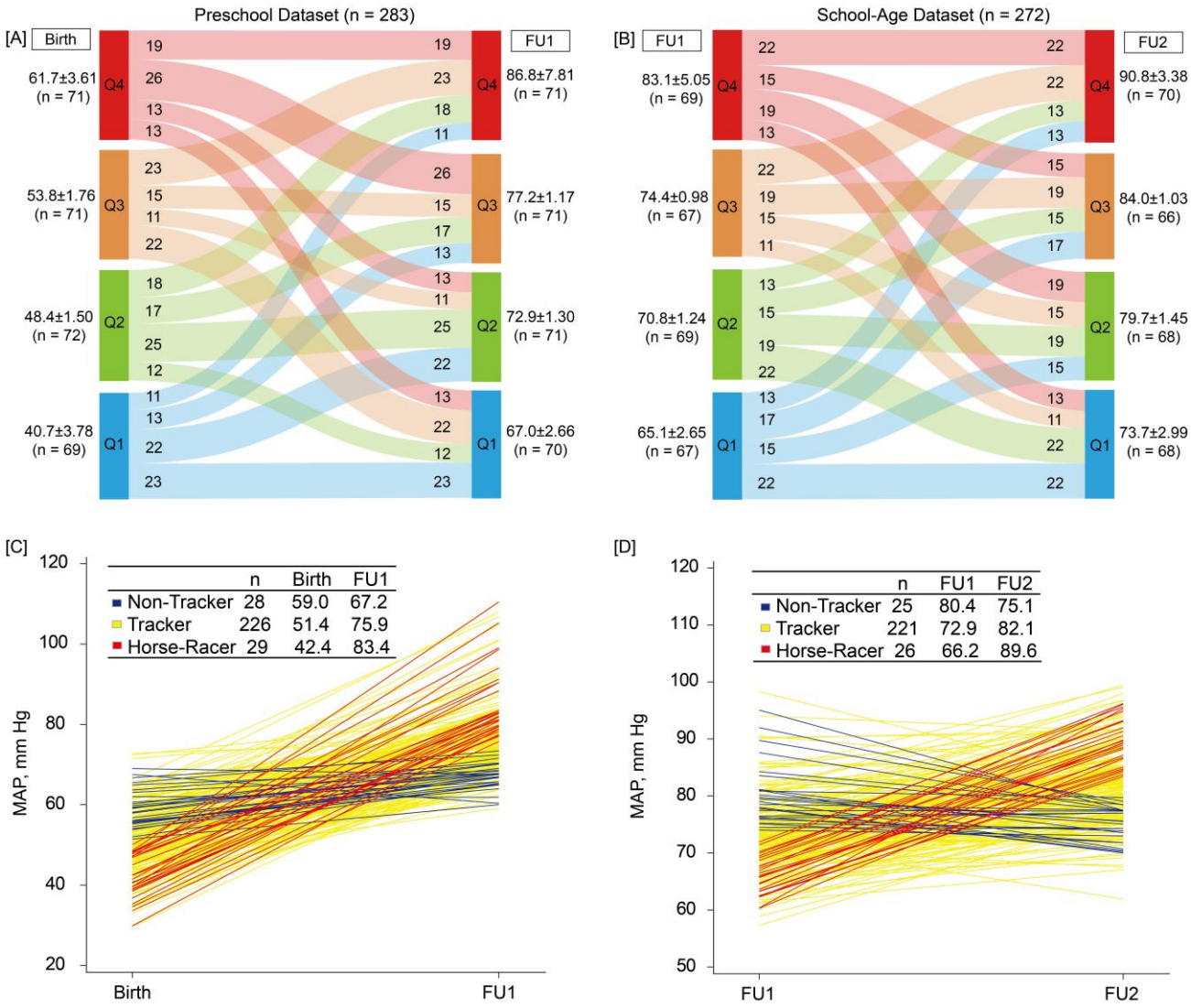


Figure 2. Tracking of Mean Arterial Pressure in the Preschool (A, C) and School-age (B, D) Datasets.

Sankey plots (A, B) depict the percentile changes of mean arterial pressure. Line graphs (C, D) depict the absolute changes of mean arterial pressure between two follow-up visits. Abbreviations: Q, quartile; FU1, the first follow-up; FU2, the second follow-up; n, number of children; MAP, mean arterial pressure.

Table 2. Association between Follow-up Blood Pressure and Initial Blood Pressure in the Preschool, School-age and Combined Datasets

Variable	Unadjusted		Adjusted [c]		Fully adjusted [d]	
	Estimate (95% CI)	*P*	Estimate (95% CI)	*P*	Estimate (95% CI)	*P*
Preschool dataset (n=283) [a] From birth to FU1						
SBP, mm Hg	2.10 (0.82, 3.38)	0.0014	1.91 (0.66, 3.16)	0.0029	1.97 (0.70, 3.24)	0.0025
DBP, mm Hg	1.69 (0.06, 3.33)	0.043	1.63 (0.01, 3.25)	0.049	1.58 (-0.05, 3.22)	0.058
MAP, mmHg	2.01 (0.55, 3.46)	0.0071	1.85 (0.41, 3.29)	0.012	1.83 (0.37, 3.28)	0.014
School-age dataset (n=272) [a] From FU1 to FU2						
SBP, mm Hg	4.73 (3.14, 6.32)	<0.0001	4.36 (2.78, 5.94)	<0.0001	3.94 (2.36, 5.52)	<0.0001
DBP, mm Hg	0.88 (-0.43, 2.19)	0.19	0.84 (-0.49, 2.17)	0.22	0.67 (-0.68, 2.03)	0.33
MAP, mm Hg	2.95 (1.12, 4.78)	0.0017	2.83 (1.00, 4.67)	0.0026	2.37 (0.52, 4.22)	0.012
Combined dataset (n=500) [b] From birth to FU1 and FU2						
SBP, mm Hg	3.02 (2.45, 3.58)	<0.0001	2.63 (1.62, 3.64)	<0.0001	2.66 (1.65, 3.67)	<0.0001
DBP, mm Hg	3.04 (2.12, 3.97)	<0.0001	1.40 (0.32, 2.49)	0.012	1.37 (0.28, 2.46)	0.015
MAP, mm Hg	4.08 (3.11, 5.04)	<0.0001	2.01 (0.88, 3.13)	0.0008	1.97 (0.85, 3.10)	0.0010

Abbreviations: CI, confidence interval; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

[a] Linear model was applied in the preschool and school-age datasets. The association size is estimate expressed as per standard deviation increase of the initial blood pressure.

[b] Mixed model was applied in the combined dataset. This model accounted for clustering of data within participants. The association size is estimate expressed as standard deviation increase of the initial blood pressure.

[c] Adjusted models accounted for sex, age, initial body mass index, change in age, and change in body height. For the analysis at birth, wakefulness of newborns (asleep *vs*. awake) was also adjusted.

[d] Fully adjusted models additionally accounted for birth weight, gestational age, maternal age at delivery, and maternal pre-pregnancy BMI.

Table 3. Risks of Elevated Blood Pressure and Incident Hypertension in Relation to Initial Mean Arterial Pressure in the Preschool, School-age and Combined Datasets

Variable	n/N	Unadjusted		Adjusted [c]		Fully adjusted [d]	
		HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Preschool dataset (n=283) [a] From birth to FU1							
Elevated blood pressure	97/283	1.18 (0.85, 1.63)	0.32	1.34 (0.97, 1.85)	0.078	1.39 (1.01, 1.91)	0.044
Hypertension	61/283	1.09 (0.72, 1.65)	.67	1.30 (0.86, 1.96)	0.22	1.36 (0.90, 2.05)	0.14
School-age dataset (n=272) [a] From FU1 to FU2							
Elevated blood pressure	89/272	2.65 (1.66, 4.24)	<0.0001	2.39 (1.50, 3.79)	0.0002	2.19 (1.35, 3.55)	0.0015
Hypertension	54/272	3.66 (2.04, 6.56)	<0.0001	3.26 (1.82, 5.83)	<0.0001	3.27 (1.79, 5.96)	0.0001
Combined dataset (n=500) [b] From birth to FU1 and FU2							
Elevated blood pressure	168/500	1.51 (1.12, 2.04)	0.0072	3.17 (1.72, 5.82)	0.0002	2.84 (1.50, 5.38)	0.0014
Hypertension	105/500	1.62 (1.11, 2.37)	0.012	3.87 (1.90, 7.90)	0.0002	3.75 (1.79, 7.86)	0.0005

Abbreviations: CI, confidence interval; HR, hazard ratio. n/N indicate the number of incident hypertension at follow-up and the number of children.

[a] Cox proportional hazard model was applied with mean arterial pressure as explanatory variable in the preschool and school-age datasets. Hazard ratios are expressed as per standard deviation increase of the initial blood pressure.

[b] Cox proportional hazard model with mixed effect was applied with mean arterial pressure as explanatory variable in the combined dataset. This model accounted for clustering of data within participants. Hazard ratios are expressed as per standard deviation increase of the initial blood pressure.

[c] Adjusted models accounted for sex, age, initial body mass index, and change in body height. For the analysis at birth, wakefulness of newborns (asleep *vs*. awake) was also adjusted.

[d] Fully adjusted models additionally accounted for birth weight, gestational age, maternal age at delivery and pre-pregnancy BMI.

Sensitivity analysis

In the pre-school dataset, the sensitivity analysis in 228 newborns at asleep status improved the significance, with the fully adjusted estimates of 2.30 mm Hg (95% CI: 0.86 to 3.74 mm Hg; *P*=0.0019) in SBP, 2.02 mm Hg (0.10 to 3.95 mm Hg; *P*=0.040) in DBP, and 2.28 mm Hg (0.58 to 3.98 mm Hg; *P*=0.0088) in MAP. The HRs were improved to 2.84 (95% CI: 1.50 to 5.38; *P=0.*0014) in elevated BP and 3.75 (1.79 to 7.86; *P*=0.0005) in hypertension. Moreover, the sensitivity analysis in 240 newborns with normal birth weight confirmed the main results, with the fully adjusted estimates of 1.64 mm Hg (95% CI: 0.19 to 3.09 mm Hg; *P*=0.027) in SBP, 1.44 mm Hg (-0.45 to 3.32 mm Hg; *P*=0.13) in DBP, and 1.64 mm Hg (-0.03 to 3.30 mm Hg; *P*=0.054) in MAP. Furthermore, among the nonobese children, the incidence of elevated BP (HR: 3.00; 95% CI: 1.56, 5.78; *P*=0.0010) and hypertension (HR: 3.83; 95% CI: 1.81, 8.08; *P*=0.0004) were significantly associated with initial MAP. In addition, after accounting for maternal postpartum BP, smoking during pregnancy, and maternal education attainment, the estimates were 1.79 mm Hg (95% CI: 0.54 to 3.04 mm Hg; *P*=0.0059) in SBP, 1.50 mm Hg (-0.03 to 3.04 mm Hg; *P*=0.054) in DBP, and 1.52 mm Hg (0.11 to 2.93 mm Hg; *P*=0.035) in MAP. The HRs were 1.49 (95% CI: 1.07 to 2.06; *P*=0.017) in elevated BP and 1.46 (0.96 to 2.22; *P*=0.074) in hypertension. Finally, after excluding the effect of regression to mean, the direction of estimates confirmed the main results in the preschool, school-age and combined datasets (**Table S6**).

Discussion

The core finding of our study is that BP at birth and earlier childhood was associated with the incidence of elevated BP and hypertension in later childhood. Children born with higher BP displayed persistently a higher BP and a higher risk of elevated BP at the age of 4-6 years. Similarly, children with higher BP at 4-6 years also experienced higher BP and risk of hypertension at 9-11 years (**Table 2** and **Table 3**). Further, our findings also demonstrated the *"tracking"* phenomena in about 80% of children. Moreover, *"horse-racing"* phenomena presented in about 10% of children and indicated that even the initial BP was low in early childhood, the risk of hypertension should not be neglected in later life. Thus, our study enriches the understanding on BP trajectory in childhood and suggests BP at birth and earlier childhood is an indicator to predict unhealthy aging in later life.

"Tracking" refers to the stability of rank of an individual's rank in the BP distribution overtime [16], while *"horse-racing"* is the correlation of the higher BP in later life with the

faster BP increase during childhood [16,17]. In the early 20th century, pioneering investigators [1,2] reported that the average level of BP in children increases with age. This increase is particularly evident for SBP and lasts until 20 years old with an average of 2 mm Hg/year in boys and 1 mm Hg/year in girls [16]. This rise was also found in DBP, though it was less pronounced [16]. In our study, the BP tracked from birth to 4-6 years and then to 9-11 years. The percentiles of BP and the estimates (**Table S2** and **Figure S4**) demonstrated the consistencies of BP distribution among the trackers.

In line with the current findings, previous observations demonstrated that BP at childhood and adolescence tracks into adulthood [18-20]. In the International Childhood Cardiovascular Cohort Consortium with 7 childhood cohorts from the United States, Finland and Australia, the SBPs between child and adolescent (r=0.48), between adolescent and young adult (r=0.40), and between child and young adult (r=0.24) were significantly correlated (all P<0.0001) [20]. A gradual increase in the frequency of adult hypertension was found across the BP change groups, which ranged from 19% in the individuals with persistent normotension from childhood to adolescence, to 80% in the individuals with elevated SBP in both childhood and adolescence [20]. Furthermore, childhood SBP and adolescent SBP ≥ 90th percentile significantly increased the risk of adult hypertension, with the odds ratio (OR) of 2.0 (95%CI: 1.6 to 2.5) and 3.0 (95% CI: 2.2 to 4.1), respectively [20]. A recent meta-analysis including 39,714 individuals from 11 articles [19] demonstrated that elevated BP in the childhood was significantly associated with hypertension in adulthood (OR: 2.02; 95% CI: 1.62 to 2.53). With 1-SD increase in SBP and DBP, the ORs for hypertension in adulthood were 1.71 (1.50 to 1.95) and 1.57 (1.37 to 1.81), respectively [19].

Hypertension is the world's leading cause of death and the strongest modifiable cardiovascular risk factor associated with complications in heart, kidneys and brain. Notably, hypertension is a *"silent"* killer, because it starts operative early in life and its target organ damages only occur after a long asymptomatic course. The global prevalence of hypertension ranks high among NCDs, accounting for 4% in children younger than 19 years [21] and 30-45% in adults [22]. The Lancet Commission on Hypertension suggested to tackle prevention and treatment of elevated BP with a lifecourse approach in consideration of early lifetime programming, long-term benefits and changing priorities with ageing [23]. Therefore, from birth to preschool- and school-age, this study observed BP trajectory across childhood and contributed to the prediction of hypertension risk in the birth cohort, and thereby emphasizing the importance to BP management early in life.

Strengths and Limitations

The strengths of our study includes the prospective birth cohort design with a relatively large sample size, and multiple visits from birth to preschool- (4-6 years) and school-age (9-11 years), so that the BP trajectory of childhood could be tracked integrally. Furthermore, at each visit, multiple consecutive BP measurements and automated techniques alleviated white-coat effect and observer bias, and therefore increase the reliability of BP readings. However, our findings should be interpreted within the context of its limitations. First, diastolic BP in young children is difficult to measure, especially for the small vessels of neonates. For this reason, we applied the validated oscillometric device [8] to avoid the misidentification of the Korotkov sounds. Second, we observed a relatively high prevalence of elevated BP at the age of 4-6 years and 9-11 years, according to the 2017 AAP guideline, which is consistent to the previous observations [24,25]. This is because of the stringent guideline was issued for a purpose of early detection and intervention. Thus, we acknowledge that hypertension diagnosis in young children requires repeated BP monitoring over time. Finally, even though BP tracks over time, the individual and parental life styles over lifecourse can influence on BP and risk of hypertension in later life. Therefore, long-term follow-up is required to assess the interaction and modification effects of BP with other environmental risk factors over children and early adulthood, which might contribute to the pediatric BP management strategies.

Conclusions

In this prospective cohort study provides evidence that BP tracks consistently over time from birth to preschool- and school-age. Furthermore, risk of elevated BP and hypertension was associated with the BP levels at birth and early childhood,highlighting its early life trajectory as a key determinant of future cardiovascular health. BP is an important modifiable risk factor operative early in life and strongly links to the health conditions in later life, particularly in cardiovascular risk. Our study underscores the importance of a lifecourse approach to BP management, demonstrating that a deeper understanding of BP trajectory and early development of cardiovascular risk is essential for timely identification and prevention of none optimal BP in childhood.

Conflict of Interest

All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and none was reported.

Funding

ENVIR*ON*AGE is supported by grants from the European Research Council (No.ERC-StG310898) and the Research Foundation Flanders (grant No. G073315N). Dr Martens holds a postdoctoral grant from the Research Foundation Flanders (FWO grant No. 12X9623N). The Non-Profit Research Association Alliance for the Promotion of Preventive Medicine, Mechelen, Belgium (www.appremed.org) received a nonbinding grant from OMRON Healthcare Co Ltd, Kyoto, Japan.

References

1. Judson CF, Nicholson P. Blood pressure in normal children. *Am J Dis Child.* 1914; 8: 257-278.
2. Stocks P. Blood pressure in early life: a statistical study. *London: Cambridge University Press.* 1924;
3. Azegami T, Uchida K, Tokumura M, Mori M. Blood pressure tracking from chilhood to adulthood. *Front Pediatr.* 2021; 9: 785356.
4. Liao YY, Ma Q, Chu C *et al*. The predictive value of repeated blood pressure measurements in childhood for cardiovascular risk in adults: the Hnagzhou Adolesscent Hypertension Study. *Hypertens Res.* 2020; 43: 969-978.
5. Sun SS, Grave GD, Siervogel RM, Pickoff AA, Arslanian SS, Daniels SR. Systolic blood pressure in childhood predicts hypertension and metabolic syndrome later in life. *Pediatrics.* 2007; 119: 237-246.
6. Tracy RE, Newman III WP, Wattigney WA, Berenson GS. Risk factors and atherosclerosis in youth: autopsy findings of the Bogalusa Heart Study. *J Med Sci.* 1995; 310 (suppl 1): S37-S41.
7. Urbina EM, Khoury PR, McCoy C, Daniels SR, Kimball TR, Dolan LM. Cardiac and vascular consequences of pre-hypertension in youth. *J Clin Hypertens.* 2011; 13: 332-342.
8. Janssen BG, Madhloum N, Gyselaers W *et al*. Cohort profile: The ENVIRonmental influence *ON* early AGEing (ENVIRO*N*AGE): a birth cohort study. *Int J Epidemiol.* 2017; 46: 1386-1387.
9. UNESCO Institute for Statistics. *International Standard Classification of Education ISCED 2011.* United Nations Educational, Scientific and Cultural Organization, Montreal, Quebec, Canada: 2012;
10. Cole TJ, Lobstein T. Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. *Pediatr Obes.* 2012; 7: 284-294.
11. Stebor AD. Basic principles of noninvasive blood pressure measurement in infants. *Adv Neonatal Care.* 2005; 5: 252-261.
12. Parati G, Stergiou GS, Asmar R *et al*. European Society of Hypertension Practice Guidelines for home blood pressure monitoring. *J Hum Hypertens.* 2010; 24: 779-785.

13. Mahieu D, Kips J, Rietzschel ER *et al*. Noninvasive assessment of central and peripheral arterial pressure (waveforms): implications of calibration methods. *J Hypertens.* 2010; 28: 300-305.
14. Flynn JT, Kaelber DC, Baker-Smith CM *et al*. Clinical practice guideline for screening and management of high blood pressure in children and adolescents. *Pediatrics.* 2017; 140: e20171904.
15. Yu R, Chen L. The need to control for regression to the mean in social psychology studies. *Front Psychol.* 2015; 5: 1574.
16. Hofman A. Blood pressure in children: an epidemiological approach to the aetiology of hypertension. *J Hypertens.* 1984; 2: 323-328.
17. Fletcher CM, Peto R. The horse-racing effect. *Lancet.* 1981; 318: 467-468.
18. Hardy ST, Urbina EM. Blood pressure in childhood and adolescence. *Am J Hypertens.* 2021; 34: 242-249.
19. Yang L, Sun J, Zhao M, Liang Y, Bovet P, Xi B. Elevated blood pressure in childhood and hypertension risk in adulthood: a systematic review and meta-analysis. *J Hypertens.* 2020; 38: 2346-2355.
20. Urbina EM, Khoury PR, Bazzano L *et al*. Relation of blood pressure in childhood to self-reported hypertension in adulthood. *Hypertension.* 2019; 73: 1224-1230.
21. Song P, Zhang Y, Yu J *et al*. Global prevalence of hypertension in children: a systematic review and meta-analysis. *JAMA Pediatr.* 2019; 173: 1154-1163.
22. Williams B, Mancia G, Spiering W *et al*. 2018 ESC/ESH guidelines for the management of arterial hypertension. *Eur Heart J.* 2018; 39: 3021-3104.
23. Olsen MH, Angell SY, Asma S *et al*. A call to action and a lifecourse strategy to address the global burden of raised blood pressure on current and future generations: the *Lancet* Commission on hypertension. *Lancet.* 2016; 388: 2665-2172.
24. Martens DS, Sleurs H, Dockx Y, Rasking L, Plusquin M, Nawrot TS. Association of newborn telomere length with blood pressure in childhood. *JAMA Network Open.* 2022; 5: e2225521.
25. Sharma AK, Metzger DL, Rodd CJ. Prevelance and severity of high blood pressure among children based on the 2017 American Academy of Pediatrics Guidelines. *JAMA Pediatr.* 2018; 172: 557-565.

Data Supplement

Table S1. Blood Pressure Classification According to the 2017 American Academy of Pediatrics Guidelines

BP category	Systolic/Diastolic BP percentile [a]
Normal BP	<90th percentile
Elevated BP	≥90th to <95th percentile or 120/80 mm Hg to <95th percentile (whichever is lower)
Stage 1 Hypertension	≥95th to <95th percentile +12 mm Hg, or 130/80 mm Hg to 139/89 mm Hg (whichever is lower)
Stage 2 hypertension	≥95th percentile +12 mm Hg, or ≥140/80 mm Hg (whichever is lower)

Abbreviation: BP, blood pressure.
[a] Percentiles are age, sex, and height matched as described by Flynn et al., 2017, and categories used for ENVIRONAGE are based on the BP categories defined for the age range of 1-13 years in children.

Table S2. Blood Pressure Levels of Non-Tracker, Tracker, and Horse-racer in the Preschool and School-age Datasets

Variables [a]	n, %	Percentiles of MAP		MAP, mm Hg		SBP, mm Hg		DBP, mm Hg	
Preschool dataset (n=283)		Birth	FU1	Birth	FU1	Birth	FU1	Birth	FU1
Non-Tracker [a]	28 (9.89)	79.5 (13.4)	13.7 (10.2)	59.0 (4.77)	67.2 (3.20)	75.7 (5.45)	93.3 (5.10)	47.9 (5.66)	49.7 (4.07)
Tracker [b]	226 (79.9)	50.7 (27.7)	50.7 (26.7)	51.4 (7.72)	75.9 (7.45)	67.3 (8.43)	101.0 (8.10)	40.7 (7.87)	59.2 (8.89)
Horse-racer [c]	29 (10.2)	19.0 (14.2)	82.9 (12.3)	42.4 (5.35)	83.4 (8.85)	58.8 (6.15)	110.0 (7.65)	31.6 (5.78)	69.0 (10.8)
School-age dataset (n=272)		FU1	FU2	FU1	FU2	FU1	FU2	FU1	FU2
Non-Tracker [a]	25 (9.19)	83.9 (11.1)	17.5 (10.7)	80.4 (5.57)	75.1 (3.01)	104.9 (6.85)	99.2 (6.05)	64.0 (7.40)	59.0 (3.76)
Tracker [b]	221 (81.3)	50.4 (27.3)	50.2 (27.4)	72.9 (6.09)	82.1 (6.38)	99.2 (7.93)	107.7 (9.29)	55.3 (7.20)	65.0 (5.96)
Horse-racer [c]	26 (9.56)	17.7 (12.7)	83.2 (12.1)	66.2 (3.32)	89.6 (4.07)	91.3 (5.52)	115.5 (6.15)	49.6 (4.00)	72.4 (5.12)

Abbreviations: BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; FU, follow-up.

[a] Non-Tracker is the child with mean arterial pressure percentiles moving down for 2 or 3 quartiles (-51% to -100%) from birth to FU1, or from FU1 to FU2.

[b] Tracker is the child with mean arterial pressure percentiles at the same quartile, or moving up or down for 1 quartile (-50% to +50%) from birth to FU1, or from FU1 to FU2.

[c] Horse-racer is the child with mean arterial pressure percentiles moving up for 2 or 3 quartiles (+51% to +100%) from birth to FU1, or from FU1 to FU2.

Table S3. Characteristics of 283 Children in the Preschool Dataset

Characteristic	ENVIRONAGE visits		Δ (95% CI)
	Birth (n=283)	FU1 (n=283)	
Age, years	NA	4.79 (0.65)	4.78 (4.70, 4.85)
Girls, n (%)	147 (51.9)	147 (51.9)	…
Gestational age, weeks	39.2 (1.57)	…	…
Birth weight, grams	3384 (515)	…	…
Body weight, kg	…	19.6 (2.95)	16.2 (15.8, 16.5)
Body height, cm	50.2 (2.24)	109.5 (6.06)	59.3 (58.6, 60.0)
BMI, kg/m^2	13.4 (1.26)	16.3 (1.42)	2.88 (2.67, 3.08)
Overweight, n (%) [b]	…	52 (18.4)	…
Obesity, n (%) [b]	…	9 (3.18)	…
Blood pressure, mm Hg			
SBP	67.3 (8.82)	101.1 (8.65)	33.8 (32.5, 35.2)
DBP	40.5 (8.34)	59.3 (9.75)	18.8 (17.4, 20.2)
MAP	51.2 (8.16)	76.0 (8.36)	24.8 (23.6, 26.1)
Hypertension category, n (%)[a]			
Normal	…	186 (65.7)	…
Elevated	…	36 (12.7)	…
Stage-1 hypertension	…	46 (16.3)	…
Stage-2 hypertension	…	15 (5.30)	…

Abbreviation: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; FU, follow-up; Δ, change between birth and FU1; CI, confidence interval.

[a] As defined by 2017 American Academy of Pediatrics guideline using age-, sex-, and height-specific BP percentiles (Table S1 in the Supplement).

[b] As defined by age- and sex-specific BMI cut-offs according to the extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity.

Table S4. Characteristics of 272 Children in the School-Age Dataset

Characteristic	ENVIRONAGE visits		Δ (95% CI) [c]
	FU1 (n=272)	FU2 (n=272)	
Age, years	4.54 (0.40)	10.1 (0.82)	5.54 (5.45, 5.64)
Girls, n (%)	151 (55.5)	151 (55.5)	…
Gestational age, weeks	38.9 (1.93)	…	…
Birth weight, grams	3347 (513)	…	…
Body weight, kg	18.7 (2.46)	36.4 (8.62)	17.7 (16.9, 18.6)
Body height, cm	107.6 (4.89)	142.0 (7.64)	34.5 (33.8, 35.1)
BMI, kg/m^2	16.1 (1.26)	17.9 (3.05)	1.78 (1.48, 2.08)
Overweight, n (%) [b]	40 (14.7)	60 (22.1)	7.35 (1.83, 12.8)
Obesity, n (%) [b]	4 (1.47)	12 (4.41)	2.94 (0.27, 5.57)
Blood pressure, mm Hg			
SBP	99.0 (8.18)	107.7 (9.45)	8.71 (7.49, 9.93)
DBP	55.6 (7.65)	65.1 (6.41)	9.52 (8.38, 10.7)
MAP	72.9 (6.58)	82.1 (6.74)	9.20 (8.19, 10.2)
Hypertension category, n (%) [a]			
Normal	203 (74.6)	183 (67.3)	-7.35 (-13.9, -0.72)
Elevated	30 (11.0)	35 (12.9)	1.83 (-3.28, 6.93)
Stage-1 hypertension	33 (12.1)	37 (13.6)	1.47 (-3.49, 6.41)
Stage-2 hypertension	6 (2.21)	17 (6.25)	4.04 (0.62, 7.41)

Abbreviation: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; FU, follow-up; Δ, change between birth and FU1; CI, confidence interval.

[a] As defined by 2017 American Academy of Pediatrics guideline using age-, sex-, and height-specific BP percentiles (Table S1 in the Supplement).

[b] As defined by age- and sex-specific BMI cut-offs according to the extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity.

[c] Δ and 95% CI of categorised variables were presented as the change in percentage.

Table S5. Characteristics of 500 Mothers in ENVIRONAGE Birth Cohort

Characteristic	Mothers (n=500)
Age at delivery, years	30.9 (3.97)
Pre-pregnancy BMI, kg/m^2	24.5 (4.43)
Smoking during pregnancy, n (%)	
Non-smoker	384 (76.8)
Stopped	81 (16.2)
Continued	35 (7.00)
Education attainment, n (%)	
Low	18 (3.60)
Middle	131 (26.2)
High	351 (70.2)
Pregnancy hypertension or preeclampsia, n (%)	28 (5.60)
Pregnancy diabetes, n (%)	23 (4.60)
Postpartum BP, mm Hg [a]	
SBP	119.8 (10.9)
DBP	75.2 (9.06)
MAP	93.0 (9.13)

Abbreviation: BMI, body mass index; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; FU, follow-up.
[a] Postpartum blood pressure (n=283) was measured ±3 days after delivery.

Table S6. Sensitivity Analysis Accounting for Regression to Mean in Association between Follow-up Blood Pressure and Initial Blood Pressure

Variable	Unadjusted		Adjusted [c]		Fully adjusted [d]	
	Estimate (95% CI)	*P*	Estimate (95% CI)	*P*	Estimate (95% CI)	*P*
Preschool dataset (n=253) [a] From birth to FU1						
SBP, mm Hg	2.05 (0.43, 3.67)	0.013	1.78 (0.21, 3.36)	0.027	1.80 (0.20, 3.41)	0.028
DBP, mm Hg	1.77 (-0.38, 3.92)	0.11	1.74 (-0.41, 3.89)	0.11	1.72 (-0.44, 3.87)	0.12
MAP, mmHg	2.12 (0.21, 4.03)	0.030	1.93 (0.05, 3.82)	0.045	1.90 (-0.01, 3.80)	0.051
School-age dataset (n=246) [a] From FU1 to FU2						
SBP, mm Hg	4.88 (2.98, 6.78)	<0.0001	4.18 (2.29, 6.06)	<0.0001	3.67 (1.81, 5.53)	0.0001
DBP, mm Hg	1.21 (-0.52, 2.95)	0.17	1.36 (-0.37, 3.10)	0.13	1.22 (-0.53, 2.96)	0.17
MAP, mm Hg	3.42 (0.80, 6.04)	0.011	3.05 (0.48, 5.62)	0.020	2.39 (-0.15, 4.93)	0.065
Combined dataset (n=451) [b] From birth to FU1 and FU2						
SBP, mm Hg	3.08 (2.46, 3.71)	<0.0001	2.60 (1.34, 3.85)	0.0001	2.66 (1.41, 3.91)	0.0001
DBP, mm Hg	3.68 (2.54, 4.82)	<0.0001	1.66 (0.23, 3.10)	0.024	1.61 (0.17, 3.05)	0.029
MAP, mm Hg	4.82 (3.63, 6.02)	<0.0001	2.04 (0.53, 3.54)	0.0094	2.02 (0.52, 3.53)	0.0097

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; CI, confidence interval.

[a] Linear model was applied in the preschool and school-age datasets. 30 and 26 children were excluded, respectively, because the initial mean arterial pressure was lower than 5th percentile or higher than 95th percentile. The association size is estimate expressed as per standard deviation increase of the initial blood pressure.

[b] Mixed model was applied in the combined dataset. 49 children were excluded, because the initial mean arterial pressure was lower than 5th percentile or higher than 95th percentile. This model accounted for clustering of data within participants. The association size is estimate expressed as per standard deviation increase of the initial blood pressure.

[c] Adjusted models accounted for sex, age, initial body mass index, change in age, change in body height. For the analysis at birth, wakefulness of newborns (asleep *vs*. awake) was also adjusted.

[d] Fully adjusted models additionally accounted for birthweight, gestational age, maternal age at delivery, and pre-pregnancy BMI.

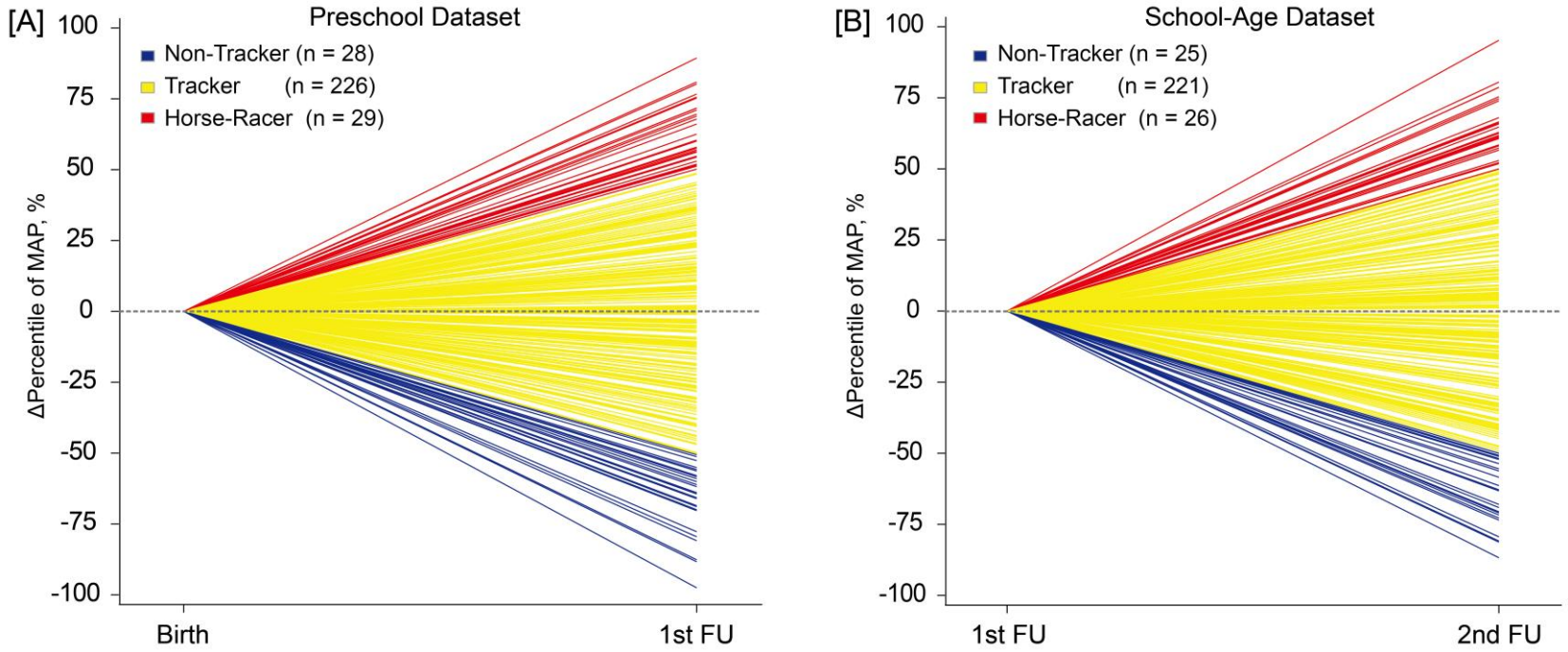


Figure S1. Changes of the Mean Arterial Pressure Percentile from Birth to the First Follow-Up (A) in the Preschool Dataset and from the First to the Second Follow-Up in the School-Age Dataset (B). Non-Tracker are the children with mean arterial pressure percentiles moving down for 2 or 3 quartiles (-51% to -100%) from birth to the 1st follow-up, and from the 1st to 2nd follow-up. Tracker are the children with mean arterial pressure percentiles at the same quartile, or moving up or down for 1 quartile (-50% to +50%) from birth to the 1st follow-up, and from the 1st to 2nd follow-up. Horse-racer are the children with mean arterial pressure percentiles moving up for 2 or 3 quartiles (+51% to +100%) from birth to the 1st follow-up, and from the 1st to 2nd follow-up. Abbreviations: Δ, change between follow-up visits. FU1, the first follow-up; FU2, the second follow-up.

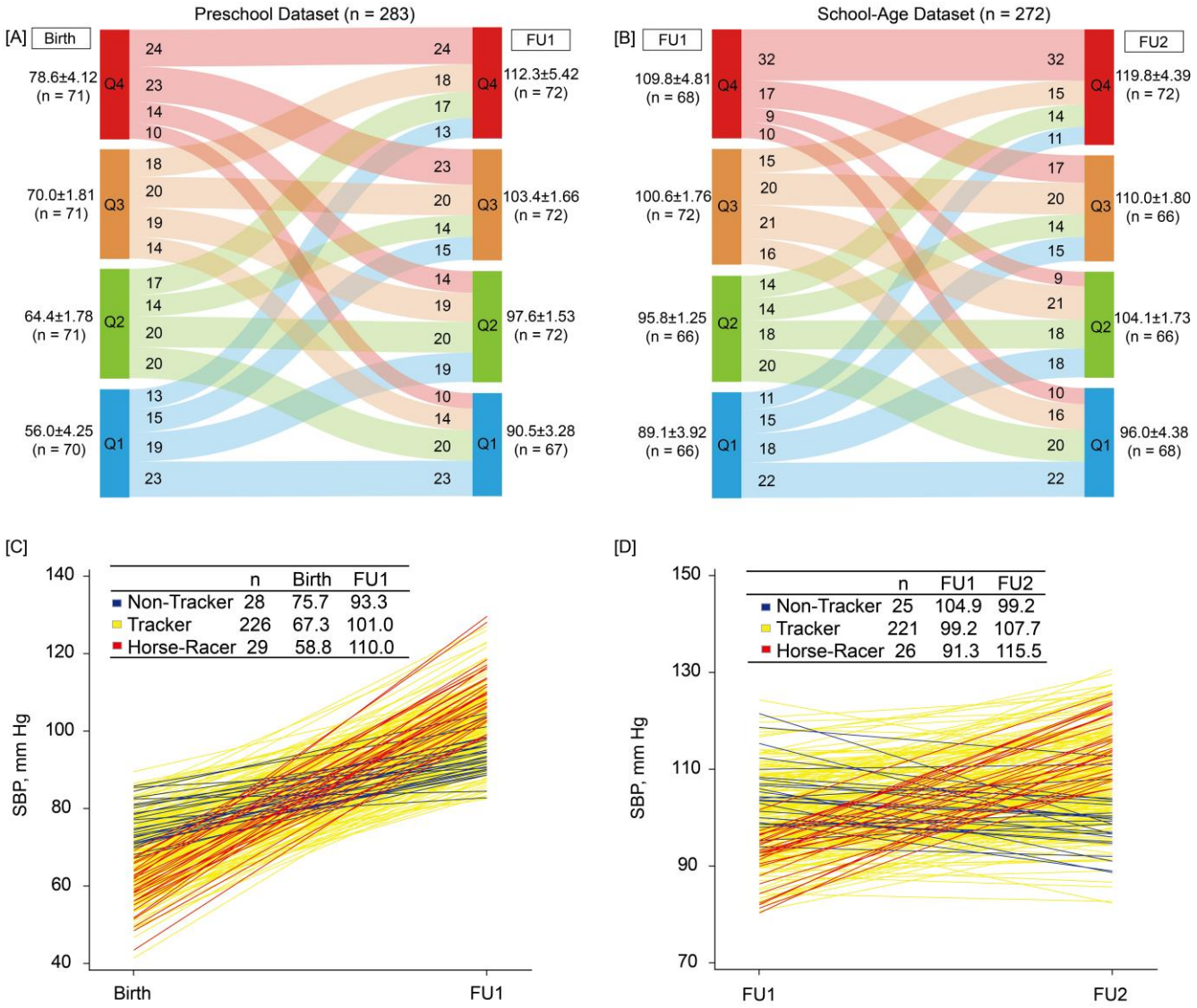


Figure S2. Tracking of Systolic Blood Pressure in the Preschool (A, C) and School-age (B, D) Datasets.

Sankey plots (A, B) depict the percentile changes of systolic blood pressure. Line graphs (C, D) depict the absolute changes of systolic blood pressure between two follow-up visits. Abbreviations: Q, quartile; FU1, the first follow-up; FU2, the second follow-up; n, number of children; SBP, systolic blood pressure.

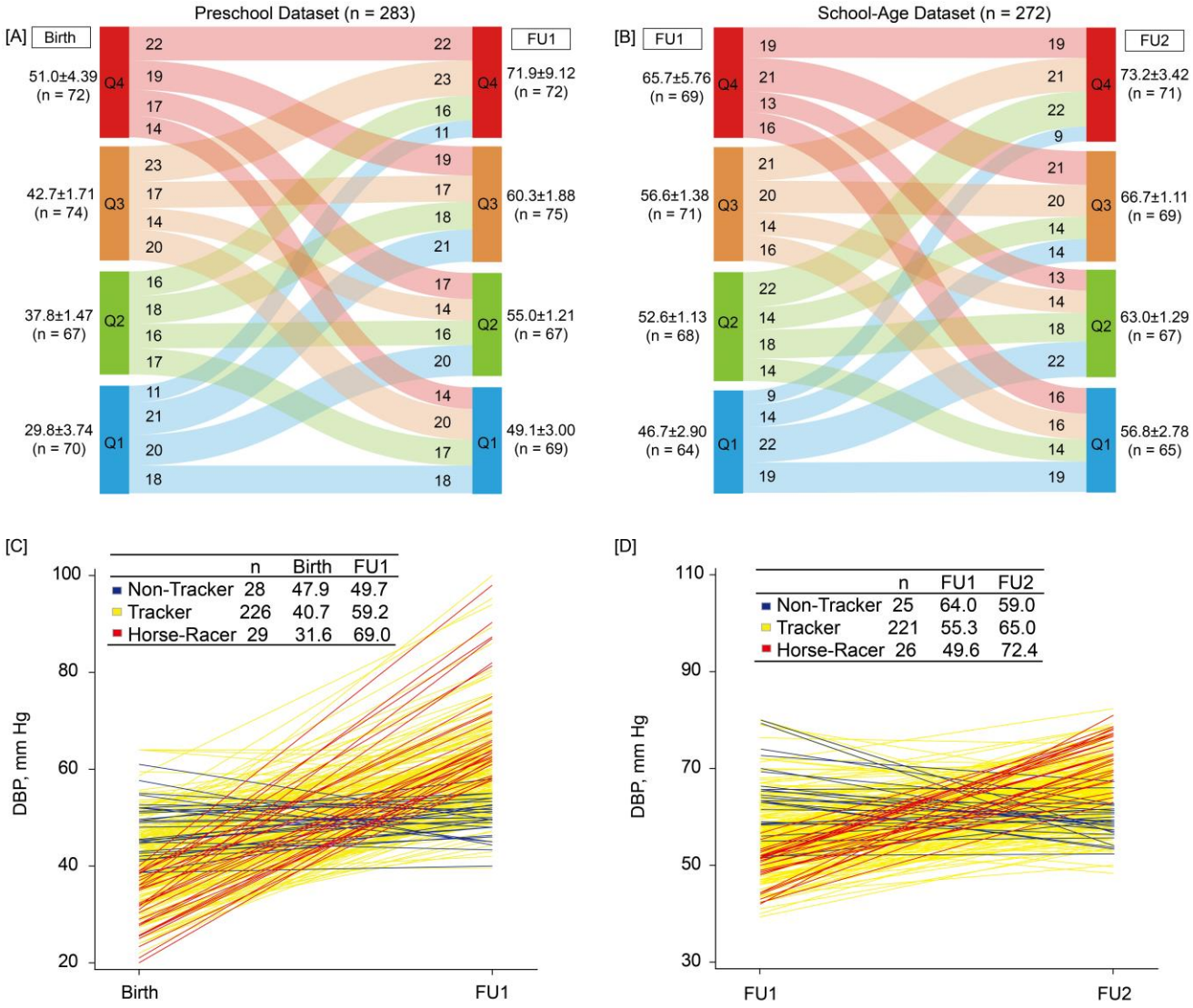


Figure S3. Tracking of Diastolic Blood Pressure in the Preschool (A, C) and School-age (B, D) Datasets.

Sankey plots (A, B) depict the percentile changes of diastolic blood pressure. Line graphs (C, D) depict the absolute changes of diastolic blood pressure between two follow-up visits. Abbreviations: Q, quartile; FU1, the first follow-up; FU2, the second follow-up; n, number of children; DBP, diastolic blood pressure.

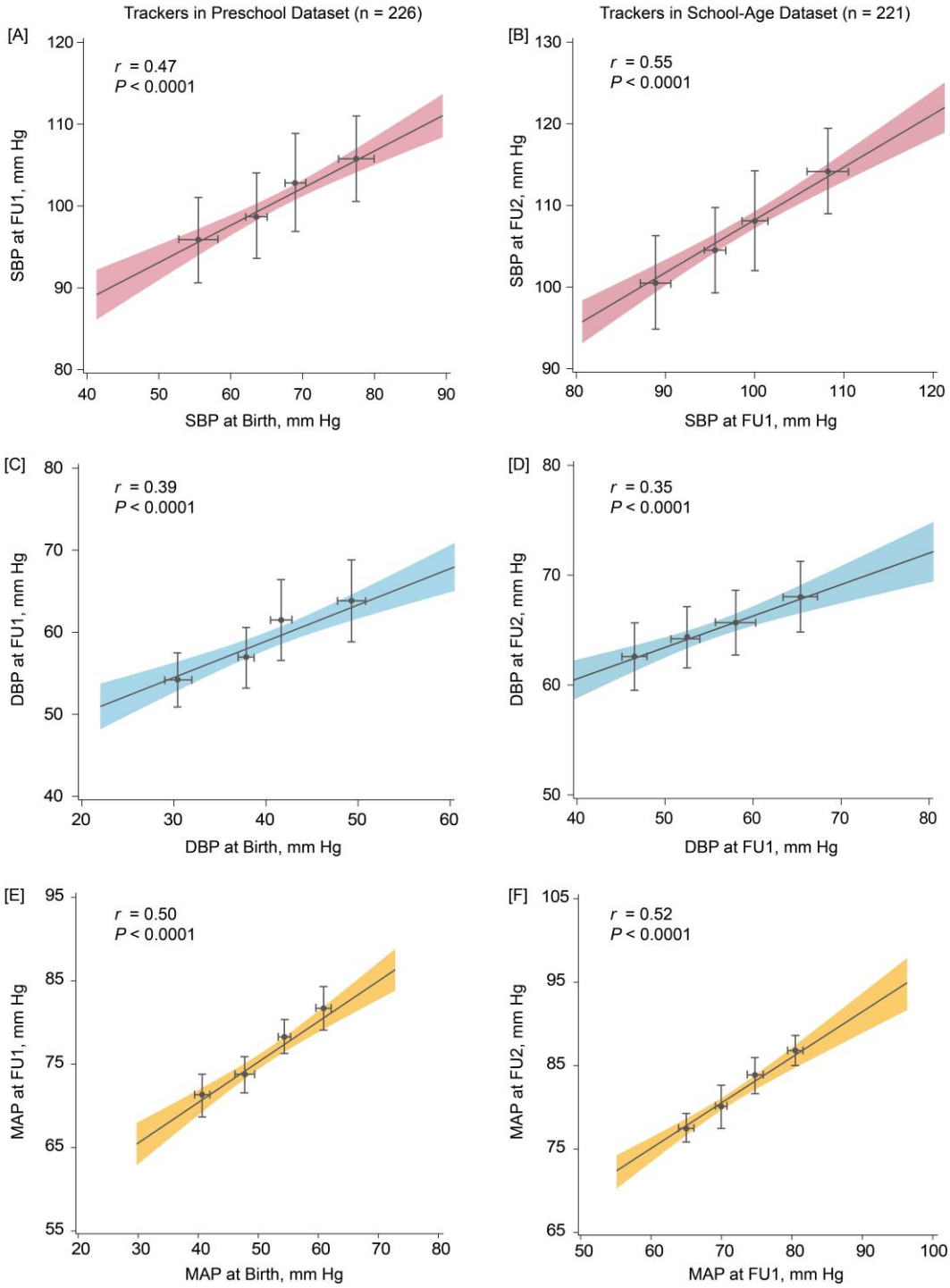


Figure S4. Relation of Systolic Blood Pressure (A,B), Diastolic Blood Pressure (C,D) and Mean Arterial Pressure (E,F) between Birth and the First Follow-Up (A,C,E) and between the First to the Second Follow-Up (B,D,F) in the trackers of the Preschool and School-Age Datasets. *n* and *r* refer to the number of children and to the correlation coefficients of the unadjusted linear regression models. To illustrate the fit of the linear model, the averages in mean arterial pressure were computed by quartiles and plotted over the regression lines given with 95% confidence interval. Categories were defined by the quartiles. Abbreviations: FU1, the first follow-up; FU2, the second follow-up; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

Chapter 4

Unhealthy ageing in relation to clinical risk factors

Part 2

Blood pressure and cardiovascular risk in relation to birth weight and urinary sodium: an individual-participant meta-analysis of European family-based population studies

Yu-Ling Yu*, Paula Moliterno*, De-Wei An, Anke Raaijmakers, Dries S. Martens, Katarzyna Stolarz-Skrzypek, Valérie Tikhonoff, Sofia Malyutina, Edoardo Casiglia, Babangida Chori, Jan Filipovský, Marek Rajzer, Karel Allegaert, Kalina Kawecka-Jaszcz, Peter Verhamme, Tim S. Nawrot, Jan A. Staessen†, José Boggia†

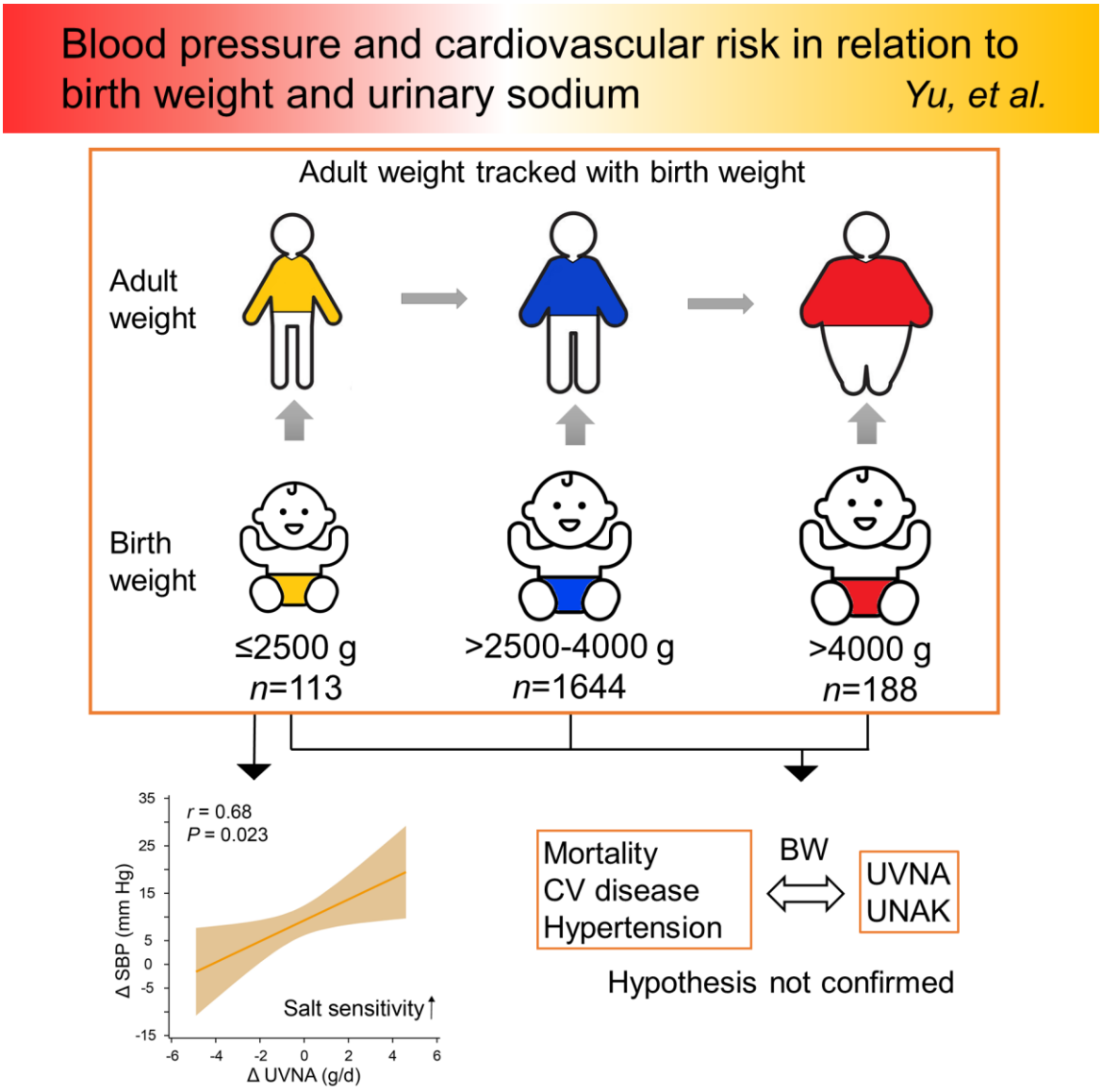
* Joint-first authors.

