Aan mama en papa,
mijn broer Peter†, en zussen Wencke en Marieke,
aan mijn vrouw Lien,
aan mijn tweelingdochters Marthe en Marit

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KU LEUVEN
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BIOMEDICAL SCIENCES

INNOVATION IN MONITORING AND TREATMENT OF NEPHROPATHIC CYSTINOSIS

Facing contemporary challenges

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Mechanisms of Human Disease

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Innovation in monitoring and treatment of nephropathic cystinosis
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To be great, be whole: nothing that's you
Should you exaggerate or exclude.
In each thing, be all. Give all you are
In the least you ever do.
The whole moon, because it rides so high,
Is reflected in each pool.

Odes – Ricardo Reis (Fernando Pessoa)

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LIST OF ABBREVIATIONS

#	number
3HA	Triple Hemagglutinin Tag (3x YPYDVPDYA amino acid sequence)
ABCB1	ATP binding cassette subfamily B member 1, MDR1, P-gp
ACTA2	Alpha-actin-2 (gene), also known as Alpha smooth muscle actin
ADIPOQ	Adiponectin (gene)
ALDH	Aldehyde Dehydrgenase
ALPL/AP	Alkaline Phosphatase (gene)
ANPEP	Alanyl Aminopeptidase (gene)
APC	Allophycocyanin
AQP1	Aquaporin 1 (gene)
AU	Arbitrary Units
BCL2	B-cell Lymphoma 2 (gene)
BMI1	BMI1 proto-oncogene, polycomb ring finger (gene)
BM-MSC	Bone-Marrow derived Mesenchymal Stem Cell
BSA	Bovine Serum Albumin
BUN	Blood Urea Nitrogen
CALB	Calbindin (gene)
CDH1	Cadherin 1 (gene), also known as Epithelial Cadherin (E-Cadherin)
CDH5	Cadherin 5 (gene), also known as Vascular Endothelial Cadherin (VE-Cadherin)
CHAT	Choline Acetyl Transferase (gene)
CI	Confidence Interval
CITED1	Cbp/p300 interacting transactivators with glutamic acid (E)/aspartic acid (D)-rich C-terminal domain
CKD	Chronic Kidney Disease
CM	Cap Mesenchyme
CNN1	Calponin-1
CrCl	Creatinine clearance
Ct	Cycle treshold
CTNS	Cystinosin
CTNS-KD	CTNS knocked down cells
CUBN	Cubilin
Cys-uKPC	Cystinotic urine-derived Kidney Progenitor Cell
DBA	Diamond-Blackfan anemia (gene)
DES	Desmin (gene)
DMEM	Dublecco's Modified Eagle's Medium
ECM1	Extracellular matrix protein 1
EF-1 alpha	Human elongation factor-1 alpha
eGFP	enhanced Green Fluorescent Protein
eGFR	estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked Immuno Sorbent Assay
EMT	Epithelial-to-Mesenchymal Transition
ENG	Endoglin (gene), also known as CD105
ESRD	End-Stage Renal Disease
ET	Epidermal thickness
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
Fura-2 AM	Fura-2 acetoxymethyl ester
GGT1	Gamma glutamyl transferase 1 (gene)
GvHD	Graft-versus-host disease
HBSS	Hanks' balanced salt solution
HD-OCT	High-Definition Optical Coherence Tomography
hAK	human Adult Kidney
hEK	human Embryonic Kidney
hFK	human Fetal Kidney
hkPSC	human kidney Perivascular Stromal Cell
HLA	Human Leucocyte Antigen
HSCs	Hematopoietic Stem Cells

HUVEC	Human Umbillical Vein Endothelial Cells
iPSC	induced Pluripotent Stem Cell
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-18	Interleukin-18
IM	Intramuscular
IQR	Inter-quartile range
JGA	Juxtaglomerular Apparatus
KDR	Kinase insert Domain Receptor (gene), also known as Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)
KLF15	Kruppel Like Factor 15
KRT	Cytokeratin
KTx	Kidney transplantation
LAMP1	Lysosomal-associated membrane protein 1
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
LRP2	Low density lipoprotein-related protein 2; megalin
LTA	Lotus tetragonolobus lectin
LTR	Long Terminal Repeats
LV	Lentiviral Vector
MAP2	Microtubule-Associated Protein 2 (gene)
MEP1B	Meprin A subunit Beta (gene)
MET	Mesenchymal-to-Epithelial Transition
MIM	Mendelian Inheritance in Man disease code
MM	Metanephric Mesenchyme
mTORC1	mammalian Target of Rapamycin Complex 1
MYH2	Myosin Heavy Chain 2
na	Not applicable
nas	Not assessed
nav	Not available
NAG	Neutral Alpha-Glucosidase
NCAM	Neural Cell Adhesion Molecule 1
NEM	N-ethylmaleimide
NEFM	Neurofilament Medium Polypeptide (gene)
NG2	Neural/glial antigen 2
NHE	Sodium hydrogen exchanger (gene)
NKCC	Sodium – potassium – chloride cotransporter (gene)
nKSPC	neonatal Kidney Stem/Progenitor cell
nKTx	non-Kidney Transplanted
NOD/SCID	Nonobese Diabetic/Severe Combined Immune Deficiency mouse model
NPHS1	Nephrin (gene)
NPHS2	Podocin (gene)
P#	Passage #
PAX2	Paired box gene 2 (gene)
PBS	Phosphate-buffered Saline
PD	Papillary dermis
PDGF-R-β	Platelet derived growth factor receptor beta
PE	Phycoerythrin
PED	Parietal Epithelial Cell
PESA	Percutaneous Epididymal Sperm Aspiration
P-gp	P-glycoprotein
PEC	Parietal Epithelial Cell
(ci)Podo	(conditionally immortalized) Podocyte
PODXL	Podocalyxin (gene)

PPARG	Peroxisome Proliferator Activated Receptor Gamma (gene)
PTA	Pre-Tubular Aggregate
(ci)PTEC	(conditionally immortalized) Proximal Tubular Epithelial Cell
PuroR	Puromycin Resistance gene
qPCR	quantitative Polymerase Chain Reaction
RFE	Relative Fold Expression
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
RRE	REV Responsive Element
rt	reverse transcriptase
RT	Room Temperature
RUNX2	Runt-related transcription factor 2 (gene)
RV	Renal Vesicle
SCNN1B	Sodium Channel Nonvoltage-gated 1 Beta subunit (gene), also known as amiloride-sensitive sodium channel subunit beta
SCID	Severe Combined Immune Deficiency
SD	Standard Deviation
SIN	Self-Inactivating
SLC5A1	Solute Carrier family 5 Member 1(gene), also known as sodium/glucose cotransporter protein (SGLT1)
SLC12A3	Solute Carrier family 12 member 3 (gene), also known as thiazide-sensitive sodium-chloride cotransporter
SSA	Sulfosalicylic Acid
SSP1	Secreted Phosphoprotein 1
STCs	Scattered Tubular Cells
SYNPO	Synaptopodin (gene)
TEX101	Testis Expressed 101
TESE	Testicular Sperm Extraction
TFEB	Transcription Factor EB
TIE2	Tyrosine-protein kinase receptor 2 (gene)
TJP1	Tight Junction Protein-1 (gene), also known as Zonula occludens -1 (ZO-1)
TRPC6	Transient receptor potential cation channel, subfamily C, member 6 (gene)
UB	Ureteric Bud
uKPC	urine-derived Kidney Progenitor Cell
UPK1A	Uroplakin 1A
US	Ultrasound
VIM	Vimentin (gene)
VRAD	Vitamin D and Retinoic Acid
VWF	Von Willebrand Factor (gene)
WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element
ZO-1	Zonula Occludens-1 (ZO-1), also known as Tight Junction Protein-1 (TJP1)

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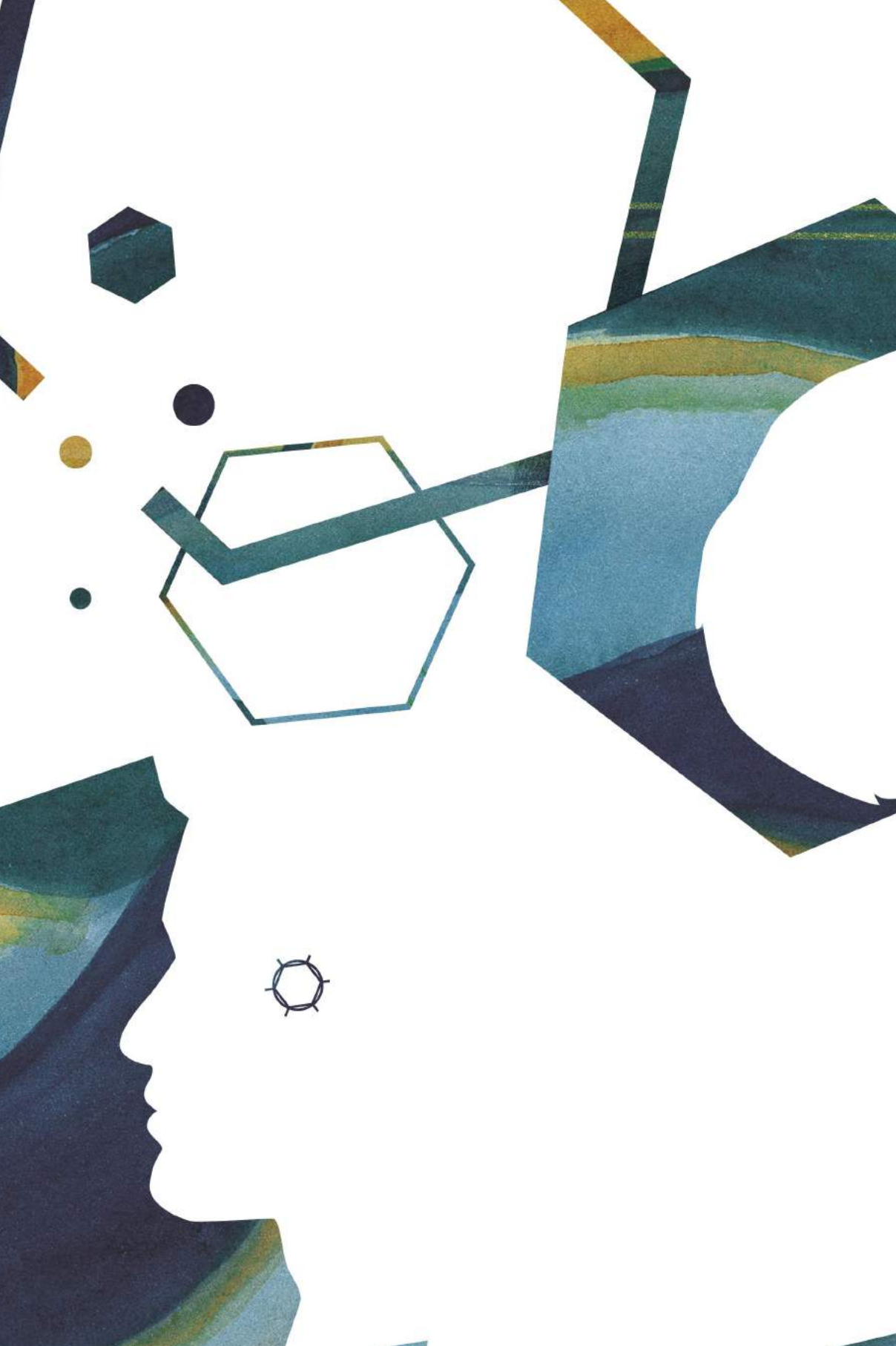

CHAPTER 1
GENERAL INTRODUCTION

Chapter based on:

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Cystinosis: a review. Orphanet J Rare Dis 2016; 11: 47.

***Veys KR**, Elmonem MA, Arcolino FO, van den Heuvel L, Levtchenko E.*
Nephropathic cystinosis: an update. Curr Opin Pediatr. 2017 29: 168-178.

Cystinosis is a rare autosomal recessive lysosomal storage disorder caused by mutations in *CTNS*, a cystine-proton cotransporter located in the lysosomal membrane [1]. Since *CTNS* is ubiquitously expressed, the absence or dysfunction of cystinosin leads to the lysosomal accumulation of cystine in all cells and tissues throughout the body. In the infantile phenotype of cystinosis, which is the most frequent and severe form, the kidney is the first and most severely affected organ. It is characterized by the renal Fanconi syndrome in infancy, followed by progressive glomerular dysfunction and end-stage renal disease by the end of the first decade of life if the disease remains untreated. Meanwhile, several extra-renal manifestations develop, including ophthalmic, endocrine (including primary hypogonadism) and neuromuscular complications. Apart from a supportive treatment for the renal Fanconi syndrome and renal replacement therapy, only one disease-modifying treatment, cysteamine, is available. Cysteamine enables the depletion of lysosomal cystine accumulation and, doing so, has shown to delay the progression of kidney disease and reduce the incidence of extra-renal complications. However, it offers no cure for the renal Fanconi syndrome, neither some of the most important extra-renal manifestations. Moreover, the currently only available modality for monitoring cystine-depleting therapy, the WBC cystine assay, suffers important limitations and drawbacks and no other means are available for assessing the extent of total body cystine tissue accumulation and severity of the disease over the long-term. Recently, attempts to explore curative therapeutic strategies for cystinosis, including hematopoietic stem cell transplantation-based gene therapy have yielded promising results in a cystinosis animal model. On the other hand, while the field of kidney regeneration and the search for its potential clinical applications is emerging, this potentially highly promising approach for developing innovative treatment options has not been investigated yet.