† Joint-last authors.

Previously published in:

Journal of Hypertension. 2023; 41:1175-1183.

Graphical abstract



Abstract

Background: While the relation of salt intake with blood pressure (BP) is linear, it is U-shaped for mortality and cardiovascular disease (CVD). This individual-participant meta-analysis explored whether the relation of hypertension, death or CVD with 24-h urinary sodium excretion (UVNA) or sodium-to-potassium (UNAK) ratio was modified by birth weight (BW).

Methods: Families were randomly enrolled in the Flemish Study on Genes, Environment and Health Outcomes (1985-2004) and the European Project on Genes in Hypertension (1999-2001). Categories of BW, UVNA and UNAK (≤2500, >2500-4000, >4000 g; <2.3, 2.3-4.6 and >4.6 g; and <1, 1-2 , >2, respectively) were coded using deviation-from-mean coding and analyzed by Kaplan-Meier survival functions and linear and Cox regression.

Results: The study population was subdivided into the Outcome (*n*=1945), Hypertension (*n*=1460) and BP cohorts (*n*=1039) to analyze the incidence of mortality and cardiovascular endpoints, hypertension and BP changes as function of UVNA changes. The prevalence of low/medium/high BW in the Outcome Cohort was 5.8/84.5/9.7%. Over 16.7 years (median), rates were 4.9%, 8.0% and 27.1% for mortality, CVD and hypertension, respectively, but were not associated with BW. Multivariable-adjusted hazard ratios were not significant for any endpoint in any of the BW, UVNA and UNAK strata. Adult body weight tracked with BW (P<0.0001). The partial r in the low BW group associating changes from baseline to follow-up in UVNA and systolic BP was 0.68 (P=0.023), but not significant in other BW groups.

Conclusion: This study did not substantiate its prior hypothesis, but showed tracking of adult with birth weight and suggest that low BW increases salt sensitivity.

Introduction

The World Health Organization [1,2], the United Nations Children's Fund [1], and the US Centers for Disease Control and Prevention [3] define birth weight (BW) of less than 1000, 1500 and 2500 gram as extremely low, very low and low, with normal and high BW ranging from 2500 up to 4000 gram and exceeding 4000 gram, respectively. High BW is a forerunner of obesity [4] and type-1 diabetes [5], which are important determinants of adult morbidity and mortality. Conversely, according to the developmental origins of disease hypothesis [6], undernutrition and an adverse intrauterine environment, on the background of genetic factors, slows fetal growth as an adaptive response that favors short-time survival at the expense of a larger susceptibility to disease later in life [7]. Furthermore, because incomplete nephron endowment [8], very low BW predisposes to hypertension. Eleven-year old children born with extremely low BW, compared with controls born at term, were 10 times more likely to have hypertension with preservation of the inverse association of plasma renin activity with blood pressure (BP), but not with 24-h urinary sodium excretion (UVNA) [9].

According to a systematic literature review [10], BP and the risk of CV complications increased when sodium intake exceeded 5g/day. In an individual-participant meta-analysis of European population studies [11], higher UVNA was associated with higher systolic BP (0.4 mm Hg per year per 2.3 g higher UVNA), but not with increased risk of hypertension or major CV events. The goal of the current analysis was to explore in the same data resource [11], however with longer follow-up of participants and updated endpoints, whether the associations of UVNA with BP and the risk hypertension, death and a composite CV endpoint were modified by BW.

Methods

Study cohorts

The population studies included in the current meta-analysis met the principles outlined in the Helsinki declaration for investigation of human participants [12]. The study protocols and the secondary analyses of anonymised data were approved by the competent local Institutional or National Review Boards. Participants gave written informed consent at recruitment and renewed consent at each follow-up visit.

Recruitment for the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) started in 1985 [13]. From August 1985 until November 1990, a random sample of the households living in a geographically defined area of Northern Belgium was investigated with the goal to recruit an equal number of participants in each of six subgroups by sex and age (20-39, 40-59, and ≥60 years). All household members with a

minimum age of 20 years were invited to take part, provided that the quota of their sex-age group had not yet been satisfied. From June 1996 until January 2004 recruitment of families continued, using the former participants (1985-1990) as index persons and also including teenagers [14]. The participants were repeatedly followed-up. In all study phases, the same standardized methods were applied to measure clinical and biochemical variables, to administer questionnaires, and to ascertain the incidence of fatal and nonfatal outcomes. The European Project on Genes in Hypertension (EPOGH) [15] recruited participants from 1999 until 2001 (**Table S1**). The EPOGH investigators were trained in Leuven, Belgium, and applied the same protocol, questionnaires and follow-up procedures, as used in FLEMENGHO. Questionnaires were translated from Dutch into Czech, Italian, Polish, and Russian and back translated into Dutch to check that all questions had the same meaning in all languages. At baseline the participants collected a 24-h urine sample. The last follow-up examination, which also included a 24-h urine sampling, took place from 1991 to 2017 in FLEMENGHO, and from 2006 until 2014 in EPOGH.

Overall, 6242 individuals were invited and 4712 participants consented to participate: 3343 in FLEMENGHO and 1369 in EPOGH (**Figure 1**). After excluding participants younger than 18 years and those with missing BW or no or inaccurate 24-h urine collection, the *Outcome Cohort* included 1945 individuals, who were available to investigate the incidence of mortality and major CV events. After applying similar exclusion criteria and excluding participants with hypertension at baseline or no follow-up BP measurement, 1460 participants were left to study the incidence of hypertension (*Hypertension Cohort*). After further exclusion of participants with no or inaccurate 24-h urine collection, the *Blood Pressure Cohort* with 1039 participants was analyzed to investigate the association between changes in BP and UVNA from baseline to follow-up. The mortality incidence rate in our study population was 3.8% in the median follow-up of 16.6 years. The mortality rate was 3.5 deaths per 1000-person per year in Belgium [16]. With α=0.05 and power of 0.95, the sample size should be 1479 or more in the study population for the analysis of mortality. In our study, the number of participants included in the *Outcome Cohort* reached the sample size. As proposed by the Framingham investigators, in participants on antihypertensive treatment at follow-up 10/5 mm Hg was added to the measured systolic/diastolic BP [17,18]. To address heaping of the reported BW and the potential overreporting of a BW of 2500 g, this threshold value was assigned to the category of low BW [19]. The predefined criteria to identify imprecise 24-h urine collections were: a volume of <300 mL per 24 h; 24-h creatinine excretion <450 mg (<4

mmol) or ≥2830 mg (≥25 mmol) in women or <680 mg (<6 mmol) or ≥3390 (≥30 mmol) in men [13]. After stratification for sex and age (<60 vs ≥60 years), urinary creatinine output was regressed in each stratum on body weight. Observations falling outside the 95% confidence interval for individual prediction of the creatinine excretion as function of body mass [20,21]. In analyses, in which the explanatory variable was the sodium-to-potassium ratio (UNAK), participants with inaccurate urine collection were not excluded, so that for the study of adverse health outcomes and hypertension, 2054 and 1516 participants were available and 1262 for the study of changes in BP in relation to UNAK changes (**Figure S1**).

Data collection at baseline and follow-up

Trained observers administered the same questionnaire at baseline and follow-up to collect each participant's self-reported BW, medical history, smoking status, drinking habits, medication use, and educational attainment. The BW questionnaire and the methods for urine collection and biochemical measurements are described in the Data Supplement. In each country, outcomes were adjudicated against relevant source documents. The vital status of FLEMENGHO and EPOGH participants was ascertained through December 31, 2016 and June 30, 2017, respectively. From the appropriate sources in each country, vital status was ascertained and the International Classification of Disease (ICD) codes for the immediate and underlying causes of death were collected. Physicians ascertained the diseases reported on the death certificates or collected by questionnaire or telephone interview against the medical records of general practitioners, hospitals or health insurers.

Endpoints were all-cause mortality, the composite of all fatal and nonfatal CV endpoints, and the incidence of hypertension. All fatal and nonfatal CV endpoints included stroke, coronary events, heart failure and other fatal and nonfatal CV events. Coronary events included sudden death, fatal and nonfatal myocardial infarction, acute coronary syndrome, hospitalization for unstable angina, and coronary revascularization. Fatal and nonfatal stroke did not include transient ischemic attacks. The other fatal or nonfatal events contributing to the mortality and the composite CV endpoint were heart failure, aortic aneurysm, pulmonary heart disease, pulmonary or arterial embolism, and peripheral arterial disease leading to amputation or revascularization. Heart failure was a clinical diagnosis or the diagnosis on the death certificate, but was in all cases validated against hospital files or the records held by family physicians. In all outcome analyses, only the first event within each category was considered. Participants free of events were censored at last follow-up.

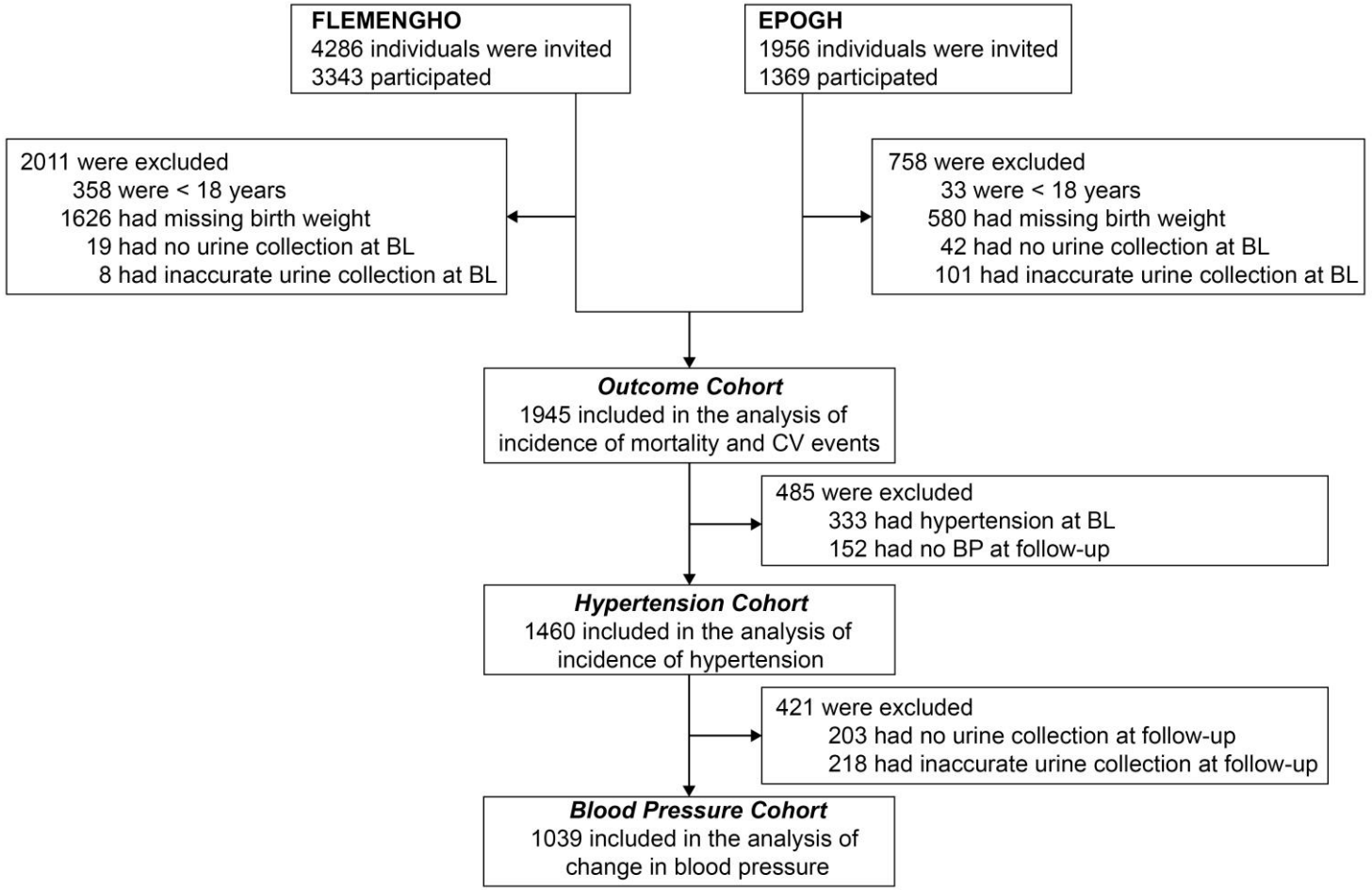


Figure 1. Flowchart for Participants in the Study. *Outcome Cohort* and *Hypertension Cohort* refer to participants used to study the incidence of mortality and morbidity of cardiovascular endpoints and the incidence of hypertension, respectively. The relation of the changes from baseline to follow-up in blood pressure with the changes in the 24-h urinary sodium excretion was investigated in the *Blood Pressure Cohort*. See Methods for the definition of inaccurate urine collections.

Statistical analysis

For database management and statistical analysis, SAS software, version 9.4 (maintenance level 5) was used. We reported the central tendency and spread of continuously distributed variables as mean and standard deviation (SD). Skewness and kurtosis were computed as the third and fourth moments about the mean divided by the cube of the SD. To compare means and proportions between groups, we applied the large sample z-test or ANOVA for continuously distributed variables, and Fisher exact test for categorical variables. Departure from normality was evaluated by the Shapiro-Wilk statistic. To assess longitudinal changes in continuously distributed and categorical variables, we used the t test and the McNemar test, respectively. After stratification for cohort and sex, we interpolated missing values of serum total and high-density lipoprotein (HDL) cholesterol, serum creatinine, blood glucose and the urinary sodium-to-potassium ratio from the regression slopes on age. In participants with unknown status of smoking or drinking and education attainment, we set the indicator (dummy) variable to the age- and sex-specific mean of the codes (0, 1). Statistical significance was a 2-sided probability of 0.05.

In exploratory analyses, we assessed the incidence of endpoints by categories of BW (≤2500, > 2500-4000, >4000 g) and the baseline UVNA (<2.3, 2.3-4.6, >4.6 g) [10] and UNAK (<1, 1-2, >2). UNAK was categorised, using similar percentile cut-offs as for the distribution of UVNA (15th and 70th percentile ranks). The 95% of rates were computed as $R \pm 1.96 \times \sqrt{(R \times [100 - R] / T)}$, where R is the rate and T is the number of participants at risk of developing an adverse outcome. Next, we plotted Kaplan-Meier survival function estimates for the endpoints under study. In Cox regression, we compared the risk in each category of BW, UVNA and the urinary sodium-to-potassium ratio with the average risk in the whole study population, using the deviation from mean coding [22]. This approach avoids to define a arbitrary reference group and generates confidence interval for all strata in the analysis [22]. All Cox models were accounted for clustering of the data within families as random effect. The covariables introduced as fixed effects in adjusted Cox models included recruitment frame (FLEMENGHO and EPOGH), sex, and the baseline variables age, mean arterial pressure, the serum total-to-HDL cholesterol ratio, blood glucose, educational attainment (elementary school *vs* secondary and high education) and the glomerular filtration rate estimated from serum creatinine. For analysis of the hypertension incidence, mean arterial pressure as baseline covariable was replaced by heart rate. In view of the small number of endpoints in some strata, covariables were combined by logistic regression in a propensity score, summarizing the probability of an endpoint as predicted by the covariables. The proportional hazards assumption was

checked by the Kolmogorov-type supremum test. To evaluate the correlation between the changes in BP and the changes in UVNA from baseline to follow-up, multivariable linear regression was applied adjusting for the aforementioned covariables.

Results

Data availability

All available data are shown within the article and the Data Supplement. Anonymised data are available from the corresponding author upon request, providing that an analysis plan is accompanying the request and that the principal investigators of each cohort approved data sharing.

Baseline characteristics in the Outcome Cohort

The number of missing values interpolated in the whole study population, which included 1332 FLEMENGHO and 613 EPOGH participants was 4 for serum total and HDL cholesterol and serum creatinine, 2 for blood glucose, and 5 for UNAK. Compared with FLEMENGHO (**Table 1**), EPOGH participants were 5.7 year younger (37.0 *vs* 31.3 y; *P*<0.001), had lower body mass index (24.9 *vs* 24.2 kg/m2; *P*=0.0018) and serum total cholesterol (202.2 *vs* 186.5 mg/dL; *P*<0.0001), but higher diastolic BP (75.2 *vs* 77.5 mm Hg; *P* < 0.0001), mean arterial pressure (90.7 *vs* 92.3 mm Hg; *P*=0.0019), heart rate (68.3 *vs* 73.7 beats per minute; *P*<0.0001) and higher glomerular filtration rate (eGFR) as estimated from serum creatinine (86.2 vs 96.7 mL/min/1.73 m^2; *P*<0.0001). Furthermore (**Table 1**), regarding the 24-h urinary measurements, volume (1.56 *vs* 1.38 L; *P*<0.0001), creatinine (1.38 *vs* 1.05 g; *P*<0.0001) and potassium (2.77 *vs* 1.84 g; *P*<0.0001) were higher in FLEMENGHO than EPOGH, whereas given similar UVNA (3.98 *vs* 4.04 g; *P*=0.40), UNAK was lower in FLEMENGHO than EPOGH (1.53 vs 2.42; *P*<0.0001). Sex differences were as reported in the literature (**Table 1**). The distributions of BW, UVNA and UNAK appear in **Figure S2**. The baseline characteristics of the *Hypertension* and *Blood Pressure Cohorts* mirrored those of the *Outcome Cohort*.

Exploratory analyses

The *Outcome Cohort*, included 185 singletons, and 1760 related participants belonging to 169 single-generation families and 221 multigeneration pedigrees. **Tables S2, S3, and S4** describe the trends of baseline characteristics across increasing categories of BW, UVNA and UNAK, respectively. A bar diagram shows the overlap of the categories of BW, UVNA and UNAK by cohort (**Figure S3**). Compared with the medium category, low BW (≤2500 g) was associated with a lower body height and eGFR (*P*≤0.039).

Table 1. Baseline characteristics by cohort and by sex (starts)

Characteristic	Cohort		Sex	
	FLEMENGHO	EPOGH	Women	Men
Number in group	1332	613	1036	909
Number with characteristic *n* (%)				
FLEMENGHO	1332 (100%)	0	683 (65.9%)	649 (71.4%)†
EPOGH	0	613 (100%)	353 (34.1%)	260 (28.6%)†
Women	683 (51.3%)	353 (57.6%)†	1036 (100%)	0
Men	649 (48.7%)	260 (42.4%)†	0	909 (100%)
Hypertension	221 (16.6%)	112 (18.3%)*	143 (13.8%)	190 (20.9%)‡
Treated hypertension	84 (6.3%)	25 (4.1%)	67 (6.5%)	42 (4.6%)
Diabetes mellitus	17 (1.3%)	6 (1.0%)	15 (1.4%)	8 (0.9%)
Smokers	356 (26.7%)	156 (25.6%)	240 (23.2%)	272 (30.0%)‡
Drinking	340 (25.5%)	198 (32.5%)†	160 (15.5%)	378 (41.7%)‡
Educational attainment				
Elementary school	81 (6.1%)	49 (8.0%)	75 (7.2%)	55 (6.1%)
Secondary school	906 (68.0%)	409 (66.7%)	680 (65.6%)	635 (69.9%)
Higher education	345 (25.9%)	155 (25.3%)	281 (27.1%)	219 (24.1%)
Mean of characteristic				
Age (y)	37.0 ± 11.5	31.3 ± 11.7‡	35.2 ± 11.6	35.2 ± 12.1
Birth weight (g)	3341 ± 580	3398 ± 573*	3275 ± 550	3454 ± 595‡
Body weight (kg)	72.7 ± 14.4	70.0 ± 14.3‡	65.2 ± 12.8	79.4 ± 12.2‡
Body height (cm)	170.7 ± 9.2	169.8 ± 9.4*	164.2 ± 6.2	177.5 ± 6.7‡
Body mass index (kg/m^2)	24.9 ± 4.1	24.2 ± 4.3†	24.2 ± 4.5	25.2 ± 3.6‡
Systolic pressure (mm Hg)	121.6 ± 13.3	121.9 ± 15.6	117.9 ± 14.1	126.0 ± 12.7‡
Diastolic pressure (mm Hg)	75.2 ± 9.9	77.5 ± 10.6‡	74.2 ± 9.7	77.9 ± 10.3‡
Mean arterial pressure (mm Hg)	90.7 ± 10.0	92.3 ± 11.5†	88.8 ± 10.4	93.9 ± 9.9‡
Heart rate (beats per minute)	68.3 ± 9.2	73.7 ± 9.7‡	71.8 ± 9.4	67.9 ± 9.6‡

Table 1. Baseline characteristics by cohort and by sex (continued)

Characteristic	Cohort		Sex	
	FLEMENGHO	EPOGH	Women	Men
Serum total cholesterol (mg/dL)	202.2 ± 40.6	186.5 ± 42.3‡	195.9 ± 39.7	198.7 ± 43.9‡
Serum HDL cholesterol (mg/dL)	55.2 ± 15.6	51.9 ± 16.2‡	59.5 ± 16.5	48.1 ± 12.7‡
Total-to-HDL cholesterol ratio	3.96 ± 1.54	3.93 ± 1.56	3.53 ± 1.22	4.43 ± 1.73‡
Blood glucose (mg/dL)	90.5 ± 20.4	85.5 ± 15.4‡	88.8 ± 20.1	89.1 ± 17.9
Serum creatinine (mg/dL)	1.00 ± 0.16	0.92 ± 0.17‡	0.89 ± 0.13	1.06 ± 0.15‡
eGFR (mL/min/1.73 m^2)	86.2 ± 15.3	96.7 ± 18.0‡	86.8 ± 17.1	92.6 ± 16.2‡
24-h urinary measurements				
Volume (L)	1.56 ± 0.69	1.38 ± 0.51‡	1.51 ± 0.68	1.50 ± 0.61
Sodium (g)	3.98 ± 1.64	4.04 ± 1.48	3.61 ± 1.40	4.44 ± 1.68‡
Potassium (g)	2.77 ± 1.04	1.84 ± 0.73 ‡	2.27 ± 0.89	2.72 ± 1.15‡
Sodium-to-potassium ratio	1.53 ± 0.68	2.42 ± 1.26‡	1.78 ± 0.98	1.84 ± 1.00
Creatinine (g)	1.38 ± 0.44	1.05 ± 0.41‡	1.05 ± 0.32	1.54 ± 0.45‡
Median follow-up IQR (y)	18.7 (15.4-22.5)	13.9 (13.1-14.4)‡	16.6 (13.9-21.4)	16.6 (13.9-21.7)

Body mass index was weight in kilograms divided by height in meters squared. BP was the average of 5 auscultatory readings. Hypertension was a BP of ≥140 mm Hg systolic or ≥90 mm Hg diastolic or being on antihypertensive treatment. Mean arterial pressure was diastolic pressure plus one third of pulse pressure. Diabetes was a self-reported diagnosis, use of antidiabetic drugs, fasting blood glucose of ≥126 mg/dL (≥7.0 mmol/L), random blood glucose of ≥200 mg/dL (≥11.1 mmol/L), or diabetes documented in practice or hospital records. Smoking was the use of smoking materials on a daily basis. Drinking was an average alcohol intake of 5 g or more per day.

SI conversion factors: to convert cholesterol from mg/dL to mmol/L, multiply by 0.0259; glucose from mg/dL to mmol/L, multiply by 0.0555, serum creatinine from mg/dL to µmol/L, multiply by 88.42; urinary sodium, potassium and creatinine from g to mmol, multiply by 43.497, 25.577, and 8.840, respectively.

Significance of the between-group differences: * $P \leq 0.05$; † $P \leq 0.01$; ‡ $P \leq 0.001$.

FLEMENGHO, Flemish Study on Environment, Genes and Health Outcomes; EPOGH, European Project on Genes in Hypertension; HDL, high-density lipoprotein; eGFR, glomerular filtration rate estimated from serum creatinine, using the Chronic Kidney Disease Epidemiology Collaboration equation; IQR, interquartile range.

Compared with the high category, low BW (≤2500 g) was associated with a higher female-to-male sex ratio, lower body weight and body height (*P*<0.0001). **Figure 2** illustrates tracking of BW with body weight during adult live; adult weight averaged 69.1 kg in the low BW category and 77.7 kg in participants with high BW.

Low UVNA (<2.3 g), compared with the medium and high UVNA categories combined, was associated with a higher female-to-male sex ratio, lower body weight, smaller body height, lower systolic BP, lower total-to HDL-cholesterol ratio, potassium excretion, UNAK, and creatinine excretion (*P*≤0.036; **Table S3**). Low UNAK (<1), compared with the medium UNAK category was associated with a higher female-to-male sex ratio and higher age (*P*≤0.030); and compared with the high UNAK category was associated with a higher female-to-male sex ratio, higher age, lower heart rate, higher blood glucose, lower eGFR, and higher urinary volume (*P*≤0.010; **Table S4**).

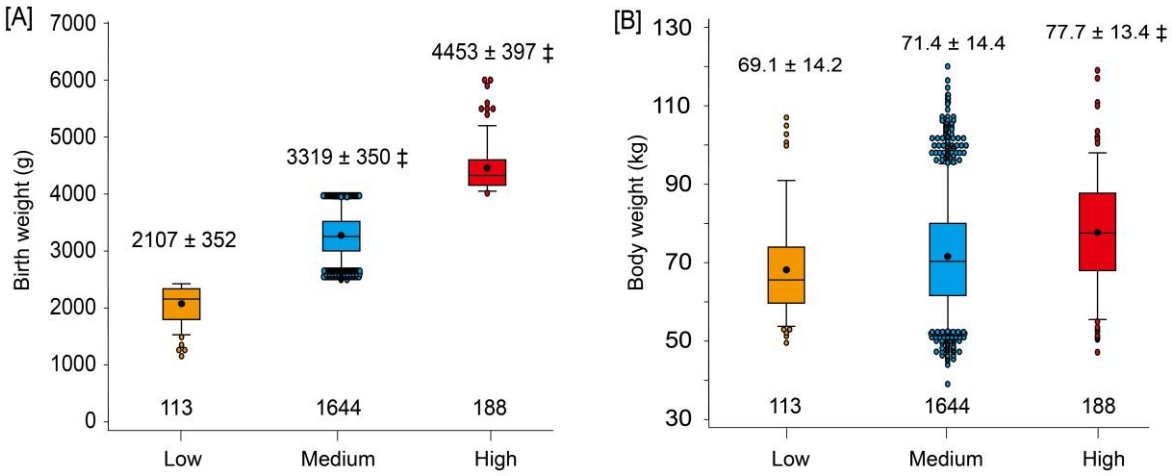


Figure 2. Boxplots showing the distributions of birth weight (A) and adult body weight (B) at baseline in the low (≤ 2500 g; yellow), medium (> 2500-4000 g; blue) and high (> 4000g; red) birth weight categories. The central line, the upper and lower lines, and the upper and lower caps represent the median, interquartile range, and the 5th to 95th percentile interval. The arithmetic means and extreme measurements are represented by circles inside the box and outside the whiskers, respectively. The arithmetic means and standard deviations are given above/beneath the whiskers. The number of the participants in each category is given along the horizontal axis. Significance of the difference with the medium group: ‡ *P* ≤ 0.001.

Incidence of endpoints

Of 1945 participants making up the *Outcome Cohort* (**Table S5**), over a median follow-up of 16.6 years (IQR: 13.9-21.6 years; 5th-95th percentile interval: 11.3-32.1 years), 73 (3.8%) died and 120 (6.2%) experienced a major CV endpoint. Of 1460 participants enrolled in the *Hypertension Cohort*, over a median follow-up of 16.8 years (IQR: 14.0-21.9 years; 5th-95th percentile interval: 12.1-32.6 years), 396 (27.1%) became hypertensive, because systolic BP was ≥140 mm Hg (n=106), or diastolic BP was ≥90 mm Hg (n=149), or both systolic and diastolic BP exceeded these thresholds (n=120), or because at follow-up they were on antihypertensive drug treatment irrespective of the BP level (n=24).

Unadjusted analyses

In unadjusted survival function analyses of the *Outcome Cohort* (**Figure 3**), participants with low or medium BW experienced lower mortality compared with the high BW group (*P*=0.004), whereas the incidence of a major CV endpoint was lower in the medium BW group, compared with the low and high birth weight groups (*P*=0.006). The gradients in absolute risk across the categories of BW, UVNA and UNAK, expressed as incidence rates per 1000 person-years, are presented in **Table S6** for women, men and both sexes combined. Among women, across increasing categories of BW, the incidence of total mortality and hypertension increased. In the low, medium and high BW categories, the death rates were 2.82, 3.21 and 3.51 fatalities per 1000 person-years (*P*=0.048) and the incidence rates of hypertension 18.9, 19.6 and 20.5 cases per 1000 person-years (*P*=0.046). In women, the hypertension incidence rates tended to decrease across increasing categories of UVNA (24.6, 19.2 and 16.1 cases per 1000 person years; *P*=0.099) and increasing UNAK (22.8, 20.0 and 16.2 cases per 1000 person-years; *P*=0.056). However, none of these trends reached significance in men (*P*≥0.14). In both sexes combined, the hypertension incidence rates decreased with higher UNAK (30.7, 26.9 and 24.1 cases per 1000 person-years; *P*=0.056).

Proportional hazards regression

Using the deviation from mean coding, hazard ratios expressed the risk compared the average risk in the BW, UVNA and UNAK categories. The unadjusted hazard ratios expressing the risk of death, a major CV endpoint or incident hypertension in relation to BW (**Table S7**), UVNA (**Table S7**) or UNAK (**Table S8**) were mostly not significant. However, in the medium BW group (**Tables S7** and **S8**), the crude hazard ratios were 0.66 (CI: 0.45-0.97; *P*=0.035) for mortality and 0.68 (CI: 0.51-0.90; *P*=0.0082) for the CV endpoint. In the high BW group, the crude hazard ratio for a CV endpoint was 1.67 (CI:

1.06-2.61; *P*=0.027). However, adjustment removed the significance of these hazard ratios.

Relation between changes in BP and UVNA

In the *Blood Pressure Cohort*, the association of the changes from baseline to follow-up in systolic BP (**Figure 4**) or diastolic BP (**Figure S6**) complied with a linear model. With adjustments applied for recruitment frame (FLEMENGHO *vs* EPOGH), sex, and the baseline variables age, heart rate, the serum total-to-HDL cholesterol ratio, blood glucose, educational attainment (elementary school vs secondary and high education) and the glomerular filtration rate estimated from serum creatinine, the partial correlation coefficient in the low BW group associating the change in systolic BP with the change in UVNA was 0.68 (*P*=0.023); the corresponding correlation coefficients in the medium and high BW categories were 0.32 (*P*=0.69), and -0.45 (*P*=0.20), respectively. In a sensitivity analysis, in which changes in systolic BP and UVNA were expressed as a percentage of the baseline value, the correlation coefficients in the low, medium and high BW categories were 0.55 (*P*=0.15), 0.30 (*P*=0.97), and -0.33 (*P*=0.27), respectively. In similar analyses correlating the changes in diastolic BP with the changes in UVNA, none of the partial correlation coefficients reached significance.

Discussion

The goal of the current study was to explore in an individual-participant meta-analysis of two family-based European population studies, whether the risk of incident hypertension, death or a composite CV endpoint were modified by BW. In unadjusted survival function analyses (**Figure 3**), participants with low or medium BW experienced lower mortality compared with the high BW group, whereas the incidence of a major CV endpoint was lower in the medium BW group, compared with the low and high birth BW groups. However, absolute risk as captured by the incidence rates of these events and adjusted relative risk as captured by hazard ratios were not significant across the categories of BW, UVNA and UNAK. However, the study generated two relevant observations, one confirmatory and one to our knowledge novel. In line with the literature [23-25], adult body weight tracked with BW (**Figure 2**). Second, in multivariable-adjusted analyses of the low BW group there was a positive association between the changes from baseline to follow-up in UVNA and the corresponding changes in systolic BP (**Figure 4**).

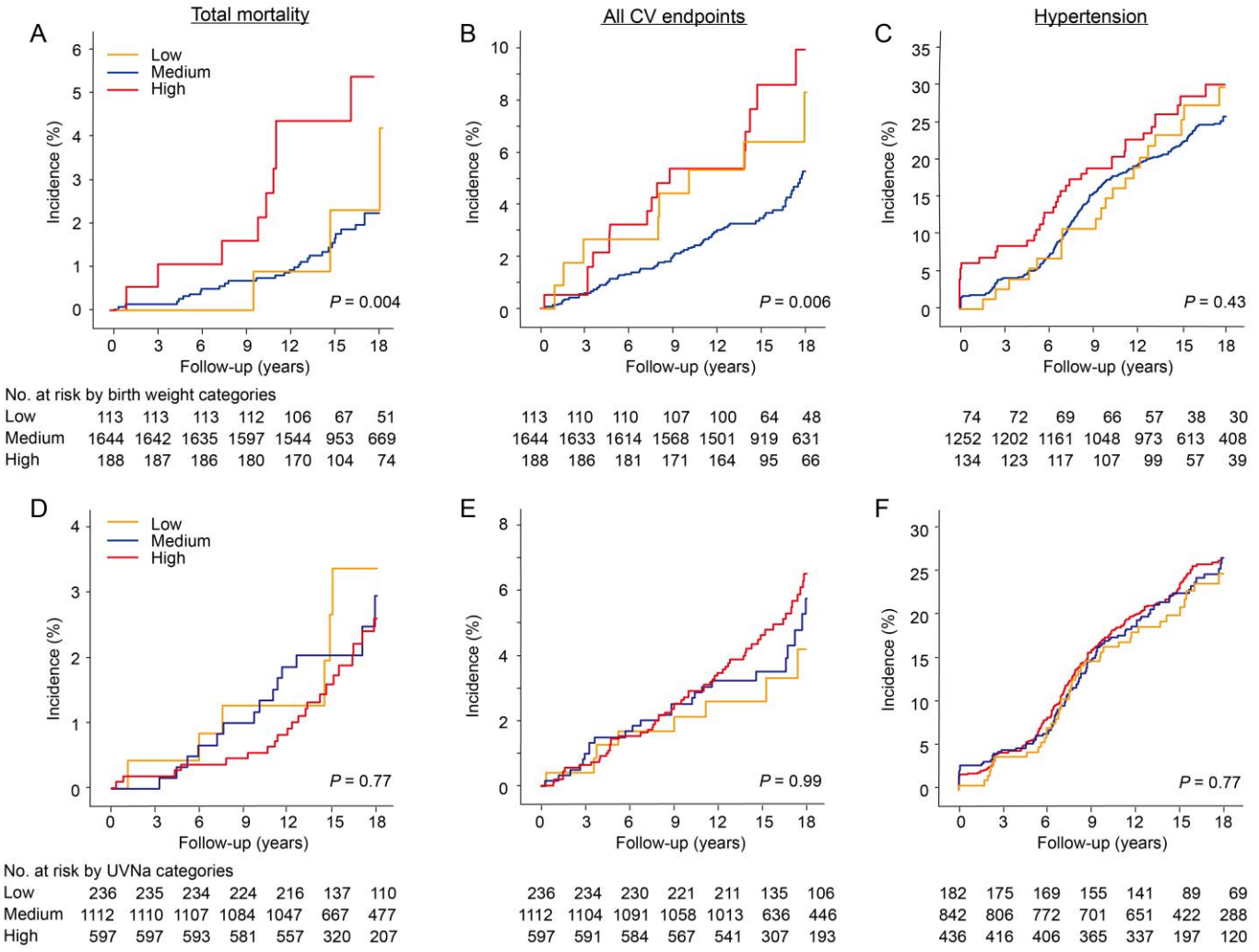


Figure 3. Kaplan-Meier survival function estimates for the incidence of total mortality, all cardiovascular endpoints and hypertension. The yellow, blue and red lines in panels A, B and C represent the low, medium and high categories of birth weight (≤2500, >2500-4000 and >4000 g). The yellow and red lines in panels D, E and F represent the low, medium and high categories of 24-h urinary sodium (<2.3, 2.3-4.6, >4.6 gram per day. The results for all-cause mortality and all cardiovascular endpoints were obtained in the *Outcome Cohort*, and the results for hypertension in the *Hypertension Cohort*. *P*-values indicate the significance of log-rank test.

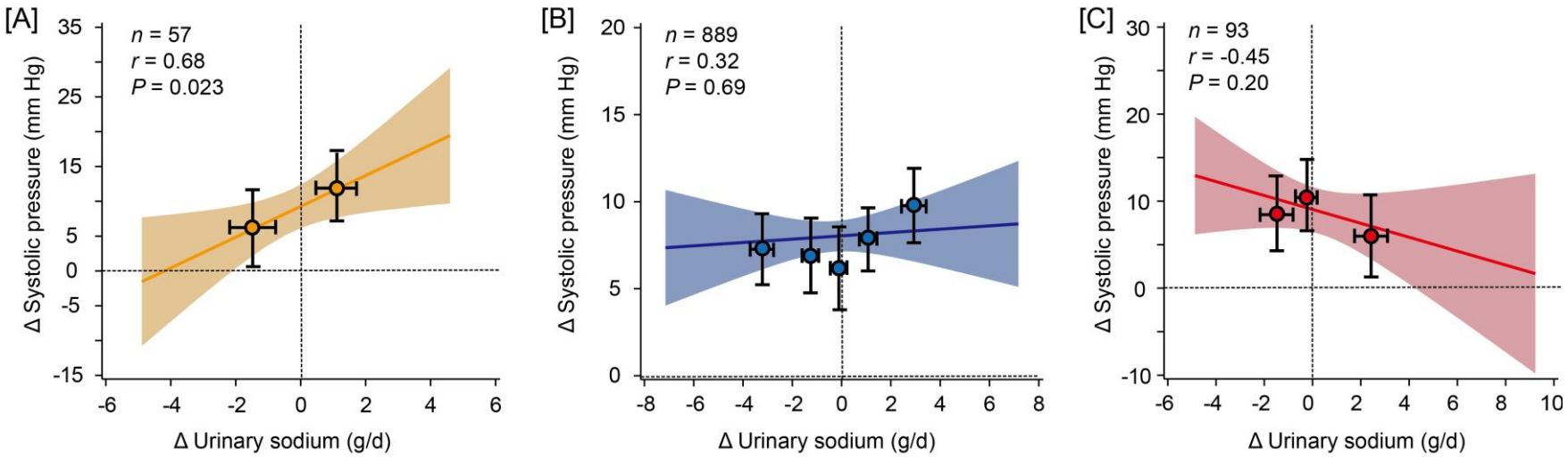


Figure 4. Relation between the changes from baseline to follow-up in systolic blood pressure and the changes in 24-h urinary sodium excretion in the low (A), medium (B), and high (C) birth weight categories (≤2500, >2500-4000, >4000 g). *n* and *r* refer to the number of participants in each group and to the partial correlation coefficients with adjustments applied for recruitment (FLEMENGHO *vs* EPOGH), sex, and the baseline variables age, heart rate, the serum total-to-HDL cholesterol ratio, blood glucose, educational attainment (elementary school vs secondary and high education) and the glomerular filtration rate estimated from serum creatinine. To illustrate the fit of the linear model, the average changes in systolic blood pressure and in 24-h urinary sodium were computed by quantiles and plotted over the regression lines given with 95% confidence interval. Categories were defined by the median, quintiles and tertiles in the low, medium and high birth weight groups, respectively. The results were obtained in the *Blood Pressure Cohort* (see Figure 1).

Prematurity as captured by low birth weight disrupts the perinatal maturation of the microcirculation and impairs arterial structure/function later in life, as exemplified by the narrower retinal arteriolar diameters and sparser retinal fractals in extremely low birth weight children and young adults compared to controls born at term [26]. Other adverse health outcomes associated with low BW are hypertension [9] and high CV disease risk in young adulthood [23-25]. In a systematic review [27], the pooled hazard ratios expressing the risk per 1000-gram BW decrements were 1.06 (CI: 1.03-1.09) for total mortality (36,834 deaths among 394,062 participants followed up) and 1.14 (CI: 1.10-1.18) for fatal CV events (11,366 deaths per 325,982 participants). Furthermore, exposure to CV risk factors in childhood promotes the development of vascular changes that launch young adults towards a trajectory of premature vascular aging. In the Young Finns Study [28], risk factor load at age 3-18 years predicted carotid intima-media thickening, higher aortic pulse wave velocity, hypertension, and loss of carotid distensibility on re-examination 21 years later. Confirmatory findings were reported in other birth cohorts [23-25,27-29]. Tracking of adult body weight with BW as observed in the current study (**Figure 2**) is in keeping with the literature [23-25].

Brenner was the first to offer a possible explanation for the inverse association between blood pressure and birth weight [30]. According to his hypothesis, hypertension supervenes when the number of nephrons decreases either by acquired disease during adult life, unilateral nephrectomy, for instance for allograft transplantation, or impaired nephron development during intra-uterine life. Two studies investigated postnatal glomerulogenesis either in premature infants weighing less than 1000 g [31] or after preterm birth with a gestational age of less than 35 weeks [32]. In 56 extremely low birth weight infants and 10 full term controls, glomerulogenesis, as measured by computer-assisted radial glomerular counts was markedly decreased in all preterm infants and correlated significantly with gestational age ($r=0.87$; $P<0.001$) [33]. Active glomerulogenesis was absent in preterm infants surviving for 40 days or longer and in all term infants [34]. In the second study [32], renal maturation accelerated in 22 infants after preterm birth, with an increased number of glomerular generations and a decreased width of the nephrogenic zone in kidneys of preterm neonates. According to Guyton's model [35], a smaller number of nephrons necessitates a higher blood pressure to excrete sodium. Our current findings as shown in **Figure 4** are in line with the concept that low BW is associated with fewer functional nephrons, thereby increasing their susceptibility to sodium driven hypertension.

Limitations

This current study must be interpreted within the context of its limitations. First, although the *Outcome Cohort* and the *Hypertension Cohort*, included 1945 and 1460 participants, respectively, the present sample size was substantially smaller than in the aforementioned meta-analysis with over 325,000 study subjects [27]. Lack of power probably explain why the trends in absolute and adjusted relative risk associated with BW, UVNA and UNAK did not reach significance. Second, BW was collected by self-administered question instead of birth certificates. In major epidemiological studies, such as the Nurses' Health Study [36], and the Health Professionals Follow-Up Study [37], BW were collected by questionnaire, telephone interviews [38] or self-administered questionnaires [36,37], which did not limit the reliability and accuracy of the self-reported data. Third, we did not assess the gestational age or evaluate the impact of post-natal growth in early life, which are the independent risk factors of CV disease [39]. Fourth, although low BW seems to enhance salt sensitivity, no objective measure of salt sensitivity was studied. Fifth, Considering individual study participants, there is substantial diurnal variation in the 24-h urinary sodium excretion [40]. However, sodium excretion averaged over a large number of individuals, as was done in the current study, does characterise the sodium excretion reproducibly, and by extension the dietary salt intake. Moreover, in our study, the gold standard for quantifying urinary sodium excretion was applied [10]. In addition, all FLEMENGHO and EPOGH participants were white Europeans, so that the current findings might not be extrapolated to other ethnicities, who tend to be more salt sensitive, such as Asian [41] and Blacks [42].

Conclusions

This individual-participant meta-analysis of two family-based European population studies, did not substantiate the prior hypothesis that the risk of incident hypertension, death or a composite CV endpoint were modified by BW. In line with the literature [23-25], adult body weight tracked with BW. In multivariable-adjusted analyses of the low BW group, there was a positive association between the changes from baseline to follow-up in UVNA and the corresponding changes in systolic BP, suggesting that low BW increases salt sensitivity.

References

1. Wardlaw T, Blanc A, Zupan J, Åhman E, World Health Organization, United Nations Children's Fund. *Low Birthweight - Country, Regional and Global Estimates*, New York, NY, USA / Geneva Switzerland: United Nation's Children's Fund / World Health Organization; 2004.
2. Branca F, De-Regil LM, Engesveen K, Garcia-Casal MN, Kennedy S, Lombardo L, et al. *Global nutrition targets 2025: low birth weight policy brief (WHO/NMH/NHD/14.5).*, Geneva, Switzerland: World Health Organization; 2014.
3. Martin JA, Hamilton BE, Osterman MJK, Curtin SC, Mathews TJ. Births: final data for 2013. *Natl Vital Stat Rep.* 2015, 64:1-65.
4. Schellong K, Schulz S, Harder T, Plagemann A. Birth weight and long-term overweight risk: systematic review and a meta-analysis including 643,902 persons from 66 studies and 26 countries globally. *PLoS One.* 2012, 7:e47776
5. Cardwell CR, Stene LC, Joner G, Davis EA, Cinek O, Rosenbauer J*, et al.* Birthweight and the risk of childhood-onset type 1 diabetes: a meta-analysis of observational studies using individual patient data. *Diabetologia.* 2010, 53:641-651.
6. Barker DJP, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. *Lancet.* 1993, 341:938-941.
7. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA*, et al.* Developmental plasticity and human health. *Nature.* 2004, 430:419-421.
8. Luyckx VA, Perico N, Somaschini M, Manfelloto D, Valensise H, Cetin I*, et al.* A developmental approach to the prevention of hypertension and kidney disease: a report from the Birth Weight and Nephron Number Working Group. *Lancet.* 2017, 390:424-428.
9. Raaijmakers A, Zhang ZY, Claessens J, Cauwenberghs N, van Tienoven TP, Wei FF*, et al.* Does extremely low birth weight predispose to low-renin hypertension? *Hypertension.* 2017, 69:443-449.
10. O'Donnell M, Mente A, Alderman MH, Brady AJB, Diaz R, Gupta R*, et al.* Salt and cardiovascular disease: insufficient evidence to recommend low sodium intake. *Eur Heart J.* 2020, 41:3363-3373.
11. Stolarz-Skrzypek K, Kuznetsova T, Thijs L, Tikhonoff V, Seidlerová J, Richart T*, et al.* Fatal and nonfatal outcomes, incidence of hypertension and blood pressure

changes in relation to urinary sodium excretion. *J Am Med Assoc.* 2011, 305:1777-1785.

12. World Medical Association. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. *J Am Med Assoc.* 2013, 310:2191-2194.
13. Staessen J, Bulpitt CJ, Fagard R, Joossens JV, Lijnen P, Amery A. Familial aggregation of blood pressure, anthropometric characteristics and urinary excretion of sodium and potassium - a population study in two Belgian towns. *J Chron Dis.* 1985, 38:397-407.
14. Staessen JA, Wang JG, Brand E, Barlassina C, Birkenhäger WH, Herrmann SM*, et al.* Effects of three candidate genes on prevalence and incidence of hypertension in a Caucasian population. *J Hypertens.* 2001, 19:1349-1358.
15. Kuznetsova T, Staessen JA, Kawecka-Jaszcz K, Babeanu S, Casiglia E, Filipovský J*, et al.* Quality control of the blood pressure phenotype in the European Project on Genes in Hypertension. *Blood Press Monit.* 2002, 7:215-224.
16. GBD 2019 Demographics Collaborators. Global age-sex-specific fertility, mortality, healthy life expectancy (HALE), and population estimates in 204 countries and territories, 1950-2019: a comprehensive demographic analysis for the Global Burden of Disease Study 2019. *Lancet.* 2020, 196:1135-1159.
17. Tobin MD, Sheehan NA, Scurrah KJ, Burton PR. Adjusting for treatment effects in studies of quantitative traits: antihypertensive therapy and systolic blood pressure. *Stat Med.* 2005, 24:2911-2935.
18. Shea S, Cook EF, Kannel WB, Goldman L. Treatment of hypertension and its effect on cardiovascular risk factors: data from the Framingham Heart Study. *Circulation.* 1985, 71:22-30.
19. Blanc AK, Wardlaw T. Monitoring low birth weight: an evaluation of international estimates and an updated estimation procedure. *Bull World Health Organ.* 2005, 83:178-185.
20. Staessen J, Bulpitt C, Fagard R, Joossens JV, Lijnen P, Amery A. Four urinary cations and blood pressure : a population study in two Belgian towns. *Am J Epidemiol.* 1983, 117:676-687.
21. Staessen J, Fagard R, Lijnen P, Amery A. Salt and blood pressure in Belgium. *J Epidemiol Community Health.* 1981, 35:256-261.

22. Hosmer DW, Jr., Lemeshow S. Polytomous independent variable. In *Applied logistic regression.*,Applied logistic regression.New York: John Wiley & Sons; 1989:47-56.

23. Liu K, Daviglus ML, Loria CM, Colangelo LA, Spring B, Moller AC*, et al.* Healthy lifestyle through young adulthood and presence of low cardiovascular disease risk in middle age: The Coronary Artery Risk Development in (Young) Adults (CARDIA) Study. *Circulation.* 2012, 125:996-1004.

24. Ferreira I, van de Laar RJ, Prins MH, Twisk JW, Stehouwer CD. Carotid Stiffness in Young Adults: A Life-Course Analysis of its Early Determinants *Hypertension.* 2012, 59:54-61.

25. Boyd A, Golding J, Macleod J, Lawlor DA, Fraser A, Henderson J*, et al.* Cohort profile: The 'children of the 90s' - the index offspring of the Avon Longitudinal Study of Parents and Children. *Int J Epidemiol.* 2013, 42:111-127.

26. Wei FF, Raaijmakers A, Zhang ZY, van Tienoven TP, Huang QF, Yang WY*, et al.* Association between cognition and the retinal microvasculature in 11-year old children born preterm or at term. *Early Hum Dev.* 2018, 118:1-7.

27. Risnes KR, Vatten LJ, Baker JL, Jameson K, Sovio U, Kajantie E*, et al.* Birthweight and mortality in adulthood: a systematic review and meta-analysis. *Int J Epidemiol.* 2011, 40:647-661.

28. Raitakari OT, Juonala M, Kähönen M, Mäki-Torkko N, Järvisalo MJ, Uhari M*, et al.* Cardiovascular risk factors in childhood and carotid artery intima-media thickness in adulthood. *J Am Med Ass.* 2003, 290:2277-2283.

29. Pollock BD, Harville EW, Mills KT, Tang W, Chen W, Bazzano LA. Cardiovascular risk and the American dream : life course observation from the BHS (Bogalusa Heart Study). *J Am Heart Assoc.* 2018, 7:e007693

30. Huxley RR, Shiell AW, Law CM. The role of size at birth and postnatal catch-up growth in determining systolic blood pressure : a systematic review of the literature. *J Hypertens.* 2000, 18:815-831.

31. Rodríguez MM, Gómez AH, Abitbol CL, Chandar JJ, Duara S, Zilleruelo GE. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. *Pediatr Dev Pathol.* 2004, 7:17-25.

32. Sutherland MR, Gubhaju L, Moore L, Kent AL, Dahlstrom JE, Horne RSC*, et al.* Accelerated maturation and abnormal morphology in the preterm neonatal kidney. *J Am Soc Nephrol.* 2011, 22:1365-1374.

33. Hall JE, Guyton AC, Brands MW. Pressure-volume regulation in hypertension. *Kidney Int Suppl.* 1996, 55:S35-S41

34. Rich-Edwards JW, Kleinman K, Michels KB, Stampfer MJ, Manson JE, Rexrode KM*, et al.* Longitudinal study of birth weight and adult body mass index in prediction risk of coronary heart disease in women. *Br Med J.* 2005, 330:1115

35. Curhan GC, Willett WC, Rimm EB, Speigelman D, Ascherio AL, Stampfer MJ. Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. *Circulation.* 1996, 94:3246-3250.

36. Leong NM, Mignone LI, Newcomb PA, Titus-Ernstoff L, Baron JA, Trentham-Dietz A*, et al.* Early life risk factors in cancer: the relation of birth weight to adult obesity. *Int J Cancer.* 2003, 103:789-791.

37. Cooper R, Atherton K, Power C. Gestational age and risk factors for cardiovascular disease: evidence from the 1958 British birth cohort followed to mid-life. *Int J Epidemiol.* 2009, 38:235-244.

38. Liu Y, Shi M, Dolan J, He J. Sodium sensitivity of blood pressure in Chinese populations. *J Hum Hypertens.* 2020, 34:94-107.

39. Morris RCJ, Sebastian A, Forman A, Tanaka M, Schmidlin O. Normotensive salt sensitivity effect of race and dietary potassium. *Hypertension.* 1999, 33:18-23.

Data Supplement

Expanded methods

Birth weight questionnaire

The questionnaire was administered to women.

- □ How many times have you been pregnant?
- □ How many miscarriages have you got?
- □ How many of your children were born alive?
- □ How many of your children were stillborn?
- □ For each of your children born alive, provide the following information: (i) date of birth; (ii) and birth weight.

Clinical and biochemical measurements

Trained nurses measured each participant's BP at baseline and follow-up by auscultation of the Korotkoff sounds. After the participants had rested for 5 minutes in the sitting position, they obtained five consecutive BP readings (phase V diastolic pressure) to the nearest 2 mm Hg, using mercury sphygmomanometers. Standard cuffs had a 12 × 24 cm inflatable portion, but if upper arm girth exceeded 31 cm, larger cuffs with 15 × 35 cm bladders were used. For analysis, the five BP readings obtained at baseline or at follow-up were averaged. Digit preference was checked at 6-month intervals [1]. Body mass index was weight in kilograms divided by height in meters squared. BP was categorized according to the contemporary European guidelines. Hypertension was a BP of ≥ 140 mm Hg systolic or ≥ 90 mm Hg diastolic or being on antihypertensive treatment. If systolic and diastolic BP were in different categories, the highest level was used to categorize participants. Heart rate rate was counted over one minute.

Venous blood samples were drawn for measurement of serum total cholesterol, serum creatinine and blood glucose. In each country (**Table S1**), a single certified laboratory did the routine biochemistry, using quality-controlled automated methods. Serum creatinine was measured, using Jaffe's method with modifications [2]. From 2004 onwards, serum creatinine measurements were calibrated by isotope-dilution mass spectrometry [2]. The estimated glomerular filtration rate was derived from serum creatinine, using the Chronic Kidney Disease Epidemiology Collaboration equation [3]. Diabetes was a self-reported diagnosis, use of antidiabetic drugs, fasting blood glucose of ≥ 126 mg/dL (≥ 7.0 mmol/L), random blood glucose of ≥ 200 mg/dL (≥ 11.1 mmol/L), or diabetes documented in practice or hospital records.

Participants collected an exactly timed 24-h h urine sample in a 2500 mL wideneck

plastic container (Sarstedt article number 77.576, Nümbrecht, Germany). The median interval between the BP readings and the start of the 24-h urine collection was 5 days at baseline (5th-95th percentile interval: 0-19 days) and 3 days (5th-95th percentile interval: 0-15 days) at follow-up. Urinary sodium and potassium were determined by flame photometry and urinary creatinine using the same methods as for serum creatinine.

References

1. Kuznetsova T, Staessen JA, Kawecka-Jaszcz K, Babeanu S, Casiglia E, Filipovský J*, et al.* Quality control of the blood pressure phenotype in the European Project on Genes in Hypertension. *Blood Press Monit.* 2002, 7:215-224.
2. Miller WG, Myers GL, Ashwood ER, Killeen AA, Wang E, Thienpont LM*, et al.* Creatinine measurement : state of the art in accuracy and interlaboratory harmonization. *Arch Pathol Lab Med.* 2005, 129:297-304.
3. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, III, Feldman HI*, et al.* A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009, 150:604-612.

Table S2. Recruitment and follow-up by cohort

Catchment area	Sampling frame	Recruitment		PR (%)	N° of participants		Follow-up	
		Timeline (years)	Invitation		In database	Analyzed	Last (year)	Median in years (5–95% interval)
Oktyabrsky, Novosibirsk, Russian Federation	Family-based random sample	1999–2001	Address list	68	306	300	2009	16.4 (8.1–17.5)
Niepolomice, Kraków, Poland	Family-based random sample	1999–2008	Address list	54	413	389	2014	13.5 (6.1–14.3)
Pilsen, Czech Republic	Family-based random sample	2000–2001	Address list	82	174	169	2015	14.1 (13.8–14.4)
Padova, Italy	Family-based random sample	1999–2007	Address list	73	314	314	2013	13.3 (12.6–14.5)
Noordkempen, Belgium	Family-based random sample	1985–2008	Address list	78	2904	1436	2016	24.5 (7.9–26.2)

The European Project on Genes in Hypertension (EPOGH) included participants recruited in Novosibirsk, Kraków, Gdańsk, Pilsen and Padova. Participants from Padova were recruited in Mirano in the province of Venice and in Torrebelvicino and Valli del Pasubio in the province of Vicenza. The participants recruited in Noordkempen were included in the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO). PR, participation rate.

Table S3. Baseline characteristics by birth weight in the Outcome Cohort (starts)

Characteristic	Birth weight			*P*-value for trend
	≤ 2500	> 2500-4000	> 4000	
Number in group	113	1644	188	N/A
Number with characteristic *n* (%)				
FLEMENGHO	81 (71.7%)	1129 (68.7%)	122 (64.9%)	0.20
EPOGH	32 (28.3%)	515 (31.3%)	66 (35.1%)	
Women	71 (62.8%)	904 (55.0%)	61 (32.4%)‡	<0.0001
Men	42 (37.2%)	740 (45.0%)	127 (67.6%)‡	
Hypertension	28 (24.8%)	267 (16.2%)†	38 (20.2%)	0.66
Treated hypertension	13 (11.5%)	85 (5.2%)†	11 (5.9%)	0.12
Diabetes mellitus	4 (3.5%)	18 (1.1%)*	1 (0.5%)	0.030
Smokers	32 (28.3%)	424 (25.8%)	56 (29.9%)	0.55
Drinking	23 (20.4%)	455 (27.7%)	60 (32.1%)	0.033
Educational attainment				
Elementary school	14 (12.4%)	94 (5.7%)†	22 (11.7%)*	0.49
Secondary school	76 (67.3%)	1126 (68.5%)	113 (60.1%)*	0.090
Higher education	23 (20.4%)	424 (25.8%)	53 (28.2%)	0.16
Mean of characteristic				
Age (y)	37.2 ± 13.8	34.7 ± 11.5*	38.8 ± 12.5‡	0.027
Birth weight (g)	2107 ± 352	3319 ± 350‡	4453 ± 397‡	N/A
Body weight (kg)	69.1 ± 14.2	71.4 ± 14.4	77.7 ± 13.4‡	<0.0001
Body height (cm)	167.4 ± 9.54	170.2 ± 9.06†	174.2 ± 9.62‡	<0.0001
Body mass index (kg/m^2)	24.7 ± 4.77	24.6 ± 4.16	25.5 ± 3.60†	0.026
Systolic pressure (mm Hg)	125.4 ± 14.1	121.1 ± 13.9†	124.4 ± 14.6†	0.73
Diastolic pressure (mm Hg)	77.3 ± 11.8	75.6 ± 10.1	78.2 ± 9.68‡	0.12
Mean arterial pressure (mm Hg)	93.4 ± 11.4	90.8 ± 10.4*	93.6 ± 10.2‡	0.24
Heart rate (beats per minute)	70.0 ± 10.6	70.0 ± 9.62	69.6 ± 9.93	0.65

Table S2. Baseline characteristics by birth weight in the Outcome Cohort (continued)

Characteristic	Birth weight			*P*-value for trend
	≤ 2500	> 2500-4000	> 4000	
Serum total cholesterol (mg/dL)	199.0 ± 42.7	196.8 ± 41.7	199.7 ± 41.8	0.72
Serum HDL cholesterol (mg/dL)	54.2 ± 16.7	54.3 ± 15.7	52.6 ± 16.3	0.27
Total-to-HDL cholesterol ratio)	3.96 ± 1.31	3.94 ± 1.57	4.11 ± 1.45	0.27
Blood glucose (mg//dL)	91.2 ± 24.7	88.8 ± 18.8	89.2 ± 17.6	0.53
Serum creatinine (mg/dL)	0.96 ± 0.16	0.96 ± 0.17	1.03 ± 0.16‡	0.0001
eGFR (mL/min/1.73 m^2)	86.6 ± 16.2	90.0 ± 17.0*	86.5 ± 15.9†	0.43
24-h urinary measurements				
Volume (L)	1546 ± 722	1497 ± 644	1545 ± 616	0.79
Sodium (g)	3.66 ± 1.26	4.01 ± 1.60*	4.08 ± 1.70	0.056
Potassium (g)	2.41 ± 0.90	2.48 ± 1.04	2.62 ± 1.12	0.067
Sodium-to-potassium ratio	1.68 ± 0.78	1.79 ± 0.80	1.71 ± 0.69	0.94
Creatinine (g)	1.17 ± 0.44	1.28 ± 0.46*	1.36 ± 0.47*	0.0004
Median follow-up IQR (y)	16.8 (14.0, 21.9)	16.6 (13.9, 21.6)	16.3 (13.9, 21.8)	0.98

Body mass index was weight in kilograms divided by height in meters squared. BP was the average of 5 auscultatory readings. Hypertension was a BP of ≥ 140 mm Hg systolic or ≥ 90 mm Hg diastolic or being on antihypertensive treatment. Mean arterial pressure was diastolic pressure plus one third of pulse pressure. Diabetes was a self-reported diagnosis, use of antidiabetic drugs, fasting blood glucose of ≥ 126 mg/dL (≥ 7.0 mmol/L), random blood glucose of ≥ 200 mg/dL (≥ 11.1 mmol/L), or diabetes documented in practice or hospital records. Smoking was the use of smoking materials on a daily basis. Drinking was an average alcohol intake of 5 g or more per day.
SI conversion factors: to convert cholesterol from mg/dL to mmol/L, multiply by 0.0259; glucose from mg/dL to mmol/L, multiply by 0.0555, serum creatinine from mg/dL to µmol/L, multiply by 88.42; urinary sodium, potassium and creatinine from g to mmol, multiply by 43.497, 25.577, and 8.842, respectively.
Significance of the difference with the adjacent left column: * $P \leq 0.05$; † $P \leq 0.01$; ‡ $P \leq 0.001$.
FLEMENGHO, Flemish Study on Environment, Genes and Health Outcomes; EPOGH, European Project on Genes in Hypertension; HDL, high-density lipoprotein; eGFR, glomerular filtration rate estimated from serum creatinine, using the Chronic Kidney Disease Epidemiology Collaboration equation; IQR, interquartile range. N/A indicates that the *P*-value for trend was not computed.

Table S4. Baseline characteristics by 24-h urinary sodium excretion in the Outcome Cohort (starts)

Characteristic	24-h sodium excretion			*P*-value for trend
	< 2.3 g	2.3-4.6 g	> 4.6 g	
Number in group	236	1112	597	N/A
Number with characteristic *n* (%)				
FLEMENGHO	160 (67.8%)	790 (71.0%)	382 (64.0%)‡	0.050
EPOGH	76 (32.2%)	322 (29.0%)	215 (36.0%)‡	
Women	174 (73.7%)	654 (58.8%)‡	208 (34.8%)‡	<0.0001
Men	62 (26.3%)	458 (41.2%)‡	389 (65.2%)‡	
Hypertension	35 (14.8%)	186 (16.7%)	112 (18.8%)	0.15
Treated hypertension	13 (5.6%)	63 (5.7%)	33 (5.5%)	0.96
Diabetes mellitus	0 (0.0%)	17 (1.5%)	6 (1.0%)	0.56
Smokers	65 (27.5%)	293 (26.4%)	154 (25.8%)	0.61
Drinking	51 (21.6%)	298 (26.9%)	189 (31.7%)*	0.0022
Educational attainment				
Elementary school	17 (7.2%)	77 (6.9%)	36 (6.0%)	0.46
Secondary school	161 (68.2%)	733 (65.9%)	421 (70.5%)	0.22
Higher education	58 (24.6%)	302 (27.2%)	140 (23.5%)	0.37
Mean of characteristic				
Age (y)	36.2 ± 12.3	35.5 ± 11.6	34.4 ± 12.1	0.033
Birth weight (g)	3311 ± 527	3342 ± 599	3408 ± 555*	0.011
Body weight (kg)	67.3 ± 13.0	70.6 ± 13.4‡	76.1 ± 15.6‡	<0.0001
Body height (cm)	166.9 ± 8.19	169.6 ± 9.11‡	173.4 ± 9.10‡	<0.0001
Body mass index (kg/m^2)	24.1 ± 4.01	24.5 ± 4.02	25.2 ± 4.40‡	<0.0001
Systolic pressure (mm Hg)	119.9 ± 15.2	121.3 ± 13.7	123.1 ± 14.0*	0.0011
Diastolic pressure (mm Hg)	75.0 ± 9.64	75.9 ± 10.1	76.3 ± 10.4	0.098
Mean arterial pressure (mm Hg)	90.0 ± 10.5	91.1 ± 10.4	91.9 ± 10.7	0.012
Heart rate (beats per minute)	70.5 ± 10.1	70.0 ± 9.47	69.8 ± 10.0	0.32

Table S3. Baseline characteristics by 24-h urinary sodium excretion in the Outcome Cohort (continued)

Characteristic	24-h sodium excretion			*P*-value for trend
	< 2.3	2.3-4.6	> 4.6	
Serum total cholesterol (mg/dL)	199.6 ± 42.0	197.7 ± 39.9	195.4 ± 44.9	0.16
Serum HDL cholesterol (mg/dL)	57.1 ± 16.6	55.0 ± 16.0	51.5 ± 14.8‡	<0.0001
Total-to-HDL cholesterol ratio)	3.75 ± 1.23	3.92 ± 1.60	4.10 ± 1.55*	0.0017
Blood glucose (mg//dL)	89.1 ± 19.4	89.9 ± 19.2	87.1 ± 18.6†	0.031
Serum creatinine (mg/dL)	0.95 ± 0.17	0.96 ± 0.16	0.99 ± 0.18†	0.0001
eGFR (mL/min/1.73 m^2)	86.6 ± 16.5	88.5 ± 16.3	92.5 ± 17.7‡	<0.0001
24 h urinary measurements				
Volume (L)	1504 ± 670	1470 ± 606	1569 ± 704†	0.031
Sodium (g)	1.82 ± 0.37	3.46 ± 0.63‡	5.86 ± 1.24‡	N/A
Potassium (g)	1.74 ± 0.77	2.37 ± 0.91‡	3.02 ± 1.12‡	<0.0001
Sodium-to-potassium ratio	1.27 ± 0.66	1.67 ± 0.70‡	2.18 ± 0.82‡	<0.0001
Creatinine (g)	0.94 ± 0.35	1.20 ± 0.39‡	1.56 ± 0.48‡	<0.0001
Median follow-up IQR (y)	16.8 (14.0, 21.9)	16.6 (13.9, 21.6)	16.3 (13.9, 21.8)	0.0093

Body mass index was weight in kilograms divided by height in meters squared. BP was the average of 5 auscultatory readings. Hypertension was a BP of ≥ 140 mm Hg systolic or ≥ 90 mm Hg diastolic or being on antihypertensive treatment. Mean arterial pressure was diastolic pressure plus one third of pulse pressure. Diabetes was a self-reported diagnosis, use of antidiabetic drugs, fasting blood glucose of ≥ 126 mg/dL (≥ 7.0 mmol/L), random blood glucose of ≥ 200 mg/dL (≥ 11.1 mmol/L), or diabetes documented in practice or hospital records. Smoking was the use of smoking materials on a daily basis. Drinking was an average alcohol intake of 5 g or more per day.

SI conversion factors: to convert cholesterol from mg/dL to mmol/L, multiply by 0.0259; glucose from mg/dL to mmol/L, multiply by 0.0555, serum creatinine from mg/dL to µmol/L, multiply by 88.42; urinary sodium, potassium and creatinine from g to mmol, multiply by 43.497, 25.577, and 8.842, respectively.

Significance of the difference with the adjacent left column: * $P \leq 0.05$; † $P \leq 0.01$; ‡ $P \leq 0.001$.

FLEMENGHO, Flemish Study on Environment, Genes and Health Outcomes; EPOGH, European Project on Genes in Hypertension; HDL, high-density lipoprotein; eGFR, glomerular filtration rate estimated from serum creatinine, using the Chronic Kidney Disease Epidemiology Collaboration equation; IQR, interquartile range. N/A indicates that the P value for trend was not computed.

Table S5. Baseline characteristics by the urinary sodium-to-potassium ratio in the Outcome Cohort (starts)

Characteristic	Urinary sodium-to-potassium ratio			*P*-value for trend
	< 1	1-2	➢ 2	
Number in group	253	1079	613	N/A
Number with characteristic *n* (%)				
FLEMENGHO	231 (91.3%)	863 (80.0%)‡	238 (38.8%)‡	<0.0001
EPOGH	22 (8.7%)	216 (20.0%)‡	375 (61.2%)‡	
Women	153 (60.5%)	571 (52.9%)*	312 (50.9%)	0.020
Men	100 (39.5%)	508 (47.1%)*	301 (49.1%)	
Hypertension	47 (18.6%)	178 (16.5%)	108 (17.6%)	0.95
Treated hypertension	14 (5.6%)	66 (6.1%)	29 (4.7%)	0.42
Diabetes mellitus	3 (1.2%)	14 (1.3%)	6 (1.0%)	0.68
Smokers	60 (23.7%)	279 (25.9%)	173 (28.2%)	0.15
Drinking	68 (26.9%)	290 (27.0%)	180 (29.4%)	0.33
Educational attainment				
Elementary school	20 (7.9%)	62 (5.7%)	48 (7.8%)	0.58
Secondary school	158 (62.5%)	717 (66.5%)	440 (71.8%)*	0.0036
Higher education	75 (29.6%)	300 (27.8%)	125 (20.4%)‡	0.0006
Mean of characteristic				
Age (y)	38.7 ± 11.7	36.5 ± 11.7†	31.6 ± 11.3‡	<0.0001
Birth weight (g)	3305 ± 591	3357 ± 582	3382 ± 566	0.089
Body weight (kg)	70.6 ± 13.0	72.5 ± 14.3*	71.3 ± 15.1	>0.99
Body height (cm)	169.3 ± 9.36	170.5 ± 9.22	170.8 ± 9.22	0.052
Body mass index (kg/m^2)	24.6 ± 3.95	24.9 ± 4.07	24.4 ± 4.37*	0.16
Systolic pressure (mm Hg)	122.5 ± 15.3	121.3 ± 13.1	122.0 ± 15.0	0.95
Diastolic pressure (mm Hg)	76.1 ± 9.88	75.6 ± 9.90	76.5 ± 10.7	0.30
Mean arterial pressure (mm Hg)	91.6 ± 10.7	90.8 ± 9.99	91.7 ± 11.3	0.48
Heart rate (beats per minute)	69.7 ± 9.13	69.0 ± 9.64	71.9 ± 9.82‡	<0.0001

Table S4. Baseline characteristics by the urinary sodium-to-potassium ratio in the Outcome Cohort (continued)

Characteristic	Urinary sodium-to-potassium ratio			*P*-value for trend
	< 1	1-2	> 2	
Serum total cholesterol (mg/dL)	200.5 ± 39.5	200.7 ± 42.3	189.8 ± 40.8‡	<0.0001
Serum HDL cholesterol (mg/dL)	56.4 ± 15.4	53.9 ± 15.7*	53.7 ± 16.3	0.060
Total-to-HDL cholesterol ratio	3.81 ± 1.29	4.05 ± 1.65*	3.85 ± 1.45*	0.51
Blood glucose (mg//dL)	91.4 ± 20.6	89.7 ± 19.7	86.6 ± 16.9†	0.0001
Serum creatinine (mg/dL)	0.97 ± 0.15	0.99 ± 0.17	0.94 ± 0.17‡	0.0006
eGFR (mL/min/1.73 m^2)	85.3 ± 14.9	87.2 ± 16.2	95.3 ± 17.4‡	<0.0001
24 h urinary measurements				
Volume (L)	1605 ± 703	1526 ± 662	1424 ± 583†	<0.0001
Sodium (g)	2.70 ± 1.07	3.87 ± 1.38‡	4.76 ± 1.71‡	N/A
Potassium (g)	3.31 ± 1.23	2.68 ± 0.93‡	1.83 ± 0.72‡	N/A
Sodium-to-potassium ratio	0.83 ± 0.20	1.47 ± 0.28‡	2.70 ± 0.66‡	N/A
Creatinine (g)	1.29 ± 0.41	1.32 ± 0.46	1.19 ± 0.46‡	<0.0001
Median follow-up IQR (y)	18.7 (14.5, 22.4)	16.9 (14.1, 22.0)†	14.2 (13.6, 17.3)‡	<0.0001

The urinary sodium-to-potassium ratio was categorized, using similar percentile cut-offs as for the distribution of the 24-h urinary sodium excretion (15th and 70th percentile ranks). Body mass index was weight in kilograms divided by height in meters squared. BP was the average of 5 auscultatory readings. Hypertension was a BP of ≥140 mm Hg systolic or ≥90 mm Hg diastolic or being on antihypertensive treatment. Mean arterial pressure was diastolic pressure plus one third of pulse pressure. Diabetes was a self-reported diagnosis, use of antidiabetic drugs, fasting blood glucose of ≥126 mg/dL (≥7.0 mmol/L), random blood glucose of ≥200 mg/dL (≥11.1 mmol/L), or diabetes documented in practice or hospital records. Smoking was the use of smoking materials on a daily basis. Drinking was an average alcohol intake of 5 g or more per day.

SI conversion factors: to convert cholesterol from mg/dL to mmol/L, multiply by 0.0259; glucose from mg/dL to mmol/L, multiply by 0.0555, serum creatinine from mg/dL to µmol/L, multiply by 88.42; urinary sodium, potassium and creatinine from g to mmol, multiply by 43.497, 25.577, and 8.842, respectively.

Significance of the difference with the adjacent left column: * $P \leq 0.05$; † $P \leq 0.01$; ‡ $P \leq 0.001$.

FLEMENGHO, Flemish Study on Environment, Genes and Health Outcomes; EPOGH, European Project on Genes in Hypertension; HDL, high-density lipoprotein; eGFR, glomerular filtration rate estimated from serum creatinine, using the Chronic Kidney Disease Epidemiology Collaboration equation; IQR, interquartile range. N/A indicates that the P value for trend was not computed.

Table S6. Incidence of endpoints in the Outcome Cohort

Endpoint	FLEMENGHO (*n* = 1332)	EPOGH (*n* = 613)	All cohorts (*n* = 1945)
Mortality	59	14	73
Cardiovascular	13	3	16
Noncardiovascular	37	11	48
Renal	1	0	1
Cause unknown	8	0	8
All cardiovascular endpoints	104	16	120
Fatal	99	13	112
Nonfatal	52	16	68
All coronary endpoints	52	16	68
Fatal	5	2	7
Nonfatal	50	14	64
Myocardial infarction	18	8	26
Fatal	3	1	4
Nonfatal	15	7	22
CABG or PCR	29	2	31
Fatal chronic IHD	0	1	1
Sudden death	2	0	2
Heart failure	9	3	12
Fatal	4	0	4
Nonfatal	7	3	10
Stroke	22	7	29
Fatal	2	1	3
Nonfatal	20	6	26
Other vascular disease	37	1	38
Fatal	2	0	2
Nonfatal	37	1	38

For each outcome, only the first occurring endpoint was considered. Nonfatal events do not add up. Other vascular disease includes peripheral arterial disease, peripheral arterial revascularization, arterial and pulmonary embolism, aortic aneurysm, and pulmonary heart disease.
FLEMENGHO, Flemish Study on Environment, Genes and Health Outcomes; EPOGH, European Project on Genes in Hypertension; CABG, coronary bypass surgery; PCR, percutaneous coronary revascularization; IHD, ischemic heart disease.

Table S7. Endpoints rates by categories of birth weight, 24-h urinary sodium excretion and urinary sodium-to-potassium ratio (starts)

Endpoints	Low		Medium		High		*P-value* for trend
	n/N	Rates (95% CI)	*n/N*	Rates (95% CI)	*n/N*	Rates (95% CI)	
Women							
Birth weight							
Categories, g		≤ 2500		> 2500-4000		> 4000	
All-cause mortality	2/71	2.82 (0.00-6.67)	29/904	3.21 (2.06-4.36)	5/61	3.51 (1.31-15.1)	0.048
All CV endpoint	2/71	2.82 (0.00-6.67)	41/904	4.54 (3.18-5.89)	3/61	4.91 (0.00-10.3)	0.23
Hypertension	10/53	18.9 (8.33-29.4)	137/700	19.6 (16.6-22.5)	9/44	20.5 (8.54-32.4)	0.046
24-h urinary sodium							
Categories, g/day		< 2.3		2.3-4.6		> 4.6	
All-cause mortality	8/174	4.60 (1.49-7.71)	21/654	3.21 (1.86-4.56)	7/208	3.37 (0.91-5.82)	0.40
All CV endpoint	9/174	5.17 (1.88-8.46)	32/654	4.89 (3.24-6.55)	5/208	2.40 (0.32-4.49)	0.27
Hypertension	34/138	24.6 (17.4-31.8)	97/504	19.2 (15.8-22.7)	25/155	16.1 (10.3-21.9)	0.099
Sodium-to-potassium ratio							
Categories		< 1		1-2		> 2	
All-cause mortality	8/156	5.13 (1.67-8.59)	18/594	3.03 (1.65-4.41)	11/331	3.32 (1.39-5.25)	0.41
All CV endpoint	11/156	7.05 (3.03-11.1)	27/594	4.55 (2.87-6.22)	12/331	3.63 (1.61-5.64)	0.17
Hypertension	29/127	22.8 (15.5-30.1)	95/476	20.0 (16.4-23.5)	36/222	16.2 (11.4-21.1)	0.056
Men							
Birth weight							
Categories, g		≤ 2500		> 2500-4000		> 4000	
All-cause mortality	3/42	7.14 (0.00-14.9)	23/740	3.11 (1.86-4.36)	11/127	8.66 (3.77-13.6)	0.83
All CV endpoint	8/42	19.0 (7.17-30.9)	48/740	6.49 (4.71-8.26)	18/127	14.2 (8.11-20.2)	0.75
Hypertension	12/21	57.1 (36.0-78.3)	195/552	35.3 (31.3-39.3)	33/90	36.7 (26.7-46.6)	0.37

Table S6. Endpoints rates by categories of birth weight, 24-h urinary sodium excretion and urinary sodium-to-potassium ratio (continued)

Endpoints	Low		Medium		High		*P-value* for trend
	n/N	Rates (95% CI)	*n/N*	Rates (95% CI)	*n/N*	Rates (95% CI)	
24-h urinary sodium							
Categories, g/day		< 2.3		2.3-4.6		> 4.6	
All-cause mortality	2/62	3.23 (0.00-7.62)	19/458	4.15 (2.32-5.97)	16/389	4.11 (2.14-6.09)	0.36
All CV endpoint	6/62	9.68 (2.32-17.0)	38/458	8.30 (5.77-10.8)	30/389	7.71 (5.06-10.4)	0.14
Hypertension	13/44	29.5 (16.1-43.0)	139/338	41.1 (35.9-46.4)	88/281	31.3 (25.9-36.7)	0.91
Sodium-to-potassium ratio							
Categories		< 1		1-2		> 2	
All-cause mortality	4/105	3.81 (0.15-7.47)	22/524	4.20 (2.48-5.92)	13/344	3.78 (1.76-5.79)	0.96
All CV endpoint	12/105	11.4 (5.34-17.5)	42/524	8.02 (5.69-10.3)	25/344	7.27 (4.52-10.0)	0.22
Hypertension	34/78	43.6 (32.6-54.6)	140/396	35.4 (30.6-40.1)	70/217	32.3 (26.0-38.5)	0.16
Both sexes							
Birth weight							
Categories, g		≤ 2500		> 2500-4000		> 4000	
All-cause mortality	5/113	4.42 (0.63-8.22)	52/1644	3.16 (2.32-4.01)	16/188	8.51 (4.52-12.5)	0.48
All CV endpoint	10/113	8.85 (3.61-14.1)	89/1644	5.41 (4.32-6.51)	21/188	11.2 (6.67-15.7)	0.74
Hypertension	22/74	29.7 (19.3-40.1)	332/1252	26.5 (24.1-29.0)	42/134	31.3 (23.5-39.2)	0.79
24-h urinary sodium							
Categories, g/day		< 2.3		2.3-4.6		> 4.6	
All-cause mortality	10/236	4.24 (1.67-6.81)	40/1112	3.60 (2.50-4.69)	23/597	3.85 (2.31-5.40)	0.59
All CV endpoint	15/236	6.36 (3.24-9.47)	70/1112	6.30 (4.87-7.72)	35/597	5.86 (3.98-7.74)	0.26
Hypertension	47/182	25.8 (19.5-32.2)	236/842	28.0 (25.0-31.1)	113/436	25.9 (21.8-30.0)	0.97
Sodium-to-potassium ratio							
Categories		< 1		1-2		> 2	
All-cause mortality	12/261	4.60 (2.06-7.14)	40/1118	3.58 (2.49-4.67)	24/675	3.56 (2.16-4.95)	0.32
All CV endpoint	23/261	8.81 (5.37-12.3)	69/1118	6.18 (4.76-7.58)	37/675	5.48 (3.76-7.19)	0.21
Hypertension	63/205	30.7 (24.4-37.0)	235/872	26.9 (24.0-29.9)	106/439	24.1 (20.1-28.1)	0.056

Rates are expressed as the number of events per 1000 person-years. CI, confidence interval; CV, cardiovascular. n/N refers to the number of endpoints and the total number of participants at risk.

Table S8. Hazard ratios of endpoints in relation to categories of birth weight and 24-h urinary sodium excretion (starts)

Variable	*n/N* (%)	Unadjusted		Adjusted		Fully adjusted	
		HR (95% CI)	*P*	HR (95%CI)	*P*	HR (95% CI)	*P*
All-cause mortality							
Birth weight							
≤ 2500 g	5/113 (4.42)	0.91 (0.49-1.68)	0.75	0.90 (0.49-1.68)	0.75	0.91 (0.49-1.71)	0.77
> 2500-4000 g	52/1644 (3.16)	0.66 (0.45-0.97)	0.035	0.74 (0.50-1.08)	0.12	0.74 (0.50-1.08)	0.12
> 4000 g	16/188 (8.51)	1.67 (1.06-2.61)	0.027	1.51 (0.96-2.38)	0.077	1.49 (0.94-2.37)	0.090
24-h urinary sodium							
< 2.3 g/day	10/236 (4.24)	1.03 (0.67-1.62)	0.89	1.10 (0.70-1.72)	0.68	1.13 (0.72-1.79)	0.59
2.3-4.6 g/day	40/1112 (3.60)	0.90 (0.65-1.24)	0.50	0.89 (0.64-1.23)	0.48	0.90 (0.65-1.24)	0.51
> 4.6 g/day	23/597 (3.85)	1.08 (0.76-1.55)	0.66	1.02 (0.72-1.46)	0.90	0.99 (0.68-1.42)	0.93
All CV endpoints							
Birth weight							
≤ 2500 g	10/113 (8.85)	1.09 (0.70-1.72)	0.70	0.97 (0.60-1.58)	0.90	0.95 (0.58-1.55)	0.83
> 2500-4000 g	89/1644 (5.41)	0.68 (0.51-0.90)	0.0082	0.89 (0.65-1.21)	0.45	0.89 (0.65-1.22)	0.46
> 4000 g	21/188 (11.2)	1.36 (0.94-1.96)	0.10	1.17 (0.79-1.73)	0.45	1.19 (0.80-1.76)	0.39
24-h urinary sodium							
< 2.3 g/day	15/236 (6.36)	0.97 (0.67-1.40)	0.88	1.07 (0.73-1.57)	0.72	1.08 (0.74-1.59)	0.68
2.3-4.6 g/day	70/1112 (6.29)	1.00 (0.77-1.30)	0.99	1.10 (0.84-1.43)	0.50	1.10 (0.84-1.43)	0.50
> 4.6 g/day	35/597 (5.86)	1.03 (0.77-1.38)	0.86	0.85 (0.62-1.16)	0.31	0.84 (0.62-1.15)	0.28

Table S7. Hazard ratios of endpoints in relation to categories of birth weight and 24-h urinary sodium excretion (continued)

Variable	n/N (%)	Unadjusted		Adjusted		Fully adjusted	
		HR (95% CI)	*P*	HR (95%CI)	*P*	HR (95% CI)	*P*
Hypertension							
Birth weight							
≤ 2500 g	22/74 (29.7)	0.90 (0.66-1.23)	0.51	1.20 (0.87-1.65)	0.27	1.18 (0.86-1.63)	0.30
> 2500-4000 g	332/1252 (26.5)	0.93 (0.77-1.12)	0.45	0.95 (0.78-1.17)	0.65	0.96 (0.78-1.18)	0.70
> 4000 g	42/134 (31.3)	1.19 (0.92-1.54)	0.18	0.88 (0.66-1.16)	0.35	0.88 (0.66-1.16)	0.37
24-h urinary sodium							
< 2.3 g/day	47/182 (25.8)	0.93 (0.76-1.15)	0.51	1.07 (0.87-1.33)	0.53	1.07 (0.86-1.32)	0.55
2.3-4.6 g/day	236/842 (28.0)	1.05 (0.91-1.21)	0.54	1.05 (0.90-1.21)	0.54	1.05 (0.90-1.21)	0.54
> 4.6 g/day	113/436 (25.9)	1.02 (0.87-1.21)	0.78	0.89 (0.75-1.06)	0.19	0.90 (0.76-1.06)	0.21

For categorical variables, the deviation from mean coding was applied, so that hazard ratios express the relative risk compared with the average risk in the study population. All Cox models accounted for clustering of the data within families as random effect. For the analysis of all-cause mortality and the composite cardiovascular endpoint, the covariables introduced as fixed effects in adjusted Cox models included cohort (FLEMENGHO *vs* EPOGH), sex, and the baseline variables age, mean arterial pressure, the serum total-to-HDL cholesterol, blood glucose, educational attainment (elementary school *vs* secondary and higher education), and the glomerular filtration rate estimated from serum creatinine. For the analysis of hypertension, mean arterial pressure as baseline covariable was replaced by heart rate. Fully adjusted models for birth weight additionally accounted for the 24-h urinary sodium excretion as continuously distributed covariable; fully adjusted models for 24-h urinary sodium accounted for birth weight as continuously distributed variable. In view of the number of endpoints *vs* the number of covariables, a propensity score derived by logistic regression was applied to combine covariables into a risk estimate. All Cox models complied with the proportional hazard assumption (Kolmogorov-type supremum test statistic: $P>0.05$).
24-H hour sodium excretion of 2.3 g corresponds with 100 mmol sodium and 5.75 g salt and 4.6 g corresponds with 200 mmol sodium and 11.5 g salt.
HR, hazard ratio; CI, confidence interval; CV, cardiovascular. n/N (%) refers to the number of endpoints and the total number of participants at risk and the percentage rate.

Table S9. Hazard ratios of endpoints in relation to categories of birth weight and the urinary sodium-to-potassium ratio (starts)

Variable	*n/N* (%)	Unadjusted		Adjusted		Fully adjusted	
		HR (95% CI)	*P*	HR (95%CI)	*P*	HR (95% CI)	*P*
All-cause mortality							
Birth weight							
≤ 2500 g	5/121 (4.13)	0.89 (0.48-1.65)	0.70	0.86 (0.46-1.61)	0.63	0.88 (0.47-1.65)	0.69
> 2500-4000 g	55/1730 (3.18)	0.69 (0.47-1.01)	0.059	0.78 (0.53-1.15)	0.20	0.79 (0.53-1.16)	0.23
> 4000 g	16/203 (7.88)	1.63 (1.03-2.56)	0.035	1.50 (0.95-2.38)	0.085	1.45 (0.90-2.32)	0.13
UNAK							
< 1	12/261 (4.60)	0.95 (0.63-1.45)	0.82	0.84 (0.55-1.29)	0.43	0.86 (0.56-1.33)	0.50
1-2	40/1118 (3.58)	0.85 (0.62-1.16)	0.29	0.88 (0.64-1.21)	0.42	0.88 (0.64-1.22)	0.45
> 2	24/675 (3.56)	1.24 (0.87-1.76)	0.23	1.35 (0.95-1.93)	0.096	1.31 (0.91-1.89)	0.14
All CV endpoints							
Birth weight							
≤ 2500 g	10/121 (8.26)	1.03 (0.66-1.62)	0.90	0.90 (0.55-1.47)	0.68	0.91 (0.55-1.49)	0.70
> 2500-4000 g	96/1730 (5.55)	0.70 (0.52-0.93)	0.013	0.90 (0.66-1.22)	0.49	0.90 (0.66-1.23)	0.50
> 4000 g	23/203 (11.3)	1.39 (0.97-1.99)	0.069	1.24 (0.84-1.83)	0.28	1.23 (0.83-1.82)	0.30
UNAK							
< 1	23/261 (8.81)	1.12 (0.82-1.52)	0.48	1.05 (0.76-1.46)	0.78	1.07 (0.77-1.49)	0.70
1-2	69/1118 (6.17)	0.88 (0.70-1.12)	0.30	0.86 (0.67-1.11)	0.25	0.87 (0.67-1.11)	0.26
> 2	37/675 (5.48)	1.02 (0.77-1.34)	0.91	1.11 (0.83-1.48)	0.50	1.08 (0.81-1.46)	0.60

Table S8. Hazard ratios of endpoints in relation to categories of birth weight and the urinary sodium-to-potassium ratio (continued)

Variable	*n/N* (%)	Unadjusted		Adjusted		Fully adjusted	
		HR (95% CI)	*P*	HR (95%CI)	*P*	HR (95% CI)	*P*
Hypertension							
Birth weight							
≤ 2500 g	22/76 (28.9)	0.88 (0.64-1.19)	0.39	1.15 (0.84-1.58)	0.38	1.17 (0.85-1.61)	0.33
> 2500-4000 g	336/1298 (25.9)	0.91 (0.75-1.10)	0.32	0.93 (0.76-1.14)	0.49	0.93 (0.76-1.13)	0.45
> 4000 g	46/142 (32.4)	1.26 (0.98-1.61)	0.072	0.93 (0.71-1.22)	0.61	0.92 (0.70-1.21)	0.56
UNAK							
< 1	63/205 (30.7)	1.04 (0.86-1.25)	0.68	0.95 (0.78-1.15)	0.60	0.94 (0.78-1.14)	0.55
1-2	235/872 (26.9)	1.00 (0.87-1.14)	0.96	0.96 (0.84-1.11)	0.61	0.96 (0.84-1.11)	0.61
> 2	106/439 (24.1)	0.97 (0.82-1.14)	0.67	1.09 (0.92-1.29)	0.30	1.10 (0.93-1.30)	0.26

For categorical variables, the deviation from mean coding was applied, so that hazard ratios express the relative risk compared with the average risk in the study population. All Cox models accounted for clustering of the data within families as random effect. For the analysis of all-cause mortality and the composite cardiovascular endpoint, the covariables introduced as fixed effects in adjusted Cox models included cohort (FLEMENGHO *vs* EPOGH), sex, and the baseline variables age, mean arterial pressure, the serum total-to-HDL cholesterol, blood glucose, educational attainment (elementary school *vs* secondary and higher education), and the glomerular filtration rate estimated from serum creatinine. For the analysis of hypertension, mean arterial pressure as baseline covariable was replaced by heart rate. Fully adjusted models for birth weight additionally accounted for the urinary sodium-to-potassium ratio as continuously distributed covariable; fully adjusted models for the urinary sodium-to-potassium ratio, accounted for birth weight as continuously distributed variable. In view of the number of endpoints *vs* the number of covariables, a propensity score derived by logistic regression was applied to combine covariables into a single risk estimate. P_{int} refers to the significance of the interaction tern between birth weight and the urinary sodium-to-potassium ratio in fully adjusted models. All Cox models complied with the proportional hazard assumption (Kolmogorov-type supremum test statistic: $P>0.05$).
HR, hazard ratio; CI, confidence interval; CV, cardiovascular. n/N (%) refers to the number of endpoints and the total number of participants at risk and the percentage rate; UNAK, urinary sodium-to-potassium ratio.

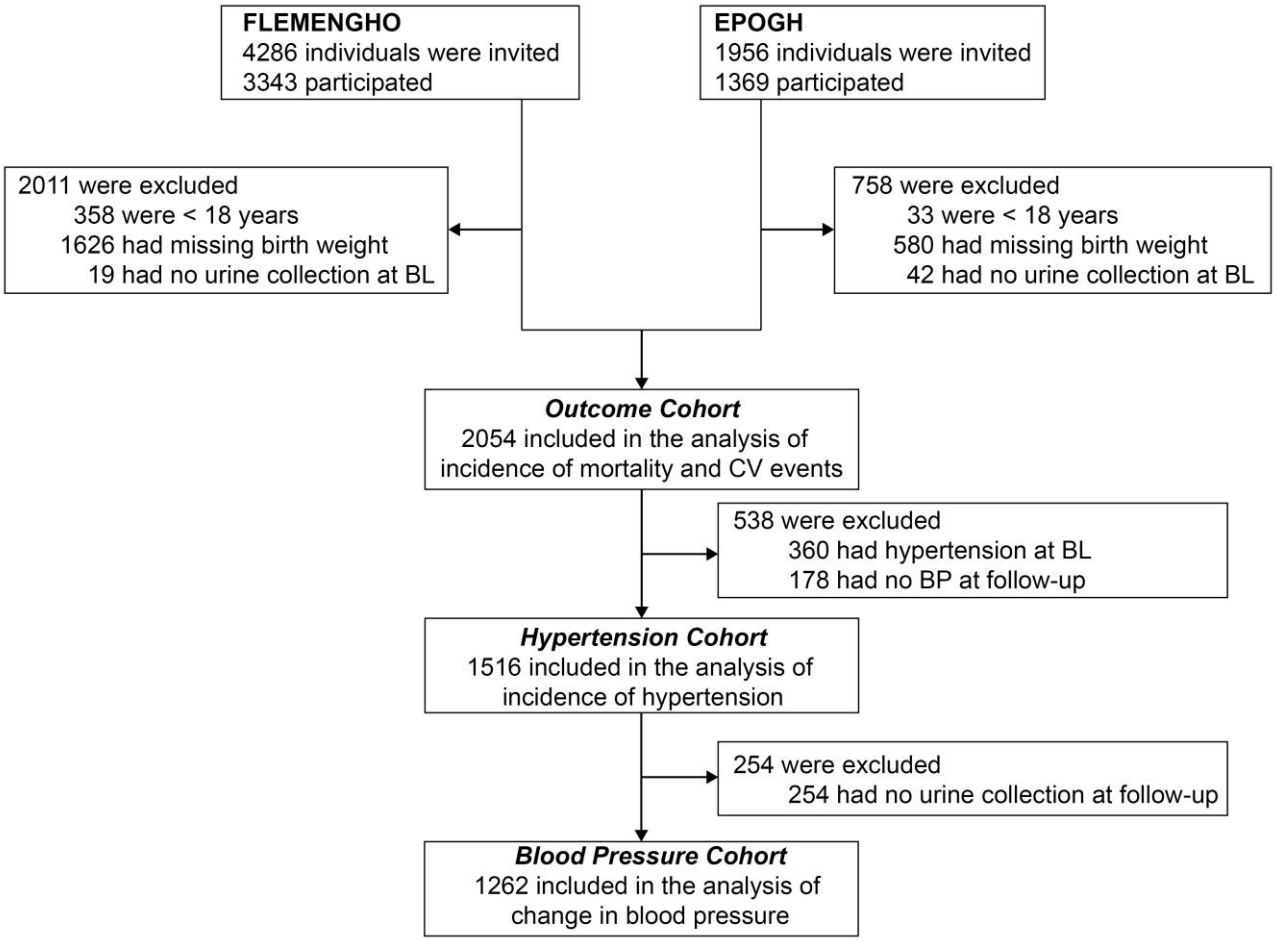


Figure S1. Flowchart of participants in analyses involving the sodium-to-potassium ratio. The *Outcome Cohort*, *Hypertension Cohort*, and *Blood Pressure Cohort* refer to participants used to study the incidence of mortality and morbidity of cardiovascular endpoints, the incidence of hypertension, and the blood pressure changes in relation to the changes in 24-h urinary sodium, respectively.