In this introduction chapter, we provide the most relevant background information on cystinosis in all its aspects in the first part. In a second part, we focus more in depth on the field of kidney regeneration.

1.1 Cystinosis

1.1.1 Background and historical perspective

Cystine, the oxidized dimer form of the amino acid cysteine was identified in 1810 by Sir W.H. Wollaston, who studied a huge 18g bladder stone in a 36-year-old male [2,3]. E. Abderhalden coined the disease entity in 1903 by giving the name of cystine to a new pathological condition found in a 21-month-old child, referred by Eduard Kaufmann (Basel, Switzerland, 1860-1931). This child died from dehydration upon a severe underlying failure to thrive and its liver and spleen contained pathological cystine crystals [4]. The Dutch pathologist George Lignac (1891-1954) was the first to provide a clear systematic description of the disease in 1924, associating it with its major clinical manifestations such as its renal disease, rickets and growth retardation [3]. Therefore initially, cystinosis was referred to as the Abderhalden-Kaufmann-Lignac syndrome. Later, Guido Fanconi (1892-1979), a Swiss pediatrician, contributed to the understanding of cystinosis by explaining the nature of the loss of various substances in urine [4].

Pioneering studies of Schneider *et al.* in 1967 revealed that cystinosis could be diagnosed by measuring elevated cystine content in peripheral blood leukocytes [5]. Altered lysosomal export of cystine as a cellular basis of cystinosis was described in 1982 [6] and opened a new era of research in understanding disease pathogenesis that was substantiated in 1998 when the underlying gene *CTNS* encoding lysosomal cystine transporter cystinosin was cloned [7]. Since then, cystinosis continued to gain attention as a prototype of a rare metabolic disorder.

1.1.2 Epidemiology

Cystinosis is an orphan disease. Worldwide, the prevalence has only been reported in few populations. In Western Europe, the overall incidence ranges from approximately 1:100.000 to 1:200.000 live births [8–12]. A higher incidence rate has been described in selected populations harbouring founder mutations, including the region of Brittany, France (1:26.000 live births) and the region of Saguenay-Lac-St-Jean in Québec, Canada (1:62.500 live births).

1.1.3 Etiology

Cystinosis is an autosomal recessive lysosomal storage disorder caused by mutations in *CTNS*, a lysosomal cystine-proton cotransporter. Until now, more than 140 pathogenic mutations have been reported (Table S1.1).

The most commonly detected pathogenic mutation is the 57kb deletion present in about 50% of *CTNS* mutant alleles of patients of North European and North American decent [13–15]. Severe or truncating mutations on both alleles are usually associated with the infantile, severe form of the

disease, while juvenile and ocular forms of cystinosis are usually associated with at least one mild mutation. In cystinosis there is a good genotype-phenotype correlation, as most detected mutations are linked phenotypically to the early infantile nephropathic cystinosis form, while a minority of mutations are linked to either juvenile or ocular cystinosis. A descriptive summary can be found in supplementary table S1.1.

1.1.4 Pathophysiology

During the past decade, it has appeared that the pathophysiology of cystinosis is more complex than a pure cell damage caused by cystine crystals. Cloning of the cystinosin gene [1], mapping of the protein to the lysosomal membrane [17], and describing its function as an ATP-dependent cystine-proton symporter [18], seemed to be the final step in understanding the disease mechanism. The detrimental role of cystine accumulation leading to enhanced cell death via apoptosis [19], mitochondrial dysfunction [20], oxidative stress [21–24] and inflammation [25,26], that could be reversed by cysteamine, depleting cells from cystine, all fitted into a logic model and supported early cysteamine administration as a cornerstone of cystinosis treatment.

However, this rational concept was challenged during the last decade by clinical data showing that not all pathological features of cystinosis could be prevented by cysteamine [27], and on the other hand, by exciting discoveries of alternative functions of cystinosin next to its cystine-transporting capacity, encompassing the regulation of vesicle trafficking, lysosomal biogenesis, mTOR signaling and autophagy [28,29,38,39,30–37]. In these studies, cysteamine treatment did not simply revert neither the alterations observed in endolysosomal trafficking, nor the impaired mTOR signaling and disturbed autophagic flux.

Cystinosis is traditionally thought to be a pure proximal tubular disorder with the secondary damage of glomeruli. Indeed, generalized proximal tubular dysfunction (renal Fanconi syndrome) is the first disease symptom in > 90% of the patients having the infantile form of cystinosis [40,41]. Moreover, renal Fanconi syndrome in cystinosis is extremely pronounced compared to the other inherited Fanconi syndromes such as in patients with Dent's disease or Lowe syndrome. Several lines of evidence, however, point to the simultaneous glomerular disease in cystinosis developing at early age. Five to 10% of all cystinosis patients present with pronounced proteinuria that can be in nephrotic range, in the absence of Fanconi syndrome. Kidney biopsies of these patients show histological lesions undistinguishable from idiopathic focal and segmental glomerular sclerosis [42]. On the other hand, electron microscopy of cystinotic renal tissue shows the presence of hypertrophic multi-nucleated podocytes with foot process effacement, that is pathognomonic for cystinosis [43]. The presence of glomerular proteinuria, increased podocyte detachment and loss into urine found in young children with yet preserved kidney function has recently been

substantiated by *in vitro* data showing alterations in podocyte cytoskeleton, adhesion and motility in cells deficient for cystinosin [44]. This information supports the administration of anti-proteinuric drugs such as inhibitors of renin-angiotensin-aldosterone system to target proteinuria-dependent kidney damage [45]. This treatment might decrease the burden of protein overload in cystinotic proximal tubular cells that are highly vulnerable to cystinosin dysfunction that cannot be improved by cysteamine [33].

1.1.5 Clinical manifestations

Depending on the age of presentation and the severity of the disease, three different phenotypes can be distinguished: the infantile (90%) and juvenile (5%) nephropathic phenotype, and a non-nephropathic ocular phenotype (5%) [40,46,47].

The infantile nephropathic phenotype is the most commonly and severe phenotype (90%). After an uneventful pregnancy and birth, patients gradually develop symptoms related to proximal tubular dysfunction. Asymptomatic aminoaciduria might already appear during the first weeks of life, being the first hallmark of an evolving severe and irreversible proximal tubular dysfunction (renal Fanconi syndrome), followed by glucosuria, urinary bicarbonate losses, phosphaturia, calciuria and low molecular weight (LMW) proteinuria in infancy (e.g. alfa-1-microglobulin, beta-2-microglobulin, retinol-binding protein) [48–50]. A full-blown generalized proximal tubular dysfunction (renal Fanconi syndrome) generally develops by the age of 6 to 12 months, presenting with failure to thrive, hypophosphatemic rickets, polyuria, polydipsia, vomiting, and episodes of severe dehydration and electrolyte imbalances in response to infections. Laboratory findings include metabolic acidosis, hypokalemia, hypophosphatemia, hypocalcemia, low carnitine levels, and sometimes hyponatriemia. A Bartter-like presentation with hypokalemic metabolic alkalosis has also been reported [51,52]. Subsequently, glomerular dysfunction gradually ensues and glomerular proteinuria may already be present from early ages. It is characterized by excessive losses of albumin and high molecular weight proteins, and may occur up to the nephrotic range [43]. Generally, serum creatinine levels remain within normal limits until the age of 5 years, and only rarely exceed 1 mg/dl below this age [40,53]. If the disease-modifying treatment, cysteamine, is not provided or initiated at a late age, and in case of severe non-compliance, renal function rapidly declines towards end-stage renal disease by the end of the first decade of life [54,55].

Being a systemic metabolic disorder, cystinosis affects several other organ systems (Table 1.2) [56,57,66–75,58,76–85,59,86–95,60,96–105,61,106–115,62,116–125,63,126–135,64,136–139,65]. Corneal cystine crystals, a pathognomonic feature of the disease, can be detected by the age of 12 months through slit lamp examination and is almost always present by the age of 18 months. Hence, the eye is the first apparently affected organ next to the kidneys [59,61–63]. Clinically, cystine crystal accumulation is characterized by a remarkable photophobia. Later, also retinopathy may develop, presenting with nyctalopia, impaired color vision or visual field restriction. Other organ systems that get affected from the second decade of life include the endocrine (primary hypothyroidism, pubertal delay, endocrine and exocrine pancreatic insufficiency), neuromuscular (peripheral myopathy, swallowing dysfunction), reproductive (primary hypogonadism in males, azoospermia), and central nervous system (idiopathic intracranial hypertension, stroke-like episodes, neurocognitive dysfunction and behavioral problems).

Almost all patients who did not receive cystine-depleting therapy or those who are not compliant, will develop several extra-renal symptoms including mainly retinal, endocrine and neuromuscular complications by the age of 30 years [140]. An overview of all extra-renal manifestations reported in cystinosis is represented in Table 1.1.

A minority of cystinosis patients (5%) is diagnosed at puberty to early adulthood, designated as the nephropathic juvenile or late-onset form (OMIM 219900). Patients present with a variable spectrum of symptoms, ranging from isolated asymptomatic proteinuria, a mild renal Fanconi syndrome, to an apparent nephrotic syndrome [40,141,142]. Generally, there is a slower progression to ESRD compared to the infantile form [42]. In the ocular, non-nephropathic form of cystinosis, only the eye is affected with corneal cystine accumulation, and patients rarely present before adulthood [143]. In one family, the co-existence of ocular and late-onset forms of cystinosis has been reported, implying the need of regular renal function controls in patients with ocular cystinosis [42]. Owing to the introduction of its specific disease-modifying treatment, cysteamine (see section 1.1.7 Treatment), and the progress in renal replacement therapy, life-expectancy of cystinosis patients has significantly increased allowing them to survive into adulthood beyond the fifth decade of life. In this increasingly older population, the phenotype of cysteamine-treated adult cystinosis patients is evolving, while some aspects have become more pronounced. As such, for example, part of the skeletal phenotype (bone pain, skeletal deformations, fractures) that is independent of the well-known hypophosphatemic rickets, has gained more attention and was recently responded by the cystinosis expert community via the establishment of a consensus guideline [144,145]. In addition, other issues related to adulthood that concern some of the most universal needs in life, including fertility and the ability of having children, are clinical features with a potentially significant impact on the quality of life of adult cystinosis patients [76].

1.1.6 Diagnostic modalities and monitoring

A high index of suspicion is of crucial importance for the diagnosis of cystinosis.

Infants presenting with failure to thrive, rickets and episodes of severe dehydration, should be suspected. Given cystinosis is the most important cause of an inherited renal Fanconi syndrome, it should always be excluded in this case. In cystinosis patients with the infantile phenotype, in the early course of the disease urine dipstick usually shows mild proteinuria, glucosuria and low specific gravidity, while serum creatinine is generally normal in young children. Cystinosis patients with the late-onset form may present with advanced stages of renal failure without evident signs of renal Fanconi syndrome.

The presence of corneal cystine crystals via a slit lamp examination is an important pathognomonic sign. However, corneal crystals appear relatively late during the second year of life, which may delay the diagnosis and the initiation of treatment [47]. Of note, their intensity cannot be used to monitor systemic cysteamine therapy, as the cornea is avascular and do not respond except to locally absorbed cysteamine.

Genetic diagnosis is an excellent confirmatory technique for cystinosis, being a monogenetic disease with a relatively small causative gene (*CTNS* has 12 exons but only 10 are coding). However, this modality has limited availability and is obviously highly time-consuming. Moreover, in small percentage of patients (5-10%) with the clinical diagnosis of cystinosis, mutations in the *CTNS* gene cannot be identified because their pathogenic mutations might be either deeply intronic or in the promoter region.

Currently, the gold standard still for both diagnosis and therapeutic monitoring is the assessment of the WBC cystine content by HPLC or LC-MS/MS, preferentially in granulocytes [25,146,147]. Granulocytes can be isolated relatively easy form venous blood samples. However, there are some important drawbacks and limitations linked to this assay: it requires a large blood sample (5 – 10ml), the technology is available in only a few specialized laboratories and granulocytes are only short living cells, thus merely representing therapeutic control over a relatively short duration [146,147]. Furthermore, cystine measurements are very sensitive to storage and transport conditions. Recently, it has been reported that the determination of cystine using immuno-magnetically purified granulocytes followed by LC-MS/MS analysis improves the inherent variability of mixed leukocyte analysis and eliminates the need for immediate sample processing after blood withdrawal [148]. Finally, the WBC cystine level is not representative for the total body tissue cystine content, and no surrogate biomarkers are currently available for assessing the overall severity or extent of the disease.