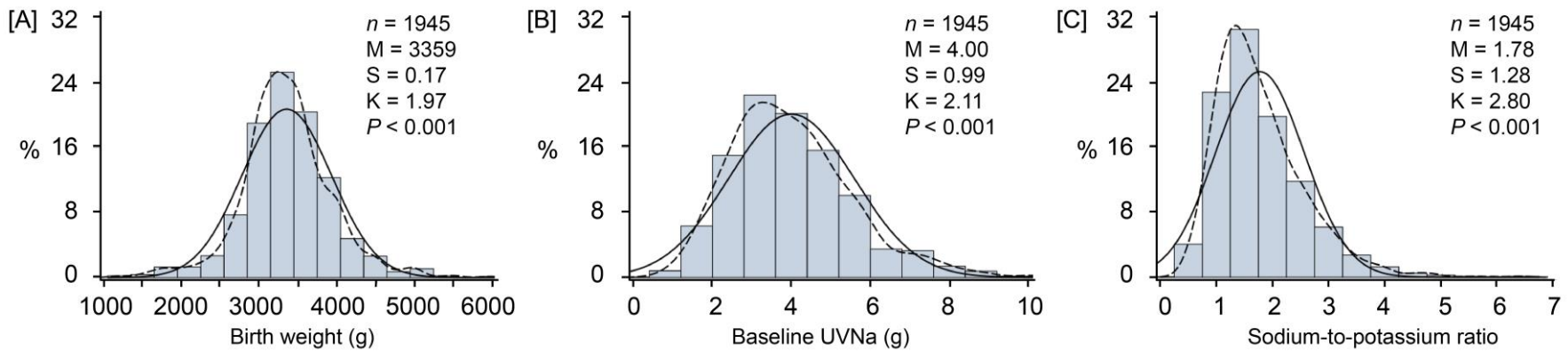


Figure S2. Distributions of birth weight (A) and the baseline 24-h urinary sodium excretion (B) and the urinary sodium-to-potassium ratio (C) in the Outcome Cohort. UVNA and UNAK indicate the 24-h urinary sodium excretion and the urinary sodium-to-potassium ratio; n, M, S and K refer to the number of participants, the arithmetic mean and the coefficients of skewness and kurtosis, respectively. Skewness and kurtosis were computed as the third and fourth moments about the mean divided by the cube of the SD. The solid and dotted lines represent the normal and kernel density distributions. The *P*-values are for departure of the actually observed distribution from normality according to the Shapiro-Wilk statistic.

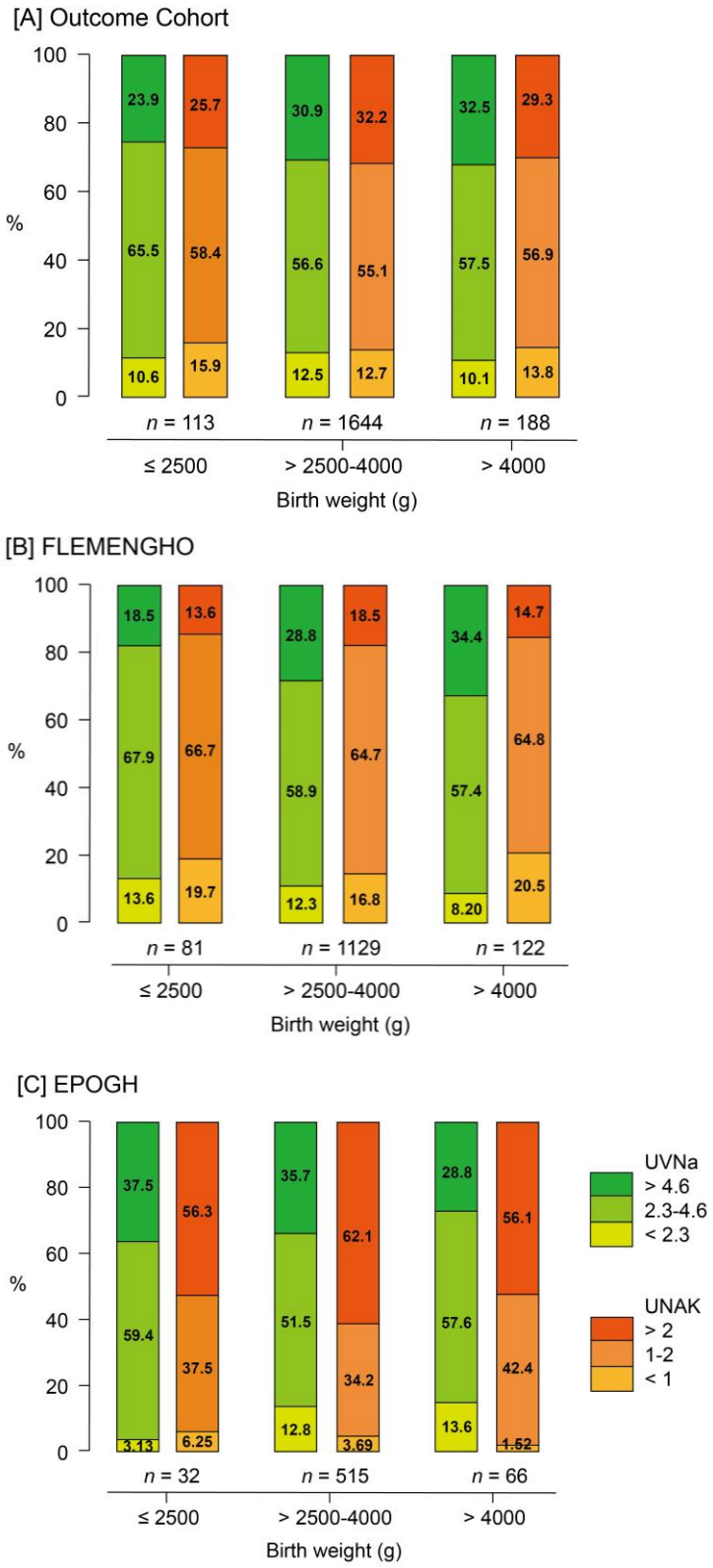


Figure S3. Bar diagrams showing the overlap between low, medium, and high categories of birth weight (BW) and the 24-h urinary sodium excretion (UVNA) and the urinary sodium-to-potassium ratio (UNAK) in the total study cohort (A), and in FLEMENGHO (B) and EPOGH (C).

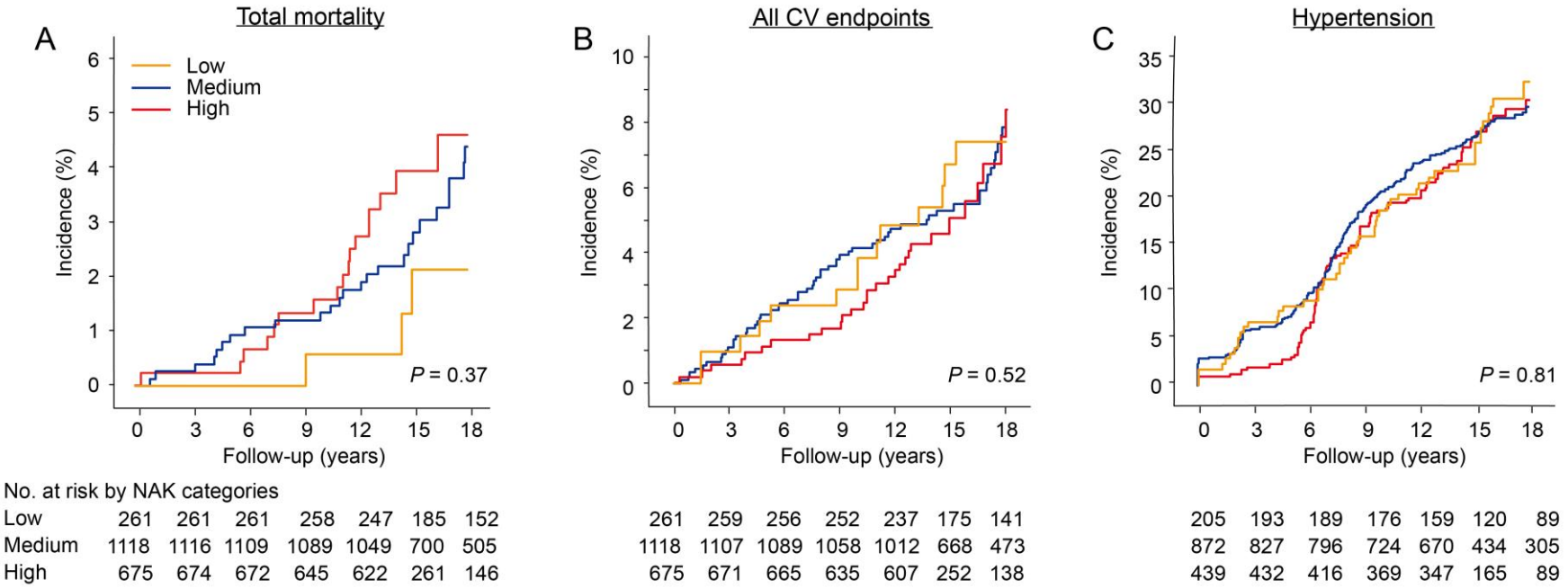


Figure S4. Kaplan-Meier survival function estimates for mortality (A), all cardiovascular events (B) and hypertension (C) in relation to the urinary sodium-to-potassium ratio at baseline. The results were obtained in the extended Outcome Cohort (see Figure S1) without exclusion of inaccurate urine collection, because urinary volume is not included in the computation of the urinary sodium-to-potassium ratio.

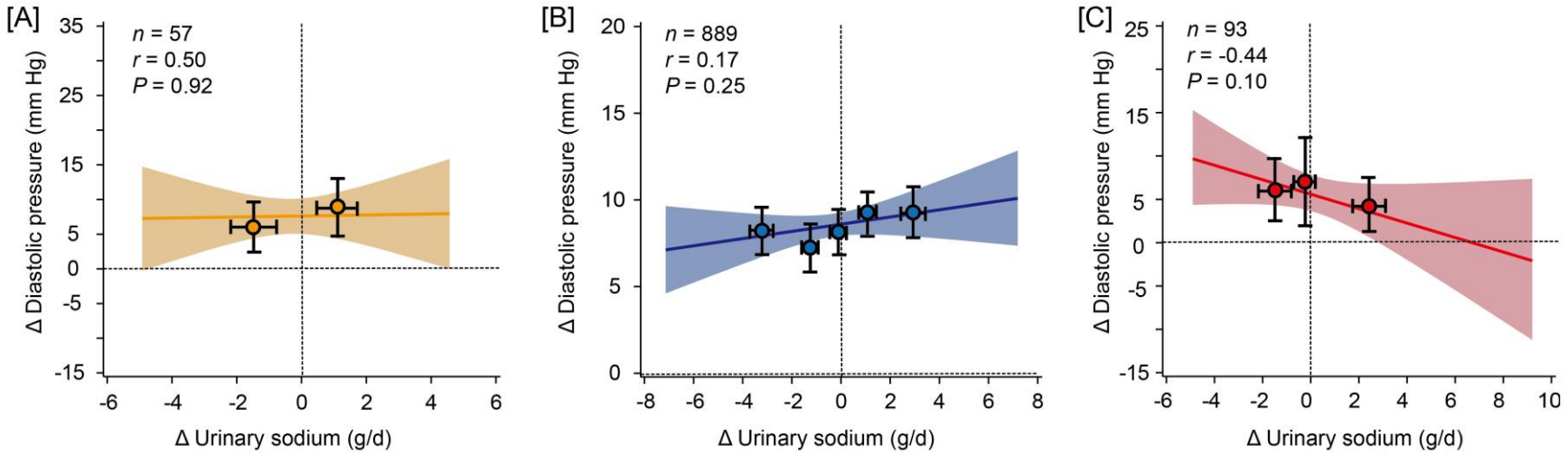


Figure S5. Relation between the changes from baseline to follow-up in diastolic blood pressure and the changes in 24-h urinary sodium excretion in the low (A), medium (B), and high (C) birth weight categories (≤2500, >2500-4000, >4000 g). *n* and *r* refer to the number of participants in each group and to the partial correlation coefficients with adjustments applied for recruitment (FLEMENGHO *vs* EPOGH), sex, and the baseline variables age, heart rate, the serum total-to-HDL cholesterol ratio, blood glucose, educational attainment (elementary school vs secondary and high education) and the glomerular filtration rate estimated from serum creatinine. To illustrate the fit of the linear model, the average changes in systolic blood pressure and in 24-h urinary sodium were computed by quantiles and plotted over the regression lines given with 95% confidence interval. Categories were defined by the median, quintiles and tertiles in the low, medium and high birth weight groups, respectively. The results were obtained in the *Blood Pressure Cohort* (see Figure 1).

Chapter 4

Unhealthy ageing in relation to clinical risk factors

Part 3

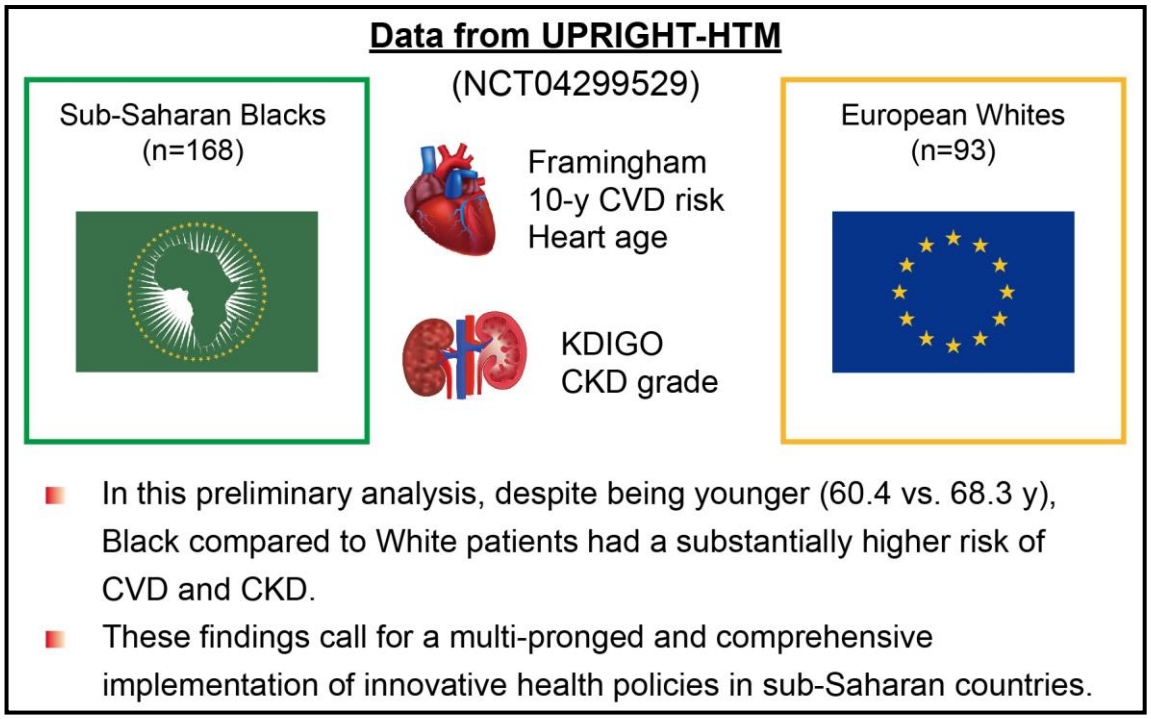
Racial and regional disparities in the risk of non-communicable disease between sub-Saharan Black and European White patients

Yu-Ling Yu, De-Wei An, Babangida S. Chori, Błażej P. Kaleta, Gontse Mokwatsi, Dries S. Martens, Olugbenga O. Abiodun, Tina Anya, Agnieszka Łebek-Szatańska, Jong-Shiuan Yeh, Catharina M.C. Mels, Agnieszka Latosinska, Ruan Kruger, Godsent Isiguzo, Krzystof Narkiewicz, Muhammad N. Shehu, Martin Salazar, Walter Espeche, Blerim Mujaj, Jana Brgulian-Hitij, Agnieszka Olszanecka, Wiktoria Wojciechowska, Peter Reyskens, Marek Rajzer, Andrzej Januszewicz, Katarzyna Stolarz-Skrzypek, Kei Asayama, Karel Allegaert, Peter Verhamme, Harald Mischak, Tim S. Nawrot, Augustine N. Odilie, Jan A. Staessen, on behalf of the UPRIGHT-HTM investigators*

* The UPRIGHT-HTM investigators are listed in the supplement.

Previously published in:
Journal of Hypertension. 2025; 42: 481-491.

Graphical abstract



Abstract

Objectives: Greater vulnerability of Black vs. White individuals to cardiovascular (CVD) and chronic kidney (CKD) disease is well charted in the United States, but studies involving sub-Saharan Blacks are scarce.

Methods: Baseline data (2021-2024) were collected in 168 sub-Saharan Blacks and 93 European Whites in an ongoing clinical trial (NCT04299529), using standardized patient selection criteria. Data included clinical and biochemical risk factors, ECG and echocardiographic traits, Framingham CVD risk, CKD grades (KDIGO 2024), self-assessed symptoms (WHO questionnaire), and urinary proteomic profiles predictive of left ventricular dysfunction (LVD) and CKD, HF1 and CKD273, respectively. Racial comparisons rested on unadjusted and multivariable-adjusted analyses.

Results: Despite being younger (60.4 vs. 68.3 years), Blacks had a worse risk profile, as evidenced by higher diabetes prevalence, higher body mass index, faster heart rate, unfavorable serum cholesterol fractions, lower estimated glomerular filtration rate, microalbuminuria and sedentary lifestyle. This resulted in Blacks having higher 10-year CVD risk, higher heart age (index of vascular ageing with chronological age as reference), and a worse CKD grades. In both races, CKD273 increased with CKD grade, but CKD273 and HF1 were not different by race. These observations were robust in subgroup and adjusted analyses.

Conclusion: This study did not differentiate host (genetic, molecular, and pathogenic) from environmental drivers of disease. Nonetheless, the findings call for a multi-pronged and comprehensive implementation of innovative health policies in sub-Saharan countries. Education, research, empowerment of stakeholders, and international learned societies connecting experts from a wide array of disciplines should vigorously sustain this effort.

Introduction

Sub-Saharan countries face persisting health challenges, such as infectious diseases, malnutrition, and child and maternal mortality, and more recently the growing burden of non-communicable diseases (NCD) [1,2]. A major cause is the demographic revolution, characterised by longer life expectancy, but also by lengthier disability-adjusted life-years and lower quality of life [3]. Hypertension, obesity, and type-2 diabetes are the drivers of the NCD burden sub-Saharan Africa [4,5], where population health indicators generally remain behind most other low- and middle-income countries [2,6]. Climate change [1], the degradation of the living environment, overcrowded metropolitan areas [1], and changing eating habits with surplus amounts of sugar and processed foods [6] increasingly contribute to the sub-Saharan NCD burden.

The racial disparity between Blacks and Whites in the disease burden due to cardiovascular (CVD) and chronic kidney (CKD) disease is well documented in the United States [7-9]. Among all racial groups, African-Americans of all ages have the highest hypertension prevalence [7,10]. The American studies are confounded by admixture, but nonetheless sub-Saharan African ancestry apparently plays a pivotal role in the high vulnerability of African-Americans to CVD and CKD [11-13]. Few studies compared the CVD and CKD predisposition of Blacks "*born and living in sub-Saharan Africa*" with European Whites [14,15]. The Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform Trial (UPRIGHT-HTM) is an ongoing clinical trial [16,17] with as objective to compare management of NCD risk based on telemonitoring of the home blood pressure (HTM) alone or HTM combined with risk assessment by urinary proteomic profiling (UPP). All UPRIGHT-HTM patients have to meet the same prespecified profile of traditional risk factors allowing the comparison of sub-Saharan Blacks and White Europeans, even though patients were recruited in different continents.

Methods

UPRIGHT-HTM (Clinicaltrials.gov identifier: NCT04299529) complies with the Helsinki declaration [18]. The Ethics Committee of University Hospitals Leuven, Belgium (Belgian registration number, B3222020000276), acting as the central Ethics Committee and, the Institutional Review Boards of all clinical sites approved the trial protocol [16]. Patients provide written informed consent using paper forms translated in the native languages of the catchment areas. These forms are the only paper documents in the trial and allow identification of the patients. However, in line with the European General Data Protection Regulations [19], these forms have to be filed in each patient's local medical record [16] and are not part of the electronic trial data.

Design of UPRIGHT-HTM

UPRIGHT-HTM is an investigator-initiated randomised clinical trial, of which the rationale and protocol [16] and first progress report [17] are published. The UPP-associated risk is communicated to caregivers and patients early during follow-up in the intervention group and only after completion of follow-up in the control group. The underlying hypothesis is that refinement of risk assessment in the intervention group by UPP on top of HTM will motivate caregivers and patients to more stringent management of risk factors and therefore to a lower incidence of the primary endpoint, a composite of intermediate target organ damage, including CKD and diastolic LVD, and hard cardiovascular and renal events [16].

Active recruitment is currently ongoing in Europe (Belgium, Kosovo, Poland and Slovenia), Asia (China), sub-Saharan Africa (Mozambique, Nigeria and South-Africa) and South America (La Plata, Argentina). Eligible patients are asymptomatic adults of either sex and must in addition to being aged (55-75 years) have five or more guideline-defined risk factors, preferably including hypertension, type-2 diabetes mellitus, or both. Furthermore, to qualify, patients must have an email address, internet access via an android smartphone, and be willing to engage in HTM during follow-up. Asymptomatic patients with CKD stage 3A [20] or diastolic LVD (stage-1 heart failure) meet the entry criteria on condition that only one of these two disorders is present. On top of the exclusion criteria common to all clinical trials, study-specific exclusion criteria comprise an impracticable echocardiographic window, atrial fibrillation or flutter or frequent extrasystoles, because these conditions interfere with the echocardiographic assessment of LVD and HTM using oscillometric devices [16]. After a screening visit and a run-in period of variable length (1-5 weeks or longer), patients are stratified by centre and sex and randomised using a fully automated web-based computerised random function [16].

Data collection

UPRIGHT-HTM is running as a patient-centred trial, mainly positioned at the patients' homes and primary care practices or out-patient clinics, using the CE-certified web-based and highly secured WiPaM platform (Wireless Patient Monitoring), developed by RDSM NV, Hasselt, Belgium (https://www.rdsm.eu). Data are collected electronically by patients using the android-based app and by their caregivers via electronic case report forms (eCRFs). Data collection is described in detail in the Data Supplement.

Mosaiques-Diagnostics GmbH, Hannover, Germany (MOS) did the UPP profiling for all patients. HF1 and CKD273 are validated urinary proteomic markers of imminent diastolic LVD and CKD. HF1 and CKD273 are expressed in normalised units and are

normally distributed. The thresholds indicating increased risk are ≥-0.350 for HF1 and >0.154 for CKD273 (Data Supplement).

Statistical analysis

The current analysis is restricted to baseline data collected at the screening and pre-randomisation visits. For data management and statistical analysis, SAS software, version 9.4, maintenance level 5 (SAS Institute, Cary, NC, USA) is used. As defined in the trial protocol [16], an intention-to-treat (ITT) and per-protocol (PP) analysis was conducted. Deviation from the normal distribution was assessed by the Shapiro-Wilk statistic. The central tendency (spread) of variables is represented by the arithmetic mean (SD) for normally distributed variables and by the median (interquartile range [IQR]) for non-normally distributed continuous variables. Categorical variables are presented as proportions.

For the current analysis, group means and medians were compared by the unpaired t-test and the Mann-Whitney U test, respectively, while proportions were compared by a χ^2-statistic or Fisher exact test, as appropriate according to the cell counts. Pearson or rank correlation coefficients, as appropriate given the distributional characteristics of the variables of interest, were computed and compared between races, using Fisher Z transform [21]. Statistical methods also included multiple linear regression (general linear model) and logistic regression for modelling binary of ordinal outcomes in relation to covariables. Statistical significance is a two-sided α-level of 0.05.

Risk of CVD and heart age were derived from the sex-specific Framingham risk score (FRS) derived from age, total serum cholesterol, HDL cholesterol, systolic blood pressure (SBP), antihypertensive treatment status (yes vs. no), smoking (yes vs. no) and diabetes (yes vs. no) [22]. Heart age reflects the biological vascular age [22]. CKD grades are categorised by cross-classification of eGFR and albuminuria according the 2024 KDIGO Guideline [20]. The traditional risk factors, cardiac function by echocardiography, and the risk of CVD and CKD grades were contrasted between sub-Saharan Blacks and European Whites. Age was categorised using ordinal 5-year groups ranging from <55 to ≥70 years.

Results

Selection of patient and data

The current study includes baseline data collected up to 19 January 2024 at screening and the pre-randomisation visit. **Figure S1** shows the number of patients screened and randomised by 2-monthly time intervals. Restrictions due to the COVID19 pandemic

were progressively – partially or completely – relaxed from the middle or end of 2022 onwards.

Across 16 centres, 440 patients were invited, of which 27 did not meet the set eligibility criteria, 12 had an incompatible mobile phone, and 4 defaulted for other reasons (**Figure 1**). Of the 397 patients screened, 274 were randomised, of whom four withdrew consent and three did not adhere to HTM during the run-in period. By 18 January 2024, 116 patients were still under screening. Divergency in the timing that centres in varying geographical locations, initiated patient enrolment explains the differences in ethnic categorisation, age and anthropometrics between patients screened but not yet randomised and randomised (**Table S1**). Furthermore, of the 274 randomised patients, 13 Asian residents of China were excluded. Given that data collection is still ongoing and therefore incomplete, the number of patients available for the Black vs. White comparisons in the ITT analysis was 261 for traditional risk factors, 227 for ECG traits and echocardiographic left ventricular structure and function, 180 for the questionnaire data, and 165 for the urinary risk markers. Finally, of 261 randomised patients, 57 did not fully comply with all entry criteria, because they were younger than 55 years (n=28), had an eGFR <45 mL/min/1.73m^2 (n=16), or both (n=1). Sensitivity analyses according to the PP approach excluded these patients.

As shown by **Table S2** for common risk factors, **Table S3** for the ECG and echocardiographic measurements, and **Table S4** for the use of medication, the characteristics of patients randomised to control and intervention were very well balanced, suggesting that assignment to randomisation group is unlikely to have had any influence on the racial and regional differences described in the following sections of the results.

Common risk factors by race

The baseline characteristics of 168 sub-Saharan Blacks and 93 European Whites are listed in **Table 1**. The median number of risk factors (IQR), including age >55 years, was 8 (7-10) in Blacks and 7 (6-9) in Whites (*P*=0.0012). Black compared to White patients, were 7.9 (SE, 0.9) years younger (60.4 vs. 68.3 years). The home and office systolic and diastolic BPs were similar in both racial groups with the exception of the self-measured diastolic BP, which was 2.4 (SE, 1.1) mm Hg lower in White than Black patients (78.3 vs. 80.7 mm Hg). The number of home BP readings analysed and details on their distribution are available in **Table S5** and **Figure S2**. As summarised in **Table S6**, antihypertensive drugs interfering with the renin-angiotensin system, either β-blockers (48.4 vs. 16.1%) or angiotensin-I converting-enzyme inhibitors (ACEIs) or angiotensin-II

receptor-I blockers (ARBs) were more frequently prescribed to White patients (86.0 vs. 70.2%), with an opposite racial trend for dihydropyridine calcium-channel blockers (58.3 vs. 47.3%; *P*=0.087). Remarkably, the heart rate on home monitoring (74.5 vs. 67.5 beats per minute) as well as on office measurement (76.3 vs. 69.3 beats per minute) was faster in Blacks compared to Whites. After excluding patients taking β-blockers and non-dihydropyridine calcium channel blockers (CCBs), the heart rate in Blacks vs. Whites was still higher on home monitoring (75.1 vs. 68.6 beats per minute; *P*=0.003) as well as on office measurement (77.5 vs. 71.7 beats per minute; *P*=0.072).

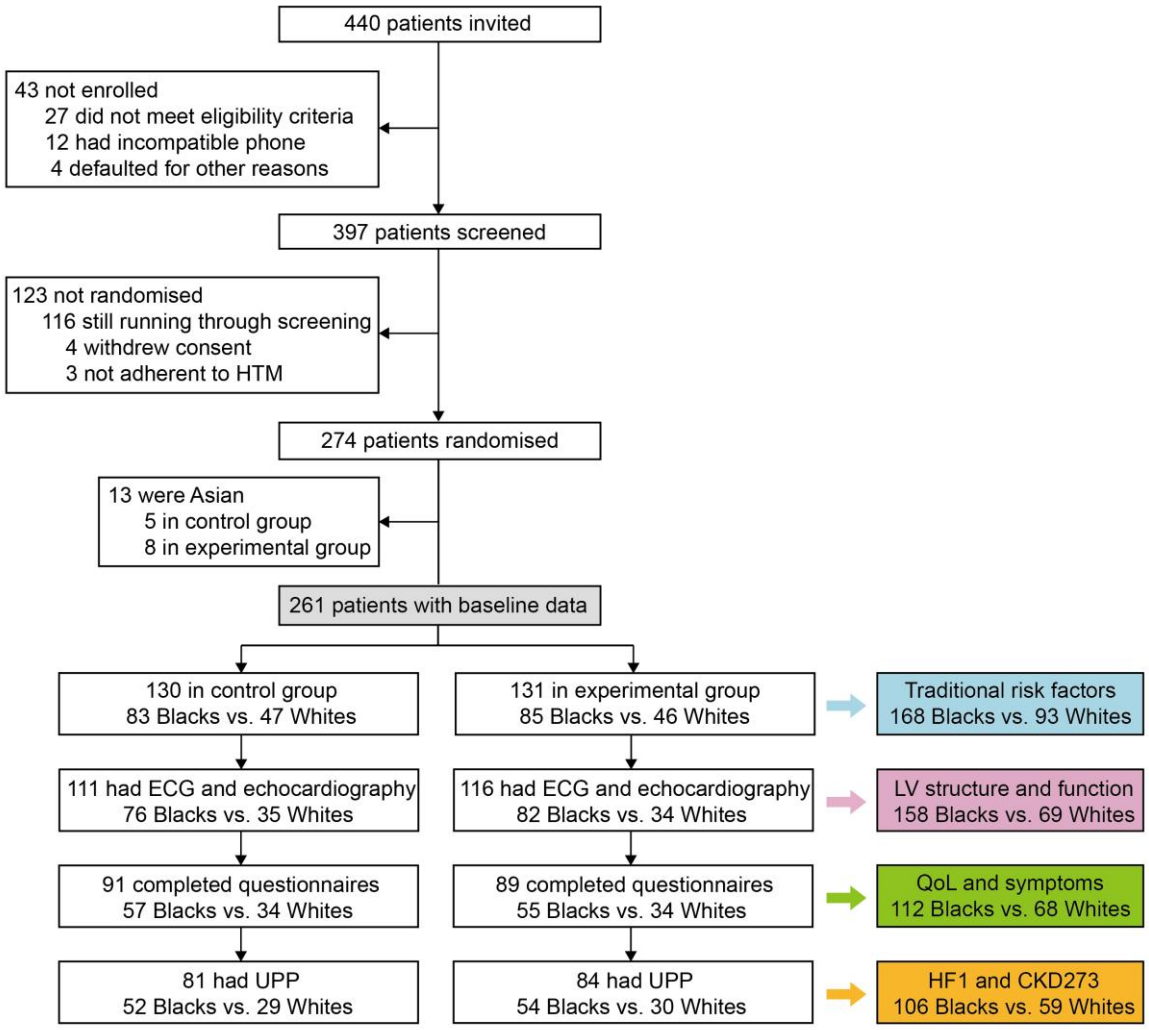


Figure 1. Consort diagram showing patient disposition, including screening, randomisation, and selection of patients for the current analyses.

Table 1. Characteristics of 261 analysed patients by race

Characteristic	Blacks		Whites		*P-value*
	N	Statistic	N	Statistic	
Female sex, n (%)	168	91 (54.2)	93	55 (59.1)	0.52
Age, years	168	60.4 (6.2)	93	68.3 (8.9)	<0.0001
Anthropometric measurements					
Body mass index, kg/m^2	168	31.0 (6.5)	93	29.1 (4.2)	0.014
Body mass index ≥30 kg/m^2, n (%)	168	80 (47.6)	93	38 (40.9)	0.30
Waist circumference, cm	168	99.2 (16.7)	93	95.4 (11.7)	0.054
Blood pressure and heart rate					
Home systolic BP, mm Hg	168	129.7 (10.8)	93	130.5 (11.2)	0.56
Home diastolic BP, mm Hg	168	80.7 (8.2)	93	78.3 (8.6)	0.026
Home heart rate, beats per minute	168	74.5 (10.4)	93	67.5 (9.07)	<0.0001
Office systolic BP, mm Hg	168	134.9 (14.8)	93	139.5 (14.5)	0.016
Office diastolic BP, mm Hg	168	82.8 (10.9)	93	83.7 (10.6)	0.52
Office heart rate, beats per minute	168	76.3 (13.1)	93	69.3 (10.9)	<0.0001
Home hypertension, n (%)	168	66 (39.3)	93	32 (34.4)	0.51
Office hypertension, n (%)	168	79 (47.0)	93	46 (49.5)	0.80
True normotension, n (%)	168	68 (40.5)	93	40 (43.0)	0.70
White-coat hypertension, n (%)	168	34 (20.2)	93	21 (22.6)	0.75
Masked hypertension, n (%)	168	21 (12.5)	93	7 (7.53)	0.30
Sustained hypertension, n (%)	168	45 (26.8)	93	25 (26.9)	>0.99
Treated hypertension, n (%)	168	161 (95.8)	93	90 (96.8)	>0.99
Renal function					
Serum uric acid, mg/dL	96	5.07 (2.03)	79	4.64 (2.30)	0.19
Serum creatinine, mg/dL	164	0.98 (0.34)	78	0.87 (0.17)	0.011
eGFR, mL/min/1.73 m^2	164	76.1 (21.5)	78	80.2 (31.2)	0.12
eGFR <60 mL/min/1.73 m^2, n (%)	164	43 (26.2)	78	6 (7.7)	<0.0001
Microalbuminuria, n (%)	167	79 (47.3)	92	0 (0.0)	<0.0001

Table 1. Characteristics of 261 analysed patients by race (continued)

Characteristic	Blacks		Whites		*P-value*
	N	Statistic	N	Statistic	
Metabolic variables					
Fasting blood glucose, mg/dL	149	106.4 (33.0)	76	102.5 (25.9)	0.36
Type-2 diabetes mellitus, n (%)	168	83 (49.4)	93	25 (26.9)	<0.0001
Treated diabetes mellitus, n (%)	168	38 (22.6)	93	18 (18.4)	0.64
Total serum cholesterol, mg/dL	165	197.6 (55.8)	80	180.7 (47.3)	0.020
HDL serum cholesterol, mg/dL	164	51.6 (19.0)	78	58.1 (12.2)	0.0064
LDL serum cholesterol, mg/dL	165	123.0 (49.7)	80	100.5 (44.5)	0.0007
Non-HDL serum cholesterol, mg/dL	165	146.8 (51.1)	80	124.1 (45.3)	0.0008
Total-to-HDL serum cholesterol ratio	164	4.35 (2.12)	78	3.19 (0.86)	<0.0001
Serum triglycerides, mg/dL	164	119.8 (61.2)	77	122.8 (45.7)	0.70
Treated dyslipidaemia, n (%)	168	72 (42.9)	93	67 (72.0)	<0.0001
Metabolic syndrome, n (%)	168	88 (52.4)	93	51 (54.8)	0.80
Other risk factors					
Current smoking, n (%)	168	1 (0.60)	93	13 (14.0)	<0.0001
Drinking alcohol, n (%)	168	11 (6.54)	93	8 (8.60)	0.62
Sedentary lifestyle, n (%)	168	103 (61.3)	93	29 (31.2)	<0.0001
Familial history of CVD, n (%)	168	24 (14.3)	93	15 (16.1)	0.72

Values are mean (SD) or number of patients with condition (%). N indicates the number of patients with data within group. Home BP is the average of all available readings up to the pre-randomisation visit. Home hypertension is a self-measured BP of ≥135 mm Hg systolic or ≥85 mm Hg diastolic. Office hypertension is an office BP of ≥40mm Hg systolic or ≥90 mm Hg diastolic. If systolic and diastolic blood pressure were in different categories, patients were classified in the highest category. White-coat hypertension is an elevated office BP combined with a normal home BP. Masked hypertension is a normal office BP combined with an elevated home BP pressure. Sustained hypertension is hypertension on home and office measurement. Home and office BP are cross-classified irrespective of antihypertensive drug treatment. Diabetes is a diagnosis provided by the caregiver and documented in local medical records. Treated diabetes refers to patients on antidiabetic drugs. Current smoking was inhaling tobacco smoke on a daily basis. Drinking alcohol was the occasional or daily consumption of ethanol containing beverages. Physical inactivity is engaging in less than 150 minutes of moderate-intensity aerobic exercise (3-6 METS) throughout the week.

As shown in **Table 1**, BMI was 1.85 (SE, 0.75) kg/m^2 higher in Blacks compared with Whites (31.0 vs. 29.1 kg/m^2) with a similar trend for waist circumference (99.2 vs. 95.4 cm; *P*=0.054). The composite profile of the serum cholesterol fractions (**Table 1**) was prognostically worse in Blacks than Whites, but treated dyslipidaemia was substantially higher in Whites than Blacks (72.9 vs. 42.9%), as evidenced by the more frequent use of lipid-lowering drugs in Whites (**Table S6**). Notwithstanding the younger age of Black patients (**Table 1**), Blacks had a higher prevalence of diabetes (49.4 vs. 26.9%), but not treated diabetes (22.6 vs. 18.4%; *P*=0.64), both risk factors possibly contributing to the higher prevalence in Blacks of eGFR <60 mL/min/1.73 m^2 (26.2 vs. 7.7%) and microalbuminuria (47.3 vs. 0%). Finally, smoking was not customary among Black patients (**Table 1**), but a sedentary lifestyle was (61.3 vs. 31.2%).

ECG and echocardiography

In Black compared to White patients (**Table 2**), the prevalence of ECG and echocardiographic left ventricular hypertrophy were similar: 13.9 vs. 8.8% and 15.8 vs. 14.4%, respectively (*P*≥0.11).

All centres performed echocardiographic imaging with General Electric (Horton, Norway) ultrasound scanners (**Table S7**). In unadjusted analyses of LV structure (**Table 2**), the aortic root diameter (29.5 vs. 26.2 mm) and relative wall thickness (49.0 vs. 45.0) were greater in Black compared to White patients (*P*<0.001), while left atrial diameter (35.6 vs. 38.6 mm) and end-diastolic LV diameter (44.8 vs. 47.6 mm) were smaller (*P*≤0.0032) with no racial difference in the end-systolic LV diameter (28.2 vs. 29.6 mm; *P*=0.15). With regard to systolic LV function (**Table 2**), stroke volume (62.1 vs. 72.0 mL) and stroke volume index (33.1 vs. 37.7 mL/m^2) were larger in Whites than Blacks. The isovolumetric relaxation time (101.0 vs. 111.8 ms) and the left atrial volume index (27.6 vs. 31.6 mL/m^2) were smaller in Black patients (*P*≤0.020) with no differences (*P*≥0.24) in the E/e' ratio (7.84 vs. 8.21) or the prevalence of diastolic LVD (36.3 vs. 55.9%).

To assess the consistency of the above unadjusted findings, the racial comparison of the echocardiographic measurements was adjusted for sex, age, BMI (not applicable to stroke volume index and left atrial volume index), diabetes, and the use of thiazide diuretics, β-blockers, inhibitors of the renin-angiotensin system (angiotensin-I converting enzyme inhibitors [ACEIs] and angiotensin-II receptor-I blockers [ARBs]) and dihydropyridine and non-dihydropyridine CCBs (**Table S8**). These adjustments confirmed the directionality of the differences between Black and White patients and only removed significance of the differences in the stroke volume index (33.6 vs. 36.5 ml/m^2; *P*=0.072) and isovolumetric relaxation time 102.6 vs. 107.7 ms; *P*=0.19).

In the ITT analysis (*P*=0.0008; **Figure S3, Panel A**), HF1 was lower in Blacks compared to Whites (-1.17 [95% CI, -1.32 to -1.02]) vs. (-0.71 [95% CI, -0.94 to -0.49]) with confirmatory results in the PP protocol analysis (*P*=0.0055; **Figure S3, Panel B**). In all patients (**Figure S3**; *P*≥0.63) and in Black (*P*≥0.35) and White (*P*≥0.87) patients analysed separately, the association of the stage of diastolic LVD was unrelated to HF1.

Framingham risk scores

Irrespective of race (**Table S9**), the median CVD risk score (**Table S9**), the 10-year CVD risk (**Figure2, Panel A**) and heart age (**Figure2, Panel B**) increased with chronological age (*P*<0.0024). The 10-year CVD risk was significantly higher in Blacks than Whites (*P*=0.010), while the between-race difference did not reach significance in other age strata (*P*≥0.18). In linear regression, the race-by-age group interaction term was not significant (*P*=0.53). In both racial groups, heart age was higher than chronological age (*P*<0.0001): 78.1 (95% CI, 76.0-80.2 years) vs. 60.6 (95% CI, 59.7-61.6 years) years in Blacks (**Figure 2, Panel C**) and 81.1 (95% CI, 78.5-83.6 years) vs. 68.4 (95% CI, 66.5-70.2 years) years in Whites (**Figure 2, Panel D**). The race-by-chronological age interaction term was also not significant (*P*=0.54). The PP analyses excluding 57 of 261 analysed patients were confirmatory (**Figure S4**).

Chronic kidney disease

The number of urine samples and the methodology of albuminuria measurement are presented in **Table S10**. In both the ITT (**Figure 3 and Table S11**; *P*=0.041) and PP (**Figure S5**; *P*=0.0085) analysis, multinomial logistic regression analysis showed that Blacks compared to Whites had an upward shift in the distribution of the CKD grade categories. Adjustment for sex, age, BMI, diabetes, and the use of thiazide diuretics, β-blockers, inhibitors of the renin-angiotensin system (ACEIs and ARBs) and dihydropyridine and non-dihydropyridine CCBs in the ITT analysis produced confirmatory results with Blacks compared to Whites having a greater risk of grade-2 CKD or higher. The odds ratios were 3.63 (95% CI, 2.27-5.79; *P*<0.0001) in Blacks and 0.28 (95% CI, 0.17-0.44; *P*<0.0001) in Whites with a significant racial difference (*P*<0.0001). In the per-protocol analysis these odds ratios were 3.82 (95% CI, 2.02-6.63; *P*<0.0001) and 0.26 (95% CI, 0.15-0.45; *P*<0.0001), respectively (*P* for racial difference, <0.0001).

In the ITT analysis (*P*=0.71; **Figure S6, Panel A**), CKD273 was not different in Blacks compared to Whites (-0.13 [95% CI, -0.20 to -0.06]) vs. (-0.15 [95% CI, -0.23 to -0.07]) with confirmatory results in the PP protocol analysis (*P*=0.80; **Figure S6, Panel B**). In all patients (**Figure S6**; *P*≤0.0014) and in Black (*P*≤0.033) and White (*P*≤0.066) patients analysed separately, CKD stage increased with CKD273.

Table 2.. ECG and echocardiographic measurements in 227 patients by race

Characteristic	Blacks		Whites		*P-value*
	N	Statistic	N	Statistic	
ECG measurements					
Sokolow-Lyon index, mV	151	2.31 (0.75)	69	2.13 (0.56)	0.074
R-wave in aVl, mV	151	0.62 (0.30)	69	0.59 (0.30)	0.46
Cornell product mV × ms	151	142.2 (75.2)	69	153.1 (76.0)	0.32
ECG LVH, n (%)	158	22 (13.9)	69	4 (5.8)	0.11
M-mode LV structure					
Aortic root diameter, mm	158	29.5 (4.4)	69	26.2 (5.7)	<0.0001
Left atrial diameter, mm	158	35.6 (5.9)	69	38.6 (4.8)	0.0003
Ratio of left atrial-to-aorta diameter	158	1.23 (0.26)	69	1.53 (0.32)	<0.0001
Interventricular septum, mm	158	10.8 (2.2)	69	10.8 (1.5)	0.97
Posterior wall thickness, mm	158	10.6 (1.8)	69	10.5 (1.6)	0.61
End-diastolic diameter, mm	158	44.8 (7.2)	69	47.6 (4.6)	0.0032
End systolic diameter, mm	158	28.2 (6.7)	68	29.6 (5.1)	0.15
Relative wall thickness	158	49.0 (11.7)	69	45.0 (7.6)	0.010
LV mass, g	158	171.1 (56.9)	69	185.2 (46.5)	0.071
LV mass index, g/m^2	158	90.8 (31.6)	69	96.3 (21.4)	0.19
Echocardiographic LVH, n (%)	158	25 (15.8)	69	10 (14.4)	0.85
LV systolic function					
Fractional shortening, %	158	36.8 (8.0)	68	37.5 (8.2)	0.58
Ejection fraction, %	158	66.6 (10.0)	69	67.5 (11.4)	0.57
Stroke volume, mL	158	62.1 (20.4)	69	72.0 (18.4)	0.0006
Stroke volume index, mL/m^2	158	33.1 (11.7)	69	37.7 (9.8)	0.0043
Cardiac output, L/min	156	4.52 (1.67)	69	4.81 (1.6)	0.23

Table 2.. ECG and echocardiographic measurements in 227 patients by race (continued)

Characteristic	Blacks		Whites		*P-value*
	N	Statistic	N	Statistic	
LV diastolic function					
Isovolumetric relaxation time, ms	155	101.0 (27.0)	57	111.8 (19.6)	0.0063
Left atrial volume index, mL/m^2	150	27.6 (13.2)	69	31.6 (7.69)	0.020
Transmitral E/A ratio	158	0.97 (0.41)	68	1.14 (1.36)	0.14
Mitral annular e'/a' ratio	154	0.81 (0.39)	67	0.81 (0.25)	0.85
E/e' ratio	156	7.84 (2.53)	68	8.21 (2.26)	0.30
LV diastolic dysfunction, n (%)	158	100 (36.3)	69	38 (55.9)	0.24
Impaired relaxation	158	22 (13.9)	69	4 (5.9)	0.077
Elevated LV filling pressure	158	53 (33.5)	69	27 (39.1)	0.42
Combined dysfunction	158	25 (15.8)	69	7 (10.1)	0.26

Values are mean (SD) or number of patients with condition (%). Diastolic LVD was classified into impaired relaxation, elevated LV end-diastolic filling pressure, and combined diastolic dysfunction (see Data Supplemental).

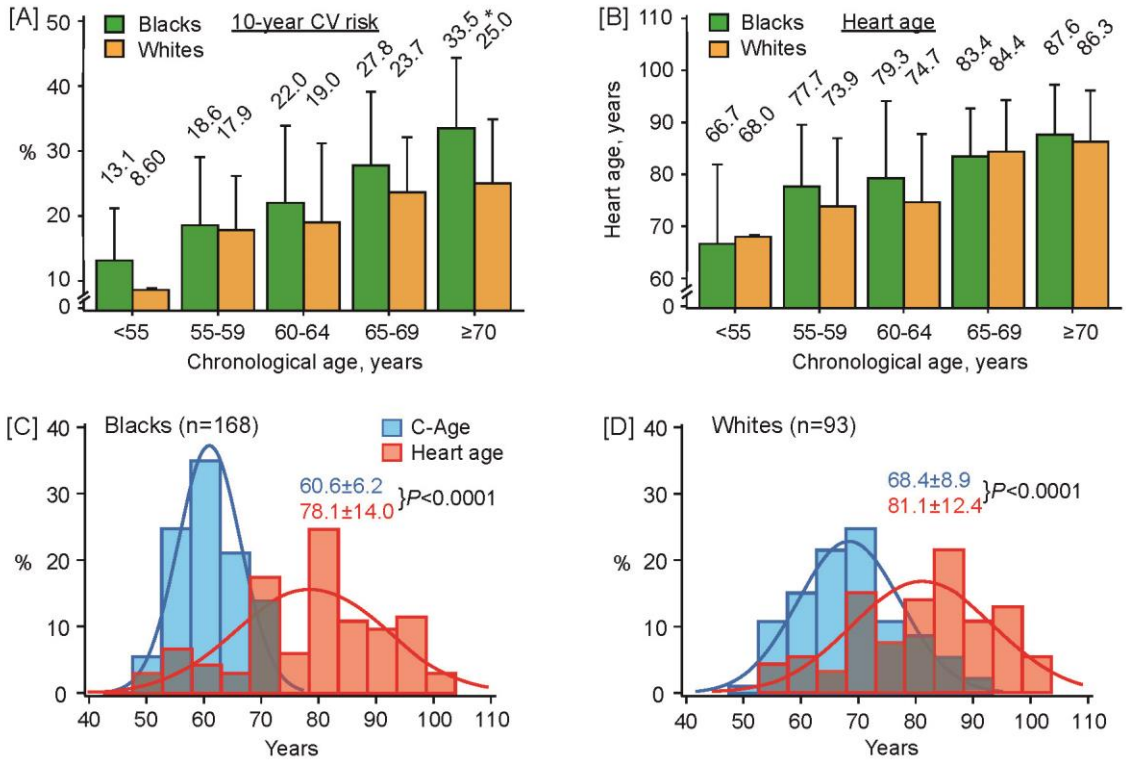


Figure 2. 10-year CV risk (A) and heart age (B) by race according to the Framingham risk scores and the distributions of chronological age and heart age in 168 Blacks (C) and 93 Whites (D). The sex-specific Framingham scores in Panels and B are derived from chronological age, total serum cholesterol, HDL serum cholesterol, SBP, antihypertensive treatment status (yes vs. no), smoking (yes vs. no) and diabetes (yes vs. no). In panels A and B, the height of the bars represents means (numerical value given) and the vertical lines the SD. The asterisk in Panel A indicates a significant difference in the 10-year CV risk between Blacks and Whites in patients aged ≥70 years. Panels C and D show the overlay in the distributions of chronological age and heart age in Blacks (C) and Whites (D). In both races, heart age was significantly higher than chronological age. The grey colour indicates the overlay in the distributions of chronological and heart age.

[A] Blacks (n=163)

eGFR, mL/min/1.73 m²			Albuminuria, mg		
			A1	A2	A3
			<30	30-300	>300
G1	≥90		28 (17)	24 (15)	1 (0.6)
G2	60-89		39 (24)	26 (16)	2 (1.2)
G3a	45-59		14 (8.6)	21 (13)	0
G3b	30-44		2 (1.2)	2 (1.2)	1 (0.6)
G4	15-29		2 (1.2)	0	1 (0.6)
G5	<15		0	0	0

[B] Whites (n=78)

eGFR, ml/min/1.73 m²			Albuminuria, mg		
			A1	A2	A3
			<30	30-300	>300
G1	≥90		21 (27)	0	0
G2	60-89		51 (65)	0	0
G3a	45-59		4 (5.1)	0	0
G3b	30-44		2 (2.6)	0	0
G4	15-29		0	0	0
G5	<15		0	0	0

CKD grade Low Moderately increased High Very high

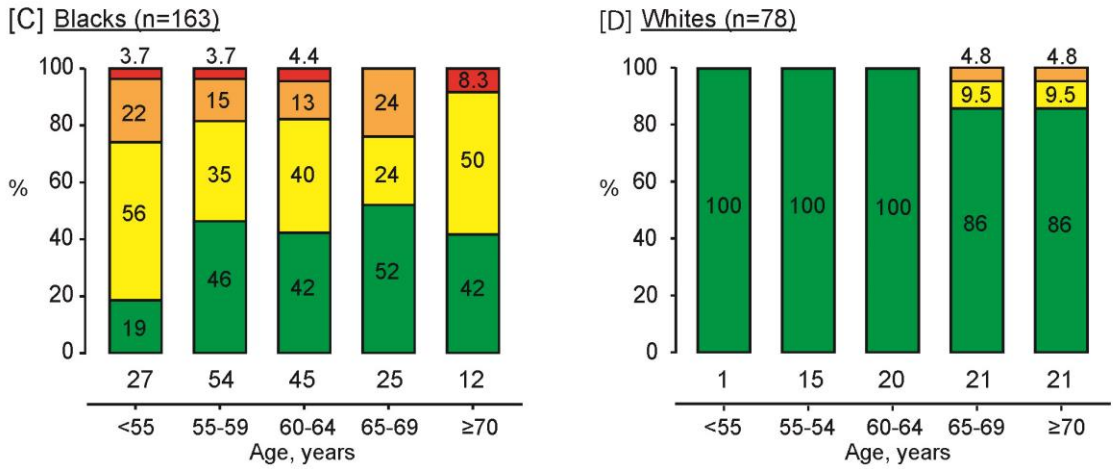


Figure 3. Grades of chronic kidney disease derived from the estimated glomerular filtration rate and albuminuria by race. The CKD grade is derived by cross-classification of eGFR and albuminuria, according to the 2024 KDIGO Guideline. Albuminuria is expressed in milligram per litre or per gram creatinine depending on the method of measurement. Panels A and B present the number of patients (%) in cross-classified cells by race. Panels C and D depict the percentage of patients at different CKD grades by age. The Spearman rank correlation coefficients between the CKD stage and age are -0.13 (*P*=0.099) in Blacks and 0.23 (*P*=0.041) in Whites.

Questionnaire data

The WHO questionnaire allowed the comparison of self-reported symptoms in Black vs. White patients. The prevalence (**Table S12**) was 10 (8.9%) vs. 6 (8.8%) for angina pectoris (*P*=0.98), 1 (0.9%) vs. 0 (0%) for claudication (*P*=0.43), 31 (27.7%) vs. 22 (32.4%) for exertional dyspnoea (*P*=0.50), 6 (5.4%) vs. 6 (8.8%) for habitual cough (*P*=0.37), and 5 (4.5%) vs. 1 (1.47%) for the production phlegm (*P*=0.28).

The prevalence of BMI ≥30 kg/m^2 (**Table 1**) was 47.6% in Blacks and 40.9% in Whites (*P*=0.30). β-blockers were more frequently prescribed to White than Black patients (48.4% vs. 16.1%; *P*<0.001; **Table S6**). Accounting for BMI as continuously distributed variable and β-blocker use, the odds of angina pectoris were 1.36 (95% CI, 0.76-2.57; *P*=0.28) in Blacks and 0.71 (95% CI, 0.39 to 1.32; *P*=0.28) in Whites, and for exertional dyspnoea 1.17 (95% CI, 0.83-1.65; *P*=0.38) and 0.86 (95% CI, 0.61-1.21; *P*=0.37), respectively. For both symptoms, the race-by-BMI and race-by-β-blockade interactions were not significant (*P*≥0.11). Finally, the median quality-of-life score (IQR), as assessed by patients on a visual analogue scale ranging from 0% to 100% was 75% (55-86%) in Blacks and 80% (67-89%) in Whites (*P*=0.76).

Discussion

The current study involved baseline data from an ongoing clinical trial and involved Blacks, born and living in sub-Saharan countries, and European Whites enrolled in Belgium, Poland, and Slovenia. All patients had to comply with the same eligibility criteria and had to be at high risk for CVD, CKD or both. Black patients had a median of 8 risk factors and White patients 7. Being younger (60.4 vs. 68.3 years), Blacks had a worse risk profile, as evidenced by higher diabetes prevalence, higher BMI, faster heart rate, unfavourable serum cholesterol fractions, lower estimated glomerular filtration rate, microalbuminuria and sedentary lifestyle. This resulted in Blacks having higher 10-year CVD risk, higher heart age (index of vascular ageing with chronological age as reference), and a worse CKD grades. In both races, CKD273 increased with CKD grade, but CKD273 and HF1 were not different by race. These observations were robust in subgroup and adjusted analyses.

A remarkable finding, which was not abolished by adjustment of the more frequent use of β-blockers in Europeans, was the higher heart rate in Blacks than Whites, both on home monitoring and at the office. The average annual temperature is likely to explain this observation, which is 5-9 ºC range for Poland, 12 ºC for Mediterranean Slovenia, but 26.9 ºC in the whole of Nigeria and around 23 ºC in the Potchefstroom area in South

Africa (World Meteorological Organization). Over the year, the climate is >10 ºC warmer in the sub-Saharan than the European catchment area of UPRIGHT-HTM patients. In an experimental setting, 37 older adults (61-80 years) rested for 8 h in 31 and 36 °C (45% relative humidity) [23]. Diabetes was the predictor of the end-exposure heart rate (with vs. without, 13 beats per minute; 95% CI, 6-23). In the present study, the prevalence of diabetes was 49.4 vs. 26.9 in Black compared to White patients. Exposure to high temperature also leads to stimulation of the sympathetic system and parasympathetic tone withdrawal [24]. In 274 randomly recruited Flemish (50% women; mean age, 40.6 years), the odds of endothelial dysfunction, as assessed by the flow-mediated vasodilatation in the brachial artery, increased by 58% (95% CI, 4-141%; *P*=0.03) for each 10 °C increment in the ambient daily temperature averaged over one week [25].

The FRS was developed and frequently applied in Whites [22,26], but was further validated in African-American [27,28]. The Jackson Heart Study [28] included 3689 Black adults (averaged 53 years, 64.8% women), among whom 270 participants experienced a first CVD event during the 9.1-year follow-up. The FRS proved to be effective in prediction risk in Black individuals with only marginal improvement of risk assessment by other risk factors, such as B-type natriuretic peptide or the ankle-brachial index [28]. Moreover, the FRS was further validated in a multi-ethnic study (n=23,424) comprising six prospectively followed US cohorts, including Whites, African-Americans, American Japanese and Hispanics, indicating reliable performance after recalibration [27]. In accordance with our study, American Blacks had a higher CVD risk profile, including higher BMI, serum uric acid, SBP and prevalence of habitual smoking compared to American Whites [29]. The National Health and Nutrition Examination Survey also highlighted the higher cardiovascular disease prevalence in Blacks than Whites with a Black-White prevalence ratio of 1.9 (95% CI,1.1-3.4) in young adulthood (35-44 years), which decreased with advancing age (*P*=0.04). Modifiable social and environmental risk factors play an important role in the racial disparity to CVD and CKD, not only in the US, but also sub-Saharan Africa [30].

Study limitations

What sets this study apart is the involvement of Blacks born and living in sub-Saharan Africa, and European Whites. While genetic admixture cannot be excluded in South African Blacks, presumably it is less of a confounding factor compared with studies conducted in the United States [7-10]. The same eligibility criteria were applicable in Africa and Europe to screen and randomise patients. Nevertheless, the present study must be interpreted within the context of several limitations. First and foremost, this study

did not differentiate host factors, which could be genetic or downstream pathogenic molecular pathways, from environmental drivers of ill health, such living in widely different climate zones, exposure to pollutants, parasitic and infectious agents, such as Anopheles mosquitos or HIV, and an unhealthy lifestyle with physical inactivity or a shifting preference of Black individuals for Westernised diets rich in refined sugars, fat and processed foods. Second, of 261 randomised patients, 57 did not fully comply with all entry criteria, because they were younger than 55 years (n=28), had an eGFR <45 mL/min/1.73m^2 (n=16), or both (n=1). This was to be expected in a trial running is so diverse regions and varying internet access. Therefore, the protocol pro-actively included the provision of an ITT and PP analysis. Both produced similar results in this preliminary analysis. As UPRIGHT-HTM is ongoing, a larger sample size will be attained at the later stage of our study. Third, not all the Black and White participants had UPP measurement. Therefore, we did not have enough power to explain the lower HF1 levels in Blacks than Whites. Finally, Asian participants were excluded from the current analysis, as there were few in number, giving that recruitment for UPRIGHT-HTM is still ongoing.

A call to action

To address the NCD burden, driven by hypertension, diabetes, and health problems related to climate change and environmental degradation a framework shift is necessary in sub-Saharan countries to deliver better health outcomes through people-centred health systems, with focuses on prevention, primary care, and public health [1]. Countries should invest more in health and do more to address inefficiencies by identification of new funding sources and movement towards prioritisation of health in domestic budgets [1]. Furthermore, an analysis of the Food and Agricultural Organization database and health indicators supplied by UNICEF and WHO revealed different dietary patterns in sub-Saharan Africa: a Westernised diet with high content of refined sugars, sweeteners, alcohol, meat, animal fats, eggs, and dairy in South Africa, and a high consumption of cereals, nuts, fish and vegetable oils in Nigeria, in particular in coastal areas [31]. The median values of health indicators explained the high prevalence of NCD in South Africa, where stunting and anaemia are higher than in other sub-Saharan regions [31]. The EAT Lancet Commission proposed that healthy diets should have an appropriate caloric intake and consist of a diversity of plant-based foods, low amounts of animal source foods, unsaturated rather than saturated fats, and small amounts of refined grains, highly processed foods, and added sugars [6].

About 40% of Nigerians live in poverty, in social conditions that create ill health, and with the ever-present risk of catastrophic expenditures from high out-of-pocket spending

for health. Even compared with countries of similar income levels in Africa, Nigeria's population health outcomes are poor, with national statistics masking drastic differences between rich and poor, urban and rural populations, and different regions [2] . The Lancet *Nigeria* Commission, in line with the mission statement of the Alliance for the Promotion of Preventive Medicine (www.appremed.org), prevention should be at the heart of health policies, given Nigeria's young population. To achieve this goal will require lifecourse approach to disease prevention [32] and the unwavering engagement to invest in prevention by the Nigerian federal and regional authorities and the community as a whole [2]. An explicit consideration of equity in the implementation of programmes and provision of social welfare, education and employment opportunities should be paramount [2].

Investment in higher education and research are essential for better health and sustainable development by enhancing research capacity for identification of challenges and devising local solutions. The International Society of Hypertension (ISH [https://ish-world.com/about-ish]) is committed to scientific innovation in the field of hypertension and in a wider context in addressing the risk factors underlying CVD and CKD. ISH also endorses the international exchange and promotion of investigators, teachers and students, the advocation of patient right, and the education of the public as well as medical professionals. Along with other national and international learned societies and in collaboration with regional health professionals, ISH is geared to take up its role in promoting health in sub-Saharan Africa.

Conclusions

In contrast to Whites in living Europe, Blacks born and living in sub-Saharan Africa had a higher risk profile of CVD and CKD, as by their risk factors load, the FRS, heart age, and their worse distribution across the KDIGO CKD grades. This study did not differentiate host (genetic, molecular, and pathogenic) from environmental drivers of disease. Nonetheless, the findings call for a multi-pronged and comprehensive implementation of innovative health policies in sub-Saharan countries. Education, research, empowerment of stakeholders, and international learned societies connecting experts from a wide array of disciplines should vigorously sustain this effort.

Funding

The Non-Profit Research Association Alliance for the Promotion of Preventive Medicine, Mechelen, Belgium (www.appremed.org) received a nonbinding grant from OMRON Healthcare Co. Ltd., Kyoto, Japan. Dries Martens holds a postdoctoral grant by the Research Foundations Flanders (FWO grant 12X9623N). The Guangci Laureate

Professorship of Jan A. Staessen is supported by the Guangci Deep Mind Project of Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China.

Conflicts of interest

Harald Mischak is the founder and co-owner of Mosaiques Diagnostics (Hannover, Germany). Agnieszka Latosinska is employed by Mosaiques Diagnostics. The other co-authors declare no conflict of interest.

References

1. Agyepong IA, Sewanjamba N, Binawaho A, Coll-Seck AM, Carrah T, Ezeh A*, et al.* The path to longer and healthier lives for all Africans by 2030: the *Lancet* Commission on future health in sub-Saharan Africa. *Lancet* 2017; 390:2803-2859.
2. Abubakar I, Dalglish SL, Angell B, Sanuade O, Abimbola S, Adamu AL*, et al.* The *Lancet* Nigeria Commission: investing in health and the future of the nation. *Lancet* 2022; 399:1155-1200.
3. GBD 2019 Investigators. Five insights from the Global Burden of Disease Study 2019. *Lancet* 2020; 396:1135-1159.
4. Keates AK, Mocumbi AO, Ntsekhe M, Sliwa K, Stewart S. Cardiovascular disease in Africa: epidemiological profile and challenges. *Nat Rev Cardiol* 2017; 14:273-293.
5. Schutte AE, Botha S, Fourie CMT, Gafane-Matemane LF, Kruger R, Lammertyn L*, et al.* Recent advances in understanding hypertension development in sub-Saharan Africa. *J Hum Hypertens* 2017; 31:491-500.
6. Willett W, Rockström J, Loken B, Springmann M, Lang T, Vermeulen S*, et al.* Food in the Anthropocene: the EAT-*Lancet* Commission on healthy diets from sustainable food systems. *Lancet* 2019; 393:447-492.
7. Tsao CW, Aday AW, Almarzooq ZI, Anderson CAM, Arora P, Avery CL*, et al.* Heart disease and stroke statistics-2023 update: a report from the American Heart Association. *Circulation* 2023; 147:e93-e621
8. Jolly S, Vittinghoff E, Chattopadhyay A, Bibbins-Domingo K. Higher cardiovascular disease prevalence and mortality among younger Blacks compared to Whites. *Am J Med* 2010; 123:811-818.
9. Lipworth L, Mumma MT, Cavanaugh KL, Edwards TL, Ikizler TA, Tarone RE*, et al.* Incidence and predictors of end stage renal disease among low-income Blacks and Whites. *PLoS One* 2012; 7:e48407
10. Nichols OI, Fuller-Rowell TE, Robinson AT, DaJuandra E, Homandberg LK. Neighborhood socioeconomic deprivation in early childhood mediates racial disparities in blood pressure in a college student sample. *J Youth Adolesc* 2022; 51:2146-2160.
11. Gafane-Matemane LF, Kruger R, Smith W, Mels CMC, van Rooyen JM, Mokwatsi GG*, et al.* Characterization of the renin-angiotension-aldosterone system in young healthy black adults: the African Prospective Study on the Early Detection and

Identification of Hypertension and Cardiovascular (African-PREDICT Study). *Hypertension* 2021; 78:400-410.

12. Parsa A, Kao L, Xie D, Astor BC, Li M, Hsu C-Y*, et al.* APOL1 risk variants, race, and progression of chronic kidney disease. *N Engl J Med* 2013; 369:2183-2196.
13. Masimango MI, Jadoul M, Binns-Roemer EA, David VA, Sumaili CA, Limou S. APOL1 renal risk variants and sickle cell trait associations with reducesd Kidney function in a large Congolese population-based study. *KI Reports* 2022; 7:474-482.
14. Odili AN, Thijs L, Hara A, Wei FF, Ogedengbe JO, Nwegbu MM*, et al.* Prevalence and determinants of masked hypertension among black Nigerians compared with a reference population. *Hypertension* 2016; 67:1249-1255.
15. Odili A, Thijs L, Yang WY, Ogedengbe JO, Nwegbu MM, Jacobs L*, et al.* Office and home blood pressures as determinants of electrocardiographic left ventricular hypertropy among Black Nigerians compared with White Flemish. *Am J Hypertens* 2017; 30:1083-1092.
16. Thijs L, Asayama K, Maestre GE, Hansen TW, Buyse L, Wei DM*, et al.* Urinary proteomics combined with home blood pressure telemonitoring for health care reform trial: rational and protocol. *Blood Press* 2021; 30:269-281.
17. Chori BS, An DW, Martens DS, Yu YL, Gilis-Malinowska N, Abubakar SM*, et al.* Urinary proteomics combined with home blood pressure telemonitoring for health care reform trial - First progress report. *J Clin Hypertens* 2023; 25:521-533.
18. World Medical Association. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. *J Am Med Assoc* 2013; 310:2191-2194.
19. Vlahou A, Hallinan D, Apweiler R, Argiles A, Beige J, Benigni A*, et al.* Data sharing under the General Data Protection Regulation: time to organize law and research ethics? *Hypertension* 2021; 77:1029-1035.
20. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2024 clinical practice guideline for the evaluation and management of chronic kidney disease. *Kidney Intern* 2024; 105:S117-S314
21. Kleinbaum DG, Kupper LL, Nizam A, Muller KE. The Correlation Coefficient and Straight-Line Regression Analysis. In *Applied Regression Analysis and Other Multivariate Methods*, 4th edn. Applied Regression Analysis and Other Multivariate MethodsAndover, Hampshire, UK: BOOKS/COLE CENGAGE Learning; 2010:91-106.

22. D'Agostino RB, Sr., Vasan RS, Pencina MJ, Wolf AM, Cobain M, Massaro JM*, et al.* General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation* 2008; 117:743-753.

23. Meade RD, Akerman AP, Notley SR, Kirby NV, Sigal RJ, Kenny GP. Exploring the contribution of inter-individual factors to the development of physiological heat strain in older adults exposed to simulated indoor overheating. *Appl Physiol Nutr Metab* 2024; 49:doi: 10.1139/apnm-2024-0135 (online ahead of print)

24. Bangalore S, Sawhney S, Messerli FH. Relation of beta-blocker-induced heart rate lowering and cardioprotection in hypertension. *J Am Coll Cardiol* 2008; 52:1482-1489.

25. Nawrot TS, Staessen JA, Fagard RH, Van Bortel L, Struijker-Boudier HA. Endothelial function and outdoor temperature. *Eur J Epidemiol* 2005; 20:407-410.

26. Cappuccio FP, Oakeshott P, Strazzullo P, Kerry SM. Application of Framingham risk estimates to ethnic minorities in the United Kingdom and implications for primary prevention of heart disease in general practice: cross-sectional population based study. *Br Med J* 2002; 325:1271-1277.

27. D'Agostino RB, Jr., Grundy S, Sullivan LM, Wilson P, for the CHD Risk Prediction Group. Validation of the Framingham coronary heart disease predictions scores. Results of a multiple ethnic groups investigation. *J Am Med Assoc* 2001; 286:180-187.

28. Fox ER, Samdarshi TE, Musani SK, Pencina MJ, Sung JH, Bertoni AG*, et al.* Development and validation of risk prediction models for cardiovascular events in black adults: the Jackson Heart Study Cohort. *JAMA Cardiol* 2016; 1:15-25.

29. Thomas SJ, Booth JN, Dai C, Li X, Allen N, Calhoun D*, et al.* Cumulative incidence of hypertension by 55 years of age in Blacks and Whites: the CARDIA study. *J Am Heart Assoc* 2018; 7:e007988

30. Mensah GA, Jaquish C, Srinivas P, Papanicolaou GJ, Wei GS, Redmond N*, et al.* Emerging concepts in precision medicine and cardiovascular diseases in racial and ethnic minority populations. *Circ Res* 2019; 125:7-13.

31. Nel JH, Steyn NP. The nutrition transition and the double-burdon of malnutrition in sub-Saharan African countries: how do these countries compare with the recommended LANCET COMMISSION global diet? *Int J Environ Res Public Health* 2022; 19:16791

32. Olsen MH, Angell SY, Asma S, Boutouyrie P, Burger D, Chirinos JA*, et al.* A call to action and a lifecourse strategy to address the global burden of raised blood pressure on current and future generations: the *Lancet* Commission on hypertension. *Lancet* 2016; 388:2665-2172.

Data Supplement

UPRIGHT-HTM investigators

Argentina-La Plata: M. Salazar, W. Espeche;
Belgium-Leuven: D.-W. An, Y.-L. Yu; J.A. Staessen, D.-Y. Zhang
Belgium-Lauwe: L. Buyse;
Belgium-Aalst: B. Mujaj;
Belgium-Mechelen: J.A. Staessen;
Kosovo-Prishtina: V. Berisha;
Mozambique-Maputo: A. Damasceno; Laurenço Chemo
Nigeria-Abakaliki: G. Isiguzo;
Nigeria-Abuja: O.O. Abiodun, T. Anya, E. Anyabolu, B. Chori, J. Ogedengbe, A.N. Odili, I. Okoye;
Nigeria-Katsina: S. Abdurrahaman, M. Gbadamosi, B.A. Kurfi, B. Sani, M.N. Sehu, B. Yusuf;
Poland-Gdańsk: N. Gilis-Malinowska, K. Narkiewicz;
Poland-Kraków: B.P. Kaleta, P. Lis, A. Olzanecka, M. Rajzer, K. Stolarz-Skrzypek, W. Wojciechowska;
Poland-Warsaw: A. Januszewicz, N. Jurzak-Mysliwy, A. Łebek-Szatańska, P. Talarowska;
Slovenia-Ljubljana: J. Brgulian-Hitij, J. Knez, N. Božič;
South Africa-Potchefstroom: G. Mokwatsi, R. Kruger, C.M.C. Mels, A.E. Schutte;
ROC-Tainan: C.-T. Liao, W.-T. Chang;
ROC-Taipei: H.-M. Cheng, C.-H. Chen, S.-H. Sung, Y.-W, Tsai, J.-S. Yeh, W.-C. Yu;
Uruguay-Montevideo: J. Boggia;
United States-Rio Grande Valley: G.E. Maestro, J.D. Melgarejo.

Data collection

Data collected by patients

For HTM, patients are using the validated [1] OMRON HEM 9210-T monitors (Omron Healthcare Co., Ltd., Kyoto, Japan) fitted with a cuff that accommodates the range of adults upper-arm circumferences. Patients are instructed to measure their BP after 5 minutes rest in the sitting position, preferably within 1 hour after awakening, before breakfast and before taking any medication, if possible, daily. At randomisation, patients complete in their native language the EQ-5D quality of life questionnaire (https://euroqol.org) and the World Health Organization questionnaire on chest pain,

claudication, dyspnoea, cough, and phlegm production [2].

Data generated by caregivers

The prerandomisation eCRF to be submitted by caregivers includes information from patients on anthropometrics, the oscillometrically measured office BP (OMRON HEM 9210-T [1]), use of medications by drug class, and medical history. Venous blood samples and mid-morning urine samples urine obtained from patients are processed by multi-analysers at certified local laboratories. Low-density lipoprotein (LDL) serum cholesterol is calculated from total serum cholesterol, high-density lipoprotein (HDL) serum cholesterol and the serum triglyceride concentration by the Friedewald formula [3]. The glomerular filtration rate is computed from serum creatinine, using the Chronic Kidney Disease Epidemiology (CKD-EPI) Collaboration formula [4]. Micro-albuminuria is the presence of albumin (30-300 mg per gram creatinine in two of three midmorning urine samples [5]. The use of semiquantitative dipstick test is allowed given that UPRIGHT-HTM involves clinical sites in low- and middle-income countries. Physicians read the ECGs and coded voltages and repolarisation changes indicative of left ventricular hypertrophy [6].

Around the time of randomisation, patients are referred to a local cardiology clinic for the baseline echocardiographic examination. All centres use General Electric echocardiographic devices interfaced with appropriate probes (**Table S1**). The Data Supplement of the open-access UPRIGHT-HTM protocol article (Supplementary Material, pp 11-15 [7]) provides a detailed description of the procedures applied for the acquisition and the off-line analysis of the echocardiographic traits, which comply with international guidelines [8,9]. Left atrial volume is calculated using the prolate ellipsoid method. The peak early (E) and peak late (A) diastolic velocities of the transmitral blood flow are derived from the pulsed wave Doppler signal and peak early (e') and peak late (a') velocities of the mitral annular movement by tissue Doppler imaging (TDI) with velocities averaged over one up to four acquisition sites (septal, lateral, inferior, and posterior). Diastolic LVD was categorised in three groups based on age-specific criteria generated in two populations studies and normal reference samples drawn from these populations [10,11] and their prognostic implications [12,12]: impaired relaxation (elevated age-specific E/A and E/e' <8.5); elevated LV end-diastolic filling pressure (E/e' >8.5 with normal age-specific E/A), and combined dysfunction (E/e' >8.5 and abnormally low age-specific E/A ratio). The intra- and inter-observer repeatability of key echocardiographic traits meet quality standards [13,14] and their publication as full-text articles is in progress.

Mosaiques-Diagnostics GmbH, Hannover, Germany (MOS) did the UPP profiling for

all patients. Multiple publications describe the methods for sample preparation, capillary electrophoresis coupled with mass spectrometry (CE-MS), peptide sequencing, and for the evaluation, calibration and quality control of the mass spectrometric data have been published [15,16]. In the CE-MS step, 29 abundant endogenous urinary peptides are used as internal standards for calibration of the signal intensity. This procedure is highly reproducible and addresses in a single calibration step both analytical and dilution variances, such as variability due to in renal function [17]. Sequenced urinary peptides allow the identification of parental proteins and their role in molecular pathogenetic processes [16]. However, the current analysis focuses on validated urinary proteomic markers of imminent diastolic LVD and CKD, respectively consisting of 85 (HF1 [18,19]) and 273 (CKD273 [20,21]) urinary peptide.

Data transfer

The HTM readings, the electronic questionnaire data collected directly from patients and the eCRFs completed by caregivers reach the study coordinating team via the WiPam platform (www.wipam.net) as CSV (coma-separated values) files. The UPP data are transferred to study coordinating team as Excel email attachments. After quality control, the data are directly imported into a SAS database, using the SAS PC File Server, version 9.4 [7].

References

1. Alpert BS. Validation of the Omron HEM-9210T by the ANSI/AAMI/ISO 81060-2:2013 with two novel cuffs: wide-range and extra-large. *Blood Press Monit* 2017, 22:166-168.
2. Rose GA, Blackburn H. Cardiovascular survey methods. *Monogr Ser World Health Organ* 1968, 56:1-188.
3. Friedewald WT, Levy RI, Frederickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge *Clin Chem* 1972, 18:499-502.
4. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, III, Feldman HI*, et al.* A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009, 150:604-612.
5. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2024 clinical practice guideline for the evaluation and management of chronic kidney disease. *Kidney Intern* 2024, 105:S117-S314.

6. Odili A, Thijs L, Yang WY, Ogedengbe JO, Nwegbu MM, Jacobs L*, et al.* Office and home blood pressures as determinants of electrocardiographic left ventricular hypertropy among Black Nigerians compared with White Flemish. *Am J Hypertens* 2017, 30:1083-1092.
7. Thijs L, Asayama K, Maestre GE, Hansen TW, Buyse L, Wei DM*, et al.* Urinary proteomics combined with home blood pressure telemonitoring for health care reform trial: rational and protocol. *Blood Press* 2021, 30:269-281.
8. Gottdiener JS, Bednarz J, Devereux R, Gardin J, Klein A, Manning WJ*, et al.* American Society of Echocardiography recommendations for use of echocardiography in clinical trials. A report from the American Society of Echocardiography's Guidelines and Standard Committee and the Task Force on Echocardiography in Clinical Trials. *J Am Soc Echocardiogr* 2004, 17:1086-1119.
9. Nagueh SF, Smiseth OA, Appleton CP, Byrd BF3, Dokainish H, Edvardsen T*, et al.* Recommendations for the Evaluation of left ventricular diastolic function by echocardiography: an update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *J,Am Soc Echocardiogr* 2016, 29:277-314.
10. Kuznetsova T, Herbots L, López B, Jin Y, Richart T, Thijs L*, et al.* Prevalence of left ventricular diastolic dysfunction in a general population. *Circ Heart Fail* 2009, 2:105-112.
11. Kloch-Badelek M, Kuznetsova T, Sakiewicz W, Tikhonoff V, Ryabikov A, González A*, et al.* Prevalence of left ventricular diastolic dysfunction in European populations based on cross-validated diagnostic thresholds. *Cardiovascular Ultrasound* 2012, 10:10.
12. Kuznetsova T, Thijs L, Knez J, Herbots L, Zhang Z, Staessen JA. Prognostic value of left ventricular diastolic dysfunction in a general population. *J Am Heart Assoc* 2014, 3:e000789.
13. Isiguzo GC, Antia SE, Ugwu CN, Ugwueze CV, Chori BS, An DW*, et al.* Intra- and inter-observer reproducibility of the echocardiographic data of the Nigerian UPRIGHT-HTM trial participants. *Blood Press Monit* 2023, 28 (Suppl 1):e5.
14. Kaleta B, Stolarz-Skrzypek K, Rajzer M. Reproducibility of the echocardiographic traits in Polish UPRIGHT-HTM participants. *Blood Press Monit* 2023, 28 (Suppl 1):e5.

15. Mavrogeorgis E, Mischak H, Latosinska A, Siwy J, Jankowski V, Jankowski J. Reproducibility evaluation of urinary peptide detection using CE-MS. *Molecules* 2021, 26:7260.

16. Yu YL, Siwy J, An DW, González A, Hansen T, Latosinska A*, et al.* Urinary proteomic signature of mineralocorticoid receptor antagonism by spironolactone: evidence from the HOMAGE trial. *Heart* 2024, 110:1180-1187.

17. Jantos-Siwy J, Schiffer E, Brand K, Schumann G, Rossing K, Delles C*, et al.* Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res* 2009, 8:268-281.

18. Kuznetsova T, Mischak H, Mullen W, Staessen JA. Urinary proteome analysis in hypertensive patients with left ventricular diastolic dysfunction. *Eur Heart J* 2012, 33:2342-2350.

19. Zhang ZY, Nkuipou-Kenfack E, Staessen JA. Urinary peptidomic biomarker for personalized prevention and treatment of diastolic left ventricular dysfunction. *Proteomics Clin Appl* 2019, 13:e1800174.

20. Pontillo C, Zhang ZY, Schanstra JP, Jacobs L, Zürbig P, Thijs L*, et al.* Prediction of chronic kidney disease stage 3 by CKD273, a urinary proteomic biomarker. *KI Reports* 2017, 2:1066-1075.

21. Tofte N, Lindhardt M, Adamova K, Bakker SJL, Beige J, Beulens JWJ*, et al.* Early detection of diabetic kidney disease by urinary proteomics and subsequent intervention with spironolactone to delay progression (PRIORITY): a prospective observational study and embedded randomised placebo-controlled trial. *Lancet Diabetes Endocrinol* 2020, 8:301-312.

Table S1. Characteristics of screened patients still progressing through the run-in period and randomised patients

Characteristic	Screened but not randomised		Randomised		*P-value*
	N	Statistic	N	Statistic	
N° with characteristic (%)					
Race	116		274		
Asian		12 (10.3)		13 (4.7)	
European Whites		32 (27.6)		93 (33.9)	0.081
Sub-Saharan Blacks		72 (62.0)		168 (61.3)	
Igbo		26 (36.1)		53 (31.6)	
Yoruba		1 (1.39)		23 (13.7)	<0.0001
Hansa-Fulani		34 (47.2)		31 (18.5)	
Other Nigerian ethnic groups		11 (15.3)		61 (36.3)	
Female sex	116	80 (48.2)	274	153 (55.8)	0.12
Current smoking	116	9 (5.4)	274	18 (6.6)	0.63
Drinking alcohol	116	11 (6.6)	274	22 (8.0)	0.59
Mean characteristic (± SD)					
Age, y	116	60.6 (10.5)	274	63.2 (8.1)	0.0074
Body height, cm	116	166.5 (8.2)	274	165.6 (8.5)	0.36
Body weight, kg	116	77.7 (15.6)	274	83.1 (17.0)	0.0038
Body mass index, kg/m^2	116	28.1 (5.7)	274	30.3 (5.9)	0.0009
Waist circumference, cm	116	94.4 (15.4)	274	97.8 (15.0)	0.041
Office blood pressure					
Systolic, mm Hg	116	135.3 (16.4)	274	135.4 (15.4)	0.92
Diastolic, mm Hg	116	81.8 (11.5)	274	82.6 (10.8)	0.47
Office heart rate, beats per minute	116	77.2 (11.5)	274	74.1 (12.6)	0.023

Current smoking is inhaling tobacco smoke on a daily basis. Drinking alcohol was the occasional or daily consumption of ethanol containing beverages. Screened patients n=116) are apparently eligible patients still proceeding through the run-in period on 6 April 2024, at the time the baseline data were frozen for the current analysis (see **Figure 1**).

Table S2. Risk factors in 261 patients by randomisation group

Characteristic	Control		Experimental		*P-value*
	N	Statistic	N	Statistic	
Female sex, n (%)	130	75 (57.7)	131	71 (54.2)	0.62
Age, years	130	63.1 (7.8)	131	63.3 (8.6)	0.79
White ethnicity, n (%)	130	47 (36.2)	131	46 (35.1)	0.86
Sub-Saharan Black ethnicity, n (%)	130	83 (63.8)	131	85 (64.9)	
Anthropometric measurements					
Body mass index, kg/m2	130	30.3 (5.3)	131	30.3 (6.4)	0.99
Body mass index ≥30 kg/m2, n (%)	130	57 (43.8)	131	61 (46.6)	0.71
Waist circumference, cm	130	97.7 (14.7)	131	97.9 (15.6)	0.95
Blood pressure and heart rate					
Home systolic BP, mm Hg	130	130.1 (11.3)	131	129.9 (10.6)	0.88
Home diastolic BP, mm Hg	130	79.3 (8.4)	131	80.5 (8.5)	0.25
Home heart rate, beats per minute	130	71.5 (10.5)	131	72.6 (10.6)	0.41
Office systolic BP, mm Hg	130	137.3 (15.9)	131	135.9 (13.9)	0.45
Office diastolic BP, mm Hg	130	83.1 (10.4)	131	83.1 (11.1)	0.96
Office heart rate, beats per minute	130	72.5 (12.7)	131	75.2 (12.8)	0.091
Home hypertension, n (%)	130	48 (36.9)	131	50 (38.2)	0.90
Office hypertension, n (%)	130	62 (47.7)	131	63 (48.1)	>0.99
True normotension, n (%)	130	57 (43.8)	131	51 (38.9)	0.45
White-coat hypertension, n (%)	130	25 (19.2)	131	30 (22.9)	0.54
Masked hypertension, n (%)	130	11 (8.5)	131	17 (13.0)	0.32
Sustained hypertension, n (%)	130	37 (28.5)	131	33 (25.2)	0.58
Treated hypertension, n (%)	130	127 (97.7)	131	124 (94.7)	0.33
Renal function					
Serum uric acid, mg/dL	83	4.96 (2.20)	92	4.80 (2.1)	0.64
Serum creatinine, mg/dL	122	0.94 (0.34)	120	0.95 (0.25)	0.89
eGFR, mL/min/1.73 m2	122	78.3 (19.5)	120	76.5 (19.1)	0.48
eGFR <60 mL/min/1.73 m2, n (%)	122	23 (18.9)	120	26 (21.7)	0.63
Microalbuminuria, n (%)	129	42 (32.6)	130	37 (28.5)	0.50

Table S2. Risk factors in 261 patients by randomisation group (continued)

Characteristic	Control		Experimental		*P-value*
	N	Statistic	N	Statistic	
Metabolic variables					
Fasting blood glucose, mg/dL	111	104.2 (27.4)	114	106.0 (33.8)	0.66
Type-2 diabetes mellitus, n (%)	130	53 (40.8)	131	55 (42.0)	0.90
Treated diabetes mellitus, n (%)	130	27 (20.8)	131	29 (22.1)	0.88
Total serum cholesterol, mg/dL	124	195.2 (56.7)	121	188.9 (50.5)	0.35
HDL serum cholesterol, mg/dL	121	53.7 (18.7)	121	53.6 (16.0)	0.96
LDL serum cholesterol, mg/dL	124	119.3 (52.5)	121	111.9 (45.3)	0.24
Non-HDL serum cholesterol, mg/dL	124	142.8 (53.8)	121	135.9 (46.5)	0.28
Total-to-HDL serum cholesterol ratio	121	4.14 (2.2)	121	3.82 (1.5)	0.19
Serum triglycerides, mg/dL	121	120.5 (51.7)	120	120.9 (61.4)	0.96
Treated dyslipidaemia, n (%)	130	66 (50.8)	131	73 (55.7)	0.46
Metabolic syndrome, n (%)	130	75 (57.7)	131	64 (48.9)	0.17
Other risk factors					
Current smoking, n (%)	130	7 (5.4)	131	7 (5.3)	>0.99
Drinking alcohol, n (%)	130	7 (5.4)	131	12 (9.2)	0.34
Sedentary lifestyle, n (%)	130	65 (50.0)	131	67 (51.1)	0.90
Familial history of CVD, n (%)	130	22 (16.9)	131	17 (13.0)	0.39

Values are mean (SD) or number of patients with condition (%). N indicates the number of patients with data within group. Home BP is the average of all available readings up to the pre-randomisation visit. Home hypertension is a self-measured BP of ≥135 mm Hg systolic or ≥85 mm Hg diastolic. Office hypertension is an office BP of ≥40mm Hg systolic or ≥90 mm Hg diastolic. If systolic and diastolic blood pressure were in different categories, patients were classified in the highest category. White-coat hypertension is an elevated office BP combined with a normal home BP. Masked hypertension is a normal office BP combined with an elevated home BP pressure. Sustained hypertension is hypertension on home and office measurement. Home and office BP are cross-classified irrespective of antihypertensive drug treatment. Diabetes is a diagnosis provided by the caregiver and documented in local medical records. Treated diabetes refers to patients on antidiabetic drugs. Current smoking was inhaling tobacco smoke on a daily basis. Drinking alcohol was the occasional or daily consumption of ethanol containing beverages. Physical inactivity is engaging in less than 150 minutes of moderate-intensity aerobic exercise (3-6 METS) throughout the week.

Table S3. ECG and echocardiographic measurements in 227 patients by randomisation group

Characteristic	Control		Experimental		*P-value*
	N	Statistic	N	Statistic	
ECG measurements					
Sokolow-Lyon index, mV	109	2.21 (0.70)	111	2.30 (0.70)	0.30
R-wave in aVl, mV	109	0.61 (0.31)	111	0.60 (0.29)	0.77
Cornell product mV × ms	109	141.9 (78.7)	111	149.3 (72.3)	0.47
ECG LVH, n (%)	111	12 (10.8)	116	14 (12.1)	0.84
M-mode LV structure					
Aortic root diameter, mm	111	28.2 (5.0)	116	28.8 (5.07)	0.32
Left atrial diameter, mm	111	36.3 (6.3)	116	36.8 (5.3)	0.50
Ratio of left atrial-to-aorta diameter	111	1.33 (0.31)	116	1.32 (0.31)	0.88
Interventricular septum, mm	111	10.8 (2.01)	116	10.8 (2.05)	0.97
Posterior wall thickness, mm	111	10.6 (1.75)	116	10.5 (1.68)	0.46
End-diastolic diameter, mm	111	44.9 (5.3)	116	46.4 (7.6)	0.094
End systolic diameter, mm	110	27.9 (5.3)	116	29.4 (7.1)	0.068
Relative wall thickness	111	48.3 (9.0)	116	47.3 (12.2)	0.48
LV mass, g	111	172.0 (52.2)	116	178.7 (56.1)	0.35
LV mass index, g/m^2	111	92.4 (30.5)	116	92.6 (27.5)	0.95
Echocardiographic LVH, n (%)	111	19 (17.1)	116	16 (13.8)	0.58
LV systolic function					
Fractional shortening, %	110	37.7 (8.0)	116	36.5 (8.0)	0.28
Ejection fraction, %	111	67.9 (10.5)	116	65.9 (10.3)	0.15
Stroke volume, mL	111	63.4 (18.7)	116	66.7 (21.6)	0.22
Stroke volume index, mL/m^2	111	34.2 (11.5)	116	34.8 (11.3)	0.71
Cardiac output, L/min	111	4.45 (1.42)	114	4.77 (1.83)	0.14
LV diastolic function					
Isovolumetric relaxation time, ms	103	103.4 (23.7)	109	104.5 (27.4)	0.75
Left atrial volume index, mL/m^2	108	29.8 (11.3)	111	27.9 (12.4)	0.23
Transmitral E/A ratio	110	1.03 (0.90)	116	1.01 (0.74)	0.82
Mitral annular e'/a' ratio	110	0.84 (0.41)	111	0.78 (0.29)	0.25
E/e' ratio	110	8.06 (2.51)	114	7.85 (2.39)	0.53
LV diastolic dysfunction, n (%)	111	70 (63.1)	116	68 (58.6)	0.49
Impaired relaxation	111	10 (9.0)	116	16 (13.8)	0.26
Elevated LV filling pressure	111	43 (38.7)	116	37 (31.9)	0.28
Combined dysfunction	111	17 (15.3)	116	15 (12.9)	0.61

Values are mean (SD) or number of patients with condition (%). Diastolic LVD was classified into impaired relaxation, elevated LV end-diastolic filling pressure, and combined diastolic dysfunction (see Methods).

Table S4. Drugs prescribed at baseline to 261 analysed patients by randomisation group

Characteristic	Control		Experimental		*P-value*
	N	Statistic	N	Statistic	
Antihypertensive drugs					
Thiazide or thiazide-like diuretics, n (%)	130	59 (45.7)	131	53 (40.5)	0.39
MRAs, n (%)	130	3 (2.3)	131	2 (1.5)	0.64
β-blocker, n (%)	130	34 (26.1)	131	38 (29.0)	0.68
ACEIs or ARBs, n (%)	130	104 (80.0)	131	94 (71.8)	0.15
Dihydropyridine CCBs, n (%)	130	76 (58.5)	131	66 (50.4)	0.19
Non-dihydropyridine CCBs, n (%)	130	4 (3.1)	131	10 (7.6)	0.11
Combination therapy, n (%)	130	99 (76.2)	131	89 (67.9)	0.17
Monotherapy, n (%)	130	28 (21.5)	131	35 (26.7)	0.39
Lipid-lowering drugs					
Statin, n (%)	130	62 (47.7)	131	72 (55.0)	0.27
Fibrate, n (%)	130	1 (0.8)	131	2 (1.5)	>0.99
Other lipid-lowering drugs, n (%)	130	5 (3.8)	131	3 (2.3)	0.50
Antidiabetic drugs					
Metformin, n (%)	130	24 (18.5)	131	26 (19.8)	0.88
Insulin, n (%)	130	3 (2.3)	131	4 (3.1)	>0.99
Other antidiabetic drugs, n (%)	130	1 (0.8)	131	2 (1.5)	>0.99
Aspirin, n (%)	130	15 (11.7)	131	18 (13.7)	0.71

N indicates the total number of patients within group. Values are number of patients (%). Abbreviations: ACEIs: angiotensin-I converting-enzyme inhibitor; ARBs: angiotensin-II type-I receptor blocker; CCBs: calcium-channel blockers; MRAs, mineralocorticoid antagonists.

Table S5. Home BP measurements at baseline in 261 randomised patients by recruitment centre

Centre	Available measurements			Home BP level, mm Hg				
	Nº patients	Nº days (%)	Median times per day	Median	P5	P25	P75	P95
001	1	14 (13.1)	1	108/72	101/67	103/70	111/75	121/80
003	1	1 (…)	1	96/65	…	…	…	…
004	7	25 (92.4)	1	140/83	106/53	124/70	154/95	171/108
050	1	1 (…)	1	123/67	…	…	…	…
052	59	36 (99.7)	1	130/79	105/61	121/72	139/85	156/96
250	10	10 (41.0)	1	143/85	111/70	131/77	152/89	169/99
300	14	38 (68.5)	1	137/75	105/60	125/69	146/82	160/92
350	102	27 (82.0)	1	129/81	109/66	120/75	139/88	154/98
351	28	29 (82.9)	1	131/80	110/63	121/71	146/89	163/100
400	11	25 (58.2)	1	122/69	107/61	116/66	130/74	143/82
401	27	9 (93.5)	1	135/88	111/70	125/81	160/113	183/129

Recruiting centres are identified by their study number. Nº of measurement days is expressed as percentage of days with measurements relative to the standard, i.e., one measurement per day. Median times per day are summarised first by patient and next by centre. The analysis includes all HTM readings from screening up to the pre-randomisation visit (see **Figure S1**). Overall, the median number of home BP readings per centre was 208 (IQR: 84-798), per patient 24 (IQR: 9-35), and per patient and centre 22 (IQR: 7-29). The number of home BP readings depends on the interval between screening and the pre-randomisation visit (1-5 days or more), which varied within and between centres. An ellipsis indicates not applicable.