Table 1.1 Overview of extra-renal manifestations reported in cystinosis

Extra-renal manifestation	Reported prevalence	Presenting age (years)	Study type (n)	Reference
Ophtalmological				
Corneal cystine crystals (photophobia, blefarospasm)	100% (U)	> 1 - 1.5	Retrospective cohort (n=8) Prospective cohort (n=170) Prospective cohort (n=8)	Kaiser-Kupfer M *et al.*, 1986 Gahl WA *et al.*, 2000 Labbé A *et al.*, 2009
Anterior segment	42% (PT)			Kaiser-Kupfer M *et al.*, 1986
Keratopathy	3 – 73%	From 2nd decade of life	Prospective cohort (n=172)	Tsilou ET *et al.*, 2002
- Filamentary keratopathy	3 – 18%	From 1st decade of life		
- Superficial punctate keratopathy	3 – 40%	From 2nd decade of life		
- Band keratopathy	10 – 73%	> 15		
Corneal erosions	20 – 40%	From 2nd decade of life		
Peripheral corneal neovascularization	5 – 53%	> 15		
Iris thickening, posterior synechiae	8 – 40%	> 15		
Cataract	6%	> 15		
Posterior segment				Broyer M *et al.*, 1981
Retinopathy (nyctalopia, impaired color vision, visual field constricton, retinal blindness)	> 5% (PT)	From 1st decade of life	Cross-sectional (n=208)	Tsilou ET *et al.*, 2006
- Peripheral retinal epithelium hypopigmentation	59%	From 1st year of life		
- Visual field constriction	± 50%	From 2nd decade of life		
- Impaired visual acuity		From 2nd decade of life		
Endocrine				
Primary hypothyroidism	> 25% (U)	From 1st decade of life	Retrospective cohort (n=16)	Chan AM *et al.*, 1970 Grunebaum A *et al.*, 1977
	> 15% (U)		Retrospective cohort (n=7)	Lucky AW *et al.*, 1977
				Burke JR *et al.*, 1978
Delayed puberty	100% (U)		Retrospective cohort (n=30)	Winkler L *et al.*, 1993
Growth retardation	> 70%	From 1st year of life		Broyer M *et al.*, 1987
			Retrospective cohort (n=245)	Van Stralen KJ *et al.*, 2011
Insulin-dependent diabetes mellitus	> 25%	Following KTx	Retrospective cohort (n=5)	Fivush B *et al.*, 1987
			Retrospective cohort (n=44)	Robert JJ *et al.*, 1999
Impaired oral glucose tolerance	± 50%	> 18	Cross-sectional (n=8)	Filler G *et al.*, 1998
Exocrine pancreatic insufficiency	*NR* (U)	From 2nd decade of life	Case report	Fivush B *et al.*, 1988
Growth hormone deficiency	11%	From 1st decade of life	Cross-sectional cohort (n=9)	Besouw M *et al.*, 2012

Table 1.1 (continued) Overview of extra-renal manifestations reported in cystinosis

Extra-renal manifestation	Reported prevalence	Presenting age	Study type (n)	Reference
Neuromuscular				
Distal vacuolar myopathy (hand muscle atrophy with muscular weakness)	24% (U) > 28%	From 2nd decade of life	Case report Cross-sectional cohort (n=54) Cross-sectional cohort (n=7) Case series (n=3)	Gahl WA, 1988 Chamas LR *et al.*, 1994 Vester U *et al.*, 1998 Cabrera-Serrano M *et al.*, 2017
Swallowing dysfunction (slow eating, dry mouth, pain and difficulty with swallowing, choking)	> 75% (PT)	From 2nd decade of life	Prospective cohort (n=101) Cross-sectional (n=20)	Sonies BC *et al.*, 2005 Van Rijssel AE *et al.*, 2019
Oral motor dysfunction (gagging, choking, difficulties chewing, coughing, swallowing multiple times for one bite of food) - Oral-pharyngeal transition difficulties	10 – 65% > 50%	From 1st decade of life	Cross-sectional (n=22) Cross-sectional (n=20)	Trauner DA *et al.*, 2001 Van Rijssel AE *et al.*, 2019
Central nervous system				
Cystinotic encephalopathy - Type 1: Cerebellar and pyramidal signs, (extra-pyramidal signs); pseudo-bulbar palsy; mental retardation - Type 2: Stroke-like episodes; hemiplegia - Cerebral atrophy; multifocal cystic necrosis; dystrophic calcification - Vasculitic lesions	14% - 30% (PT)	From 3rd decade of life	Cross-sectional cohort (=14) Case report Retrospective cohort (n=26) Case report	Fink JK *et al.*, 1989 Vogel DG *et al.*, 1990 Broyer M *et al.*, 1996 Cazals X *et al.*, 2013
Posterior reversible encephalopathy	*NR*	From 3rd decade of life	Case report	Marquardt L *et al.*, 2013
Cerebrovascular disease: - Ischemic strokes (recurrent) - Vasculopathy of spinal cord	*NR*	From 3rd decade of life	Case report Case report	Neutel D *et al.*, 2013 Berger JR *et al.*, 2009
Idiopathic intracranial hypertension (pseudotumor cerebri)	*NR*	From 1st decade of life	Retrospective case series	Dogulu CF *et al.*, 2004
Chiari 1 malformation/tonsillar ectopia	18,9%	From 2nd decade of life	Cross-sectional cohort (n=53)	Kavya I *et al.*, 2015
Neurocognitive Intellectual impairment - IQ in lower range of normal; lower compared to siblings & parents and age and SES-matched controls - Lower arithmetic and spelling performance	*na*	From 1st decade of life	Cross-sectional cohort (n=15) Cross-sectional cohort (n=19) Cross-sectional cohort (n=20)	Williams BL *et al.*, 1994 Ballantyne AO *et al.*, 1997 Spilki AM *et al.*, 2007
Behavioral problems - Social problems	20%	From 1st decade of life	Cross-sectional cohort (n=186)	Delgado G *et al.*, 2005

Table 1.1 (continued) Overview of extra-renal manifestations reported in cystinosis

Extra-renal manifestation	Reported prevalence	Presenting age	Study type (n)	Reference
Central nervous system				
Neurocognitive (continued)				
Non-verbal deficits	*na*	From 1st decade of life	Cross-sectional cohort (n=20)	Spilki AM *et al.*, 2007
- Lower performing and processing speeds (WPPSI-III)				
Specific cognitive defects	*na*	From 1st decade of life	Cross-sectional cohort (n=25)	Trauner DA *et al.*, 2007
- Impaired visual spatial & visual motor function				
Reproductive				
Primary hypogonadism (males)	70% (U)	From adult age	Cross-sectional cohort (n=10)	Chik CL *et al.*, 1993
Azoospermia	100%	From adult age	Cross-sectional cohort (n=7)	Besouw M *et al.*, 2010
Pulmonary				
Extraparenchymal restrictive lung disease	100% (U) 30 – 90% (U) 5 – 80% (T)	From 3rd decade of life	Cross-sectional cohort (n=15) Retrospective cohort (n=100)	Anikster Y *et al.*, 2001 Gahl WA *et al.*, 2007 Simon RH *et al.*, 2016
Skeletal (excluding vitamin D resistant hypophosphatemic rickets)				
Skeletal deformities and bone impairment (scoliosis, genua valgum, walking impairment, stress fractures, leg and joint pain)	70%	From 3rd decade of life	Cross-sectional cohort (n=9) Case series (n=3) Prospective cohort (n=7)	Zimakas PJA *et al.*, 2003 Bacchetta J *et al.*, 2016 Bertholet-Thomas A *et al.*, 2018
Cardiovascular				
Coronary artery and other vascular calcifications	32% (PT)	From 3rd decade of life	Retrospective cohort (n=41)	Ueda M *et al.*, 2006
Pregnancy-associated cardiomyopathy	*na*		Case report	Ramappa AJ *et al.*, 2010
Gastro-intestinal				
Hepatomegaly	*NR*	From 2nd decade of life		
Noncirrhotic portal hypertension; hepatic fibrosis	*NR*	From 2nd decade of life	Case report	Klenn PJ *et al.*, 1994
			Case report	DiDomenico P *et al.*, 2004
			Case report	Rossi S *et al.*, 2005
Cholestatic liver disease; scloerosing cholangitis	*NR*	From 3rd decade of life	Case series (n=2)	Cornelis T *et al.*, 2008
Splenomegaly	*NR*	From 2nd decade of life		
Hematological				
Cystine crystal laden macrophages in bone marrow	*NR*	From 2nd year of life	Case report	Abdulsalam AH *et al.*, 2013
			Case report	Lyou Y *et al.*, 2015
			Case report	Monier L *et al.*, 2015
			Case report	Vicari P *et al.*, 2015

Table 1.1 (continued) Overview of extra-renal manifestations reported in cystinosis

Extra-renal manifestation	Reported prevalence	Presenting age	Study type (n)	Reference
Cutaneous				
Decreased sweat production	> 60% (U & PT)	From 2nd year of life	Cross-sectional cohort (n=13)	Gahl WA *et al.*, 1984
Congenital hypopigmentation of skin and hair	*NR*	From birth	Case series (n=4) Case report Research article	Guillet G *et al.*, 1998 Patel R *et al.*, 2010 Chiavérini C *et al.*, 2012
Signs of premature skin aging	*NR*	From 3rd decade of life	Case series (n=4)	Guillet G *et al.*, 1998
Facial papules	*NR*	From 2nd decade of life	Case report Case report	Patel R *et al.*, 2010 Stevens S *et al.*, 2015

Abbreviations: U: cysteamine untreated; never received cysteamine treatment; PT: partially treated with cysteamine; has received cysteamine treatment for a part of life but not from diagnosis; T: treated with cysteamine since diagnosis; SES: socio-economical status; WPPSI-III: Wechsler Preschool and Primary Scale of Intelligence-Third Edition; *na*: not applicable

Therefore, the development of alternative biomarkers for the monitoring of cysteamine therapy are essential for achieving a better outcome for cystinosis patients. Recently, the role of macrophages and their role in cystine crystal-induced inflammation was highlighted [26]. Also, novel means for assessing the total body tissue cystine content could be useful as an alternative monitoring method for cystine-depleting therapy. In this regard, *in vivo* confocal reflectance microscopy has recently been put forward as a promising tool for assessing the cutaneous cystine crystal deposition in a small descriptive study [134]. However, a systematic quantitative study in a substantial cohort of cystinosis patients, using the most advanced non-invasive optical imaging technology for assessing the cutaneous manifestations of cystinosis in all its aspects, including the specific skin layers where cystine crystal depositions have been observed to accumulate, is still lacking.

1.1.7 Treatment

The management of cystinosis consists of (1) a supportive treatment with substitution of urinary lost compounds, nutritional support and hormonal replacement therapy, and (2) the specific disease-modifying cystine-depleting therapy by use of cysteamine [47]. An overview of the current pharmacological treatment of cystinosis is represented in Table 1.2.

Supportive treatment is aimed at restoring the electrolyte and acid-base balance, preventing rickets and improving nutritional status and growth. Cysteamine bitartrate (Cystagon®, Mylan Pharma, UK) is currently the most widely used formulation since its approval for use in the mid 1990's.

Substantial evidence indicates that adequate cystine-depleting therapy holds the potential to delay the progression towards end-stage renal disease, to reduce the incidence of extra-renal manifestations and to improve growth [54,149–153].

In contrast, it does not cure the renal Fanconi syndrome and systemic administration of cysteamine has no effect on the corneal accumulation of cystine crystals [154,155].

Renal replacement therapy is indicated in case of end-stage renal disease and parallels the management of ESRD due to other causes.

In order to overcome the burden imposed by the immediate-release, (IR) cysteamine bitartrate, a new extended-release (ER) formulation has been developed during the past decade. This formulation is an enteric-coated capsule containing microspheronized beads of cysteamine (RP103, Procysbi® - Horizon Pharma, USA). Notably, in a recently published short term follow-up study in 12 infantile cystinosis patients who had switched from IR-cysteamine to ER-cysteamine, a significant improvement of halitosis was reported in the majority of patients, and

a discontinuation of proton pump inhibitor therapy and reduction of the median dose of cysteamine seemed to be possible in some [156].

After kidney transplantation cysteamine should be administered as soon as possible as it does not interfere with the reabsorption of immunosuppressive agents [157].

Corneal cystine crystal deposition is mainly treated with cysteamine hydrochloride topical aqueous solutions (eye drops). Cystaran® containing cysteamine hydrochloride 0.44%, (Sigma-Tau Pharmaceuticals, Gaithersburg, MD, USA) is currently approved by the FDA for this indication [47]. In Europe 0.55% cysteamine hydrochloride gel formulation (Cystadrops®) has been approved by the European Medical Agency in 2016 [158]. New ocular (pre)-formulations and drug delivery systems are being developed, aimed at increasing the therapeutic efficiency and drug stability [159,160]. For example, sodium hyaluronate has recently been identified as a new suitable preformulation for topical ocular cystinosis treatment [159]. Furthermore, a cysteamine delivery nanowafer has recently been developed showing a higher drug bioavailability and therapeutic efficiency at a lower drug loading dose, and an extended drug stability without inducing any inflammatory response [160]. Since the nanowafer is fabricated out of a polymer biomaterial that are being used in artificial tear eye drops, it can serve as an extra lubricant and has an established safety profile.

Finally, as life expectancy and outcome of cystinosis patients has improved, transition of care from pediatric to adult medicine is increasingly gaining attention among treating physicians. In some highly specialized reference centres, multi-disciplinary cystinosis clinics are set up, while models for transition of care to adult physicians are being implemented, aiming to improve patient empowerment and a continuum of care [161].

Future treatment perspectives

Currently, strategies for developing novel treatments for cystinosis mainly focus on hematopoietic stem cell-based gene therapy, drug development aimed at other pathogenic mechanisms and improvement of the current cystine-depleting therapy by reducing its adverse effects. Indeed, now about a decade ago, bone-marrow and hematopoietic stem cell transplantation in the cystinosis mouse model showed impressive results in terms of significant reduction in cystine tissue content of various relevant organs, preservation of kidney function and improvement of keratopathy as an important extra-renal complication [162–164]. Similarly, the concept of autologous HSCT following *ex vivo* gene repair via lentiviral vector technology, has proven to be successful [165].

In contrast, while the field of kidney regeneration is emerging, few is known about potential mechanisms of kidney repair in cystinosis and how to beneficially affect this process, leaving an enormous opportunity to the research field.

1.1.8 Conclusion

In summary, cystinosis continues to gain attention as a prototype of a rare disorder in which growing insights in pathophysiology have provided novel information into molecular and cell biological mechanisms in general, and produced new opportunities for cystinosis patients in specific.

While substantial progress in the treatment of cystinosis during recent decades has transformed the disease into a chronic metabolic disorder allowing patients to survive into adulthood, important challenges for the further improvement of the management and outcome of cystinosis patients are being faced.

The search for novel, additional biomarkers for cystine-depleting therapy could further ameliorate the outcome of cystinosis patients. Likewise, the exploration of cutting-edge innovative strategies for the treatment of cystinosis in all its aspects, ranging from HSCT to cell-based gene therapy and novel drug development, harbors significant potential and raises hope for advancing towards a final cure.

Table 1.2 Treatment of nephropathic cystinosis

Indication	Drug	Daily dose	Frequency of administration	Remarks
A. Cystine-depleting therapy				
Systemic treatment	Immediate-release (IR) cysteamine bitartrate (Cystagon® - Mylan Laboratories Inc., UK)	< 12 years of age: 1.30 – 1.95 g/m^2/day > 12 years of age: 2 g/day	/6h - QID	Start at a low dose (1/6th of the target dose) and increase gradually over 6 – 8 weeks Do not exceed max dose of 1.95 g/m2/day Regular assessment of the leucocyte cystine level (LCL) is recommended (children 3-4 times per year, adults: 1-2 times per year). Aim for LCL < 0,6 nmol cystine/mg protein In case of side –effects: - Gastro-intestinal complains: add proton pump inhibitor -Skin striae, angioendotheliomatosis: perform dose reduction with 25-50%
	Extended-release (ER) Cysteamine bitartrate (Procysbi® - Horizon Pharma Inc., USA)	Start with 80% of dose of the IR formulation	/12h - BID	
Corneal cystinosis Topical treatment	Cysteamine hydrochloride 0.44 – 0.55% ophtalmic topical solution (eye drops) (Cystaran® - Leadiant Biosciences, USA)		/2h – 6-12 times daily	Yearly ophthalmological follow-up is recommended. with slit-lamp examination, OCT Careful for eye burning symptoms in case of corneal erosions
	Cysteamine ophthalmic topical gel (eye gel) (Cystadrops® - Orphan Europe, Recordati group, France)		/6h - QID	
B. Supportive treatment				
Polyuria	Free access to water and toilet			Avoid prolonged exposure to sun and heat; special attention to hydration in case of diarrhea, vomiting
Sodium losses	Sodium potassium citrate	2 – 10 mmol K/kg/day	/6h – QID	Administer in between meals
	Sodium bicarbonate	2 – 15 mmol Na/kg/day	/6h - QID	
Potassium losses	Potassium citrate	2 – 10 mmol K/kg/day	/6h - QID	Aim for a potassium level > 3 mmol/L
	Potassium phosphate	0.6 – 2 mmol P/kg/day	/6h - QID	Trough levels should be monitored before administration of the next dose
Alkali losses	Citrate *or* bicarbonate as sodium and potassium salts	5 – 15 mmol/kg/day	/6h - QID	Aim for a normal bicarbonate level (21 – 24 mmol/L)
Phosphate losses	Phosphate *in* potassium or sodium salts	30 – 60mg elementary phosphate/day	QID	Aim for age-related normal phosphate levels High doses of phosphate supplements can cause or aggravate nephrocalcinosis Trough levels should be monitored before administration of next dose

Table 1.2 (continued) Treatment of nephropathic cystinosis

Indication	Drug	Daily dose	Frequency of administration	Remarks
Difficult to control electrolyte losses and polyuria	Indomethacin	0.5 – 3 mg/kg/day	/8h - TID	Monitor renal function Discontinue in case of dehydration Concomitant use of ACE-inhibition is contra-indicated
Proteinuria	ACE-inhibition: enalapril	0.10 – 0.25 mg/kg/day	QD	Monitor renal function and serum potassium Preferable administration at night to avoid hypotension Concomitant use of Indomethacin is contra-indicated
Rickets	1-alpha-hydroxycholecalciferol	0.5 – 2 ug/kg/day	Single dose	Beware of hypercalcemia; monitor serum calcium
Carnitine losses	L-carnitine	20 – 30mg/kg/day	/8h – TID	
Copper deficiency	Copper supplementation			1 – 10mg/day depending on age and serum copper levels Chlorophyllin tablets that are used to mitigate halitosis contain 4mg of elemental copper per tablet
B.2 Nutritional support				
Malnutrition	High caloric intake	130% of RDI		Consider tube feeding in young infants
B.3 Hormonal replacement				
Short stature	rhGH			Only to be considered for early initiation in case of optimal feeding schedule, adequate control of electrolyte balance and treatment of rickets, and normal eGFR Higher doses of phosphate may be needed
Primary hypothyroidism	Levothyroxin	< 12 years: 5 ug/kg/day > 12 years: 2 – 3 ug/kg/day Adults: 1.7 ug/kg/day	QD	Start at 25% of the daily recommended dose, and increase gradually to a full dose in 4 weeks
Insulin-dependent diabetes mellitus	Insulin	According to insulin need		Blood glucose monitoring; Regular assessment of HbA1c

Supplementary data

Table S1.1 Overview of all reported mutations in the *CTNS* gene

Missense/nonsense mutations						
No.	**mutation**	**protein**	**exon**	**phenotype**	**country**	**Reference**
1	c.1A>C	p.M1L	3	Infantile	France	Kalatzis et al., 2002, Hum Mut
2	c.2T>C	p.M1T	3	Infantile	Spain	Macias-Vidal et al., 2009, Clin Genet
3	c.3G>A	p.M1I	3	Infantile	France	Kalatzis et al., 2002, Hum Mut
4	c.3 G>C	p.M1I	3	Infantile	Turkey	Topaloglu et al., 2017, CJASN
5	c.15G>A	p.W5*	3	Infantile	France, Egypt	Kalatzis et al., 2002, Hum Mut Soliman et al., 2014, JIMD Rep
					Turkey	Topaloglu et al., 2017, CJASN
6	c.124G>A	p.V42I	4	Juvenile	UK	Attard et al., 1999, Hum Mol Genet
					Qatar	Rodriguez-Flores et al., 2014, Hum Mut
7	c.283G>T	p.G95*	6	Infantile	UK, France	Town et al., 1998, Nat Genet
8	c.382C>T	p.Q128*	7	Infantile	UK, France	Town et al., 1998, Nat Genet
					UK	Attard et al., 1999, Hum Mol Genet
9	c.397A>T	p.I133F	7	Infantile	Canada	McGowan et al., 1999, Eur J Hum Genet
10	c.414G>A	p.W138*	7	Infantile	UK, France	Town et al., 1998, Nat Genet
					Canada	McGowan et al., 1999, Eur J Hum Genet
11	c.416C>T	p.S139F	7	Juvenile	UK	Attard et al., 1999, Hum Mol Genet
					France	Servais et al., 2008, CJASN
					Spain	Macias-Vidal et al., 2009, Clin Genet
12	c.422C>T	p.S141F	7	Infantile	France	Kalatzis et al., 2004, Hum Mol Genet
					Saudi Arabia	Aldahmesh et al., 2009, Ophthalm Genet
13	c.433C>T	p.145*	7	Infantile	Iran	Sadeghipour, 2017, Hum Genom Var
14	c.451A>G	p.R151G	7	Infantile	Turkey	Topaluglu et al., 2012, Pediatr Nephrol
15	c.470G>A	p.G157D	8	Infantile	Turkey	Topaluglu et al., 2012, Pediatr Nephrol
16	c.473T>C	p.L158P	8	Infantile	Canada	McGowan et al., 1999, Eur J Hum Genet
17	c.506G>A	p.G169D	8	Infantile	USA	Shotelersuk et al., 1998, Am J Hum Genet
18	c.517T>C	p.Y173H	8	Infantile	Iran	Sadeghipour, 2017, Hum Genom Var
19	c.518A>G	p.Y173C	8	Infantile	Turkey	Topaluglu et al., 2012, Pediatr Nephrol
20	c.530A>G	p.N177S	8	Infantile	Saudi Arabia	Aldahmesh et al., 2009, Ophthalm Genet
21	c.530A>C	p.N177T	8	Juvenile	France	Kalatzis et al., 2002, Hum Mut
22	c.544T>C (•)	p.W182R	8	Infantile	USA	Shotelersuk et al., 1998, Am J Hum Genet
23	c.560A>G	p.K187R	8	Infantile	Egypt	Soliman et al., 2014, JIMD Rep
24	c.589G>A	p.G197R	9	Ocular	USA	Anikster et al., 1999, Hum Mut
25	c.599C>T	p.P200L	9	Juvenile	France	Kalatzis et al., 2002, Hum Mut
26	c.613G>A	p.D205N	9	Infantile	USA	Shotelersuk et al., 1998, Am J Hum Genet
					Turkey	Topaloglu et al., 2017, CJASN
					Iran	Sadeghipour, 2017, Hum Genom Var
27	c.647C>G	p.T216R	9	Infantile	Mexico	Alcantra-Ortigoza, 2008, Hum Genet
28	c.664C>T	p.Gln222*	9	Infantile	Turkey	Topaloglu et al., 2017, CJASN
29	c.665A>G	p.Q222R	9	Infantile	France	Kalatzis et al., 2002, Hum Mut
30	c.704G>A	p.W235*	10	Infantile	Italy	Pennesi et al., 2005, Pediatr Nephrol
31	c.734G>A	p.W245*	10	Infantile	Egypt	Soliman et al., 2014, JIMD Rep
32	c.809C>T	p.S270F	10	Infantile	Spain	Macias-Vidal, 2009, Clin Genet
33	c.809C>A	p.S270Y	10	Infantile	South Africa	Owen et al., 2015, Pediatr Nephrol
34	c.839A>G (•)	p.K280R	10	Juvenile	USA	Thoene et al., 1999, Mol Genet Metab

Table S1.1 (continued) Overview of all reported mutations in the *CTNS* gene

Missense/nonsense mutations (continued)						
No.	**mutation**	**protein**	**exon**	**phenotype**	**country of diagnosis**	**Reference**
					France	Servais et al., 2008, CJASN
35	c.850C>T	p.Q284*	10	Infantile	Thailand	Yeetong et al., 2012, Gene
36	c.861G>A	p.M287I	11	Infantile	Italy	Mason et al., 2003, Eur J Hum Genet
37	c.864C>A (•)	p.N288K	11	Infantile	France	Kalatzis et al., 2002, Hum Mut
38	c.870C>G	p.Y290*	11	Infantile	USA	Shotelersuk et al., 1998, Am J Hum Genet
39	c.878G>T	p.S293I	11	Infantile	Turkey	Topaloglu et al., 2017, CJASN
40	c.890G>A	p.W297*	11	Infantile	Italy	Mason et al., 2003, Eur J Hum Genet
41	c.893G>A (•)	p.S298N	11	Infantile	USA	Shotelersuk et al., 1998, Am J Hum Genet
42	c.914A>G	p.D305G	11	Infantile	USA	Shotelersuk et al., 1998, Am J Hum Genet
43	c.913G>T	p.D305Y	11	Infantile	UK	Attard et al., 1999, Hum Mol Genet
44	c.922G>A	p.G308R	11	Infantile	USA	Shotelersuk et al., 1998, Am J Hum Genet
					UK	Attard et al., 1999, Hum Mol Genet
					Italy	Mason et al., 2003, Eur J Hum Genet
					Spain	Macias-Vidal et al., 2009, Clin Genet
					Saudi Arabia	Aldahmesh et al., 2009, Ophthalm Genet
					Egypt	Soliman et al., 2014, JIMD Rep
45	c.922G>C	p.G308R	11	Infantile	Turkey	Topaloglu et al., 2017, CJASN
46	c.923G>A	p.G308E	11	Infantile	Iran	Shahkarami, 2013, Nephrologia
47	c.923G>T	p.G308V	11	Infantile	Germany, Switzerlands	Kiehntopf, 2002, Hum Mut
48	c.926G>A	p.G309D	11	Infantile	Thailand	Yeetong et al., 2012, Gene
49	c.926G>T	p.G309V	11	Infantile	Spain	Macias-Vidal, 2009, Clin Genet
50	c.969C>G (•)	p.N323K	11	Juvenile	USA	Thoene et al., 1999, Mol Genet Metab
					Thailand	Yeetong et al., 2012, Gene
51	c.1001C>A	p.T334N	12	Juvenile	France	Servais et al., 2008, CJASN
52	c.1009G>A	p.G337R	12	Infantile	Italy	Mason et al., 2003, Eur J Hum Genet
53	c.1013T>G	p.L338R	12	Infantile	Saudi Arabia	Aldahmesh et al., 2009, Ophthalm Genet
54	c.1013T>C	p.L338P	12	Infantile	UK	Attard et al., 1999, Hum Mol Genet
					Canada	Rupar et al., 2001, J Med Genet
					Italy	Mason et al., 2003, Eur J Hum Genet
					Spain	Macias-Vidal, 2009, Clin Genet
					Egypt	Soliman et al., 2014, JIMD Rep
					Turkey	Topaloglu et al., 2017, CJASN
					Iran	Sadeghipour, 2017, Hum Genom Var
56	c.1036G>A	p.D346N	12	Juvenile	UK	Attard et al., 1999, Hum Mol Genet
57	c.1084G>A	p.G362R	12	Infantile	Egypt	Soliman et al., 2014, JIMD Rep