Table S6. Drugs prescribed at baseline to 261 analysed patients by race

Characteristic	Blacks		Whites		*P-value*
	N	Statistic	N	Statistic	
Antihypertensive drugs					
Thiazide or thiazide-like diuretics, n (%)	168	67 (40.1)	93	45 (48.4)	0.20
MRAs, n (%)	168	3 (1.8)	93	2 (2.2)	0.84
β-blocker, n (%)	168	27 (16.1)	93	45 (48.4)	<0.0001
ACEIs or ARBs, n (%)	168	118 (70.2)	93	80 (86.0)	0.0040
Dihydropyridine CCBs, n (%)	168	98 (58.3)	93	44 (47.3)	0.087
Non-dihydropyridine CCBs, n (%)	168	9 (5.4)	93	5 (5.4)	0.99
Combination therapy, n (%)	168	117 (69.6)	93	71 (76.3)	0.31
Monotherapy, n (%)	168	44 (26.2)	93	19 (20.4)	0.37
Lipid-lowering drugs					
Statin, n (%)	168	72 (42.7)	93	62 (66.7)	0.0002
Fibrates, n (%)	168	0 (0)	93	3 (3.23)	0.044
Other lipid-lowering drugs, n (%)	168	2 (1.2)	93	6 (6.4)	0.025
Antidiabetic drugs					
Metformin, n (%)	168	35 (20.8)	93	15 (16.1)	0.41
Insulin, n (%)	168	5 (3.0)	93	2 (2.2)	>0.99
Other antidiabetic drugs, n (%)	168	1 (0.6)	93	2 (2.2	0.29
Aspirin, n (%)	168	19 (11.4)	93	14 (15.2)	0.44

N indicates the total number of patients within group. Values are number of patients (%). Abbreviations: ACEIs: angiotensin-I converting-enzyme inhibitor; ARBs: angiotensin-II type-I receptor blocker; CCBs: calcium-channel blockers; MRAs, mineralocorticoid antagonists.

Table S7. Echocardiographic equipment by centre

Centre	Echocardiographic device	Interfaced probe
001	GE, Vivid 7 Pro	2.5-to 3.5-MHz phased-array probe
052	GE, Vivid S60N with 0-2 cm minimum field-of-view range, 0-36 cm maximum field-of-view range, 10-120° width range, M-modes (tissue velocity, anatomical and curved anatomical) and doppler modes (pulsed wave and continuous wave)	3SC with 1.3-4.0 MHz scanner range, 90° field of view and 30cm depth of field
250	Vivid iq Premium R4 machine	M5Sc-RS cardiac probe, 12L-RS linear probe, C1-5-RS abdominal probe
350	GE, Vivid E95 with 0-5 cm minimum field-of-view range, 0-50 cm maximum field-of-view range, 0-120° width range, M- (tissue, anatomical & curved anatomical), doppler (pulsed, continuous and tissue) and tissue tracking modes	4Vc with 1.5-4.0 MHz scanner range, 90° field of view and 30cm depth of field
	GE, Vivid S70N with 0-2 cm minimum field-of-view range, 0-36 cm maximum field-of-view range, 10-120° width range, M- (tissue, anatomical and curved anatomical), doppler (pulsed, continuous and tissue) and tissue tracking mode	M5Sc-D with 1.5-4.5 MHz scanner range, 120° field of view and 30 cm depth of field
351	GE, Vivid E9 with 0-2 cm minimum field-of-view, 30cm maximum field-of-view, 2D, M-(anatomical), M-colour, trapezoidal and doppler (SCW, pulse and tissue) modes	M5S with 1.5-4.5 MHz scanner range, 120° field of view and 30cm depth of field
	GE S6 with 0-2 cm minimum field-of-view range, 30cm maximum. Field of view, 2D, M mode (anatomical), M-colour mode and doppler (SCW, pulse and tissue)	M5S with 1.5-4.5 MHz scanner range, 120° field of view and 30cm depth of field
400	GE, Vivid T8 v 203 (Jiangsu, China) with 0-5cm minimum field of view range, 0-50 maximum field of view, 0-120o width range 0-120o width range, (tissue, anatomical & curved anatomical), doppler (pulsed, continuous and tissue) and tissue tracking modes	1-7/3.4 MHz 3Sc-RS scanner, 90o field of view and 16cm depth of field
401	GE Vivid T9 (General Electric, Jiangsu China). Minimum field of view 1cm, Maximum field of view 33cm and width range of 10 to 168°. Field of view are 2D, M-mode, continuous, pulse waves and Doppler	3Sc-RS 30cm depth, 120°field of view, 1.3-4.0 MHZ scanner frequency

Centres are sorted by their study identification number.
GE indicates General Electric with headquarters in Horton, Norway.

Table S8. Multivariable-adjusted echocardiographic measurements in 227 patients by race

Characteristic	Blacks		Whites		*P-value*
	N	Statistic	N	Statistic	
M-mode LV structure					
Aortic root diameter, mm	158	29.0 (3.7)	69	27.4 (5.3)	0.0090
Left atrial diameter, mm	158	35.8 (5.8)	69	38.2 (4.3)	0.0022
Ratio of left atrial-to-aorta diameter	158	1.84 (0.24)	69	0.73 (0.16)	0.0006
End-diastolic diameter, mm	158	45.0 (6.8)	69	47.2 (4.6)	0.0143
Relative wall thickness	158	48.9 (11.4)	69	45.3 (7.6)	0.017
LV systolic function					
Stroke volume, mL	158	63.1 (19.5)	69	69.8 (18.3)	0.016
Stroke volume index, mL/m^2	158	33.6 (11.5)	69	36.5 (9.8)	0.072
LV diastolic function					
Isovolumetric relaxation time, ms	155	102.6 (26.7)	57	107.7 (19.3)	0.19
Left atrial volume index, mL/m^2	150	27.4 (13.0)	69	31.7 (7.55)	0.013

Values are least square mean (SD). Covariables included sex, age, BMI (not applicable to stroke volume index and left atrial volume index), diabetes, and the use of thiazide diuretics, β-blockers, inhibitors of the renin-angiotensin system (ACEIs and ARBs) and dihydropyridine and non-dihydropyridine CCBs.

Table S9. Ten-year cardiovascular risk and heart age by race and age group

Category	N	CVD risk score		CVD risk, %		Heart age, years	
		Median	IQR	Mean	SD	Mean	SD
Blacks (n=168)							
<55 y	27	13.0	9.00-14.0	13.1	8.1	66.7	15.3
55-59 y	53	15.0	13.0-17.0	18.6	10.5	77.7	11.9
60-64 y	47	16.0	12.0-19.0	22.0	11.9	79.3	14.8
65-69 y	27	18.0	16.0-19.0	27.8	11.3	83.4	9.3
≥70 y	14	18.0	18.0-20.0	33.5	10.8	87.6	9.6
P for trend		*<0.0001*		*<0.0001*		*<0.0001*	
Whites (n=93)							
<55 y	1	12.0	…	8.6	…	68.0	…
55-59 y	16	14.5	12.0-17.5	17.9	8.3	73.9	13.1
60-64 y	20	14.0	11.5-18.0	19.0	12.2	74.7	13.1
65-69 y	20	17.5	15.5-19.0	23.7	8.4	84.4	9.9
≥70 y	36	17.0	16.0-19.5	25.0	9.9	86.3	9.8
P for trend		*<0.0001*		*0.0024*		*<0.0001*	

N indicates the total number of patients within group. The Framingham 10-year CVD risk and heart age by race are derived from the sex-specific Framingham risk scores, derived from chronological age, total serum cholesterol, HDL serum cholesterol, SBP, antihypertensive treatment status (yes vs. no), smoking (yes vs. no) and diabetes (yes vs. no). An ellipsis denotes not applicable.

Table S10. Number of urine samples and methodology of albuminuria of 241 analysed patients by race

Characteristic	Blacks		Whites		*P-value*
	N	Statistic	N	Statistic	
Number of urine samples					
1 sample per patient	163	3 (1.84)	78	0 (0)	
2 samples per patient	163	95 (58.3)	78	49 (62.8)	0.42
3 samples per patient	163	65 (39.9)	78	29 (37.2)	
Methodology					
Sample 1					
ACR	163	74 (45.4)	78	70 (89.7)	<0.0001
Dipstick	163	89 (54.6)	78	8 (10.3)	
Sample 2					
ACR	160	57 (35.6)	78	70 (89.7)	<0.0001
Dipstick	160	103 (64.4)	78	8 (10.3)	
Sample 3					
ACR	65	17 (26.2)	29	21 (72.4)	<0.0001
Dipstick	65	48 (73.9)	29	8 (27.6)	

N indicates the number of patients with data within group. Values are number of patients (%). Twenty participants had no albuminuria measurements. Overall, 3, 144 and 94 participants had 1, 2 and 3 measurements, respectively. Abbreviation: ACR. Albumin-to-creatinine ratio.

Table S11. Estimated glomerular filtration rate and albuminuria by race and age group

Category	N	Mean eGFR (SD), mL/min/1.73 m^2	Number of patients by albuminuria category (%)		
			<30	30-300	>300
Blacks (n=168)					
<55 y	27	80.4 (24.4)	8 (29.6)	17 (63.0)	2 (7.41)
55-59 y	54	77.9 (23.6)	29 (53.7)	25 (46.3)	0 (0)
60-64 y	45	74.6 (20.4)	25 (55.6)	18 (40.0)	2 (4.44)
65-69 y	25	69.5 (16.9)	18 (72.0)	7 (28.0)	0 (0)
≥70 y	12	78.4 (16.8)	5 (41.7)	6 (50.0)	1 (8.33)
P for trend		*0.16*		*0.088*	
Whites (n=93)					
<55 y	1	104.2	1 (100)	0 (0)	0 (0)
55-59 y	15	89.9 (9.08)	15 (100)	0 (0)	0 (0)
60-64 y	20	82.9 (8.58)	20 (100)	0 (0)	0 (0)
65-69 y	21	74.9 (14.1)	21 (100)	0 (0)	0 (0)
≥70 y	21	74.7 (13.5)	21 (100)	0 (0)	0 (0)
P for trend		*<0.0001*		…	

N indicates the total number of patients within group. An ellipsis denotes not applicable.

Table S12. WHO questionnaire of 261 analysed patients by race

Characteristic	Blacks		Whites		*P-value*
	N	Statistic	N	Statistic	
Angina pectoris	168	10 (8.9)	93	6 (8.8)	0.98
Claudication	168	1 (0.9)	93	0 (0)	0.43
Exertional dyspnoea	168	31 (27.7)	93	22 (32.4)	0.50
Habitual cough	168	6 (5.4)	93	6 (8.8)	0.37
Production phlegm	168	5 (4.5)	93	1 (1.47)	0.28

N indicates the number of patients with data within group. Values are number of patients (%).

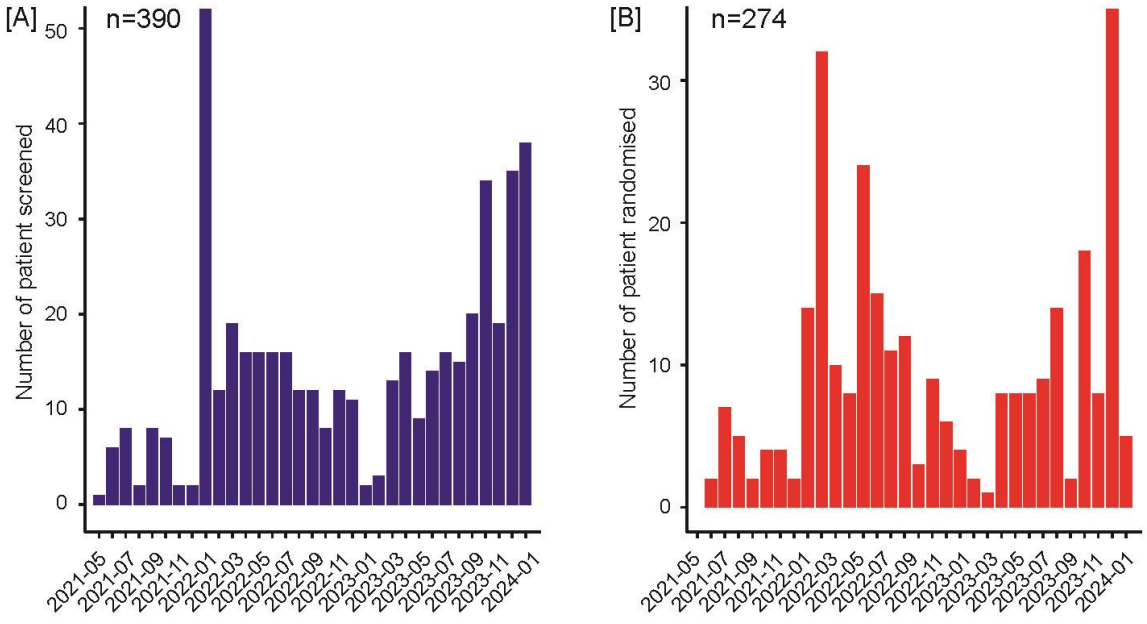


Figure S1. Number of patients screened (A) and patients randomised (B) by 2-monthly time intervals. Restrictions due to the COVID19 pandemic were progressively – partially or completely – relaxed from the middle or end of 2022 onwards.

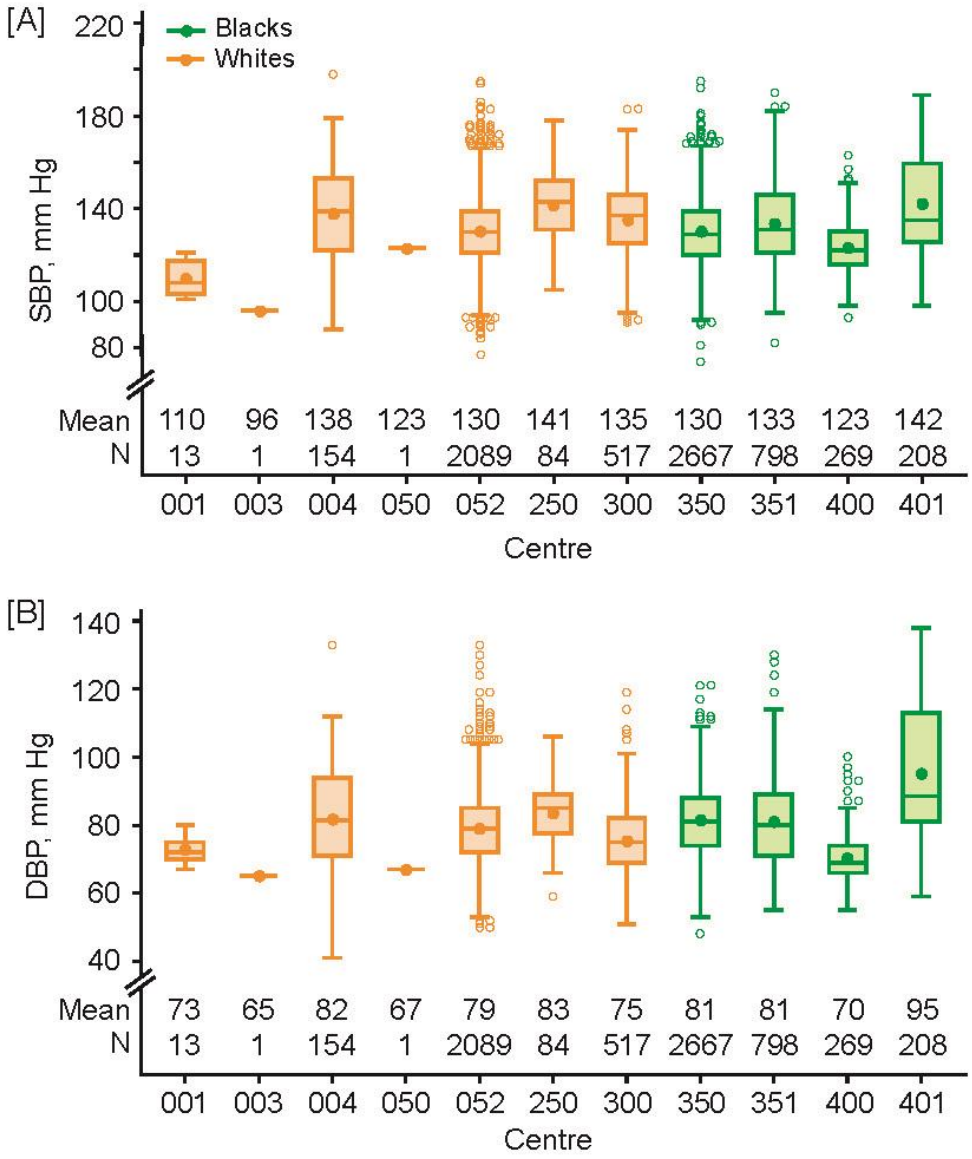


Figure S2. Distribution of the self-measured systolic and diastolic home BP from screening to the pre-randomisation visit by centre. The central line, the upper and lower lines, and the upper and lower caps represent the median, interquartile range, and the 10th to 90th percentile interval. The arithmetic means and extreme measurements are represented by circles inside the box and outside the whiskers, respectively. The arithmetic means and the number of data points contributing to each whisker plot is given within the boxes.

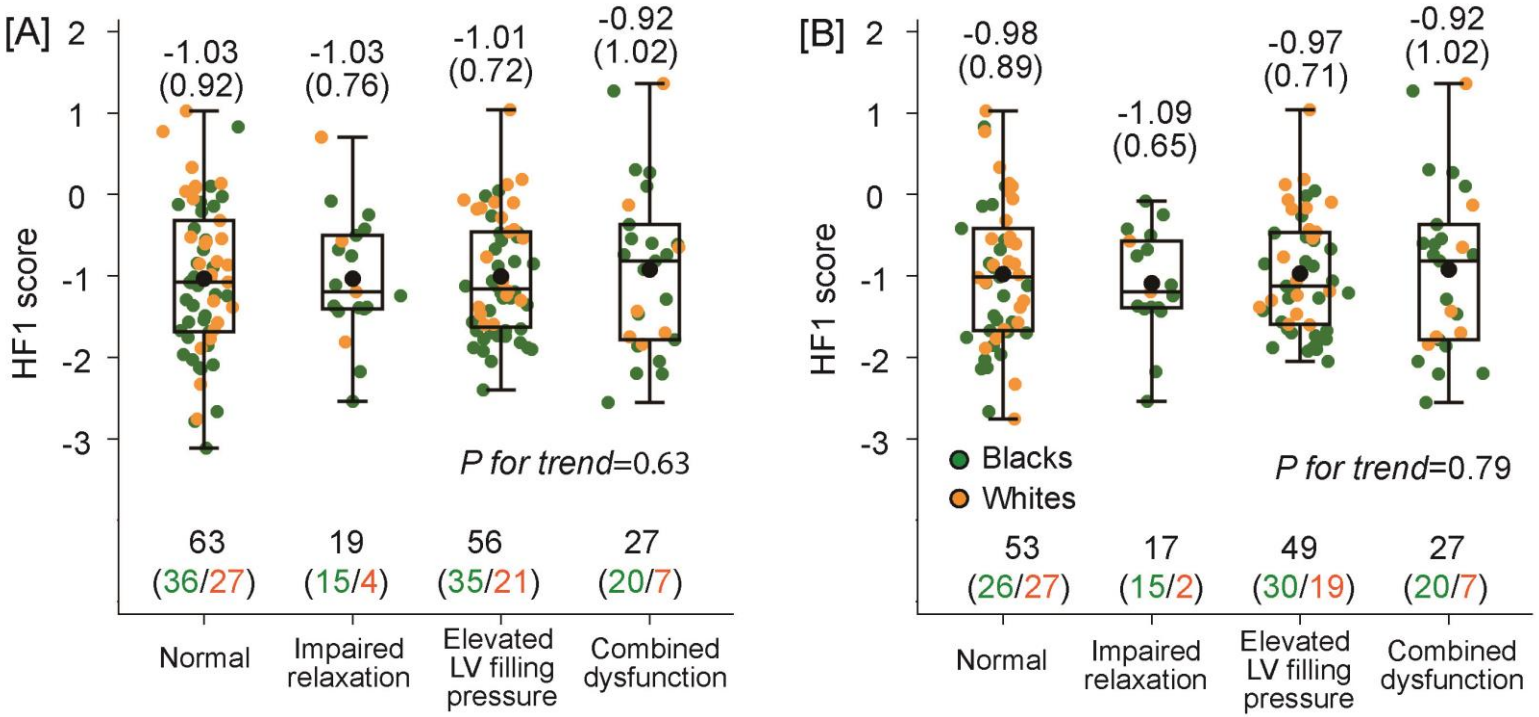


Figure S3. HF1 score by stage of diastolic LVD and race. The central line, the upper and lower lines, and the upper and lower whiskers represent the median, interquartile range, and the 10th to 90th percentile interval. Means (SD) are given above the upper whisker and the number of patients (Blacks/Whites) below the lower whisker. Panel A includes all randomised patients in the ITT analysis and Panel B randomised patients who complied with all entry criteria in the PP approach.

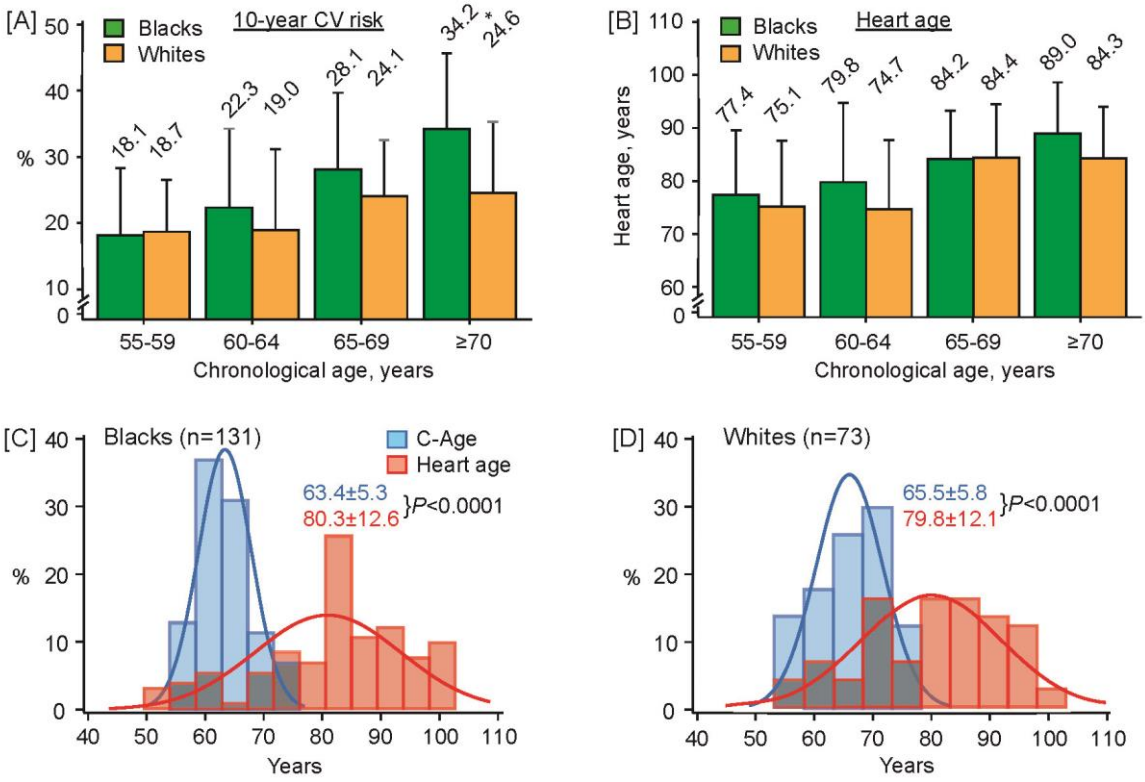


Figure S4. 10-year CV risk (A) and heart age (B) by race according to the Framingham risk scores and the distributions of age and heart age in 131 Blacks (C) and 73 Whites (D), who complied with all entry criteria (per-protocol approach). The sex-specific Framingham scores in Panels and B are derived from chronological age, total serum cholesterol, HDL serum cholesterol, SBP, antihypertensive treatment status (yes vs. no), smoking (yes vs. no) and diabetes (yes vs. no). In panels A and B, the height of the bars represents means (numerical value given) and the vertical lines the SD. The asterisk in Panel A indicates a significant difference in the 10-year CV risk between Blacks and Whites in patients aged ≥70 years. Panels C and D show the overlay in the distributions of chronological age and heart age in Blacks (C) and Whites (D). In both races, heart age was significantly higher than chronological age. The grey colour indicates the overlay in the distributions of chronological and heart age.

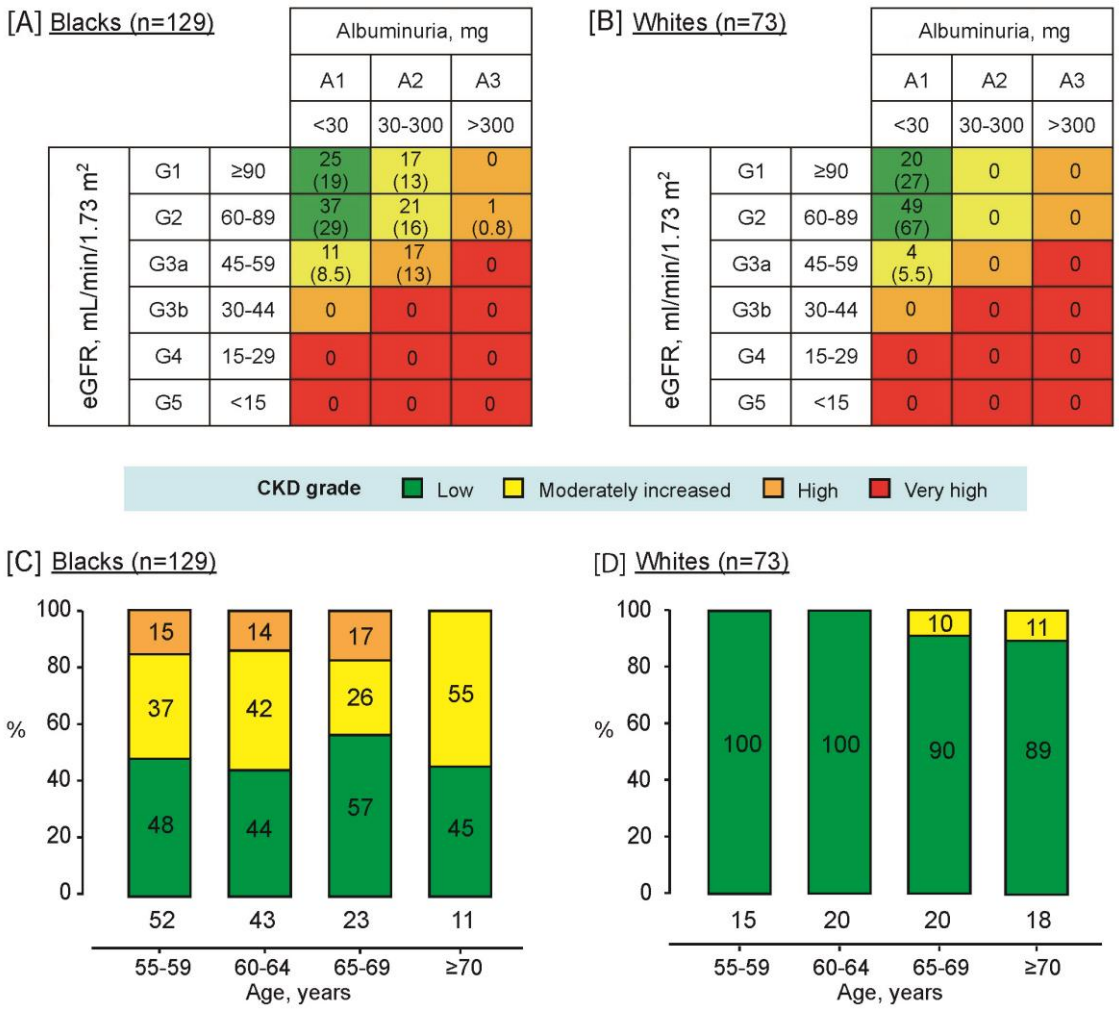


Figure S5. CKD grades derived from eGFR and albuminuria by race in a sensitivity analysis of 202 randomised patients who complied with all entry criteria (PP analysis). The CKD grade is derived by cross-classification of eGFR and albuminuria, according to the 2024 KDIGO Guideline. Albuminuria is expressed in milligram per litre or per gram creatinine depending on the method of measurement. Panels A and B present the number of patients (%) in cross-classified cells by race. Panels C and D depict the percentage of patients at different CKD grades by age. The Spearman rank correlation coefficients between the CKD grade and age are -0.030 (*P*=0.74) in Blacks and 0.20 (*P*=0.0085) in Whites.

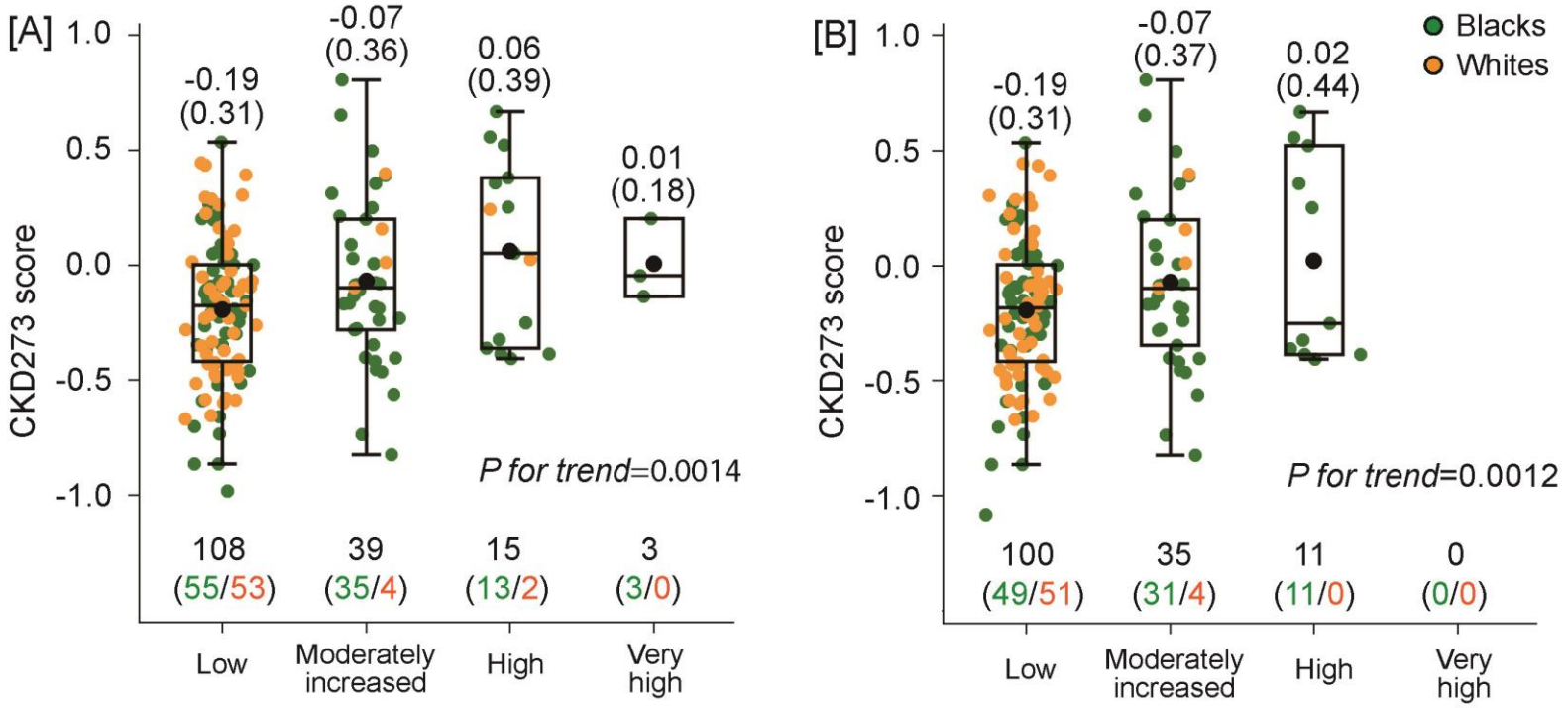


Figure S6. CKD273 score by stage of CKD and race. The central line, the upper and lower lines, and the upper and lower whiskers represent the median, interquartile range, and the 10th to 90th percentile interval. Means (SD) are given above the upper whisker and the number of patients (Blacks/Whites) below the lower whisker. Panel A includes all randomised patients in the ITT analysis and Panel B randomised patients who complied with all entry criteria in the PP approach.

Chapter 5
General discussion

Risk factors, originating in the environment or innate, determine differentiate unhealthy ageing from the average or ideal lifecourse. These risk factors, in the context of this dissertation termed external and internal drivers of ill health, operate “silently” in the perinatal phase of life, during childhood and young adulthood, but if unattended ultimately cause target organ damage and debilitating health problems. Risk factor identification is the key approach for implementing preventive interventions, especially in individuals with high risk profile [1]. In line with the stated objectives, we conducted our research revolving around three themes: exposure to lead as the prototype of a ubiquitously distributed environmental pollutant and therefore an external driver of ill health (***Chapter 2***); studies focusing on the urinary proteome, which reflects body-wide pathogenic processes that are fuelled by internal drivers (***Chapter 3***); and finally clinical risk factors, which arise through the interplay between external and internal drivers of ill health (***Chapter 4***). With our access to multiple cohorts, we conducted these studies in children, young lead workers, and population and patients, which covered the whole human life span (**Figure 5.1**).

5.1 Lead exposure

As suggested by the Lancet Commission on Pollution and Health, pollution is the largest environmental cause of disease and death worldwide, responsible for an estimated 9 million premature deaths in 2015 [2]. Approximately 92% of pollution-related mortality occurred in low- and middle-income countries [2]. Among toxic metals contributing to unhealthy ageing, lead is the dominating contaminant in this class. Historically, before the implementation of these regulations, lead exposure was a driver of hypertension [3,4] and renal dysfunction [5-7]. Over the past decades, environmental and occupational exposure fell drastically [8-10], because lead-containing materials were phased out and because in developed countries regulations to protect workers were substantially tightened. However, it is still under debate whether at the current-day lower exposure level, lead remains a contaminant responsible for sizable adverse health effects [4,11-13].

In light of the knowledge gap, ***Chapter 2*** (**Table 5.1**) mainly focused on environmental and occupational lead exposure. In the SPHERL cohort [14], the newly hired workers without known previous lead exposure and employed at lead recycling plants in the United States were recruited. Based on the follow-up visits at 2 years (***Chapter 2.1***) and up to 6 years in the final examination cycle (***Chapter 2.2***), we investigated the co-primary endpoints, hypertension and renal dysfunction, in relation blood lead (BL) concentration after longitudinal occupational exposure. In addition to these findings, we summarised the toxicokinetics, legislation and the state-of-the-art evidence in environmental and occupational lead exposure (***Chapter 2.3***).

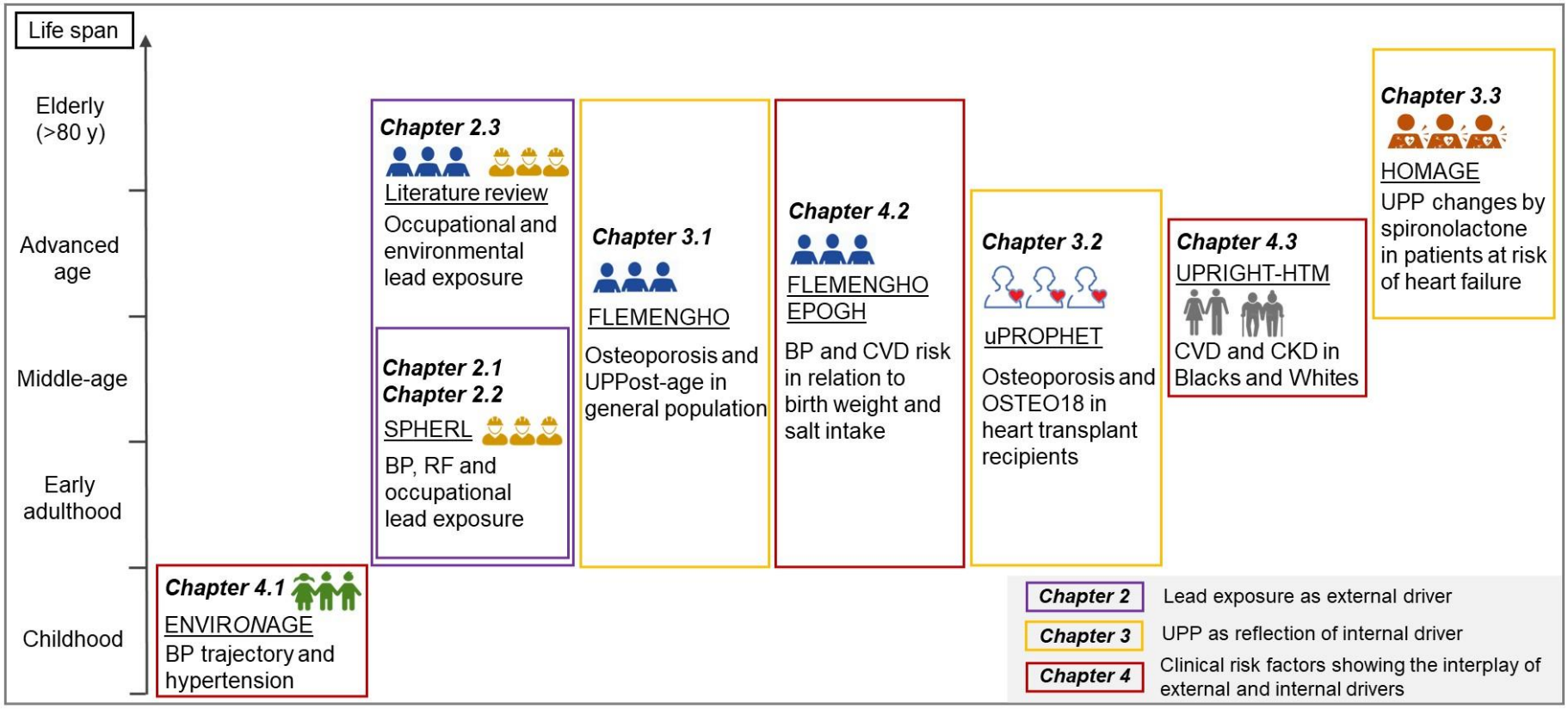


Figure 5.1 Summary of the doctoral dissertation. This dissertation investigated children, young lead workers, general population and patients, which covered the whole human life span. Abbreviations: BP, blood pressure; RF, renal function; CVD, cardiovascular disease; CKD, chronic kidney disease; UPP, urinary proteomic profiling. UPPost-age and OSTEO18 are multidimensional UPP biomarkers indicating risk of osteoporosis. *Chapter 2.3* reviewed several lead-related studies, including SPHERL. The height of the box indicates the age range of the participants in each study.

5.1.1 Renal function responses to lead exposure for two years

In ***Chapter 2.1***, we reported that renal function decline was not significantly associated with two-year occupational lead exposure. In fully adjusted mixed models, accounting for covariables and the within-participant clustering of the 1- and 2-year follow-up data, a 3-fold BL increment generated the estimates of the lead-related eGFR changes amounting to -0.86, -1.58 and -1.32 ml/min/1.73 m^2 for eGFRcrt, eGFRcys or eGFRcc, respectively. These eGFR changes were comparable to the expected age-related decline in eGFRs in current study. The responses of serum osmolality, urine specific gravity, urinary albumin-to-creatinine ratio and the haematological measurements were not significantly associated to the increment in lead exposure. However, given the confidence intervals around the point estimates of the changes in eGFRcc and eGFRcys, the decrease in eGFRs could not be excluded for certainty. A strength of the SPHERL study design is that the eGFRcys, as derived from serum cystatin C, is a better biomarker reflecting glomerular filtration with higher reliability than eGFRcrt, because eGFRcys is less confounded by ethnicity, sex, age, muscle mass and protein intake [15]. However, the small sample size and the 2-year follow-up of the current SPHERL cohort warrant a cautious interpretation of the findings. Nevertheless, our study demonstrated that the 2-year changes in renal function were unrelated to the BL increase, and confirmed the safety of workers under the contemporary low-level lead exposure.

5.1.2 Blood pressure and renal function responses to lead exposure for up to six years

To consolidate the above findings, we extended the SPHERL follow-up visit for up to six years. In ***Chapter 2.2***, we found that the BL concentration increased by 3 fold at the last follow-up and reached a stable level after the first year. The over 3-fold BL increment was not significantly associated with increases in systolic/diastolic blood pressure (BP), mean arterial pressure, or higher risk of hypertension. Similarly, the increment in BL was not associated with renal function decline as captured by eGFRcrt, eGFRcys, serum osmolality, urine specific gravity and urinary albumin-to-creatinine ratio. As depicted by heat maps, baseline BP, and baseline eGFRcrt and eGFRcys were the principal determinants contributing to their changes during follow-up, while the BL increment did not significantly produce elevated BP or renal function decline. Due to regression to mean, as revealed in all clinical measurements [16], the workers with lower BP at baseline were more likely to have an increased BP level at follow-up, and *vice versa*.

The current observations are consistent with our previous research. In a meta-analysis of summary statistics extracted from 31 studies involving 58 518 participants, all

published before February 2001 [4], doubling of BL was associated with a marginally higher BP. The summative estimates averaged 1.0 mm Hg (confidence interval [CI], 0.5-1.4 mm Hg) systolic and 0.6 mm Hg (CI, 0.4-0.8 mm Hg) diastolic. However, several studies of populations [17-21] or workers [22] reported an inverse association between eGFR and BL or an increased risk of chronic kidney disease in relation to BL. Because approximately 70% of lead excretion occurs via the urine [23,24], these observational studies could not demonstrate whether the reduced eGFR is associated with lesser urinary lead excretion and higher BL, or higher lead exposure as reflected by BL causes reduced eGFR.

Compared to ***Chapter 2.1***, ***Chapter 2.2*** included the data in a longer follow-up period and indicated a stable BL level after the first year, which could provide a more reliable outcome interpretation after starting the first occupational exposure. In a nutshell, our findings could not demonstrate a significant association between hypertension, renal function decline and occupational lead exposure in newly hired workers under the protection of tightened regulations. These findings also confirmed the previously cross-sectional analyses at baseline [25,26] and longitudinal analyses at 2-year follow-up [27,28] of the SPHERL project.

5.1.3 Planning the future

Lead is worldwide a ubiquitous metal with high toxicity affecting cardiovascular system, kidneys, neurocognitive system and other organs, in both adults and children. Even though lead exposure has substantially declined in developed countries, the long-term health effects of lead exposure in low- and middle-income countries still remain a matter of concern [29], because regulations are still being developed or insufficiently reinforced [30,31]. As we suggested in ***Chapter 2.3***, policy makers should prioritise the legislations for reducing the occupational and environmental exposure to the particulate loaded with lead deposited in the previous century [32]. Education, improvement in living conditions and empowerment of deprived social groups are also the essential steps to alleviate the impact from heavy metal exposure, particularly in low- and middle-income countries. From a clinical point of view, environmental pollutants and their exposure vectors should always be considered in the pathogenic work-up of unexplained hypertension or chronic kidney disease. If lead exposure is suspected, and given that co-exposure is common, blood or urinary lead and cadmium should be measured, but only at a certified laboratory qualified for the measurement of heavy metals.

Table 5.1 Summary of *Chapter 2*

Study	What is known	What this study adds	Overall interpretations
Chapter 2.1	• Whether in developed countries lead exposure still contributes to renal impairment is uncertain, because blood lead (BL) is declining towards pre-industrial levels. • Longitudinal studies correlating renal function (RF) and BL changes over time are scarce.	• In a real-world experiment, there was no association between the 2-year changes in RF and the over 3-fold increment of BL in the workers without know previous lead exposure.	• The 2-year changes in renal function were unrelated to the BL increase. • Our findings confirmed the safety of workers under the current low-level lead exposure.
Chapter 2.2	• Previous publications have demonstrated the significant associations of lead exposure with the risks of hypertension or RF decline.	• In the worker exposed to lead for up to six years, the blood pressure (BP) and RF responses to an over 3-fold BL increment were small and not significant.	• This study confirmed the safety of modern lead-handing facilities operating under current safety rules.
Chapter 2.3	• Lead is a ubiquitous toxic metal. • BL in the general population declined drastically, approaching pre-industrial levels, at least in developed nations.	• This review summarised the toxicokinetics and toxicology of lead, the methods to measure BL, summarised recently published studies in low BL levels, and provided an appraisal of recent regulations to protect exposed workers.	• There is large progress in reducing occupational and environmental lead exposure in the past decades. • However, policy makers should prioritise the design and reinforcement of stricter regulations to reduce lead exposure, in particular in low- and middle-income countries.

5.2 Urinary proteomic profiling

Urinary proteomic profiling (UPP) is an effective predictor of aging-associated disabilities [33]. Single sequenced urinary peptides allow identifying the parental proteins [34], thereby providing body-wide information on the molecular mechanisms of internal disease. In ***Chapter 3*** (**Table 5.2**), using the novel *"omics"* technology, we developed and validated multidimensional biomarkers to assess the risk of osteoporosis in general population (***Chapter 3.1***) and heart transplant recipients (***Chapter 3.2***). Furthermore, we demonstrated the UPP changes in response to spironolactone in patients at high risk of developing HF (***Chapter 3.3***).

5.2.1 Osteoporosis in general population

Osteoporosis is a silent disease with minimal or even no symptoms until fractures occur. In general population, the prevalence of osteoporosis raises with increasing age [35], and women are more likely to experience osteoporotic fracture than men [36]. In terms of the deterioration in quality of life and increase in socio-economic burden, osteoporosis represents a major public health issue [37] and therefore should be diagnosed and prevented in a timely manner.

In ***Chapter 3.1***, we constructed a novel aging-related biomarker indicative of osteoporosis, UPPost-age, and validated UPPost-age cross-sectionally and longitudinally in the large-scale family-based FLEMENGHO study. Participants were invited for a follow-up examination and had UPP measured in 2005-2010 and re-invited in 2009-2013. Our findings are as follows. Chronological age was associated with bone-related UPP fragments, including collagens, IGF2 and MGP in the derivation and validation datasets. Among the 99 bone-related urinary peptides associated with chronological age, 32 (32.3%) showed a sex-specific distribution probably explaining sexual heterogeneity in the molecular pathogenic mechanisms in osteoporosis. Body-height-to-arm-span ratio (HASR) and HASD ≤ -3 cm, anthropometric measurements reflecting osteoporosis, were associated with UPPost-age. Over median follow-up of 4.9 years, the risk of osteoporosis increased by 36% in per 10-year increment in UPPost-age and the risk of osteoporotic fracture by 44% in women.

On top of these findings, a positive correlation was found between the plasma levels of dp-ucMGP and UPPost-age, and the risk of osteoporosis. Plasma dp-ucMGP is a marker of vitamin K deficiency [38] and participates in the differentiation and gene expression in osteoblasts and in the mineralization of the bone extracellular matrix [39]. In addition, collagens type I, III, and V are the most abundant constituents of the organic extracellular matrix in bone tissue [40,41]. The inverse association between chronological age and

type-I collagen fragments indicated that collagen I fragments decreased in urine with advancing age. Furthermore, the up-regulated IGF2 in women might be an upregulation for bone repair and remodeling [42]. The sex specificity of UPP is supported by a populational study, in which 51 (<45 years) and 66 (>55 years) urinary peptides had different levels in men and women, in particular in women after menopause [43].

Our findings in FLEMENGHO illustrated how a urinary multidimensional urinary biomarker, an internal driver of ill health, predicts osteoporosis. For patients, clinicians and trialists, the UPPost-age biomarker provides a novel approach to predict the risk of osteoporosis at an early stage, long before the incidence of osteoporotic fracture.

5.2.2 Osteoporosis in heart transplant recipients

Organ transplant recipients experience a higher risk of osteoporosis, due to immunosuppressants and glucocorticosteroids to prevent allograft rejection [44]. In ***Chapter 3.2***, we developed OSTEO18, a novel 18-peptide multidimensional urinary biomarker indicative of osteoporosis in the case-control study of heart transplant recipients, replicated and validated in the whole uPROPHET cohort. On top of relevant osteoporosis risk factors, OSTEO18 improved the AUC as categorized variable with an optimised threshold of 0.095 as well as continuously distributed biomarker. Moreover, the OSTEO18 urinary biomarker includes eight type-I collagen fragments (44.4%) and two type-III collagen fragments (11.1%), reflecting the turnover of the extracellular bone matrix. Furthermore, PIGR functions in transporting locally produced dimeric IgA across mucosal epithelia, and in serving as the precursor of secretory component and plays a key role in mucosal immunity [45]. The presence of a PIGR-derived urinary peptide in OSTEO18 might be explained by dysregulation of its role by immunosuppression.

Osteoporosis represents a major public health issue [37], in terms of the worsening quality of life and social health cost. In ***Chapter 3.2***, we further reviewed the socio-economic studies focusing on medical costs of osteoporosis in European countries [46-48] and the United States [49]. Importantly, we found that the costs of osteoporotic treatment, surgery for osteoporotic fracture and the medical management are far higher than the cost of a UPP test for analysing OSTEO18. Currently, OSTEO18 is being certified as an in vitro diagnostic and is therefore ready to enter the clinical management of heart transplant recipients. Given that the current findings in generated in a single-centre cohort, the performance of OSTEO18 still needs to be validated in other organ transplant cohorts and perspective studies.