Table S1.1 (continued) Overview of all reported mutations in the *CTNS* gene

Intronic mutations / Splice-site mutations

No.	mutation	intron	phenotype	country of diagnosis	Reference
1	c.61+5g>a	4	Infantile	Mexico	Alcantra-Ortigoza, 2008, Hum Genet
				Spain	Macias-Vidal, 2009, Clin Genet
				Canada	Mucaki et al., 2013, Hum Mut
2	c.61+5g>t	4	Infantile	Egypt	Soliman et al., 2014, JIMD Rep
3	c.141-22a>g	5	Infantile	Turkey	Topaloglu et al., 2017, CJASN
4	c.141-24t>c	5	Infantile	Italy	Taranta et al., 2010, pediatr Nephrol
5	c.140+1g>t	5	Infantile	UK, France	Town et al., 1998, Nat Genet
				Turkey	Topaloglu et alv, 2017, CJASN
6	c.225+3a>t	6	Infantile	Italy	Mason et al., 2003, Eur J Hum Genet
7	c.225+5_225+6 delgtinscc	6	Infantile	Australia	Cabrera-Serrano, 2017, Neuromuscul Disord
8	c.226-3c>g	6	Infantile	Australia	Cabrera-Serrano, 2017, Neuromuscul Disord
9	c.329g>t	7	Juvenile	France	Kalatzis et al., 2002, Hum Mut
10	c.329g>c	7	Infantile	Japan	Higashi et al., 2017, BMC Nephrol
11	c.329+2t>c	7	Infantile	Japan	Higashi et al., 2017, BMC Nephrol
12	c.329+1delg	7	Infantile	China	Yang, 2015, ScientificWorld Journal
13	c.462-10c>g	8	Juvenile	UK	Attard et al., 1999, Hum Mol Genet
14	c.562-1g>c	9	Infantile	UK, France	Town et al., 1998, Nat Genet
				Saudi Arabia	Aldahmesh et al., 2009, Ophthalm Genet
15	c.681delgt	10	Infantile	China	Yang, 2015, ScientificWorld Journal
16	c.682-1g>t	10	Infantile	Spain	Macias-Vidal, 2009, Clin Genet
17	c.681g>a (•)	10	Infantile	Saudi Arabia	Aldahmesh et al,. 2009, Ophthalm Genet
				Egypt	Soliman et al., 2014, JIMD Rep
				Iran	Ghazi et al., 2017, Nephrologia
				Turkey	Topaloglu et al., 2017, CJASN
18	c.681+1g>a	10	Infantile	UK	Attard et al., 1999, Hum Mol Genet
				Italy	Mason et al., 2003, Eur J Hum Genet
19	c.853-3c>g	11	Ocular	USA	Anikster et al., 1999, Hum Mut
				Russia	Bryzgalov, 2013, PLoS One
20	c.853-2a>g	11	Infantile	France	Kalatzis et al., 2002, Hum Mut
21	c.853-1g>a	11	Infantile	Turkey	Topaloglu et al., 2017, CJASN
22	c.971-12g>a (•)	12	Infantile	UK	Attard et al., 1999, Hum Mol Genet
				Thailand	Yeetong et al., 2012, Gene
				South Africa	Owen et al., 2015, Pediatr Nephrol
				Iran	Sadeghipour, 2017, Hum Genom Var
23	c.970+2t>c	12	Juvenile	USA	Thoene et al., 1999, Mol Genet Metab

Promotor mutations

No.	position	phenotype	country of diagnosis	Reference
1	–295 g>t (–50 relative to transcription initiation site)	Infantile	USA	Phornphutkul et al., 2001, Am J Hum Genet
			Italy	Mason et al., 2003, Eur J Hum Genet
2	–295 inst (–50 relative to transcription initiation site)	Ocular	USA	Phornphutkul et al., 2001, Am J Hum Genet
3	–303 g>c (–42 relative to transcription initiation site)	Ocular	USA	Phornphutkul et al., 2001, Am J Hum Genet

Indels

No.	mutation	protein	exon	phenotype	country of diagnosis	Reference
1	c.225+5_225+6delGTinsCC	Splicing		Infantile	USA	Anikster et al., 1999, Hum Mut
2	c.751_754delACCAinsCG	T251Rfs*44	10	Juvenile	Mexico	Alcantra-Ortigoza, 2008, Hum Genet
3	c.1028_1035delTCGTCTTCinsA	I343Kfs*22	12	Infantile	USA	Shotelersuk et al., 1998, Am J Hum Ge
4	c.1032delCinsTG	F345Vfs*20	12	Infantile	Egypt	Soliman et al., 2014, JIMD Rep

Table S1.1 (continued) Overview of all reported mutations in the *CTNS* gene

Deletions						
No.	**mutation**	**protein**	**exon**	**phenotype**	**country of diagnosis**	**Reference**
1	c.18_21delGACT	T7Ffs*7	3	Infantile	UK, France	Town et al., 1998, Nat Genet Heil et al., 2001, Nephron Mason et al., 2003, Eur J Hum Genet Alcantra-Ortigoza, 2008, Hum Genet
					France	Servais et al., 2008, CJASN
					Spain	Macias-Vidal, 2009, Clin Genet
					Thailand	Yeetong et al., 2012, Gene
					Iran	Shahkarami, 2013, Nephrologia
					Turkey	Topaloglu et al., 2017, CJASN
2	c.36delT	L14*	3	Infantile	UK, France	Town et al., 1998, Nat Genet
3	c.40delC	L14*	3	Infantile	Mexico	Alcantra-Ortigoza, 2008, Hum Genet
4	c.60_61delTG	Splicing			UK, France	Town et al., 1998, Nat Genet
5	c.61_61+2delGGT	Splicing			Germany, Switzerlands	Kiehntopf, 2002, Hum Mut
6	c.120delC	N41Tfs*10	4	Infantile	Iran	Ghazi et al., 2017, Nephrologia
7	c.198_218del21 (•)	ITILELP67-73del	5	Juvenile	USA	Shotelersuk et al., 1998, Am J Hum Genet
8	c.206_210delTCCTT	I69Rfs*5	5	Infantile	USA	Shotelersuk, 1998, Am J Hum Genet
9	c.225+5_225+8delGTAA	Splicing			France	Kalatzis et al., 2002, Hum Mut
10	c.257_258delCT	S86Ffs*38			Iran	Ghazi et al., 2017, Nephrologia
11	c.260_261delTT	F87Sfs*37	6	Infantile	Egypt	Soliman et al., 2014, JIMD Rep
12	c.280delG	V94Lfs*24	6	Infantile	Germany, Switzerlands	Kiehntopf, 2002, Hum Mut
13	c.291_294delTACT	T98Ffs*19	6	Infantile	Turkey	Topaloglu et al., 2017, CJASN
14	c.295_310del16	V99Ifs*14	6	Infantile	Spain	Macias-Vidal, 2009, Clin Genet
15	c.314_317delACTC	H105Pfs*12	6	Infantile	USA	Shotelersuk, 1998, Am J Hum Genet
16	c.320_323delATCA	N107Rfs*10	6	Infantile	Spain	Macias-Vidal, 2009, Clin Genet
17	c.323delA	Q108Rfs*10	6	Infantile	Iran	Ghazi et al., 2017, Nephrologia
18	c.325_329del	Splicing	6	Infantile	Turkey	Doneray, 2017, Eurasian J Med
19	c.423delC	F142Sfs*5	7	Infantile	UK	Attard et al., 1999, Hum Mol Genet
20	c.492_515del24	FVALNLTGdel162-169	8	Infantile	Iran	Sadeghipour, 2017, Hum Genom Var
21	c.518_519delAC	Y173*	8	Infantile	Spain	Macias-Vidal, 2009, Clin Genet
22	c.519_520delCA	Y173*	8	Infantile	UK, France	Town et al., 1998, Nat Genet

Table adapted from David D et al, Nephron 2019; reproduced with permission [16].

Table is updated up to July 2018.

(•), mutations cited in the text; * data based on public HGMD database

Table S1.1 (continued) Overview of all reported mutations in the *CTNS* gene

Deletions (continued)						
No.	**mutation**	**protein**	**exon**	**phenotype**	**country of diagnosis**	**Reference**
23	c.561+1delG	Splicing			UK, France	Town et al., 1998, Nat Genet
24	c.569_577delTTCTCCTCA	F190*D205del	9	Infantile	UK, France	Town et al., 1998, Nat Genet Mason et al., 2003, Eur J Hum Genet
25	c.614_616delACG		9	Infantile	Italy USA	Shotelersuk, 1998, Am J Hum Genet
26	c.659_665delTCGTGCA	I220Sfs*31	9	Infantile	UK Italy	Attard et al., 1999, Hum Mol Genet Mason et al., 2003, Eur J Hum Genet
27	c.699_700delGT	S234Lfs*61	10	Infantile	Italy	Mason et al., 2003, Eur J Hum Genet
28	c.741delC	F247Lfs*6	10	Juvenile	USA	Shotelersuk, 1998, Am J Hum Genet
29	c.770_792del23	G258Sfs*30	10	Infantile	UK Tunisia	Attard et al., 1999, Hum Mol Genet Chkioua et al., 2015, Metagene
30	c.809_811delCTC	E270del	10		UK	Attard et al., 1999, Hum Mol Genet
31	c.834_842del	279-281del	10	Infantile	Egypt Turkey	Soliman et al., 2014, JIMD Rep Topaloglu et al., 2017, CJASN
32	c.898-IVS8+24del27	Splicing			UK	Attard et al., 1999, Hum Mol Genet
33	c. 960del	Y321Tfs*8	11	Infantile	Turkey	Topaloglu et al., 2017, CJASN
33	c.992delG	G331Efs*36	12	Infantile	UK	Attard et al., 1999, Hum Mol Genet
34	c.1018_1041del24	VFSIVFDV340-347del	12	Infantile	Saudi Arabia	Aldahmesh et al., 2009, Ophthalm Genet
35	c.1027_1038del12	343-346del	12	Infantile	UK Italy	Attard et al., 1999, Hum Mol Genet Mason et al., 2003, Eur J Hum Genet
36	c.1036_1047del12	344-347del	12	Infantile	France	Kalatzis et al., 2002, Hum Mut
37	>1.7kb del		1, 2	Infantile	Germany, Switzerland	Kiehntopf, 2002, Hum Mut
38	>4194bp del		1–3	Infantile	USA	Aradhya, 2012, Genet Med
39	~13 kb del		1–3	Infantile	UK, France	Town et al., 1998, Nat Genet
40					Italy	Mason et al., 2003, Eur J Hum Genet
41	~20 kb del		1–5	Infantile	Tunisia	Chkioua et al., 2015, metagene
42	~57 kb del (•)		1–10	Infantile	UK, France	Town et al., 1998, Nat Genet
					USA	Shotelersuk, 1998, Am J Hum Genet
					UK	Attard et al., 1999, Hum Mol Genet
					USA	Anikster et al., 1999, Hum Mut
					Canada	McGowan et al., 1999, Eur J Hum Genet
					Netherlands	Heil et al., 2001, Nephron
					Germany, Switzerland	Kiehntopf, 2002, Hum Mut
					France	Kalatzis et al., 2002, Hum Mut
					Italy	Mason et al., 2003, Eur J Hum Genet

Table S1.1 (continued) Overview of all reported mutations in the *CTNS* gene

Deletions (continued)						
No.	**mutation**	**protein**	**exon**	**phenotype**	**country of diagnosis**	**Reference**
					France	Servais et al., 2008, CJASN
					Mexico	Alcantra-Ortigoza, 2008, Hum Genet
					Spain	Macias-Vidal, 2009, Clin Genet
					Netherlands	Wamelink et al., 2011, Mol Genet Metab
					Belgium	Besouw et al., 2012, Pediatr nephrol
					UK	Attard et al., 1999, Hum Mol Genet
					Netherlands	Heil et al., 2001, Nephron
					Mexico	Alcantra-Ortigoza, 2008, Hum Genet
43	~40 kb del		1–12	Infantile	Brazil	Villela et al., 2013, Genet Mol Biol
44	ex.4-5 del		4, 5	Infantile	USA Mexico	Anikster et al., 1999, Hum Mut Alcantra-Ortigoza, 2008, Hum Genet
45	10 kb del, c.62-1083_551		4–8	Infantile	Turkey	Topaluglu et al., 2012, Pediatr Nephrol
Insertions						
No.	**mutation**	**protein**	**exon**	**phenotype**	**country of diagnosis**	**Reference**
1	c.92insG	V31Gfs*29	4	Infantile	Iran	Ghazi et al., 2017, Nephrologia
2	c.152_153insCT	A52Lfs*5	5	Infantile	Iran	Shahkarami, 2013, Nephrologia
3	c.292dupA	T98Nfs*27	6	Infantile	Germany, Switzerland	Kiehntopf, 2002, Hum Mut
4	c.346_347insACTTC	L116Hfs*4	7	Infantile	Germany, Switzerland	Kiehntopf, 2002, Hum Mut
5	c.516dupC	Y173Lfs*55	8	Infantile	Italy	Mason et al., 2003, Eur J Hum Genet
6	c.577_578insA	N196Kfs*32	9	Infantile	Greece	Bitsori et al., 2018, CEN Case rep
7	c.646dupA	T216Nfs*12	9	Juvenile	USA	Shotelersuk, 1998, Am J Hum Genet
					UK	Attard et al., 1999, Hum Mol Genet
					Mexico	Alcantra-Ortigoza, 2008, Hum Genet
					France	Servais et al., 2008, CJASN
					Spain	Macias-Vidal, 2009, Clin Genet
8	c.661insT	Q222Afs*6	9	Infantile	Iran	Ghazi et al., 2017, Nephrologia
9	c.696_697dupCG	V233Rfs*21	10	Infantile	USA	Shotelersuk, 1998, Am J Hum Genet
					UK	Attard et al., 1999, Hum Mol Genet
10	c.696dupC	V233Rfs*63	10	Infantile	USA	Shotelersuk, 1998, Am J Hum Genet
					UK	Attard et al., 1999, Hum Mol Genet
					Canada	McGowan, 1999, Eur J Hum Genet
					Italy	Mason et al., 2003, Eur J Hum Genet
11	c.829dupA	T277Nfs*19	10	Infantile	Belgium	Besouw et al., 2012, Pediatr nephrol
					Egypt	Soliman et al., 2014, JIMD Rep
					Turkey	Topaloglu et al., 2017, CJASN
12	c.926dupG	S310Qfs*55	11	Infantile	USA	Shotelersuk, 1998, Am J Hum Genet
					Netherlands	Heil et al., 2001, Nephron
13	c.1047_1048ins12	DVEF349-350ins	12	Infantile	France	Kalatzis et al., 2002, Hum Mut

1.2 Kidney development and repair

1.2.1 Kidney development and the nephron progenitor cell niche

In humans, the development of the kidney and urological tract is exclusively restricted to the period of gestation, starting at 5 to 6 weeks and completing at approximately 32 to 36 weeks of gestational age [166–168]. The formation of the new nephrons, the functional units of the kidney, a process appointed as nephrogenesis, is finalized before birth and the total number of nephrons reached at this stage will not increase over lifetime.

During renal development three sets of excretory organs develop sequentially: the pronephros and mesonephros which are transitory organs that precede the formation of the final kidney precursor, the metanephros. All originate from the intermediate mesoderm; the intermediate part of the mediolaterally patterned mesoderm that is derived from the posterior primitive streak which arises during gastrulation at the epiblast stage [169]. The intermediate mesoderm is characterized by the expression of key specification markers, including, amongst others, odd-skipped related gene (*OSR1*) and paired-box genes 2 (*PAX2*). The anterior part of the intermediate mesoderm will give rise to the nephric duct (Wolffian duct), which is the precursor of the urine collecting duct, the renal calyces and pelvis, and the ureter. Depending on the stage of renal development, the nephric duct is referred to as the pronephric, mesonephric or metanephric duct. The posterior part of the intermediate mesoderm gives rise to the mesonephric and metanephric mesenchyme (MM); the latter also referred to as the metanephric blastema. While the pronephros is a temporary nonfunctional organ regressing at the fourth week of gestation, induction of the nephric duct during this stage is crucial for the further development of the final kidneys as it induces tubule formation in the mesonephric mesenchyme and it gives rise to the ureteric bud (UB); a single epithelial outbranching of the nephric duct that is of key importance for the further specification of the metanephric mesenchyme. In contrast, the mesonephros is the real first functional excretory organ that degenerates only by the end of the first trimester of gestation, after the metanephros has started to develop which is at about the fourth to fifth week of gestation. As the ureteric bud outgrows the nephric (Wolffian) duct and penetrates the MM, an aggregation of mesenchymal cells will occur adjacent to the tip of the advancing ureteric bud and a crucial extensive reciprocal inductive signaling between the MM and the UB will be established. This condensation of mesenchymal cells of the part of the MM closest to the ureteric bud into a cap-like structure, is referred to as the cap mesenchyme (CM). The multipotent mesenchymal cells of the CM are the actual nephron progenitors: they give rise to all epithelial cells of the nephron, ranging from the tubular epithelium of the proximal and distal convoluted tubule and the loop of Henle (tubulogenesis), to the podocytes and parietal epithelial cells of the Bowman's

capsule of the glomerulus (glomerulogenesis) [168,170,171]. It is a heterogeneous population in which subsets of cells differentially express transcriptional key regulators including *OSR1*, *PAX2*, sine oculis homeobox protein 2 (*SIX2*), Wilm's tumor protein 1 (*WT1*) and *CITED1* [172,173]. A subset of the loosely organized mesenchymal cells surrounding the CM express the transcription factor winged-fork head transcription factor (*FOXD1*) and gives rise to the stromal cells of the kidney, including the glomerular mesangial cells, pericytes, vascular smooth muscle cells, fibroblasts and renin-secreting cells.

The nephron progenitors of the CM that co-express *OSR1*, *SIX2*, and *CITED1*, mainly localized at the inner part of the cortical side of the ureteric tip, remain undifferentiated and self-renew to maintain the nephron progenitor pool. In contrast, the CM nephron progenitor cells at the medullary side of the branching ureteric tip undergo differentiation and initiation of mesenchymal-to-epithelial transition (MET) during which clustering occurs to form the pre-tubular aggregate (PTA), followed by the sequential formation of the Renal Vesicle (RV), Comma-shaped body and S-shaped body and the renal corpuscle [168,170,171].

Of note, the region in the outer part of the cortex of the fetal kidney comprising these renal developmental structures (RV, C- & S-shaped body, renal corpuscle) is referred to as the nephrogenic zone. During differentiation, the *OSR1*+ *SIX2*+ *CITED1*+ nephron progenitors lose expression of *CITED1* and later *OSR1* [174]. Hence, expression *CITED1* is specifically associated with the phenotype of multipotent, undifferentiated and self-renewing nephron progenitor cells of the CM, and represent a stage prior to MET (pre-MET). In this stage, all *SIX2*+ expressing cells have the ability to differentiate to all various cell types of the mature nephron. Expression of *SIX2* and *WT1* is kept up to the stage of the RV, where it is downregulated at the distal side and kept at the proximal side, and further regresses thereafter. Since MET occurs at the moment of formation of the first epithelial structures at the stage of the PTA, post-MET a heterogenous population of multiple mixed, lineage-restricted progenitor cells develop, that show clonal proliferation but can only give rise to specific segments of the nephron. The reciprocal interactive signaling between the CM and the UB is of pivotal importance for both the proliferation of the CM nephron progenitors and induction of nephrons (nephrogenesis), as the branching and extension of the UB into a ureteric tree (branching morphogenesis).

Key signaling systems involved in/required for the differentiation of nephron progenitors involve/comprise several Wnt signals (Wnt7, Wnt9b, Wnt11) produced by the ureteric epithelium, while glial cell-derived neurotrophic factor (GDNF) and fibroblast growth factor (FGF) signals produced by the CM are needed for the branching of the ureteric bud [175–177].

The self-renewal of the nephron progenitor pool of the CM is maintained by bone morphogenetic protein (BMP) and FGF signaling pathways including BMP7, FGF9 and FGF20 [178,179]. Also

Smad4, a co-Smad required for transforming growth factor (TGF-ß) and BMP signaling, and c-Myc, a factor for pluripotency, have been identified as important additional factors for retaining stemness in nephron progenitor cells [180–183]. Importantly, the canonical ß-Catenin Wnt9b signaling pathway and the corresponding responsiveness of the nephron progenitors to Wnt9b, together with Six2 both coordinate the balance between nephron progenitor differentiation and self-renewal, which is probably attenuated by signals from the interstitium [184].

Table 1.3 Overview of cell-type specific markers of kidney stem/progenitor cells during nephrogenesis

Gene	Protein	Alias	Expression	Function	Reference
Kidney stem/progenitor cells					
OSR1	Osr1	Odd-Skipped-Related Transcription Factor-1	- hFK: cells of early intermediate mesoderm, cap mesenchyme	- Zinc-finger protein; transcriptional regulator Involved in: - Nephrogenesis: specification of mesoderm to intermediate mesoderm; essential for development of multiple IM-derived structures; OSR1+ cells are possible precursors for SIX2 and FOXD1 cells, but also maintain the renal progenitor cell pool in conjunction with SIX2; inactivation of OSR1 causes premature differentiation - MET: Downregulated on MET	So PL *et al.*, 1999 Mugford JW *et al.*, 2008 Brown AC *et al.*, 2013 Xu J *et al.*, 2014 173,174,185,186
PAX2	PAX2	Paired box gene 2	- hFK: intermediate mesoderm, in actively branching tips of ureteric bud, nephron progenitor cells of the cap mesenchyme - hAK: medullairy region (collecting duct, thin ascending limb of Henle's loop); expressed in tissue-derived multipotent putative renal stem cells, in co-expression with CD133	- (Homeobox) transcriptional regulator Involved in: - Nephrogenesis: crucial for kidney development, initiating cascade from undifferentiated mesenchyme to early stages of differentiated kidney epithelium - Regulates action of GDNF - Activates expression of WT1 in CM - Essential for MET - Expression of PAX2 and WT1 in S-shaped body are mutually exclusive to each other - Ectopic expression of PAX2 in WT1+ cells causes downregulation of WT1, leading to renal failure - PAX2 reexpression in mature podocytes leads to dedifferentiation	Dehbi *et al.*, 1996 Winyard PJ *et al.*, 1996 McConnell MJ *et al.*, 1997 Wagner KD *et al.*, 2006 Dressler GR *et al.*, 2011 Ye Y *et al.*, 2011 187–192
SIX2	Six2	Sine oculis Homeobox protein 2	- hFK: nephron epithelium progenitor cells of the CM, and early stage renal vesicle	- Transcription factor - Most expressed transcription factor in CM Involved in: - Nephrogenesis: (1) critical for maintaining the CM multipotent nephron progenitor cells in the undifferentiated progenitor state (2) co-operates with WNT9B to promote self-renewing of the progenitor cell population, (3) opposes inductive signals from the ureteric bud, (4) can differentiate via the Wnt4-mediated MET - Downregulation upon differentiation (can remain in RV)	Self M *et al.*, 2006 Kobayashi A *et al.*, 2008 Humphreys BD *et al.*, 2008 Boyle S *et al.*, 2008 Mugford JW *et al.*, 2009 Park JS *et al.*, 2012 Brunskill EW *et al.*, 2012 193–199

Table 1.3 (continued) Overview of cell-type specific markers of kidney stem/progenitor cells during nephrogenesis

Gene	Protein	Alias	Expression	Function	Reference	
Kidney stem/progenitor cells (continued)						
FOXD1	FOXD1	Forkhead Box D1	- hFK: cortical stroma: stromal cells adjacent to nephron progenitor cells of CM	Transcriptional regulator Involved in: - Nephrogenesis: development of interstitial cells: pericytes, mesangial cells: FOXD1+ stromal cells promote nephron progenitor differentiation	Levinson RS *et al.*, 2005 Fetting JL *et al.*, 2014 Fanni D *et al.*, 2016	200–202
CITED1	CITED1	CBP/p300-interacting transactivators with glutamic acid (E) / aspartic acid (D) rich C-terminal domain	- hFK: exclusively in nephrogenic progenitor cells of (cortical part of) cap mesenchyme; co-expressed with subset of SIX2+ cells	Transcriptional regulator Involved in: - Nephrogenesis: expression restricted to nephron progenitor cells of CM - Downregulation before MET - Not critical for nephrogenesis: downregulation does not impair kidney development	Boyle S *et al.*, 2007 Boyle S *et al.*, 2008	196,203
WT1	Wt1	Wilm's tumor protein 1	- hFK: nephron progenitor cells of CM, RV, S-shaped body; differentiating podocytes	Transcriptional regulator Binds promotors of SALL1, PAX2 and BMP7 Involved in: - Nephrogenesis: nephron progenitor differentiation & Wilm's tumor prevention - Wt1 represses expression of PAX2 in presumptive precursors of podocytes in S-shaped body	Ryan G *et al.*, 1995 Georgas K *et al.*, 2009 Kreidberg JA, 2010 Hartwig S *et al.*, 2010 Huff V, 2011 Essafi A *et al.*, 2011	204–209
NCAM1	Neural cell adhesion molecule 1	- CD56 - NCAM - MSK39	- hFK: nephron progenitor cells of CM, nephrogenic zone, Wilm's tumor progenitor blastema: co-expression with CD133, CD24, PAX2, WT1, VIM - hAK: reactivation upon injury - Other: brain, adrenal tissue, heart, endometrium, ovary, prostate, thyroid, urine bladder	Cell-adhesion protein Involved in: - Cell-cell and cell-matrix adhesion - Nephrogenesis: expression during EMT and MET - Re-expression in adult human kidney upon injury; reactivation of NCAM1 defines subpopulation of hAK epithelial cells with clonogenic and progenitor properties - Removal of NCAM1+ cells from during regenerative respons following AKI worsens peak injury	Abbate M *et al.*, 1999 Benigni *et al.*, 2011 Harrari-Steinberg O *et al.*, 2013 Buzhor R *et al.*, 2013	210–213

Table 1.3 (continued) Overview of cell-type specific markers of various kidney cell types