5.2.3 Risk of heart failure

Fibrosis is the common pathological response to inflammation and chronic tissue injury. In the HOMAGE-RCT trial, patients at high risk of heart failure were randomised to spironolactone 25-50 mg/day on top of usual therapy or usual therapy alone and followed up for 9 months. In ***Chapter 3.3***, we analysed the between-group differences in the UPP and the associations of urinary with serum fibrosis markers, carboxyterminal propeptide of procollagen I (PICP) and carboxyterminal telopeptide of collagen I (CITP) [50,51]. To the best of our knowledge, this was the first study to assess UPP changes in response to spironolactone in patients at high HF risk because of coronary heart disease. We summarised the key observations as follows. The UPP differences between control and spironolactone were exclusively confined to 27 collagen fragments in line with the observation that collagen fragments constitute >70% of the UPP [33]. Of these 27 fragments, 16 were downregulated on spironolactone and 11 upregulated. Moreover, compared to control, serum PICP and the serum PICP/CITP ratio decreased on spironolactone, which replicates the previous HOMAGE-RCT publications [50]. Furthermore, the correlations and regression slopes between the changes from baseline to follow-up in the urinary peptides and the corresponding changes in CITP were similar in patients randomised to control and spironolactone, but these correlations only reached significance, if the COL1A1 fragments were upregulated during follow-up.

The serum PICP reflects collagen synthesis and serum CITP reflects the degradation of mature collagen I. Therefore, the downregulation of urinary collagen fragments in ***Chapter 3.3*** might demonstrate the reduced synthesis of COL1A1 and by extension a lower body-wide pool of collagens available for degradation [52,53]. With regard to the upregulation in 11 collagen fragments (seven COL1A1 fragments), two mechanisms might be involved. First, a certain degree of collagen turnover is always required to maintain physiological collagen scaffoldings. Second, the degradation of longer fragments by proteases along the nephron and the lower urinary tract, might also explain this phenomenon. However, the latter mechanism is unlikely to have played a role. Indeed, in a placebo-controlled study of a dipeptidyl peptidase-4 inhibitor [54], the UPP included pairs of peptide chains, i.e., the substrate for the protease activity (e.g., PPGPPGKNGDDGEAGKPG) and the resulting breakdown product (e.g., GPPGKNGDDGEAGKPG). In ***Chapter 3.3***, we reached the clinically relevant conclusion that combining urinary and serum fibrosis markers provides new avenues for discovery of antifibrotic drugs and refines insight in the action of antifibrotic drugs.

Table 5.2 Summary of *Chapter 3*

Study	What is known	What this study adds	Overall interpretations
Chapter 3.1	• Osteoporosis is an age-related skeletal disorder with higher prevalence in elderly and women. • Urinary peptidomic profile (UPP) is an effective predictor aging-associated disabilities.	• Chronological age was associated with 99 bone-related urinary peptides. • The body-height-to-arm-span ratio was negatively associated with UPPost-age. • Over 4.89 years (median), the risk of osteoporosis associated with UPPost-age increased by 36% overall and osteoporotic fracture by 44% in women.	• An aging-related UPP signature with focus on peptide fragments derived from bone-related proteins is associated with osteoporosis risk. • UPPost-age a novel approach to predict the risk of osteoporosis at an early stage.
Chapter 3.2	• Immunosuppressive treatment in heart transplant (HTx) recipient causes osteoporosis. • UPP includes peptide fragments derived from the bone extracellular matrix.	• A novel 18-peptide multidimensional urinary biomarker indicative of osteoporosis was developed, replicated and validated in HTx recipients. • On top of relevant osteoporosis risk factors, OSTEO18 improved the area under curve as categorized and continuously distributed biomarker.	• OSTEO18 is a clinically meaningful novel biomarker indicative of osteoporosis in HTx recipients and is being certified as in-vitro diagnostic.
Chapter 3.3	• Spironolactone reduces plasma markers reflecting collagen-1 synthesis and decreases myocardial fibrosis by inhibiting activation of the mineralocorticoid receptors. • Over 70% of urinary peptides are derived from collagens.	• In patients prone to heart failure because of coronary heart disease, spironolactone compared to control reduced 16 urinary collagen fragments and increased 11 with no other differential changes in UPP. • Spironolactone did not affect the relation between urinary and serum fibrosis markers.	• Combining urinary and serum fibrosis markers opens new avenues for discovery of antifibrotic drugs and refines insight in the action of antifibrotic drugs.

5.3 Clinical risk factors

The screening and management of clinical risk factors play an important role in timely prevention and intervention to promote healthy ageing. Along the line of searching and validating drivers of unhealthy ageing, ***Chapter 4*** (**Table 5.3**) focused on the major risk factors of cardiovascular disease. Across the whole human life span, we investigated children (***Chapter 4.1***), the general adult population (***Chapter 4.2***) and the patients at risk of CVD or CKD (***Chapter 4.3***).

5.3.1 Hypertension as risk factor in childhood

Hypertension remains the number one cause of death in the world and the major modifiable risk factor for cardiovascular disease (CVD) worldwide [55]. From this point of view, in this dissertation is both an outcome variable (***Chapter 4.1***) and a major risk factor, for which analyses have to be adjusted. According the Developmental Origins of Health and Disease concepts [56], perinatal factors affect the micro- and macrocirculation and the heart and predispose to cardiovascular disease, renal dysfunction and heart failure later in life. In view of this, ENVIR*ON*AGE) birth cohort study focuses on risk factors present at birth and their trajectory in early (4-6 years) and later (9-11 years) childhood.

In ***Chapter 4.1***, we indicated that BP tracked over time, meaning that most infants as they age through childhood keep their percentile position in the blood pressure distribution. Systolic/diastolic BP tracked from 67.3/40.5 mm Hg at birth to 100.2/57.5 mm Hg at 4-6 years and further to 107.7/65.1 mm Hg at 9-11 years. While accounting for confounders, at the last follow-up, systolic/diastolic BP increased by 2.6/1.4 mm Hg per 1-SD increment in the baseline BP. Moreover, the increasing risk of elevated BP and hypertension was significantly associated with the mean arterial pressure at baseline. In addition, approximately 10% of children were so-called horse-racers, in whom the BP increase was faster than expected on tracking. To the best of our knowledge, our findings in the ENVIR*ON*AGE birth cohort is the first observation highlighting that the BP at birth determines the BP rise through childhood. Based on these findings, we suggest that paediatric BP management should be routinely implemented, and timely CVD risk identification and prevention should be provided early in life.

5.3.2 Birth weight and salt intake

Prematurity and dietary are two important risk factors of CVD. Prematurity as indicated by low birth weight disrupts the perinatal maturation of the microcirculation and impairs arterial structure/function later in life, as exemplified by the narrower retinal arteriolar diameters and sparser retinal fractals in extremely low birth weight children and young adults compared to controls born at term [57]. The literature is unanimously showing that

low birth weight is associated with hypertension [58] and high CVD risk later in life [59-61]. Besides, a systematic literature review [62] indicated that BP and CVD risk increased when sodium intake exceeded 5 g/day. Another individual-participant meta-analysis [63] demonstrated that higher UVNA was associated with minor systolic BP increment, but not with increased risk of hypertension or CVD. Following the research line initiated in children (***Chapter 4.1***), we moved the focus to adults and explored how birth weight and salt intake interact to determine BP and CVD risk among adults (***Chapter 4.2***).

In ***Chapter 4.2***, we investigated birth weight and salt intake as reflected by urinary sodium excretion (UVNA) measured in the 24-h urine samples, the gold standard for quantifying urinary sodium excretion [62]. We performed an individual-participant meta-analysis in two family-based European population studies, FLEMENGHO and EPOGH, and investigated whether the risk of incident hypertension, death or a composite CV endpoint in relation to UVNA was modified by birth weight. In unadjusted survival function analyses, participants with low or medium birth weight experienced lower mortality compared with the high-birth-weight group, whereas the incidence of a major CV endpoint was lower in the medium-birth-weight group, compared with the low- and high-birth-weight groups. However, absolute risk as captured by the incidence rates of adverse outcomes, and adjusted relative risk as captured by hazard ratios, were not significant across the categories of birth weight, UVNA and urinary sodium-to-potassium ratio. Importantly, this study generated two relevant observations. One confirmatory is that, in line with the previous publications [59-61], adult body weight tracked with birth weight. Another novel observation is that, in multivariable-adjusted analyses of the low-birth-weight group, there was a positive association between the changes from baseline to follow-up in UVNA and the corresponding changes in systolic BP. The findings in ***Chapter 4.2*** are in line with Guyton's model [64] that low birth weight is associated with fewer functional nephrons, thereby increasing their susceptibility to sodium driven hypertension.

Unhealthy life style and poor diet quality are strongly related to the increased risk of CV morbidity and mortality [65,66]. Dietary-related chronic illness are prevalent over whole life span. Linking to developmental origins, undernutrition and an adverse intrauterine environment limits fetal growth and leads to premature birth [67]. This is an adaptive response with a price of a larger susceptibility to disease later in life [67], for instance, the increased salt sensitivity in the individuals born with low birth weight, as indicated in ***Chapter 4.1***. As suggested by the 2021 Dietary Guidance to Improve Cardiovascular Health [68], efforts to achieve and sustain healthy dietary and life style

behaviours from birth throughout lifecourse should be prioritised to reduce adverse cardiometabolic conditions, including obesity, elevated BP and metabolic syndrome.

5.3.3 Racial and regional inequity

Racial and regional inequity is an comprehensive risk driver of ill health in low- and middle-income countries, involving gene background, distribution of aetiological agents, ethical and socio-economic disparities, life styles, medical resource allocations, and climatic differences. Sub-Saharan countries face persisting health challenges, such as infectious diseases, malnutrition, premature death, and the growing burden of non-communicable diseases [69,70]. To assess the health risk profile between races living in different regions, ***Chapter 4.3*** analysed the baseline data from the ongoing UPRIGHT-HTM trail, involving Blacks, "*born and living in sub-Saharan countries*", and European Whites enrolled in Belgium, Poland, and Slovenia. All patients were at high risk for CVD, chronic kidney disease (CKD) or both. Black patients had a median of 8 risk factors and White patients had 7. Being younger (60.4 vs. 68.3 years), Blacks had a worse risk profile, as evidenced by higher diabetes prevalence, higher BMI, faster heart rate, unfavourable serum cholesterol fractions, lower estimated glomerular filtration rate, microalbuminuria and sedentary lifestyle. Therefore, Blacks had higher 10-year CVD risk assessed by Framingham risk score, higher heart age (index of vascular ageing with chronological age as reference), and a worse CKD grades. In both races, CKD273 increased with CKD grade, but CKD273 and HF1 were not different by race. In addition, the heart rate was higher in Blacks than Whites, both on home monitoring and at the office after accounting for the use of β-blockers, given that average annual temperature is higher in sub-Saharan Africa than in Europe. In conclusion, Blacks born and living in sub-Saharan Africa, in contrast to Whites in living Europe, had a higher risk profile of CVD and CKD.

Racial and regional inequity leads to the high non-communicable diseases burden sub-Saharan Africa [71,72], in particular the poor health outcomes in Nigeria [70]. A potential limitation of ***Chapter 4.3*** is that the host factors (genetic, molecular, and pathogenic) could not be differentiated from environmental drivers of disease. Nevertheless, the *"born and living"* background of our patients excluded genetic admixture [73-76], which is the major confounder in most studies of African-Americans. The findings in ***Chapter 4.3***, in line with the Lancet Nigeria Commission, call for a comprehensive implementation of innovative health policies, and higher investment in education and research in sub-Saharan countries, to achieve the goal of prioritising disease prevention, which should be emphasised in public health management to reach healthy lifecourse [55].

Table 5.3 Summary of *Chapter 4*

Study	What is known	What this study adds	Overall interpretations
Chapter 4.1	• BP is an essential modifiable risk factor and associated with cardiovascular disease in adulthood. • The modifiable effect of BP might initiates from birth and operates across the whole life span.	• In the Belgian birth cohort with BP measured at birth, preschool- (4-6 years) and school-age (9-11 years), BP tracked over time. • Risk of elevated BP and hypertension was associated with the BP levels at birth and earlier childhood.	• BP is a modifiable risk factor operative from birth. • Our findings suggest that monitoring and managing BP from birth is a potential strategy to reduce cardiovascular risk later in childhood.
Chapter 4.2	• While the relation of salt intake with BP is linear, it is U-shaped for mortality and cardiovascular disease (CVD). • Low birth weight (BW) leads to a larger susceptibility to disease later in life.	• In two family-based European population studies, the risks of incident hypertension, all-cause mortality or CVD were not modified by BW. • Adult body weight tracked with BW. • In the low BW group, the changes from baseline to follow-up in 24-h urinary sodium was associated with the corresponding changes in systolic BP.	• This individual-participant meta-analysis confirmed the tracking of adult body weight with BW. • Low BW is associated with fewer functional nephrons, thereby increasing the susceptibility to sodium driven hypertension.
Chapter 4.3	• Greater vulnerability of Black vs. White individuals to CVD and chronic kidney disease (CKD) is well charted in the United States, but studies involving sub-Saharan Blacks are scarce.	• Despite being younger (60.4 vs. 68.3 years), Blacks born and living in sub-Saharan Africa had a worse risk profile compared to European Whites. • Blacks had higher 10-year CVD risk as assessed by Framingham risk score, higher heart age (index of vascular ageing), and a worse CKD grades.	• The findings call for a multi-pronged and comprehensive implementation of innovative health policies in sub-Saharan countries. • Education, research, empowerment of stakeholders, and international learned societies connecting experts from a wide array of disciplines should vigorously sustain this effort.

5.4 Strengths and limitations

In this thesis, we utilised the state-of-art technologies and the multiple cohorts covering the whole human life span, to identify and validate the internal and external drivers of unhealthy ageing. However, our findings should be interpreted within the context of potential limitations. First, we focused on lead exposure (***Chapter 2***), while the co-exposure of other heavy metals (e.g., cadmium) should not be neglected in occupational and environmental settings.

Second, the workers recruited in SPHERL are young and healthy (***Chapters 2.1 and 2.2***). Therefore, the healthy worker effect might lead to underestimating the true risk of occupational lead exposure due to the generally better health of the workforce compared with general population. Third, the multidimensional UPP biomarkers of osteoporosis (***Chapters 3.1*** and ***3.2***) and the differential levels of UPP by spironolactone (***Chapter 3.3***) should be further validated different populations. Fourth, at the time of the thesis writing, the follow-up visit of ENVIR*ON*AGE (***Chapter 4.1***) was still ongoing. Therefore, a more complete follow-up of the ENVIR*ON*AGE cohort is required to consolidate the current findings. Fifth, although we included two large population cohorts, FLEMENGHO and EPOGH, in the analysis of birth weight and salt intake (***Chapter 4.2***), this sample size was substantially smaller than in the previously published meta-analysis with over 325,000 study subjects [77]. Lack of power probably explain why the trends in absolute and adjusted relative risk associated with birth weight, UVNA and UNAK did not reach significance. Finally, in UPRIGHT-HTM (***Chapter 4.3***), Asian participants were excluded from the current analysis, as there were few in number. As UPRIGHT-HTM is ongoing, a larger sample size including multiple races will be attained at the later stage of our study.

5.5 General conclusion and perspectives

With its focus on external and internal drivers of ill health and their interaction, this dissertation fulfilled its stated objectives covering the whole human life span. We addressed exposure to lead as the prototype of an external driver of ill health, urinary proteome reflecting body-wide internal pathogenic processes, and traditional risk factors arising through the interplay between external and internal drivers of ill health. This dissertation highlights the importance of risk factor identification, prevention and management, and endorses the worldwide health mission aiming at reducing disease burden of population and improving quality of life. As an ultimate goal, I hope these findings will be put in practice and research with the timely prevention of unhealthy ageing early in life, long before usually irreversible target organ damage and associated complication lead to a decrease in quality-full years of life and premature mortality.

5.6 Individual development plan

After the completion of my doctoral training, I will continue to pursuit my research career in cardiovascular sciences and epidemiology. Moreover, I will apply the scientific expertise that I attained during my doctoral training at KU Leuven to raise funding, conduct cohort studies and produce novel research findings. My scientific mission will still focus on the understanding the ageing mechanisms, developing preventive strategies to reduce age-related disorders. As a researcher in Biomedical Sciences, I will also devote myself to the dissemination of scientific knowledge and the improvement of public health and wellbeing.

5.7 Scientific acknowledgement

The International Lead Association (www.ila-lead.org) provided an unrestricted grant to support data collection and management and statistical analysis of the SPHERL project. The Non-Profit Research Association Alliance for the Promotion of Preventive Medicine, Mechelen, Belgium (URL, www.appremed.org) received a nonbinding grant from OMRON Healthcare Co Ltd, Kyoto, Japan. uPROPHET was supported by the European Research Council (Advanced Researcher Grant-2011-294713-EPLORE and Proof-of-Concept Grant-713601-uPROPHET). HOMAGE was funded by the European Union Seventh Framework Program (HEALTH-F7-305507). ENVIR*ON*AGE is supported by grants from the European Research Council (No.ERC-StG310898) and the Research Foundation Flanders (No. G073315N).

For the clinical data collection, we were supported by Prof. Stefan Janssens, Prof. Johan Van Cleemput and the heart transplant team of the University Hospitals Leuven, Belgium. The UPP measurements were performed by Prof. Harald Mischak and his team at Mosaiques Diagnostics GmbH, Germany.

5.8 Personal contribution

I performed data cleaning, statistical analyses and wrote the first draft of all the manuscripts assembled in this dissertation. I contributed to the data cleaning and management of SPHERL. I read the medical histories of participants, collected the clinical data in UZ Leuven and updated the database of uPROPHET. I visited Mosaiques Diagnostics GmbH (Hannover, Germany) to gain a deeper understanding in urinary proteomics and bioinformatic analysis. Additionally, I also presented our research findings at internal meetings and seminars, and the conferences organised by international and European scientific societies, with the support of my supervisors and co-authors. During my doctoral training, I shared my knowledge and expertise with my colleagues and provided insights to our common projects.

5.9 Conflict of interest

The funding sources had no role in study design, data collection, statistical analysis, results interpretation or writing the study reports. The doctoral candidate and other co-authors of the manuscripts assembled in this dissertation declare no conflict of interest.

5.10 Statement of the use of generative AI

Generative AI was not involved in any part of this dissertation.

References

1. Wilson N, Blakely T, Foster RH, Hadorn D, Vos T. Prioritizing risk factors to identify preventive interventions for economic assessment. *Bull World Health Organ.* 2012; 90: 88-96.
2. Landrigan PJ, Fuller R, Acosta NJR *et al*. The Lancet Commission on pollution and health. *Lancet.* 2018; 391: 462-512.
3. Wedeen RP. Bone lead, hypertension, and lead nephropathy. *Environ Health Perspect.* 1988; 78: 57-60.
4. Nawrot TS, Thijs L, Den Hond EM, Roels HA, Staessen JA. An epidemiological re-appraisal of the association between blood pressure and lead : a meta-analysis. *J Hum Hypertens.* 2002; 16: 123-131.
5. Kim R, Rotnitzky A, Sparrow D, Weiss ST, Wagner C, Hu H. A longitudinal study of low-level lead exposure and impairment of renal function. The Normative Aging Study. *J Am Med Assoc.* 1996; 275: 1177-1181.
6. Chowdhury R, Darrow L, McClellan W, Sarnat S, Steenland K. Incident ESRD among participants in a lead surveillance program. *Am J Kidney Dis.* 2014; 64: 25-31.
7. Staessen JA, Lauwerys RR, Buchet JP *et al*. Impairment of renal function with increasing blood lead concentrations in the general population. *N Engl J Med.* 1992; 327: 151-156.
8. Pirkle JL, Brody DJ, Gunter EW *et al*. The decline in blood lead levels in the United States. The National Health and Nutrition Examination Surveys (NHANES). *J Am Med Assoc.* 1994; 272: 284-291.
9. Muntner P, Menke A, DeSalvo KB, Rabito FA, Batuman V. Continued decline in blood lead levels among adults in the United States : the National Health and Nutrition Examination Surveys. *Arch Intern Med.* 2005; 165: 2155-2161.
10. Hara A, Thijs L, Asayama K *et al*. Blood pressure in relation to environmental lead exposure in the National Health and Nutrition Examination Survey 2003 to 2010. *Hypertension.* 2015; 65: 62-69.
11. Yang WY, Zhang ZY, Mujaj B, Thijs L, Staessen JA. Environmental exposure to lead : old myths never die. *Lancet Public Health.* 2018; 3: e362.

12. Navas-Acien A, Guallar E, Silbergeld EK, Rothenberg SJ. Lead exposure and cardiovascular disease — a systematic review. *Environ Health Perspect.* 2007; 115: 472-482.

13. Staessen JA, Bulpitt CJ, Fagard R *et al*. Hypertension caused by low-level lead exposure : myth or fact ? *J Cardiovasc Risk.* 1994; 1: 87-97.

14. Hara A, Gu YM, Petit T *et al*. Study for promotion of health in recycling lead - rationale and design. *Blood Press.* 2015; 24: 147-157.

15. Inker LA, Schmid CH, Tighiouart H *et al*. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med.* 2012; 367: 20-29.

16. Gasowski J, Li Y, Kuznetsova T *et al*. Is "usual" blood pressure a proxy for 24-hour ambulatory blood pressure in predicting cardiovascular outcomes ? *Am J Hypertens.* 2008; 21: 994-1000.

17. Staessen JA, Lauwerys RR, Buchet JP *et al*. Impairment of renal function with increasing blood lead concentrations in the general population. *N Engl J Med.* 1992; 327: 151-156.

18. Åkesson A, Lundh T, Vahter M *et al*. Tubular and glomerular kidney effects in Swedish women with low environmental cadmium exposure. *Environ Health Perspect.* 2005; 113: 1627-1631.

19. Harari F, Sallsten G, Christensson A *et al*. Blood lead levels and decreased kidney function in a population-based cohort. *Am J Kidney Dis.* 2018; 72: 381-389.

20. Muntner P, He J, Vupputuri S, Coresh J, Batuman V. Blood lead and chronic kidney disease in the general United States population: results from NHANES III. *Kidney Int.* 2003; 63: 1044-1050.

21. Kim R, Rotnitzky A, Sparrow D, Weiss ST, Wagner C, Hu H. A longitudinal study of low-level lead exposure and impairment of renal function. The Normative Aging Study. *J Am Med Assoc.* 1996; 275: 1177-1181.

22. Chowdhury R, Darrow L, McClellan W, Sarnat S, Steenland K. Incident ESRD among participants in a lead surveillance program. *Am J Kidney Dis.* 2014; 64: 25-31.

23. O'Flaherty EJ. Physiologically based methods for bone-seeking elements. IV. Kinetics of lead disposition in humans. *Toxicol Appl Pharmacol.* 1993; 118: 16-29.

24. Leggett RW. An age-specific kinetic model of lead metabolism in humans. *Environ Health Perspect.* 1993; 101: 598-616.

25. Yang WY, Efremov L, Mujaj B *et al*. Association of office and ambulatory blood pressure with blood lead in workers before occupational exposure. *J Am Soc Hypertens.* 2017; 12: 14-24.
26. Mujaj B, Yang WY, Zhang ZY *et al*. Renal function in relation to low-level environmental lead exposure. *Nephrol Dial Transplant.* 2018; 34: 941-946.
27. Yu YL, Yang WY, Thijs L *et al*. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *Hypertension.* 2020; 76: 1299-1307.
28. Yu YL, Thijs L, Wei DM *et al*. Two-year responses of renal function to first occupational lead exposure. *KI Reports.* 2022; 7: 1198-1209.
29. Hu H, Aro A, Payton M *et al*. The relationship of bone and blood lead to hypertension. The Normative Aging Study. *J Am Med Assoc.* 1996; 275: 1171-1176.
30. Yan LD, Rouzier V, Pierre JL *et al*. High lead exposure associated with higher blood pressure in Haiti: a warning sign for low-income country. *Hypertension.* 2022; 79: 283-290.
31. Obadia PM, Kayembe-Kitenge T, Haufroid V, Nkulu CBL, Nemery B. Preeclampsia and blood lead (and other metals) in Lubumbashi, DR Congo. *Environ Res.* 2024; 167: 468-471.
32. Resongles E, Dietze V, Green DC, Weiss DJ. Strong evidence for the continued contribution of lead deposited during the 20th century to the atmospheric environment in London today. *Proc Natl Acad Sci.* 2021; 118: e2102791118.
33. Martens DS, Thijs L, Latosinska A *et al*. Urinary peptidomics to address age-related disabilities: a prospective population study with replication in patients. *Lancet Healthy Longev.* 2021; 2: e690-e703.
34. Klein J, Papadopoulos T, Mischak H, Mullen W. Comparison of CE-MS/MS and LC-MS/MS sequencing demonstrates significant complementarity in natural peptide identification in human urine. *Electrophoresis.* 2014; 35: 1060-1064.
35. Veiras LC, Girardi ACC, Curry J *et al*. Sexual dimorphic pattern of renal transporters and electrolyte homeostasis. *J Am Soc Nephrol.* 2017; 28: 3504-3517.
36. Bruge R, Dawson-Hughes B, Solomon DH, John BW, Alison K, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. *J Bone Miner Res.* 2007; 22: 465-475.

37. Bliuc D, Nguyen ND, Milch VE, Nguyen TV, Eisman JA, Center JR. Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women. *J Am Med Assoc.* 2009; 301: 513-521.

38. Wei FF, Trenson S, Verhamme P, Vermeer C, Staessen J. Vitamin K-dependent matrix Gla protein as multifaceted protector of vascular and tissue integrity. *Hypertension.* 2019; 73: 1160-1169.

39. Akbari S, Rasouli-Ghahroudi AA. Vitamin K and bone metabolism: a review of the latest evidence in preclinical studies. *Biomed Res Int.* 2018; 4629383: 1-5.

40. Lin X, Patil S, Gao YG, Qian A. The bone extracellular matrix in bone formation and regeneration. *Front Phrmacol.* 2020; 11: 757.

41. Licini C, Vitale-Brovarone C, Mattioloni-Belmonte M. Collagen and non-collagenous proteins molecular crosstalk in the pathophysiology of osteoporosis. *Cytokine Growth Factor Rev.* 2019; 49: 59-69.

42. Min SK, Kim M, Park J-B. Insulin-like growth factor 2-enhanced osteogenic differentiation of stem cell spheroids by regulation of Runx2 and Col1 expression. *Exp Ther Med.* 2021; 383: 1-6.

43. Laurent S, Chatellier G, Azizi M *et al*. SPARTE Study: normalization of arterial stiffness and cardiovascular events in patients with hypertension at medium to very high risk. *Hypertension.* 2021; 78: 983-995.

44. Kovvuru K, Kanduri SR, Vaitla P *et al*. Risk factors and management of osteoporosis post-transplant. *Medicina (Kaunas).* 2020; 56: 302.

45. Johansen FE, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. *Mucosal Immunol.* 2011; 4: 598-602.

46. Dehanne F, Gourdin M, Devleesschauwer B *et al*. Cost-DALY comparison of hip replacement care in 12 Belgian hospitals. *BMJ Open Quality.* 2021; 10: e001263.

47. Dunnewind T, Dvortsin EP, Smeets HM *et al*. Economic consequences and potentially preventable costs related to osteoporosis in the Netherlands. *Value Health.* 2017; 20: 762-768.

48. Thomas T, Tubach F, Bizouard G *et al*. The economic burden of severe osteoporotic fractures in the French healthcare database: the FRACTOS study. *JBMR.* 2022; 37: 1811-1822.

49. Williams SA, Daigle SG, Weiss R, Wang Y, Arora T, Curtis JR. Economic burden of osteoporosis-related fractures in the US medicasre population. *Ann Pharmacother.* 2021; 55: 821-829.

50. Cleland JGF, Ferreira JP, Mariottoni B *et al*. The effect of spironolactone on cardiovascular function and markers of fibrosis in people at increased risk of developing heart failure: the heart 'OMics' in AGEing (HOMAGE) randomized clinical trial. *Eur Heart J.* 2021; 42: 684-696.

51. Ravassa S, Kuznetsova T, Varo N *et al*. Biomarkers of cardiomyocyte injury and stress identify left atria and left ventricular remodelling and dysfunction: a population-based study. *Int J Cardiol.* 2015; 185: 177-185.

52. Magalhães P, Pejchinovski M, Markoska K *et al*. Association of kidney fibrosis with urinary peptides: a path towards non-invasive liquid biopsies? *Sci Rep.* 2017; 7: 16915.

53. Mavrogeorgis E, Mischak H, Latosinska A *et al*. Collagen-derived peptides in CKD: a link to fibrosis. *Toxins.* 2022; 14: 10.

54. Siwy J, Klein T, Rosler M, von Eynatten M. Urinary proteomics as a tool to identify kidney responders to dipeptidyl peptidase-4 inhibition: a hypothesis-generating analysis of the MERLINA-T2D trial. *Proteomics.* 2019; 13: 1800144.

55. Olsen MH, Angell SY, Asma S *et al*. A call to action and a lifecourse strategy to address the global burden of raised blood pressure on current and future generations: the *Lancet* Commission on hypertension. *Lancet.* 2016; 388: 2665-2172.

56. Barker DJP, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. *Lancet.* 1993; 341: 938-941.

57. Wei FF, Raaijmakers A, Zhang ZY *et al*. Association between cognition and the retinal microvasculature in 11-year old children born preterm or at term. *Early Hum Dev.* 2018; 118: 1-7.

58. Raaijmakers A, Zhang ZY, Claessens J *et al*. Does extremely low birth weight predispose to low-renin hypertension? *Hypertension.* 2017; 69: 443-449.

59. Liu K, Daviglus ML, Loria CM *et al*. Healthy lifestyle through young adulthood and presence of low cardiovascular disease risk in middle age: The Coronary Artery Risk Development in (Young) Adults (CARDIA) Study. *Circulation.* 2012; 125: 996-1004.

60. Ferreira I, van de Laar RJ, Prins MH, Twisk JW, Stehouwer CD. Carotid Stiffness in Young Adults: A Life-Course Analysis of its Early Determinants. *Hypertension.* 2012; 59: 54-61.

61. Boyd A, Golding J, Macleod J *et al*. Cohort profile: The 'children of the 90s' - the index offspring of the Avon Longitudinal Study of Parents and Children. *Int J Epidemiol.* 2013; 42: 111-127.

62. O'Donnell M, Mente A, Alderman MH *et al*. Salt and cardiovascular disease: insufficient evidence to recommend low sodium intake. *Eur Heart J.* 2020; 41: 3363-3373.

63. Stolarz-Skrzypek K, Kuznetsova T, Thijs L *et al*. Fatal and nonfatal outcomes, incidence of hypertension and blood pressure changes in relation to urinary sodium excretion. *J Am Med Assoc.* 2011; 305: 1777-1785.

64. Hall JE, Guyton AC, Brands MW. Pressure-volume regulation in hypertension. *Kidney Int Suppl.* 1996; 55: S35-S41.

65. Visseren FLJ, Mach F, Smulders YM *et al*. 2021 ESC guidelines on cardiovascular disease prevention in clinical practice. *Eur Heart J.* 2021; 42: 3227-3337.

66. Dai H, Much AA, Maor E *et al*. Global, regional, and national burden of ischaemic heart disease and its attributable risk factors, 1999-2017: results from the Global Burden of Disease Study 2017. *European Heart Journal - Quality of Care and Clinical Outcomes.* 2022; 8: 50-60.

67. Bateson P, Barker D, Clutton-Brock T *et al*. Developmental plasticity and human health. *Nature.* 2004; 430: 419-421.

68. Lichtenstein AH, Appel LJ, Vadiveloo M *et al*. 2021 Dietary Guidance to Improve Cardiovascular Health: a scientific statement from the American Heart Association. *Circulation.* 2021; 144: e472-e487.

69. Agyepong IA, Sewanjamba N, Binawaho A *et al*. The path to longer and healthier lives for all Africans by 2030: the *Lancet* Commission on future health in sub-Saharan Africa. *Lancet.* 2017; 390: 2803-2859.

70. Abubakar I, Dalglish SL, Angell B *et al*. The *Lancet* Nigeria Commission: investing in health and the future of the nation. *Lancet.* 2022; 399: 1155-1200.

71. Keates AK, Mocumbi AO, Ntsekhe M, Sliwa K, Stewart S. Cardiovascular disease in Africa: epidemiological profile and challenges. *Nat Rev Cardiol.* 2017; 14: 273-293.

72. Schutte AE, Botha S, Fourie CMT *et al*. Recent advances in understanding hypertension development in sub-Saharan Africa. *J Hum Hypertens.* 2017; 31: 491-500.

73. Tsao CW, Aday AW, Almarzooq ZI *et al*. Heart disease and stroke statistics-2023 update: a report from the American Heart Association. *Circulation.* 2023; 147: e93-e621.

74. Jolly S, Vittinghoff E, Chattopadhyay A, Bibbins-Domingo K. Higher cardiovascular disease prevalence and mortality among younger Blacks compared to Whites. *Am J Med.* 2010; 123: 811-818.

75. Lipworth L, Mumma MT, Cavanaugh KL *et al*. Incidence and predictors of end stage renal disease among low-income Blacks and Whites. *PLoS One.* 2012; 7: e48407.

76. Nichols OI, Fuller-Rowell TE, Robinson AT, DaJuandra E, Homandberg LK. Neighborhood socioeconomic deprivation in early childhood mediates racial disparities in blood pressure in a college student sample. *J Youth Adolesc.* 2022; 51: 2146-2160.

77. Risnes KR, Vatten LJ, Baker JL *et al*. Birthweight and mortality in adulthood: a systematic review and meta-analysis. *Int J Epidemiol.* 2011; 40: 647-661.

Summary

Risk factors, irrespective of whether they originate from the environment or are innate, differentiate unhealthy ageing from the average or optimal lifecourse. These risk factors, in the context of this dissertation termed external and internal drivers of ill health, operate *"silently"* in the perinatal phase of life, during childhood and young adulthood, but if unattended ultimately cause target organ damage and debilitating health problems. The objective of my doctoral research was to study these external and internal drivers of unhealthy ageing. In line with the title of this dissertation, I structured my research around three themes: (i) exposure to lead as the prototype of a ubiquitously distributed environmental pollutant and therefore an external driver of ill health; (ii) studies focusing on the urinary proteome, which reflects body-wide pathogenic processes that are fuelled by internal drivers, and (iii) finally clinical risk factors, which arise through the interplay between external and internal drivers of ill health. These studies were conducted in children, young lead workers, and population and patient cohorts, thereby covering the whole human life span.

Lead exposure as prototype of an external driver of ill health

Over the past 40 years, environmental and occupational exposure fell drastically, because lead-containing materials were phased out and because in developed countries regulations to protect workers were substantially tightened. Historically, before these regulations became law and were reinforced, lead exposure was a cause of hypertension and renal dysfunction. The question remained whether at the much lower current-day exposure, lead remains a contaminant responsible for sizable adverse health effects. In The Study for Promotion of Health in Recycling Lead (SPHERL), I studied newly hired workers without known previous lead exposure, who were employed at lead recycling plants in the United States. The co-primary endpoints were hypertension and renal dysfunction. Follow-up spanned 2 years (n=251) in the first examination cycle (***Chapter 2.1***) and up to 6 years (n=234) by the end of the project (***Chapter 2.2***). Over follow-up, blood lead increased over three-fold. However, blood pressure, a wide range of renal function measures did not significantly change and likewise the incidence of hypertension or worsening of the stage of renal function were not significant. Extrapolating the findings to the population, I concluded that that at current exposure levels, lead cannot be a primary cause of hypertension or renal dysfunction. However, given the wide confidence interval of some findings, I could not exclude that low-level lead exposure might still be an external driver of ill health, in particular in susceptible individuals with comorbidities or in low- and middle-income countries where regulations are still being drafted or insufficiently

reinforced. For this reason, in ***Chapter 2.3***, I reviewed the exposure vectors, the toxicokinetics, the pathogenic mechanisms driven by lead, and the legislation put in place in the United States and Europe to protect workers. An important observation was that fine particulate matter remains contaminated by lead deposited in the previous century, leading to the conclusion that policy makers, particularly in low- and middle-income countries, should continue designing and reinforcing stricter regulations to reduce lead exposure of the general population and workers.

Urinary proteomic profiling to unveil internal drivers of ill health

By sequencing the peptide fragments in the urinary proteomic profile, parental proteins can be identified that play a role in the pathogenesis of age-related chronic disease. I focused on osteoporosis in the general population (***Chapter 3.1***) and in heart transplant recipients (***Chapter 3.2***) and the system-wide fibrotic signature as can be detected in patients at high heart failure risk because of coronary heart disease (***Chapter 3.3***).

The Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) is a large-scale family-based study (1985-2019). Participants were invited for a follow-up examination in 2005-2010 and re-invited in 2009-2013. In ***Chapter 3.1***, the participants in both 2005-2010 and 2009-2013 follow-up examinations constituted the derivation dataset (n=519), which included the 2005-2010 data, and the time-shifted validation dataset (n=519), which included the 2009-2013 follow-up data. The remaining participants, who only took part in the 2005-2010 examination, constituted the synchronous validation dataset (n=187). Chronological age was associated with 99 urinary peptides (16 proteins). Peptides derived from IGF2 and MGP were upregulated in women compared to men, whereas COL1A2, COL3A1, COL5A2, COL10A1 and COL18A1 were downregulated. Via application of a 1000-fold bootstrapped elastic regression procedure, finally, 29 peptides (10 proteins) constituted the UPPost-age biomarker, replicated across datasets. The body-height-to-arm-span ratio is an index of osteoporosis of the axial skeleton, resulting in a decrease in body height, while arm span is constant throughout life. This marker was negatively associated with UPPost-age. Over median follow-up of 4.9 years, the risk of osteoporosis increased by 36% in per 10-year increment in UPPost-age and the risk of osteoporotic fracture by 44% in women. FLEMENGHO illustrated how a urinary multidimensional urinary biomarker, an internal driver of ill health, predicts osteoporosis.

Heart transplant recipients are prone to osteoporosis as a consequence of corticosteroid treatment. I therefore analysed heart transplant patients enrolled in the

Urinary Proteomics in Predicting Heart Transplantation (uPROPHET) Study. In ***Chapter 3.2***, cases with osteoporosis and matching controls were randomly selected from all available 389 patients. A multidimensional urinary proteomic biomarker for osteoporosis, named OSTEO18, was developed, replicated and validated. On top of relevant osteoporosis risk factors, OSTEO18 improved the area under the curve as categorised (optimised threshold 0.095) and continuously distributed biomarker. Although further studies in other transplant cohorts required, OSTEO18 is a clinically meaningful novel biomarker indicative of osteoporosis in heart transplantation recipients and is registered as an in-vitro diagnostic.

The “Omics” in Ageing Study (HOMAGE) is a multicentre open-label randomised clinical trial including patients at high risk of developing heart failure, because of coronary heart disease. These patients were randomised to spironolactone 25-50 mg/day on top of usual therapy or usual therapy alone and followed up for 9 months. In ***Chapter 3.3*** (n=290), the between-group differences in the urinary proteomic profile were confined to 27 collagen fragments. Of these 27 fragments, 16 were downregulated on spironolactone and 11 upregulated. Moreover, compared to control, serum PICP (a marker of collagen synthesis) and the serum PICP/CITP ratio (a marker of the balance between collagen synthesis and degradation) decreased on spironolactone. This HOMAGE-RCT findings confirm the antifibrotic action of mineralocorticoid receptor antagonists. With regard to the upregulation in 11 collagen fragments, the most likely explanation is that a certain degree of collagen synthesis is required to maintain the structural integrity of vital organs. Combining urinary and serum fibrosis markers refines the insight in the action of antifibrotic drugs in support of the clinical management of heart failure patients and the discovery of antifibrotic drugs.

Hypertension as risk factor in childhood

Hypertension is the major modifiable cardiovascular risk factor. From this point of view, in this dissertation is both an outcome variable (***Chapter 4.1***) and a major risk factor, for which analyses have to be adjusted. According the Developmental Origins of Health and Disease concepts, perinatal factors affect the micro- and macrocirculation and the heart and predispose to cardiovascular disease, renal dysfunction and heart failure later in life. Keeping this idea in mind, the Environmental Influence on Early Ageing (ENVIR*ON*AGE) birth cohort study focuses on risk factors present at birth (n=283) and their trajectory in early (4-6 years, n=500) and later (9-11 years, n=272) childhood. In ***Chapter 4.1***, I found that blood pressure tracked over time, meaning that most children maintained their percentile position in the blood pressure distribution as they aged. Systolic/diastolic blood

pressure tracked from 67.3/40.5 mm Hg at birth to 100.2/57.5 mm Hg at 4-6 years and further to 107.7/65.1 mm Hg at 9-11 years. While accounting for confounders, at the last follow-up, systolic/diastolic blood pressure increased by 2.6/1.4 mm Hg per 1-SD increment in the baseline blood pressure. Approximately 10% of children were so-called "*horse-racers*", in whom the blood pressure increase was faster than expected on tracking. The ENVIR*ON*AGE observations for the first time highlight that the blood pressure at birth determines the blood pressure rise through childhood.

Birth weight and salt intake

In ***Chapter 4.2***, I continued the line of research initiated in the ENVIR*ON*AGE birth cohort (***Chapter 4.1***), but I moved the focus to adults. I investigated how salt intake (external driver), estimated from exactly time 24-h urine collections (UVNA) and birth weight (internal driver) interact to determine blood pressure and cardiovascular risk among adults. The European Project on Genes in Hypertension (EPOGH) is a family-based population cohort using the same protocol, questionnaires and follow-up procedures, as in FLEMENGHO. The study population was subdivided into the Outcome (n=1945), Hypertension (n=1460) and Blood Pressure cohorts (n=1039) to analyse the incidence of cardiovascular endpoints, hypertension and changes in blood pressure in relation to UVNA changes and how birth weight modified these associations. Adult body weight tracked with birth weight, but the incidence of cardiovascular disease and hypertension were not associated with UVNA or birth weight. However, in the low-birth-weight group (≤2500 g), the relation between changes in blood pressure and UVNA reached significance. This study showed tracking of adult with birth weight and suggests that low birth weight increases salt sensitivity. The latter observation is in keeping with the abundant literature reporting that low birth weight is associated with fewer functional nephrons, thereby increasing salt sensitivity.

Environmental and innate risk factors in Blacks and Whites

Blacks are more susceptible to hypertension and associated complications. However, most – if not all – previous reports focused on African-Americans compared with White adults living in the United States. I analysed the data of the ongoing Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform Trial (UPRIGHT-HTM). High-risk patients are being recruited applying the same selection and exclusion criteria across study sites in Europe and sub-Saharan Africa. This study allowed to address external drivers as captured by the region of residence and internal drivers as captured by race (***Chapter 4.3***). Using the data available by January 2024

(Blacks: n=168; Whites: n=93), I demonstrated that, although Blacks born and living in sub-Saharan Africa were younger (60.4 versus 68.3 years), they had a worse risk profile compared to European Whites. In addition, Blacks had a higher 10-year cardiovascular risk as assessed by Framingham risk score, a higher heart age (index of vascular ageing), and a worse renal function. This study did not differentiate host (genetic, molecular, and pathogenic) from environmental drivers of disease. Nonetheless, the findings call for a multipronged and comprehensive implementation of innovative health policies in sub-Saharan countries. Education, research, empowerment of stakeholders, and international learned societies connecting experts from a wide array of disciplines should vigorously sustain this efforts.

Conclusions and perspectives

With its focus on external and internal drivers of ill health and their interaction, this dissertation fulfilled its stated objectives. As an ultimate goal, I hope that these findings will be translated in to practice and future research with the aim of the timely prevention of unhealthy ageing early in life, long before usually irreversible target organ damage and the associated morbidity lead to a decrease in quality-adjusted life years and premature mortality.

Samenvatting

Risicofactoren, ongeacht of ze afkomstig zijn uit het leefmilieu of aangeboren zijn, differentiëren of mensen volgens een ziekelijk, gemiddeld of héél gezonde traject ouder worden. Deze risicofactoren, die in de context van dit proefschrift als externe en interne oorzaken van minder goede gezondheid worden benoemd, zijn reeds actief op stille wijze in de perinatale fase van het leven en tijdens de groei van kinderen tot jonge volwassenen. Als ze niet tijdig behandeld worden, kunnen ze aanleiding geven tot aantasting van vitale organen, slopende gezondheidsproblemen en voortijdig overlijden. Het doel van mijn doctoraal onderzoek was om deze externe en interne oorzaken van ongezond ouder worden te bestuderen. In lijn met de titel van dit proefschrift, heb ik mijn onderzoek gestructureerd rond drie thema's: (i) blootstelling aan lood als het prototype van een alomtegenwoordige milieuverontreinigende stof en daarom een externe aanstoker van gezondheidsdeficit; (ii) studies gericht op het urinaire proteoom, dat lichaamsbrede pathogene processen weerspiegelt, en (iii) ten slotte klinische risicofactoren, die ontstaan door de wisselwerking tussen externe en interne oorzakelijke factoren, die leiden tot een minder goede gezondheid. Mijn onderzoek werd uitgevoerd bij kinderen, jonge arbeiders blootgesteld aan lood, met behulp van bevolkingsstudies en bij patiënten met diverse ziekten, waardoor het hele bereik van het menselijk leven werd bestreken.

Lood als prototype van een contaminant met negatieve invloed op de gezondheid

De afgelopen 40 jaar daalde de blootstelling aan lood via het leefmilieu en via de werkomgeving drastisch, omdat loodhoudende materialen werden uit gefaseerd en omdat zeker in ontwikkelde landen de regelgeving ter bescherming van de bevolking en werknemers aanzienlijk werd aangescherpt. Historisch gezien, voordat deze strenge regelgeving van kracht werd, veroorzaakte blootstelling aan lood hypertensie en nierfunctiestoornissen. De vraag blijft echter of lood bij de huidige veel lagere blootstelling niveaus nog steeds verantwoordelijk blijft voor nadelige gezondheidseffecten. In The Study for Promotion of Health in Recycling Lead (SPHERL) bestudeerde ik nieuw aangeworven werknemers zonder eerdere blootstelling aan lood, die als nieuwe arbeiders werk startten in loodrecyclage fabrieken in de Verenigde Staten. De co-primaire eindpunten waren hypertensie en nierfunctiestoornissen. De follow-up besloeg 2 jaar (n=251) in de eerste cyclus van het onderzoek (***Hoofdstuk 2.1***) en tot 6 jaar (n=234) aan het einde van de studie (***Hoofdstuk 2.2***). Tijdens de follow-up nam het bloedloodgehalte meer dan drie keer toe, echter zonder significante beïnvloeding van de bloeddruk, een breed scala aan nierfunctiemetingen en de incidentie van hypertensie of achteruitgang

van de nierfunctie. Door de bevindingen te extrapoleren naar de algemene bevolking, concludeerde ik dat lood aan de huidige blootstellingsniveaus geen primaire oorzaak van hypertensie of nierfunctiestoornissen kan zijn. Gezien het brede betrouwbaarheidsinterval van sommige bevindingen in SPHERL (***Hoofdstuk 2.1*** en ***Hoofdstuk 2.2***), blijft er het caveat dat de huidige lage loodblootstelling nog steeds nadelige gezondheidseffecten kan veroorzaken, met name bij vatbare personen met comorbiditeiten of in landen met een laag en gemiddeld inkomen, waar een strengere regelgeving nog in de maak is of onvoldoende wordt toegepast. Om deze reden heb ik in ***Hoofdstuk 2.3*** de blootstellingsvectoren, de toxicokinetiek, de pathogene mechanismen die door lood worden aangestuurd en de wetgeving die in de Verenigde Staten en Europa van kracht is om werknemers te beschermen, samengevat. Een belangrijke waarneming is dat fijnstof nog steeds verontreinigd is door lood dat in de vorige eeuw is afgezet. Beleidsmakers, met name vooral in ontwikkelingslanden, moeten daarom verder werken aan het opstellen en opleggen van een strengere regelgeving.

Ziekte-gerelateerde kenmerken van het urinair proteoom

Door de volgorde van de aminozuren in urinaire peptiden te bepalen kan men de proteïnen identificeren, die een rol spelen in de pathogenese van leeftijdsgebonden chronische ziekten. Ik bestudeerde osteoporose bij de algemene bevolking (***Hoofdstuk 3.1***) en bij harttransplantatiepatiënten (***Hoofdstuk 3.2***) en de systeembrede fibrotische urinaire proteomische signatuur zoals die kan worden gedetecteerd bij patiënten met een hoog risico op hartfalen vanwege coronaire hartziekte (***Hoofdstuk 3.3***).

The Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) is een grootschalige bevolkingsstudie met aselectieve bemonstering van families uit een afgelijnd geografisch gebied in het noorden van Vlaanderen (1985-2019). Deelnemers werden uitgenodigd voor een vervolgonderzoekingen in 2005-2010 en opnieuw in 2009-2013. In ***Hoofdstuk 3.1*** vormden 519 personen, die deelnamen in 2005-2010, de dataset voor de primaire analyse. De overige 187 personen, die in 2005-2010 deelnamen vormden de tijd synchrone replicatiedataset. De resultaten van de 519 personen, die een tweede maal onderzocht werden in 2009-2009, werden geanalyseerd als een in de tijd verschoven dataset. Chronologische leeftijd was geassocieerd met 99 urinaire peptiden afkomstig van 16 proteïnen. Peptiden afkomstig van IGF2 en MGP waren opwaarts gereguleerd bij vrouwen vergeleken met mannen, terwijl COL1A2, COL3A1, COL5A2, COL10A1 en COL18A1 neerwaarts gereguleerd waren. Via toepassing van een 1000-voudige herhaling van de elastische regressieprocedure met herbemonstering van de primaire analyse dataset werd de multidimensionale urinaire biomerker voor

leeftijdsgebonden osteoporose geconstrueerd en benoemd als UPPost-age. Deze biomerker bestond uit 29 urinaire peptiden afkomstig van 10 proteïnen. De verhouding tussen gestalte en armlengte is een index voor osteoporose van het axiale skelet. Inderdaad, de lichaamslengte neemt af met ouder worden door vermindering van de rugwervelhoogte, terwijl de armlengte gedurende het hele leven constant blijft. Deze merker was negatief geassocieerd met UPPost-leeftijd. Gedurende een mediane follow-up van 4,9 jaar nam het risico op osteoporose toe met 36% per 10 jaar-toename in UPPost-age in alle studiedeelnemers terwijl bij vrouwen de kans op osteoporotische fracturen met 44%. FLEMENGHO illustreerde hoe een multidimensionale urinaire biomerker osteoporose voorspelt en hoe een specifieke signatuur van het urinair proteoom een imminent gezondheidsprobleem kan reflecteren.

Harttransplantpatiënten zijn vatbaar voor osteoporose als gevolg van de behandeling met corticosteroïden en immunosuppressiva. Daarom analyseerde ik harttransplantpatiënten die beschikbaar waren in The Urinary Proteomics in Predicting Heart Transplantation (uPROPHET) Study. In ***Hoofdstuk 3.2*** werden patiënten met osteoporose en controlepatiënten met een gelijkaardig osteoporose risicoprofiel op aselecte wijze geselecteerd uit alle beschikbare 389 patiënten. Een multidimensionale urinaire proteomische biomerker voor osteoporose, genaamd OSTEO18, werd ontwikkeld, gerepliceerd en gevalideerd. Bovenop relevante osteoporose risicofactoren verbeterde OSTEO18 de diagnostische precisie zowel wanneer de geoptimaliseerde drempelwaarde van 0,095 werd toegepast als wanneer OSTEO18 geanalyseerd werd als biomerker met een continue distributie. Hoewel verdere studies in andere transplantatiecohorten vereist zijn, is OSTEO18 een klinisch betekenisvolle nieuwe biomerker die indicatief is voor osteoporose bij harttransplantpatiënten. OSTEO18 is geregistreerd als een in-vitro diagnostische test.

De "Omics" in Ageing Study (HOMAGE) is een multicenter open-label klinische studie. Patiënten met een hoog risico op het ontwikkelen van hartfalen omwille van onderliggend coronair lijden werden gerandomiseerd worden tot spironolacton 25-50 mg per dag bovenop de gebruikelijke therapie of alleen tot de gebruikelijke therapie. De patiënten werden 9 maanden opgevolgd. In ***Hoofdstuk 3.3*** (n=290), vond ik dat de verschillen in het urinair proteoom tussen beide groepen beperkt ware tot 27 collageenfragmenten. Van deze 27 fragmenten waren 16 neerwaarts gereguleerd op spironolacton en 11 opwaarts gereguleerd. Bovendien namen, vergeleken met de controlegroep, serum PICP (een marker van collageensynthese) en de serum PICP/CITP-ratio (een marker van de balans tussen collageen synthese en afbraak) af op spironolacton. Deze HOMAGE-RCT-

bevindingen bevestigden de antifibrotische werking van mineralocorticoïde receptorantagonisten. Met betrekking tot de opwaartse regulatie in 11 collageenfragmenten is de meest waarschijnlijke verklaring dat een bepaalde mate van collageensynthese vereist blijft om de structurele integriteit van vitale organen te behouden. Meer in het algemeen, verfijnt het combineren van urinaire en serumfibrosemarkers het inzicht in de werking van antifibrotische geneesmiddelen. Deze bevinding ondersteunt het klinische management van hartfalenpatiënten en opent nieuwe mogelijkheden in het onderzoek naar nieuwe antifibrotische geneesmiddelen.

Hypertensie als risicofactor bij kinderen

Hypertensie is de belangrijkste beïnvloedbare cardiovasculaire risicofactor. Vanuit dit oogpunt is in dit proefschrift bloeddruk zowel een uitkomstvariabele (***Hoofdstuk 4.1***) als een belangrijke risicofactor, waarvoor analyses moeten worden aangepast. Volgens de concepten van The Developmental Origins of Health and Disease beïnvloeden perinatale factoren de micro- en macrocirculatie en het hart en maken risicofactoren die reeds aanwezig op jeugdige leeftijd kinderen vatbaar voor hart- en vaatziekten, nierfunctiestoornissen en hartfalen op latere leeftijd. Met dit idee in gedachten, bestudeert The Environmental Influence on Early Ageing (ENVIR*ON*AGE) geboortecohortstudie risicofactoren die reeds bij de geboorte aanwezig zijn (n=283) en hun verder traject in de vroege (4-6 jaar, n=500) en latere (9-11 jaar, n=272) kindertijd. In ***hoofdstuk 4.1***, vond ik dat de bloeddruk dat de meeste kinderen hun percentielpositie in de bloeddrukverdeling behouden bij het opgroeien. De gemiddelde systolische/diastolische bloeddruk gemeten bij de geboorte was 67,3/40,5 mm Hg en nam toe tot 100,2/57,5 mm Hg op 4-6 jaar en verder tot 107,7/65,1 mm Hg op 9-11 jaar. Rekening houdend met verstorende factoren, steeg de systolische/diastolische bloeddruk bij de laatste follow-up met 2,6/1,4 mm Hg per 1 SD-verhoging in de basisbloeddruk. Ongeveer 10% van de kinderen waren zogenaamde "*horse racers*", bij wie de bloeddruk sneller steeg dan verwacht op basis van hum percentiel in de bloeddrukdistributie bij een eerdere meting. De ENVIR*ON*AGE-beschrijven voor de eerste maal het bloeddruktraject vanaf de geboorte tot kinderen de schoolgaande leeftijd bereikt hebben.

Geboortegewicht en zoutinname

In ***Hoofdstuk 4.2***, zette ik de onderzoekslijn voort die was ingezet in het ENVIR*ON*AGE-geboortecohort (***Hoofdstuk 4.1***), maar ik verlegde de focus naar volwassenen. Ik heb onderzocht hoe zoutinname (externe risicofactor), geschat op basis van exact getimed 24-uurs urinecollecties (UVNA) en geboortegewicht (interne risicofactor) samenwerken

om bloeddruk en cardiovasculair risico bij volwassenen te bepalen. The European Project on Genes in Hypertension (EPOGH) is een Europese bevolkingscohorte, die gerekruteerd, onderzocht en opgevolgd werd met hetzelfde protocol als toegepast in FLEMENGHO. De deelnemers werden onderverdeeld in de Outcome (n=1945), Hypertension (n=1460) en Blood Pressure cohorten (n=1039) om de incidentie van cardiovasculaire eindpunten, hypertensie en veranderingen in bloeddruk in relatie tot UVNA-veranderingen te analyseren en hoe geboortegewicht deze associaties modificeerde. Volwassen lichaamsgewicht percentielsgewijze spoorde met geboortegewicht, maar de incidentie van hart- en vaatziekten en hypertensie waren niet geassocieerd met UVNA of geboortegewicht. Echter, in de groep met een laag geboortegewicht (≤2500 g), was de relatie tussen de veranderingen in de bloeddruk en in UVNA statistisch betekenisvol. Deze subgroep analyse suggereert dat een laag geboortegewicht de zoutgevoeligheid verhoogt. Deze observatie past in de literatuur die suggereert dat een laag geboortegewicht geassocieerd is met minder functionele nefronen, waardoor een de zoutgevoeligheid toeneemt en een hogere bloddruk nodig is om natrium urinair uit te scheiden.

Leefmilieu gebonden en aangeboren risicofactoren bij zwarte en blanke mensen

Zwarte personen zijn vatbaarder voor hypertensie en bijbehorende complicaties. Echter, de meeste – zo niet alle – eerdere rapporten richtten zich op Afro-Amerikanen in vergelijking met blanke volwassenen die in de Verenigde Staten wonen. Ik analyseerde de gegevens van de lopende Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform Trial (UPRIGHT-HTM). Patiënten met een hoog risico worden gerekruteerd met toepassing van dezelfde selectie- en exclusiecriteria op alle onderzoek locaties in Europa en Sub-Saharisch Afrika. Deze studie maakte het mogelijk om externe risicofactoren te bestuderen gerelateerd aan de regio van residentie tezamen met aangeboren raciale risicofactoren (***Hoofdstuk 4.3***). Met behulp van de gegevens die beschikbaar waren in januari 2024 (zwarte personen: n=168; blanke deelnemers: n=93), toonde ik aan dat, hoewel zwarten geboren en wonend in Sub-Saharisch Afrika jonger waren (60,4 versus 68,3 jaar), ze een slechter risicoprofiel hadden vergeleken hun Europese blanke deelgenoten. Bovendien hadden zwarten een hoger 10-jarig cardiovasculair risico, zoals beoordeeld door de Framingham-risicoscore, een hogere hartleeftijd (index van vasculaire veroudering) en een slechtere nierfunctie. Deze studie maakte geen onderscheid tussen gastheer-gebonden (genetische, moleculaire en pathogene) en omgevingsfactoren van ziekte. Niettemin vragen de bevindingen om een veelzijdige en uitgebreide implementatie van innovatief

gezondheidsbeleid in Sub-Saharisch landen. Onderwijs, onderzoek, empowerment van belanghebbenden en internationale geleerde genootschappen die experts uit een breed scala aan disciplines samenbrengen, zouden deze inspanningen krachtig moeten ondersteunen.

Besluit en perspectief

Met zijn focus op externe en interne risicofactoren als moter van slechte gezondheid en hun interactie, voldoet dit proefschrift aan zijn gestelde doelen. Ik hoop dat mijn bevindingen zullen worden vertaald naar de klinische praktijk en toekomstig onderzoek zullen inspireren. Het ultieme doel is het tijdig voorkomen van een ongunstig verouderingstraject. Preventie op jonge leeftijd, lang voordat doorgaans onomkeerbare doelorgaanschade zich manifesteert is zonder twijfel de weg voorwaarts met als doel morbiditeit met een afname van het aantal kwalitatief gecorrigeerde levensjaren en vroegtijdige sterfte te verminderen.

总结

无论是外源性还是先天性的危险因素，都是将不健康衰老与平均或健康生命历程区分开来的关键因素。在本论文中，我们将这些危险因素称为外部危险因素或内部危险因素。这些危险因素在围产期、儿童时期和青年时期“悄无声息”地起作用。如果它们不能及时被发现和治疗，最终会导致靶器官损害和严重健康问题。因此，我的博士课题旨在研究导致不健康衰老的外部和内部危险因素。从论文标题出发，我的研究围绕着三个主题展开：(一)重金属铅：铅是一种广泛分布的环境污染物，也是导致疾病的外部危险因素；(二)尿蛋白质组学：尿蛋白质组学能反映由内部危险因素推动的疾病机制；(三)临床危险因素：临床危险因素是由外部和内部危险因素相互作用而产生的。在此框架下，本研究在儿童、接触重金属铅的年轻工人、普通人群和患者群体中进行，从而涵盖整个人类生命周期。

重金属铅作为不健康衰老的外部风险因素

在过去的 40 年里，由于含铅材料被逐步淘汰，且发达国家的工人保护法规日益严格，使环境和职业重金属铅暴露量急剧下降。然而，在相关法律法规颁布实施之前，铅暴露是高血压和肾功能障碍的病因之一。尽管如今铅暴露量远低于过去，其健康风险尚未明确。在 SPHERL（Study for Promotion of Health in Recycling Lead）研究中，我探讨了美国铅回收厂工人的健康情况。他们在该工厂工作前无明确铅职业暴露史。该研究的共同主要终点是高血压和肾功能障碍。第一个检查周期（**第 2.1 章**）的随访期为 2 年（n=251），到研究结束时（**第 2.2 章**），随访期长达 6 年（n=234）。在随访期间，工人们的血铅比基线水平上升三倍以上。然而，血压和多个肾功能指标并无显著变化，高血压的发病率或肾功能恶化程度也并不显著。根据统计结果推断，在目前的暴露水平下，铅不是导致高血压或肾功能障碍的危险因素。然而，鉴于少部分统计结果的宽置信区间，我不能排除低水平铅暴露为危险因素的可能性，特别是在患有合并症的易感人群或法律有待完善的中低收入国家。因此，在**第 2.3 章**中，我回顾了铅的暴露媒介、毒代动力学、致病机制以及欧美国家的工人保护法规。值得注意的是，如今环境中的细颗粒物仍然受到上个世纪沉积的铅的污染。因此，我们呼吁世界各国，特别是中低收入国家的立法部门，进一步完善法律法规，以减少普通民众和工人的铅暴露。

尿蛋白质组学反映内部风险因素导致的疾病机制

我们通过对尿蛋白质组学谱中的肽片段进行测序，可以识别在衰老相关的慢性疾病发病机制中发挥作用的亲本蛋白。在本章节，我重点研究了普通人群（**第 3.1 章**）和心脏移

植受体（**第 3.2 章**）中的骨质疏松症，以及在因冠心病而存在心力衰竭高风险的患者中检测到的纤维化特征（**第 3.3 章**）。

FLEMENGHO（Flemish Study on Environment, Genes and Health Outcomes）研究是一项大规模、基于家系信息的普通人群研究(1985-2019)。参与者于 2005-2010 年和 2009-2013 年受邀参加随访检查。在**第 3.1 章**中，我们将参加了两次随访的参与者的 2005-2010 年数据构成衍生数据集(n=519)，2009-2013 年的随访数据构成时间验证数据集(n=519)，其余仅参加了 2005-2010 年检查的参与者数据构成同步验证数据集(n=187)。统计结果显示，参与者的年龄与 99 种尿肽(16 种蛋白质)显著相关。与男性相比，女性中 IGF2 和 MGP 衍生肽上调，而 COL1A2、COL3A1、COL5A2、COL10A1 和 COL18A1 下调。通过应用 1000 次循环的弹性网络回归，由 29 种肽（10 种蛋白质）构成了 UPPost-age 生物标志物，并在验证数据集中得到重复。此外，骨质疏松症导致身高下降，而臂展在整个生命过程中保持不变。因此，身高与臂展之比是骨质疏松症的体格指标。统计结果显示，该指标与 UPPost-age 呈负相关。在 4.9 年的中位随访期内，UPPost-age 每增加 10 年，骨质疏松症在总人群的发病风险增加 36%，而女性患骨质疏松性骨折的风险增加 44%。综上所述，FLEMENGHO 阐述了尿液蛋白质组学多维生物标志物如何反映疾病的内在风险因素，并预测骨质疏松症。

心脏移植受体因皮质类固醇治疗而患骨质疏松症的风险更高。因此，在 uPROPHET（Urinary Proteomics in Predicting Heart Transplantation）研究中，我分析了心脏移植受体的尿蛋白质组学谱。在**第 3.2 章**中，我在 389 名受体中随机选择了骨质疏松症病例并匹配对照组。利用这些数据，我构建、重复和验证了一种可用于检测骨质疏松症风险的尿蛋白质组学多维生物标志物，名为 OSTEO18。在已调整骨质疏松症的相关危险因素的情况下，作为分类变量（优化阈值 0.095）和连续性变量，OSTEO18 可改善曲线下面积。虽然还需要在其他移植队列进行进一步验证，但 OSTEO18 确是一种具有临床意义的新型生物标志物，可提示心脏移植接受体的骨质疏松症风险，并已被注册为体外诊断方法。

HOMAGE（“Omics” in Ageing Study）研究是一项多中心、开放标签、随机对照临床试验。该研究纳入了因冠心病而存在心力衰竭高风险的患者。这些患者被随机分为干预组和对照组：干预组在常规治疗的基础上加用螺内酯（25-50mg/天）；对照组仅接受常规治疗。所有患者在第 9 个月时进行随访。在**第 3.3 章**（n=290）中，我们发现尿蛋白质组学谱的组间差异仅限于 27 个胶原蛋白片段。在这 27 个片段中，干预组有 16 个片段下调，11 个上调。此外，与对照组相比，干预组的血清 PICP（胶原合成标志物）和血清 PICP/CITP 比率（胶原合成与降解平衡标志物）降低。这一结果证实了盐皮质激素受体拮抗剂的抗纤维化作用。而关于上述 11 种胶原蛋白片段的上调，最可能的解释是，机体需要一定程度的

胶原蛋白合成来维持重要器官的结构完整性。结合尿液和血清纤维化标志物，我们可以完善对抗纤维化药物作用机制的了解，从而有助于心力衰竭患者的临床管理和抗纤维化药物的开发。

高血压是儿童期的危险因素

高血压是重要的、可控的心血管危险因素。因此，在本论文中，高血压既是结果变量（**第 4.1 章**），也是主要危险因素，在统计分析中需要进行调整。根据健康和疾病的发展起源学说，围产期风险因素会影响微循环、大循环和心脏，并导致个体成年后易患心血管疾病、肾功能障碍和心力衰竭。因此，ENVIR*ON*AGE（Environmental Influence on Ageing in Early Life）出生队列研究侧重于出生时存在的危险因素（n=283）及其在童年早期（4-6 岁，n=500）和晚期（9-11 岁，n=272）的发展轨迹。在**第 4.1 章**中，我发现血压会随时间推移而变化，这就意味着大多数儿童在年龄增长时血压分布的百分位保持不变。儿童的收缩压/舒张压从出生时的 67.3/40.5 mm Hg 增至 4-6 岁时的 100.2/57.5 mm Hg，再增至 9-11 岁时的 107.7/65.1 mm Hg。调整混杂因素后，基线收缩压/舒张压每增加 1 个标准差，末次随访血压相应增加 2.6/1.4 mm Hg。此外，大约 10%的儿童是所谓的“加速者”，即他们的血压增长速度比预期更快。因此，ENVIR*ON*AGE 的观察结果首次强调，出生时的血压决定了儿童时期的血压。

出生体重和盐摄入量

在**第 4.2 章**中，我延续了 ENVIR*ON*AGE 出生队列（**第 4.1 章**）中研究思路，但我将研究重点转向成年人。我研究了根据 24 小时尿液估算的盐摄入量（UVNA，外部危险因素）和出生体重（内部危险因素）如何相互作用以决定成年人的血压和心血管疾病风险。EPOGH（European Project on Genes in Hypertension）研究是一个基于家系信息的普通人群队列，使用与 FLEMENGHO 相同的方案、问卷和随访程序。我们将研究人群分为心血管事件数据集（n=1945）、高血压事件数据集（n=1460）和血压变化数据集（n=1039），以分析心血管事件、高血压发病率和血压水平变化与 UVNA 变化的关系，以及出生体重如何影响上述关系。统计结果显示，成人体重与出生体重呈正相关，但心血管疾病和高血压发病率与 UVNA 或出生体重无关。然而，在低出生体重组（≤2500 克）中，血压变化与 UVNA 显著相关。该研究阐述了出生体重与成年体重的相关性，并表明低出生体重会增加盐敏感性。后者与大量文献报道一致，即低出生体重导致功能性肾单位较少，从而增加盐敏感性。

黑人和白人的环境和先天危险因素

黑人更容易患高血压和相关并发症。然而，目前的多数研究仅侧重于非裔美国人与居住在美国的白人。基于此背景，我分析了 UPRIGHT-HTM（Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform Trial）的研究数据。该研究采用相同的纳入和排除标准，在欧洲和撒哈拉以南的非洲招募了存在高危因素的患者。这项研究探讨的是由居住地区差异介导的外部风险因素和由种族差异介导的内部风险因素对心血管疾病和慢性肾脏疾病的影响（**第 4.3 章**）。截止至 2024 年 1 月的数据（黑人：n=168；白人：n=93）显示，尽管撒哈拉以南的非洲黑人更年轻（60.4 岁对 68.3 岁），但他们较欧洲白人存在更高的健康风险。此外，黑人的 Framingham 十年心血管风险更高，心脏年龄（血管老化指数）更大，且肾功能更差。然而，这项研究并未区分疾病的宿主（遗传、分子和致病）和外部环境因素。尽管如此，本研究呼吁撒哈拉以南的国家多管齐下，全面实施创新卫生政策。教育、研究、立法部门、各领域的专家学者和国际学术团体应该共同努力。

结论和展望

综上所述，本论文通过研究导致健康问题的外部与内部危险因素及其相互作用，实现了本博士课题的研究目的。本课题的最终目标是，希望这些研究成果能够转化为临床实践和进一步的科学研究，从而在生命早期及时预防不健康衰老。这些预防措施应在不可逆的靶器官损害、质量调整生命年减少和过早死亡发生之前予以实施。

Curriculum Vitae

Yuling Yu 余钰玲

Nationality: Chinese

Date of birth: 15 March 1994

Email: yuling.yu@kuleuven.be; yyl0603@sina.cn

ORCID: 0000-0002-8255-3770

EDUCATION

KU Leuven, Belgium *Sep. 2021 – Present*

PhD candidate in Biomedical Sciences

Field: hypertension; epidemiology; urinary proteomics; environmental exposure

Dissertation: External and internal drivers of unhealthy ageing

Guangdong Cardiovascular Institute, China *Sep. 2018 – Jun. 2021*

Master in Internal Medicine

Field: cardiology; hypertension; epidemiology

Dissertation: Association between inter-arm blood pressure difference and atherosclerotic cardiovascular disease in hypertensive population

Southern Medical University, China *Sep. 2013 – Jun. 2018*

Bachelor in Clinical Medicine

TRAINING

UZ Leuven, Belgium

Good clinical practice (GCP) certificate *Apr. 2024*

KU Leuven, Belgium

Kickstart your clinical research *Feb. 2024*

Exploitation of research – technology & knowledge transfer *Oct. 2023 – Apr. 2024*

Medical writing training *May. 2023*

Visual communication in academic publishing *Sep.2022*

Visiting scholar in Cardiovascular Sciences *Nov. 2019 – Oct. 2020*

Mosaiques Diagnostics GmbH, Germany

Lab visit *Jan. 2023*

Cochrane Belgium

Cochrane systematic review course *May. 2022*

Guangdong Provincial People's Hospital, China

Clinical rotation internship *Jan. 2017 – Jan. 2018*

ADWARDS

Travel grant for European Meeting on Hypertension and Cardiology Protection, 2024

Travel grant for European Meeting on Hypertension and Cardiology Protection, 2023

Chinese award for outstanding student abroad, 2023

Travel grant for Scientific Meeting of the International Society of Hypertension, 2022

Excellent presentation in ESH summer school, 2022

PhD scholarship grated by APPREMED, 2022-2025

PhD scholarship grated by KU Leuven, 2021

Excellent postgraduate of Guangdong Province, China, 2021

Excellent postgraduate of Guangdong Provincial People's Hospital, 2021

National scholarship of China, 2020

Visiting scholarship grated by KU Leuven, 2019

Excellent student scholarship of Southern Medical University, 2015, 2016 and 2017

MEMBERSHIPS & APPOINTMENT

Member of ESH Working Group of BP Monitoring and Cardiovascular Variability

Member of European Society of Hypertension Young Investigator Forum

ESH Annual Meeting ambassador of 2023

LANGUAGES

Chinese (Mandarin): Native speaker

Cantonese: Native speaker

English: Fluent

Dutch: Intermediate

List of publications

Publications of PhD thesis

1. **Yu YL**, Siwy J, An DW, González A, Hansen TW, Latosinska A, Pellicori P, Ravassa S, Mariottoni B, Verdonschot JAJ, Petutschnigg J, Rossignol P, Heymans S, Cuthbert J, Girerd N, Clark AL, Verhamme P, Nawrot TS, Janssens S, Cleland JGF, Zannad F, Díez J, Mischak H, Ferreira JP, Staessen JA. Urinary proteomic signature of mineralocorticoid receptor antagonism by spironolactone: evidence from the HOMAGE trial. *Heart. 2024*. 110: 1180-1187.
2. **Yu YL**, Martens DS, An DW, Chori B, Latosinska A, Siwy J, Odili AN, Stolarz-Skrzypek K, Maestre GE, Asayama K, Li Y, Verhamme P, Allegaert K, Mischak H, Nawrot TS, Staessen JA. Osteoporosis in relation to a bone-related aging biomarker derived from the urinary proteomic profile: a population study. *Aging and Disease. 2024*; 16: 633-644.
3. **Yu YL**, Huang QF, An DW, Raad J, Martens DS, Latosinska A, Stolarz-Skrzypek K, Van Cleemput J, Feng YQ, Mischak H, Allegaert K, Verhamme P, Janssens S, Nawrot TS, Staessen JA. OSTEO18, a novel urinary proteomic signature, associated with osteoporosis in heart transplant recipients. *Heylion. 2024;* 10:e24867.
4. **Yu YL**, An DW, Chori BS, Kaleta BP, Mokwatsi G, Marterns DS, Abiodun OO, Anya T, Łebek-Szatańska A, Yeh JS, Mels CMC, Latosinska A, Kruger R, Isiguzo G, Narkiewicz K, Shehu MN, Salazar M, Espeche W, Mujaj B, Brgulian-Hitij J, Olszanecka A, Wojciechowska W, Reyskens P, Rajzer M, Januszewicz A, Stolarz-Skrzypek K, Asayama K, Allegaert K, Verhamme P, Mischak H, Nawrot TS, Odili AN, Staessen JA, on behalf of the UPRIGHT-HTM investigators. Racial and regional disparities in the risk of non-communicable disease between sub-Saharan Black and European White patients. *Journal of Hypertension.* 2025; 42: 481-491.
5. **Yu YL**, Moliterno P, An DW, Raaijmakers A, Martens DS, Stolarz-Skrzypek K, Tikhonoff V, Malyutina S, Casiglia E, Chori B, Filipovský J, Rajzer M, Allegaert K, Kawecka-Jaszcz K, Verhamme P, Nawrot TS, Staessen JA. Blood pressure and cardiovascular risk in relation to birth weight and urinary sodium: an individual-participant meta-analysis of European family based population studies. *Journal of Hypertension. 2023*; 41:1175-1183.
6. **Yu YL**, An DW, Yang WY, Verhamme P, Allegaert K, Nawrot TS, Staessen JA. Blood

pressure and renal function responses in workers exposed to lead for up to six years. *Journal of Clinical Hypertension. 2023*; 25: 1086-1095.

7. **Yu YL**, Thijs L, Wei DM, Melgarejo JD, Yu CG, Yang WY, Roels HA, Zhang ZY, Nawrot TS, Staessen JA. Two-year responses of renal function to first occupational lead exposure. *Kidney International Report.* 2022; 7:1198-1209.
8. **Yu YL**, An DW, Yang WY, Zhang DY, Martens DS, Nawrot TS, Staessen JA. Health risks related to environmental and occupational lead exposure. *Kardiologia Polska. 2025;* doi: 10.33963/v.phj.104575. (Literature review, online ahead of print)
9. **Yu YL**, Renaers E, Sleurs H, Massot A, Berden D, Plusquin M, Alfano R, Martens DS, Zhang DY, An DW, Raaijmakers A, Allegaert K, Verhamme P, Staessen JA, Nawrot TS. Blood pressure trajectory from birth to preschool- and school-age: evidence from ENVIRONAGE birth cohort. (In preparation)

Other publications

1. **Yu YL**, An DW, Staessen JA. Hypertension and exposure to toxic metals: lead as the prime toxicant in this class. *Cardiovascular Research.* 2024. (Under review)
2. **Yu YL**, An DW, Yang WY, Verhamme P, Allegaert K, Nawrot TS, Staessen JA. Blood pressure and renal function responses in workers exposed to lead for up to six years. *Journal of Hypertension.* 2024; 42 (e-Suppl 1): e49.
3. NCD Risk Factor Collaboration (NCD-RiskC). Worldwide trends in the double burden of underweight and obesity from 1990 to 2022: a pooled analysis of 3663 population-representative studies with 222 million children, adolescents and adults. *The Lancet*. 2024; 403: 1027-1050.
4. An DW, **Yu YL**, Hara A, Martens DS, Yang WY, Cheng YB, Huang QF, Asayama K, Stolarz-Skrzypek K, Raizer M, Verhamme P, Nawrot TS, Li Y, Staessen JA. Lead-associated mortality in the US 1999-2020: a time-stratified analysis of a national cohort. *Journal of Hypertension*. 2024; 42: 1322-1330.
5. Norton GR, An DW, Aparicio LS, **Yu YL**, Wei FF, Niiranen TJ, Liu C, Stolarz-Skrzypek K, Wojciechowska W, Jula AM, Raijzer M, Martens DS, Verhamme P, Li Y, Kawecka-Jaszcz K, Nawrot TS, Staessen JA, Woodiwiss AJ, The International Database of Central Arterial Properties for Risk Stratification Investigators. Mortality and cardiovascular end points in relation to the aortic pulse wave components: an individual-participant meta-analysis. *Hypertension*. 2024; 81: 1065-1075.

6. Huang QF, An DW, Aparicio LS Cheng YB, Wei FF, **Yu YL**, Sheng CS, Yang WY, Niiranen TJ, Boggia J, Stolarz-Skrzypek K, Tikhonoff V, Gilis-Malinowski N, Wojciechowska W, Casiglia E, Narkiewicz K, Filipovský J, Kalina Kawecka-Jaszcz K, Nawrot TS, Wang JW, Li Y, Staessen JA, The International Dabase of Central Arterial Properties for Risk Stratification (IDCARS) Investigators. An outcome-driven threshold for pulse pressure amplification. *Hypertension Research*. 2024; 47: 2478-2488.
7. Wei FF, Mariottoni B, An DW, Pellicori P, **Yu YL**, Verdonschot JAJ, Liu C, Ahmed FZ, Petutschnigg J, Rossignol P, Heymans S, Cuthbert J, Girerd N, Li Y, Clark AL, Nawrot TS, Ferreira JP, Zannad F, Cleland JGF, Staessen JA, on behalf of the HOMAGE investigators. Heart rate reactivity, recovery, and endurance of the incremental shuttle walk test in patients prone to heart failure. *ESC Heart Failure*. 2024; 11: doi: 10.1002/ehf2.15000. (Online ahead of print)
8. Wei FF, Mariottoni B, An DW, Pellicori P, **Yu YL**, Verdonschot JAJ, Liu C, Ahmed FZ, Petutschnigg J, Rossignol P, Heymans S, Cuthbert J, Girerd N, Li Y, Clark AL, Nawrot TS, Ferreira JP, Zannad F, Cleland JGF, Staessen JA, on behalf of the HOMAGE investigators. Effects of spironolactone on exercise blood pressure in patients atincreased risk of developing heart failure: report from the HOMAGE trial. *Hypertension Research*. 2024; 47: doi: 10.1038/s41440-024-01843-z (Online ahead of print)
9. NCD Risk Factor Collaboration (NCD-RisC). General and abdominal adiposity and hypertension in eight world regions: a pooled analysis of 837 population-based studies with 7.5 million participants. *The Lancet*. 2024; 404: 851-863.
10. **Yu YL**, An DW, Chori BS, Nawrot TS, Staessen JA. Blood pressure and hypertension in relation to lead exposure updated according to present-day blood lead levels. *Kardiologia Polska. 2023*; 81:675-683. (Literature review)
11. An DW, **Yu YL**, Martens DS, Latosinska A, Zhang ZY, Mischak H, Nawrot TS, Staessen JA. Statistical approaches applicable in managing OMICS data: urinary proteomics as exemplary case. *Mass Spectrometry Reviews*. 2023; 1-18. (Literature review)
12. An DW, Hansen TW, Aparicio LS, Chori B, Huang QF, Wei FF, Cheng YB, **Yu YL**, Sheng CS, Gilis-Malinwoska N, Boggia J, Woiciechowska W, Niiranen TS, Tikhonoff V, Casiglia E, Narkiewicz K, Stolarz-Skrzypek K, Kawecka-Jaszcz, Jula AM, Yang WY, Woodiwiss AJ, Filipovský J, Wang JG, Rajzer M, Verhamme P, Nawrot TS,

Staessen JA, The International Database of Central Arterial Properties Investigators. Derivation of an outcome-driven threshold for aortic pulse wave velocity: an individual-participant meta-analysis. *Hypertension*. 2023; 80: 1949-1959.

13. Jacobs JEJ, L'Hoyes W, Lauwens L, **Yu YL**, Brusselmans W, Weltens C, Voigt JU, Wildiers H, Neven P, Herrmann J, Thijs L, Staessen JA, Janssens S, Van Aelst LNL. Mortality and major adverse cardiac adverse events in patients with breast cancer receiving radiotherapy: the first decade. *Journal of the American Heart Association*. 2023; 12: e027855.
14. Chori BS, An DW, Martens DS, **Yu YL**, Gilis-Malinowska N, Abubakar MS, Ibrahim EA, Ajanya O, Abiodun OO, Anya T, Tobechukwu I, Isiguzo G, Cheng HM, Chen CH, Liao CT, G Mokwatsi, Stolarz-Skrzypek K, Wojciechowska W, Narkiewicz K, Rajzer M, Brguljan-Hitij J, Nawrot TS, Asayama K, Reyskens P, Mischak H, Odili AN, Staessen JA, The UPRIGHT-HTM Investigators. Urinary proteomics combined with home blood pressure telemonitoring for health care reform trial—First progress report. *Journal of Clinical Hypertension*. 2023; 25: 521-533.
15. Grobman B, Tuckson-Ocran RA, Staessen JA, **Yu YL**, Lipsitz LA, Mukamal KJ, Juraschek SP. Body position and cardiovascular events in hypertensive adults: an observational analysis of the Syst-Eur Trial. *Hypertension*. 2023; 80: 820-827.
16. NCD Risk Factor Collaboration (NCD-RisC). Diminishing benefits of urban living for children and adolescent's growth and development. *Nature*. 2023; 615: 874-883.
17. Martens DS, An DW, **Yu YL**, Chori BS, Wang C, Silva AI, Wei FF, Liu C, Stolarz-Skrzypek K, Rajzer M, Latosinska A, Mischak H, Staessen JA, Nawrot TS, the FLEMENGHO Investigators. Association of air pollution with a urinary biomarker of biological aging and effect modifiers in a prospective population study: protective role of antioxidant vitamin K. *Environmental Health Perspectives*. 2023; 131: 127011.
18. **Yu YL**, Yang WY, Hara A, Asayama K, Roels HA, Nawrot TS, Staessen JA. Public and occupational health risks related to lead exposure updated according to present-day blood lead levels. *Hypertension Research. 2022*; 46:395-407. (Literature review)
19. Laenens D, **Yu YL**, Santens B, Jacobs J, Beuselinck B, Bechter O, Wauters E, Staessen JA, Janssens S, Van Aelst L. Incidence of cardiovascular events in patients treated with immune checkpoint inhibitors. *Journal of Clinical Oncology*. 2022; 40: 3430-3438.

20. Staessen JA, Wendt R, **Yu YL**, Kalbitz S, Thijs L, Siwy J, Raad J, Metzger j,Neuhaus B, Papkalla A, von der Leyen H, Mebazaa A, Dudoignon E, Spasovski G, Milenkova M, Canevska-Taneska A, Salgueira Lazo M, Psichogiou M, Rajzer MW, Fulawka L, Dzitkowska-Zabielska M, Weiss G, Feldt T, Stegemann M, J Normark, A Zoufaly, Schmiedel S, Salgueira Lazo M, Seilmaier M, Rumpf B, Banasik M, Krajewska M, Czerwienska B, Peters B, Nilsson Å, Rothfuss K, Lübbert C, Mischak H, Beige J, the CRIT-Cov investigators. Predictive performance and clinical application of COV50, a urinary proteomic biomarker in early COVID-19 infection: a cohort study. *The Lancet Digital Health*. 2022; 4: e727-e737.
21. Cheng YB, Thijs L, Aparicio LS, Huang QF, Wei FF, **Yu YL**, Barochiner J, Sheng CS, Yang WY, Niiranen TJ, Boggia J, Zhang ZY, Stolarz-Skrzypek K, Gilis-Malinowska N,Tikhonoff V, Wojciechowska W, Casiglia E, Narkiewicz K, Filipovský J, Kawecka-Jaszcz K, Wang JG, Li Y, Staessen JA, International Database of Central ArterialProperties for Risk Stratification (IDCARS) Investigators. Risk stratification by cross-classification of central and brachial systolic blood pressure. *Hypertension*. 2022; 79:1101-1111.
22. Salaets T, Raaijmakers A, Zhang ZY, **Yu YL**, Wei DM, Staessen JA, Allegaert K. QTc intervals are not prolonged in former ELBW infants at pre-adolescent age. *Pediatric Research*. 2022; 92: 848-852.
23. **Yu YL**, Huang YQ, Liu L, Liu XC, Tan QH, Chen CL, Huang JY, Feng YQ, Tang ST. Association between vascular overload index and new-onset ischemic stroke in elderly population with hypertension. *Clinical Interventions in Aging.* 2021;16:1293-1301.
24. **Yu YL**, Thijs L, Yu CG, Yang WY, Melgarejo JD, Wei DM, Wei FF, Nawrot TS, Verhamme P, Roels HA, Staessen JA, Zhang ZY. Two-year responses of heart rate and heart rate variability to first occupational lead exposure. *Hypertension*. 2021;77:1775–1786.
25. Huang YQ, Liu L, **Yu YL**, Chen CL, Huang JY, Lo K, Feng YQ. The relationship between famine exposure during early life and carotid plaque in adulthood. *European Journal of Clinical Nutrition*. 2021;75:546-554.
26. Zhang ZY, **Yu YL**, Asayama K, Hansen TW, Maestre GE, Staessen JA. Starting antihypertensive drug treatment with combination therapy: controversies in hypertension - con side of the argument. *Hypertension*. 2021;77:788-798.
27. Huang JY, Liu L, Huang YQ, Lo K, **Yu YL**, Chen CL, Tang ST, Zhang B, Feng YQ.

Association between pulse pressure and ischaemic stroke in elderly patients with hypertension. *Postgraduate Medical Journal*. 2021;97:222-226.

28. He TL, Melgarejo JD, Clark AL, **Yu YL**, Thijs L, Diéz J, López B, González A, Cleland JG, Schanstra JP, Vlahou A, Latosinska A, Mischak H, Staessen JA, Zhang ZY, Jankowski V. Serum and urinary biomarkers of collagen type-I turnover predict prognosis in patients with heart failure. *Clinical and Translational Medicine*. 2021; 11: e267.
29. Huang J, Liu L, **Yu YL**, Chen CL, Lo K, Huang YQ, Tang ST, Feng YQ. Relationship between body mass index and ischaemic stroke in Chinese elderly hypertensive patients. *Postgraduate Medical Journal*. 2021;97:217-221.
30. Huang YQ, Liu L, **Yu YL**, Lo K, Chen CL, Huang JY, Zhou YL, Feng YQ. The relationship between famine exposure in early life and left atrial enlargement in adulthood. *Journal of Human Nutrition and Dietetics*. 2021;34:356-364.
31. **Yu YL**, Yang WY, Thijs L, Melgarejo JD, Yu CG, Wei DM, Wei FF, Nawrot TS, Zhang ZY, Staessen JA. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *Hypertension.* 2020;76:1299-1307.
32. **Yu YL**, Liu L, Lo K, Tang ST, Feng YQ. Prevalence and associated factors of inter-arm blood pressure difference in Chinese community hypertensive population. *Postgraduate Medicine*. 2020;1826184.
33. **Yu YL**, Liu L, Huang JY, Shen G, Chen CL, Huang YQ, Zhang B, Tang ST, Feng YQ. Association between systolic blood pressure and first ischemic stroke in the Chinese older hypertensive population. *Journal of International Medical Research.* 2020;48:1-12.
34. **Yu YL**, Thijs L, Saenen ND, Melgarejo JD, Wei DM, Yang WY, Yu CG, Roels HA, Nawrot TS, Maestre GE, Staessen JA, Zhang ZY. Two-year neurocognitive responses to first occupational lead exposure. *Scandinavian Journal of Work, Environment & Health*. 2020;45:298-307.
35. **Yu YL**, Feng WL. COVID-19: perspectives from a clinical doctor and health authority officer. *Iranian Journal of Public Health.* 2020;49:127-129. (Letter to the editor)
36. Huang YQ, Liu XC, Lo K, Liu L, **Yu YL**, Chen CL, Huang JY, Feng YQ, Zhang B. The U shaped relationship between high-density lipoprotein cholesterol and all-cause or cause-specific mortality in adult population. *Clinical Interventions in Aging*. 2020;15:1883-1896.

37. Chen CL, Liu L, Huang JY, **Yu YL**, Shen G, Lo K, Huang YQ, Feng YQ. Thigh circumference and risk of all-cause, cardiovascular and cerebrovascular mortality: a cohort study. *Risk Management and Healthcare Policy*. 2020;13:1977-1987.
38. Chen CL, Liu XC, Liu L, Lo K, **Yu YL**, Huang JY, Huang YQ, Chen JY. U-shaped association of high-density lipoprotein cholesterol with all-cause and cardiovascular mortality in hypertensive population. *Risk Management and Healthcare Policy*. 2020;13:2013-2025.
39. Liu L, Chen CL, Lo K, Huang JY, **Yu YL**, Huang YQ, Feng YQ. Trends of status of hypertension in southern China, 2012-2019. *International Journal of General Medicine*. 2020;13:599-608.
40. Chen CL, Liu L, Huang JY, **Yu YL**, Lo K, Huang YQ, Feng YQ. Systolic blood pressure, cardiovascular mortality, and all-cause mortality in normoglycemia, prediabetes, and diabetes. *Diabetes, Metabolic Syndrome and Obesity*. 2020;13:2375-2388.
41. Liu XC, Liu L, **Yu YL**, Huang JY, Chen CL, Lo K, Huang YQ, Feng YQ. The association of subscapular skinfold with all-cause, cardiovascular and cerebrovascular mortality. *Risk Management and Healthcare Policy*. 2020;13:955-963.
42. Lo K, Huang YQ, Shen G, Huang JY, Liu L, **Yu YL**, Chen CL, Feng YQ. Effects of waist to height ratio, waist circumference, body mass index on the risk of chronic diseases, all-cause, cardiovascular and cancer mortality. *Postgraduate Medical Journal*. 2020:postgradmedj-2020-137542.
43. Huang YQ, Liu L, Huang C, **Yu YL**, Lo K, Huang JY, Chen CL, Zhou YL, Feng YQ. Impacts of pre-diabetes or prehypertension on subsequent occurrence of cardiovascular and all-cause mortality among population without cardiovascular diseases. *Diabetes, Metabolic Syndrome and Obesity*. 2020;13:1743-1752.
44. Lo K, Huang YQ, Liu L, **Yu YL**, Chen CL, Huang JY, Feng YQ. Serum vitamin D, Sleep pattern and cardiometabolic diseases: findings from the National Health and Nutrition Examination Survey. *Diabetes, Metabolic Syndrome and Obesity*. 2020;13:1661-1668.
45. Huang YQ, Liu L, Huang JY, Lo K, Chen CL, **Yu YL**, Li J, Feng YQ. Prehypertension and risk for all-cause and cardiovascular mortality by diabetes status: results from the national health and nutrition examination surveys. *Annals of Translational Medicine*. 2020;8:323.

46. Huang JY, Liu L, **Yu YL**, Chen CL, Lo K, Zhang B, Tang ST, Huang YQ, Feng YQ. A nonlinear relationship between low-density-lipoprotein cholesterol levels and atrial fibrillation among patients with hypertension in China. *Annals of Palliative Medicine*. 2020;9:2953-2961.
47. Chen CL, Huang JY, Liu L, **Yu YL**, Shen G, Lo K, Huang YQ, Tang ST, Feng YQ. Relationship between diastolic blood pressure and the first ischaemic stroke in elderly patients with hypertension. *Postgraduate Medical Journal*. 2020;96:525-529.
48. **Yu YL**, Huang YQ, Feng YQ. Research progress on the association between inter-arm blood pressure and cardio-cerebrovascular diseases and target-organ damage. *Chinese Journal of Cardiology*. 2019;47:244-248. (Literature review in Chinese)
49. Liu L, Shen G, Huang JY, **Yu YL**, Chen CL, Huang YQ, Feng YQ. U-shaped association between low-density lipid cholesterol and diabetes mellitus in patients with hypertension. *Lipids in Health and Disease*. 2019;18:163.
50. **Yu YL**, Shang QW, Chen NN, Xu SF. NF-κB inhibitor bortezomib for the prevention and treatment of graft-versus-host disease. *Chinese Journal of Clinicians (Electronic Edition)*, 2018;12:49-52. (Literature review in Chinese)

Conference contributions

1. Blood pressure and renal function responses in workers exposed to lead for up to six years. *The 33nd European Meeting on Hypertension and Cardiology Protection (ESH 2024)*, Berlin, Germany. (Oral presentation; ESH Travel Grant)
2. Chairperson of the E-poster session with short presentation – *Epidemiology. The 33nd European Meeting on Hypertension and Cardiology Protection (ESH 2024*), Berlin, Germany.
3. Urinary proteomic signature of mineralocorticoid receptor antagonism by spironolactone: evidence from the HOMAGE trial. *European Society of Cardiology Congress (ESC 2024)*, London, UK. (Oral presentation)
4. Blood pressure and renal function responses in workers exposed to lead for up to six years. *European Society of Cardiology Congress (ESC 2024)*, London, UK. (Poster presentation)
5. OSTEO18, a novel urinary proteomic signature, associated with osteoporosis in heart transplant recipients. *European Society of Cardiology Congress (ESC 2024),* London, UK. (Poster presentation)

6. Blood pressure and cardiovascular risk in relation to birth weight and urinary sodium: an individual-participant meta-analysis of European family-based population studies. *European Society of Cardiology Congress (ESC 2024)*, London, UK. (Poster presentation)
7. Urinary proteomic signature of mineralocorticoid receptor antagonism by spironolactone: evidence from the HOMAGE trial. *Belgian Hypertension Committee 2024*, Brussels, Belgium. (Oral presentation)
8. Blood pressure and cardiovascular risk in relation to birth weight and urinary sodium. *The 32nd European Meeting on Hypertension and Cardiology Protection (ESH 2023),* Milan, Italy. (Poster presentation; ESH Travel Grant)
9. Chairperson of the E-poster session (with short presentation) – Lifestyle and Comorbidities. *The 32nd European Meeting on Hypertension and Cardiology Protection (ESH 2023)*, Milan, Italy.
10. Blood pressure and renal function responses in workers exposed to lead for up to six years. *Conference on Health and Environmental Change 2023*, Leuven, Belgium. (Oral presentation)
11. Blood pressure and renal function responses in workers exposed to lead for up to six years. *Belgian Hypertension Committee 2023, Brussels, Belgium*. (Oral presentation)
12. Two-year responses of heart rate and heart rate variability to first occupational lead exposure. *The 31st European Meeting on Hypertension and Cardiology Protection (ESH 2022)*, Athens, Greece. (Poster presentation)
13. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *ESH summer school 2022*, Arandjelovac, Serbia. (Poster presentation; top 3 excellent presentation)
14. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *The 29th Scientific Meeting of the International Society of Hypertension 2022*, Kyoto, Japan. (Oral presentation; ISH Travel Grant)
15. Cardiovascular risk in relation to birth weight and urinary sodium excretion — A Population Study. *Belgian Hypertension Committee 2022*, Brussels, Belgium. (Oral presentation)
16. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *Belgian Society of Cardiology 2021*, Brussels, Belgium. (Poster

presentation)

17. Inter-arm blood pressure difference and atherosclerotic cardiovascular disease in hypertensive population. *Oriental Congress of Cardiology 2021*, Shanghai, China. (Oral presentation; the 3rd place of excellent presentation)
18. Inter-arm blood pressure difference and atherosclerotic cardiovascular disease in hypertensive population *South China International Congress of Cardiology 2021,* Guangzhou, China. (Poster presentation)
19. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *Chinese Hypertension Meeting 2021*, Fuzhou, China. (Oral presentation)
20. Changes in office and ambulatory blood pressure in response to occupation lead exposure. *ESH-ISH 2020*, Glasgow, UK. (Poster presentation)
21. Association between vascular overload index and ischemic stroke in elderly patients with hypertension in Chinese community. *ESH-ISH 2020*, Glasgow, UK. (Poster presentation)
22. Two-year responses of office and ambulatory blood pressure to first occupational lead exposure. *Belgian Hypertension Committee 2020*, Brussels, Belgium. (Oral presentation)
23. Intervention for prehypertension in adults with type 2 diabetes: protocol introduction of clinical trial. *Congress of hypertension in Chinese medical doctor association 2019*, Guangzhou, China. (Oral presentation)
24. Aminopeptidase A inhibitor: the possible therapy for resistant hypertension. *Great wall international congress of cardiology 2019*, Beijing, China. (Congress article)
25. Prevalence and associated factors of inter-arm blood pressure difference in Chinese community hypertensive population. *Oriental Congress of Cardiology 2019*, Shanghai, China. (Poster presentation)