Gene	Protein	Alias	Expression	Function	Reference	
Kidney stem/progenitor cells (continued)						
PROM1	CD133	Prominin-1		Transmembrane glycoprotein	Sagrinati C *et al.*, 2006	214–220
			- hFK: S-shaped body - hAK: renal progenitor cells of the parietal epithelium of the Bowman's capsule, scattered tubular cells in proximal & distal tubule and inner medullary papilla region (loop of Henle, S3 sement of proximal tubule) - Other: hematopoietic stem cells, stem cells of various tissues (incl. neuronal, glial stem cells), endothelial progenitor cells	Involved in: - Glucose uptake; establishment of anaerobic metabolism - Inhibition of endocytosis of transferrin, hereby inhibiting iron uptake & mitochrondrial activity Associated with: - Increased resistance to injury and cell death - Tissue repair of tubular compartment - Glomerular disease In normal healthy kidney tissue: - Co-expression with vimentin, cytokeratin 7, cytokeratin 19 in Bowman's capsule & proximal tubule In context of repair following acute kidney injury: - Co-expression with vimentin, kidney-injury molecule 1	Coskun V *et al.*, 2008 Griguer C *et al.*, 2008 Ronconi E *et al.* 2009 Bourseau-Guilmain E *et al.*, 2011 Smeets B *et al.*, 2013 Hansson J *et al.*, 2014	
CD24	CD24		- hFK: early epithelial aggregates of MM, cells committed to tubular epithelia of PTA, RV, S- and comma-shaped bodies - hAK: putative renal progenitor cells at parietal epithelium of Bowman's capsule/urinary pole of Bowman's capsule, scattered tubular cells	GPI-linked, heat-stable sialoglycoprotein - Ligand for CD62P (P-selectin) Involved in: - In CD133+ CD24+ co-expressing cells: regeneration of podocytes and tubular epithelium - Development of hyperplastic lesions of podocytopathies and crescentic glomerulonephritis	Sagrinati C *et al.*, 2006 Smeets B *et al.*, 2009 Ronconi E *et al.*, 2009 Sallustio F *et al.*, 2010 Ivanova L *et al.*, 2010 Lindgren D *et al.*, 2011 Angelotti ML *et al.*, 2012 Lazzeri E *et al.*, 2015	214,217, 221–226

Abbreviations: Sc Loc: subcellular localization: cytoplasma membrane (M), cytoplasma (C), nucleus (N); CD: Cluster of Differentiation; GPI: glycosyl-phosphatidylinositol; (BM-)MSC: (bone marrow-derived) mesenchymal stem cells; HSC: hematopoietic stem cells; ISCT: International Society for Cell Therapy; hgl-MSC: human glomerular MSCs; BMP: bone morphogenetic protein; RMEC: renal microvascular endothelial cells; MMPs: matrix metalloproteinases; FSGS: focal segmental glomerulosclerosis; MCD: minimal changes diseases; hFK: human fetal kidney; hAK: human adult kidney

1.2.2 Kidney repair and the putative kidney stem/progenitor cell niche

Undoubtly, the kidney holds a certain potential for regeneration, depending on the type of cell affected, the extent of the injury and the number of recurrent damaging events. Apart from observations in the human clinical context, the endogenous reparative potential of the kidney has been demonstrated in several experimental animal models of both acute and chronic kidney injury [227,228].

Upon injury, the renal tubular epithelium can regenerate to restore its structure and function [229,230]. Recently, a specific phenotype of tubular cells, scattered distributed in the convoluted and some parts of the straight proximal tubule (S3 segment) has been described and associated with renal tubular cell repair in human kidney (scattered tubular cells, STCs) [224,231].

In contrast, in podocytes, the highly organized structure of the cytoskeleton and podocyte foot processes in these terminally differentiated cells, impair their ability to undergo mitosis without jeopardizing their function in safeguarding the glomerular permselectivity. Also, despite a higher number of podocytes in the larger adult glomeruli compared to the smaller glomeruli of children, the total number of podocytes decreases with age, which indicates that insufficient repair modalities are present for podocytes [232–234]. Therefore, it can be presumed that podocytes do not divide in the healthy adult kidney and do not contribute to genuine repair of podocytes by division and proliferation [235–240]. Only in some (glomerular) pathologies a limited proliferation of podocytes has been postulated in compromise with a significant and enduring reduced glomerular function, including glomerulonephritis with crescent formation, and collapsing glomerulopathy [241–245]. Here, the origin of the regenerated podocytes has been attributed to migrating parietal epithelial cells from the Bowman's capsule.

The putative kidney stem/progenitor cell niche

Both the origin of kidney repair and the existence of a potential kidney stem/progenitor cell niche in adult human kidney, has been a matter of extensive research for more than a decade [230,245–250]. Several hypotheses for an endogenous source for kidney repair have been explored, ranging from (1) the recruitment of bone-marrow derived stem cells (BMSCs) as an extrarenal source, to several intrarenal sources including (2) transdifferentiation of stromal cells to epithelium, (3) dedifferentiation followed by proliferation and redifferentiation of tubular epithelium in response to injury, and (4) the replenishment of nephron epithelium by a resident kidney stem/progenitor cell pool.

Bone-marrow derived stem cells (BMSCs) have been found not to provide a major contribution to kidney regeneration. While a beneficial effect of BMSCs in kidney repair has been shown in

some animal models, comprising kidney engraftment and differentiation to parenchymal cells, the actual contribution seemed to be very limited [251–257].

Therefore, the search for the origin of kidney regeneration focused on finding proof for a resident intrarenal stem/progenitor cell pool in the human kidney. This research has yielded the identification of potential renal progenitors for podocytes in the glomerulus and interstitium, for renal tubular epithelial cells within the tubuli, and for stromal cells within the interstitium [213,214,217,222,224,225,257–260]. Table 1.3 describes the most important kidney stem/progenitor cell markers relevant to this chapter and thesis. Table 1.4 provides an overview of human kidney-derived stem/progenitor cells reported in literature.

Importantly, most of these studies relied on the analysis of cell surface stem cell markers (CD133, CD24), in human kidney tissue specimes and in *in vitro* developed clonal cell lines derived from several segments of human kidney tissue (whole kidney, renal cortex deprived of glomeruli, Bowman's capsule-deprived glomeruli) [213,214,217,222,225,258,259,261] (Table 1.4). Few studies focused on nephron progenitor specific gene expression for the characterization of progenitor cells, and only some aimed at deriving progenitor cells from urine [262–264].

In parallel, pioneering studies on human embryonic or fetal kidney have aided in the biomarking of a potential renal progenitor cell niche in adult human kidney [212,261,265–267].

However, in light of this research, it should be noted that important caveats are associated with the use of presumed stem cell membrane markers for the identification of an *in vivo* resident stem/progenitor cell pool. It has been shown that some of these markers are re-expressed in human kidney tissue in certain pathological settings [192,219–221,229,268,269]. Moreover, *in vitro* it is possible that certain cell culture conditions drive selection of subsets of cells expressing specific markers, and/or induce marker expression [270].Also, CD133 could be contested for being a marker of nephron progenitor cells, since in fetal kidney, nephron progenitor cells of the CM were identified as NCAM+ CD133- [212,213]. Furthermore, strategies based on the sorting of some markers (CD24, CD133) and the assumption of a single multipotent renal progenitor cell nich could be questioned since in adult mammalian kidney, segment specific lineage-restricted clonal expansions within nephron epithelia have been demonstrated, suggesting the existence of multiple fate-restricted progenitors organized in mosaic tapestry-like fashion along the renal tubuli [271,272]. In order to overcome the limitations linked to these stem cell markers, lineage tracing studies have been applied to explore the fate of potential progenitor cells in support of the findings observed in the human studies [273–276]. Unfortunately, also the interpretation of some of these studies has been hampered by differences in marker expression and potential alternative mechanisms of kidney repair between mouse and human which finally kept some questions unresolved.

Podocyte repair

In general, upon podocyte injury, the damaged glomerulus seems to be able to ensue two different strategies for achieving a certain level of repair of the damaged podocytes: while *hypertrophy* can only achieve a limited functional recovery, (2) *regeneration* of podocytes has the potential to restore the functional and structural integrity of the glomerular filtration barrier.
In summary, *regeneration* of podocytes has been attributed to two putative intrarenal sources of kidney progenitors: a bipotent CD133+ CD24+ CD106+ PODXL- renal progenitor population at the urinary pole of the Bowman's capsule, and cells of renin lineage (CoRL) in the interstitium [225,277–280].

It has been proposed that, depending on the *de novo* expression of podocalyxin (*PODXL*), a podocyte-specific marker, and the downregulation of expression of CD106, a CD133+ CD24+ CD106+ PODXL+ podocyte-precursor, or CD133+ CD24+ CD106- PODXL- tubular progenitor can arise from the CD133+ CD24+ CD106+ PODXL- bipotent renal progenitor [225]. This CD133+ CD24+ CD106+ PODXL+ podocyte-precursor can differentiate towards a podocyte during migration via the vascular stalk to the glomerular vascular tuft. However, whether these presumed podocyte-progenitor cells are already committed to become a podocyte given the high levels of expression of other podocyte-specific genes (WT1, synaptopodin), has been questioned [231,281]. Here, it cannot be excluded that changes in cell surface marker expression are due to injury and indicative for a transitory phenomenon of de-differentiation and re-differentiation [282]. Nevertheless, the contribution of parietal epithelial cells (PECs) to the regeneration of podocytes has been put forward as a significant mechanism for repair, in contrast to podocyte hypertrophy. In fact, the balance between podocyte hypertrophy versus regeneration has been shown to determine the fate between remission or persistent proteinuria in animal models of glomerular injury [276,283,284]. Also, insufficient differentiation of the parietal epithelial progenitor cells into podocytes has been associated with the development of focal segmental glomerulosclerosis [221,284–289]. Hence, strategies to improve genuine podocyte repair are aimed at promoting the recruitment of the podocyte-committed PEC progenitor pool, and supporting their differentiation potential. Recently, retinoic acid has been put forward as an important mediator for differentiation of PEC progenitor cells, and the potential for pharmacological enhancement of this process (GSK3 inhibitor, CXCL12 antagonist) is a subject of recent and ongoing research [276,290,291].
In addition, in lineage tracing studies cells of renin lineage (CoRL) which originate from the juxtaglomerular apparatus (JGA) have shown to give rise to non-renin producing cells and to repopulate the glomerulus upon podocyte injury [278–280]. Moreover, in the latter, a subset of cells showed the acquisition of podocyte-specific markers WT1, nephrin, podocin and synaptopodin, suggesting CoRL as a potential source for regeneration of podocytes [278].

Table 1.4 Overview of human kidney stem/progenitor cells isolated from human kidney, reported in literature

Publication	S	Origin	H/D	Acronym	Characterization	Potency	Fate	*In vitro* properties	*In vivo* properties
Bussolati B *et al.*, 2005	T	hAK (Cortex; deprived of glomeruli)	H	na	CD133+ CD73+ CD44+ CD29+ HLA-ABC+ PAX2+	Multipotent	Tub	- Self-renewal - Clonogenic - Differentiation potential: tubular epithelium (*TJP1, CDH1, ALPL, ANPEP, SLC12A3*, polarization of cell layer, apical microvilli, junctional complexes), endothelial cells (*KDR, CD105/ENG, VWF, CDH5*)	Human kidney tissue biopsy: - CD133+ in cortex, interstitium Xenotransplantation in SCID mice (SC injection; in Matrigel): - CD133+ cells: Differentiation to epithelial tubular structures - CD133+ differentiated to endothelium in vitro: In vivo organization into functional vessels Glycerol-induced AKI SCID mouse model (IV injection): - CD133+ cells engraftment in proximal and distal tubuli
Sagrinati C *et al.*, 2006	T	hAK (Cortex: Glomeruli)	H	APEMP	Adult Parietal Epithelial Multipotent Progenitor cells Subset of parietal epithelial cells at urinary pole of Bowman's capsule CD133+ CD24+ OCT4+ BMI1+ VIM+	Multipotent	Tub	- Self-renewal - Clonogenic - Multilineage differentiation potential: mature proximal and distal tubular epithelial cells (*ANPEP, NHE, SLC5A1, GGT1, AQP1, SLC12A3, AQP3*), cells of osteogenic (*AP, RUNX2*), adipogenic (*ADIPOQ*) and neurogenic lineage (*NF200, NEFM, CHAT, MAP2*)	Glycerol-induced AKI SCID mouse model - Engraftment in kidney - Regeneration of tubular epithelium - Restoration of kidney function (BUN)

Table 1.4 (continued) Overview of human kidney stem/progenitor cells isolated from human kidney, reported in literature

Publication	S	Origin	H/D	Acronym	Characterization	Potency	Fate	*In vitro* properties	*In vivo* properties
Lazzeri E *et al.*, 2007	T	hEK	H	REC	Renal embryonic cells CD133+ CD24+	Multipotent	Tub	- Self-renewal - Clonogenic - Multilineage differentiation potential: tubular-like cells (*AP, SLC5A1, AQP1, AQP3, SLC12A3, CALB, SCNN1B, LRP2*), osteocyte-like cells (*RUNX2, SPP1, AP*), adipocyte-like cells (*ADIPOQ, PPARG*), endothelial-like cells (*KDR, TIE2, CDH5, VWF*), stromal-like cells (*SMA, CNN1*) - Similar phenotypic markers as APEMP	Human embryonic kidney tissue specimens: - CD133+ CD24+ co-expression in renal vesicles, S-shaped bodies; during development progressive decrease in presence & restricted only in urinary pole of Bowman's capsule Glycerol-induced AKI SCID mouse model: (Rhabdomyolysis-induced AKI model): - Engraftment & proliferation in vivo - Generation of renal tubular-like cells - Reduction of tissue necrosis and fibrosis - Improvement in kidney function
Zhang Y *et al.*, 2008	U	Urine from paediatric & adult patients	H & D	UPC	Urine-derived progenitor cells c-Kit+ SSEA4+ CD44+ CD73+ CD90+ CD105+ CD133+ alfa-SM Actin+ Vimentin+	Multipotent	*na*	- Self-renewal - Clonogenic - Multilineage differentiation: urothelial-like (*UPK1A, KRT7, KRT13, KRT17, KRT19*), smooth-muscle-like (*ACTA2, VIM, CNN1, DES, MYH2*) endothelial-like (*VWF, CD31*), interstitial-like cells (*VIM, CD177*)	*na*
Ronconi E *et al.*, 2009	T	hAK (whole kidney)	H	*na*	CD133+ CD24+	Bipotent	Podo, Tub	* CD133+ CD24+ PODXL- - Self-renewal - Clonogenic - Differentiation: tubular epithelial cells (*ANPEP, AQP1, AQP3, NHE, SLC5A1, GGT1, SLC12A3*) and podocyte (*WT1, NPHS1, NPHS2, PODXL*) * CD133+ CD24+ PODXL+ - No self-renewal - Unipotent differentiation potential: podocyte	Doxorubicin-induced KI SCID mouse model (FSGS nephrotic syndrome model): * CD133+ CD24+ PODXL-: - Engraftment in kidney (glomeruli & tubuli) - Reduction of glomerular and tubulointerstitial injury - Reduction of proteinuria (acute and chronic)

Table 1.4 (continued) Overview of human kidney stem/progenitor cells isolated from human kidney, reported in literature

Publication	S	Origin	H/D	Acronym	Characterization	Potency	Fate	*In vitro* properties	*In vivo* properties
Bruno S *et al.*, 2009	T	hAK (Bowman's capsule-derived glomeruli)	H	hgl-MSCs	Human glomerular mesenchymal stem cells CD73+ CD90+ CD105+ CD44+ CD29+ CD146+ CD24+ PAX2+ VIM+	Multipotent	Podo	- Differentiation to: podocytes (*NPHS1, NPHS2, SYNPO*), osteocytes, chondrocytes, adipocytes, endothelial cells, mesangial cells	
Sallustio F *et al.*, 2010	T	hAK (Cortex: glomerular & tubular fractions)	H*	ARPC	Adult renal progenitor cells CD133+ CD24+ PAX2+ BMI1+ OCT4+ CD44+	Multipotent	Tub	- Self-renewal - Clonogenic - Differentiation to tubular-like cell (*KRT19, TJP1*), osteoblast-like cell (*BSP*), adipocyte-like cell (*ACRP30*), endothelial (*VWF, CD105*) - Similar gene-expression profiles in glomerular and tubular ARPCs	Human kidney tissue specimens: - CD133+ CD24+ co-expressing cells in proximal and distal tubular epithelium
Lindgren D *et al.*, 2011	T	hAK (Cortex; tubular)	H	na	Scattered tubular renal progenitor cells $ALDH^{High}$ CD133+ CD24+ VIM+ KRT7+ KRT19+ BCL2+	na	Tub	- Anchorage-independent growth - Sphere formation - Clonogenic	Human kidney biopsies with clinical and pathological diagnosis of AKI in remission: - CD133+ VIM+ cells in tubules of regenerating kidneys in contrast to normal kidneys

Table 1.4 (continued) Overview of human kidney stem/progenitor cells isolated from human kidney, reported in literature

Publication	S	Origin	H/D	Acronym	Characterization	Potency	Fate	*In vitro* properties	*In vivo* properties
Angelotti ML *et al.*, 2012	T	hAK (Glomerular & tubular)	H	*na*	Bipotent renal progenitor cells CD133+ CD24+ CD106+ Tubular-committed renal progenitors CD133+ CD24+ CD106-	Bipotent	Podo, Tub	* CD133+ CD24+ CD106+ Bipotent renal progenitor / urinary pole Bowman's capsule cell: - High proliferative rate - Differentiation potential towards tubular epithelial cells (*NKCC, SLC12A3, MEP1B, NHE, GGT1*) and podocyte (*WT1, NPHS1, NPHS2, PODXL*) * CD133+ CD24+ CD106-: Scattered tubular cells / Tubular-commited renal progenitors - Low proliferative rate - Committed to tubular epithelial cells	Glycerol-induced AKI SCID mouse model & Doxorubicin-induced KI SCID mouse model (FSGS nephrotic syndrome model): * CD133+ CD24+ - Engraftment in kidney - Reduction of renal fibrosis - Restoration of kidney function (BUN) * CD133+ CD24+ CD106+ - Regeneration of tubular epithelium (proximal & distal) & podocytes * CD133+ CD24+ CD106- - Regeneration of tubular epithelium (proximal & distal) Human kidney biopsies of acute and chronic tubular injury: * Active proliferation of CD133+ CD24+ CD106- following injury Chorio-allantoic membrane of chick embryo - Engraftment on chorio-allantoic membrane - Generation of tubule-like structures with mature nephron cell types including markers for proximal, loop of Henle and distal tubular epithelial cells 5/6 nephrectomy NOD/SCID mouse model: Via direct intraparenchymal injection: - Engraftment in kidney (tubular, intestitial) - Regeneration of proximal (*LTA*) and distal (*DBA*) tubular epithelium - Limitation of disease progression (CrCl)
Harari-Steinberg O *et al.*, 2013	T	hFK	H*	hFK hNPC	Human Fetal Kidney/ Human Nephron Progenitor Cell NCAM1+ OSR1+ PAX2+ SALL1+ SIX2+ VIM+ WT1+ CITED1+	na	Tub	- Self-renewal - Clonogenic	

Table 1.4 (continued) Overview of human kidney stem/progenitor cells isolated from human kidney, reported in literature

Publication	S	Origin	H/D	Acronym	Characterization	Potency	Fate	*In vitro* properties	*In vivo* properties
Buzhor E *et al.*, 2013	T	hAK (total kidney)	H‡	hKEpCs	Adult human kidney epithelial cells CD133+ CD24+ NCAM1+ PAX2+ SALL1+ SIX2+ WT1+ VIM+	Multipotent	na	- Clonogenic - Differentiation to mesenchymal lineages (osteogenic, adipogenic) - Generation of epithelial kidney spheres and epithelial renal tissue on single-cell grafting in chick CAM and mouse	Glycerol-induced AKI SCID mouse model - Elimination of NCAM1+ cells worsens peak renal injury
Lazzeri E *et al.*, 2015	U	Urine of paediatric healthy & glomerular disease	H, D	u-RPC	Urine-derived Renal Progenitor Cell CD133+ CD24+ CD106+	Bipotent	Podo, Tub	CD133+ CD24+ (28-70%) CD106+ - High (exponential) proliferative rate - Clonogenic - Identical transcriptome as tissue-derived renal progenitor cells - Differentiation potential to tubular cells (*AQP3, NHE, SLC3A1, NKCC, EMA-1*) and podocytes (*NPHS1, NPHS2, PODXL, KLF15*)	Doxorubicin-induced KI SCID mouse model (FSGS nephrotic syndrome): - Engraftment in kidney (glomeruli, tubuli); acquisition of podocyte-specific markers (*NPHS1, NPHS2*), tubular markers (*LTA*) - Reduction of proteinuria
Arcolino FO *et al.*, 2016	U	Urine of preterm neonate	H*	nKSPC	Neonatal Kidney Stem Progenitor Cell SIX2+ CITED1+ VIM+ CD73+ CD90+ CD105+ CD29+ HLA-ABC+	Bipotent	Podo, Tub	- Differentiation potential to functional podocytes (*PODXL, SYNPO, CD2AP, NPHS1*) and functional proximal tubular cells (*CD13, ABCB1, CUBN, LRP2*) - Protective effect against cisplatin-induced apoptosis in co-culture with ciPTECs	*na*

Table 1.4 (continued) Overview of human kidney stem/progenitor cells isolated from human kidney, reported in literature

Publication	S	Origin	H/D	Acronym	Characterization	Potency	Fate	*In vitro* properties	*In vivo* properties
Leuning DG *et al.*, 2016	T	hAK (transplant grade)	H'	hkPSC	Clinical grade human kidney Perivascular Stromal Cells NG2+ PDGF-R-β+ CD146+ CD73+ CD90+ CD105+ HLA-ABC+	Multipotent	*na*	- High transcriptomal similarity with BM-MSC, with exception of high upregulation of Hox10, Hox11 - Immunomodulatory properties - Pericytic properties: stabilization of vascular network formation in co-culture with HUVEC - Enhanced renal epithelial wound repair - Differentiation potential to osteocytes & chondrocytes	Glycerol-induced AKI C57Bl/6 mouse model: Via IV or renal SC route: - Integration in cortical interstitium (not medullar; not in tubular structures) - Preservation of kidney function (BUN)
Da Sacco S *et al.*, 2017	T	hFK	H"	hFK SIX2+ CITED1+ cells	Human Fetal Kidney derived nephron progenitor cells SIX2+ CITED1+	Bipotent	Podo Tub	- Expression of genes suppressing MET - Differentiation potential towards tubular epithelial cells (*AQP1, AQP2*) and podocyte-like cells (*WT1, SYNPO, NPHS2, FOXD2*) - Formation of tubular-like structures in 3D collagen system	*na*

Exclusively histopathological studies on renal/kidney progenitor cells in human kidney, not including *in vitro* characterization of the cells, were excluded from this overview.

Abbreviation: S: source; T: tissue, U: urine; hAK: human adult kidney; hEK: human embryonic kidney; hFK: human fetal kidney; $ALDH^{High}$: High Aldehyde Dehydrogenase activity; Podo: Podocyte; Tub: Tubular; BUN: blood urea nitrogen; BM-MSC: bone-marrow mesenchymal stem cell; HUVEC: human umbilical vein endothelial cells; IV: intravenous; renal SC: renal subcapsular route; NOD/SCID: Nonobese diabetic/Severe Combined Immune Deficiency; CrCl: Creatinine clearance

H* Portions of normal-appearing cortex of patients with renal carcinoma, were isolated surgically and examined histologically to exclude the presence of carcinoma.

H° Human fetal kidney tissue specimens were obtained from fetuses that were electively aborted; gestational age range 15-23 weeks.

$H^{‡}$ Human fetal kidney tissue specimens obtained from fetuses with a gestational age of approximately 17 weeks.

$H^{\cdot}$ Healthy preterm born neonates: no renal abnormalities on ultrasound, no nephrotoxic drugs administered

H' Transplant-grade kidneys discarded for surgical reasons; average donor age 62 years with estimated creatinine clearance (Cockgroft) of 105 ml/min.

H" Human fetal kidney specimens of approximately 17 weeks of gestational age

Regeneration of tubular epithelium

The regeneration of the renal tubular epithelium has been attributed to an intra-tubular source by *in vivo* lineage tracing and label-retaining animal studies, and human studies including kidney histopathological data in acute kidney injury, and the identification of kidney tissue-derived tubular progenitor cells [195,224,225,292,293].

This intra-tubular renal progenitor cell niche has been substantiated in the phenotype of scattered tubular cells (STCs). STCs have been described to harbor an important proliferative capacity, resistance to cell death and typical morphological characteristics including smaller size, lack of the brush border and fewer mitochondria [219,220].

In humans, it was first described by Lindgren et al based on a high *in vitro* aldehyde dehydrogenase activity, clonogenic properties and the propensity to form three-dimensional structures when grown in Matrigel® [224]. Angelotti and coworkers identified a unipotent CD133+ CD24+ CD106- PDXL- tubular cell progenitor, which appeared to be present in a scattered fashion along the proximal and distal convoluted tubule, next to the aforementioned bipotent CD133+ CD24+ CD106+ PODXL- renal progenitor at the urinary pole of the Bowman's capsule [225].

It is not fully clear yet whether these STCs represent a resident fixed pool of progenitor cells or a transient phenotype in the process of de-differentiation to redifferentiation. While this has been a matter of debate in the field during the last years, substantial evidence is in favour of the last option [247,274,294,295]. First, for more than two decades it has been shown that when exposed to injury, tubular cells can show the expression of several mesenchymal markers which are not expressed in differentiated tubular epithelial cells (PTECs) [192,229,268,288]. Second, PTECs can go immediately into the S-phase of the cell cycle [219,294,295]. And third, lineage tracing studies have shown that upon injury no significant contribution of a progenitor cell pool outside of differentiated PTECs exists, and tubular progenitor cells show a high proliferative capacity [247,274]. The latter data exclude the possibility of an intrinsic progenitor population and suggests that any surviving PTEC can give rise to tubular progenitor-like cell, which can subsequently proliferate substantially while being the only source for future differentiated PTECs. These data are compatible with the observation of the aforementioned injury-induced tubulogenesis via segment specific lineage-restricted clonal expansions within the nephron tubular epithelia of adult mammalian kidney [271].

Regardless of their origin, STCs represent the cell niche of a reparative potential of the renal tubular epithelium. Currently, in parallel to improving podocyte regeneration, efforts are put in identifying agents to pharmacologically enhance tubular regeneration [296].

CHAPTER 2
OBJECTIVES

In this thesis, we aimed to address some of the most important challenges faced in the current clinical management of nephropathic cystinosis through the development of novel modalities for therapeutic monitoring and the exploration of innovative treatment strategies.

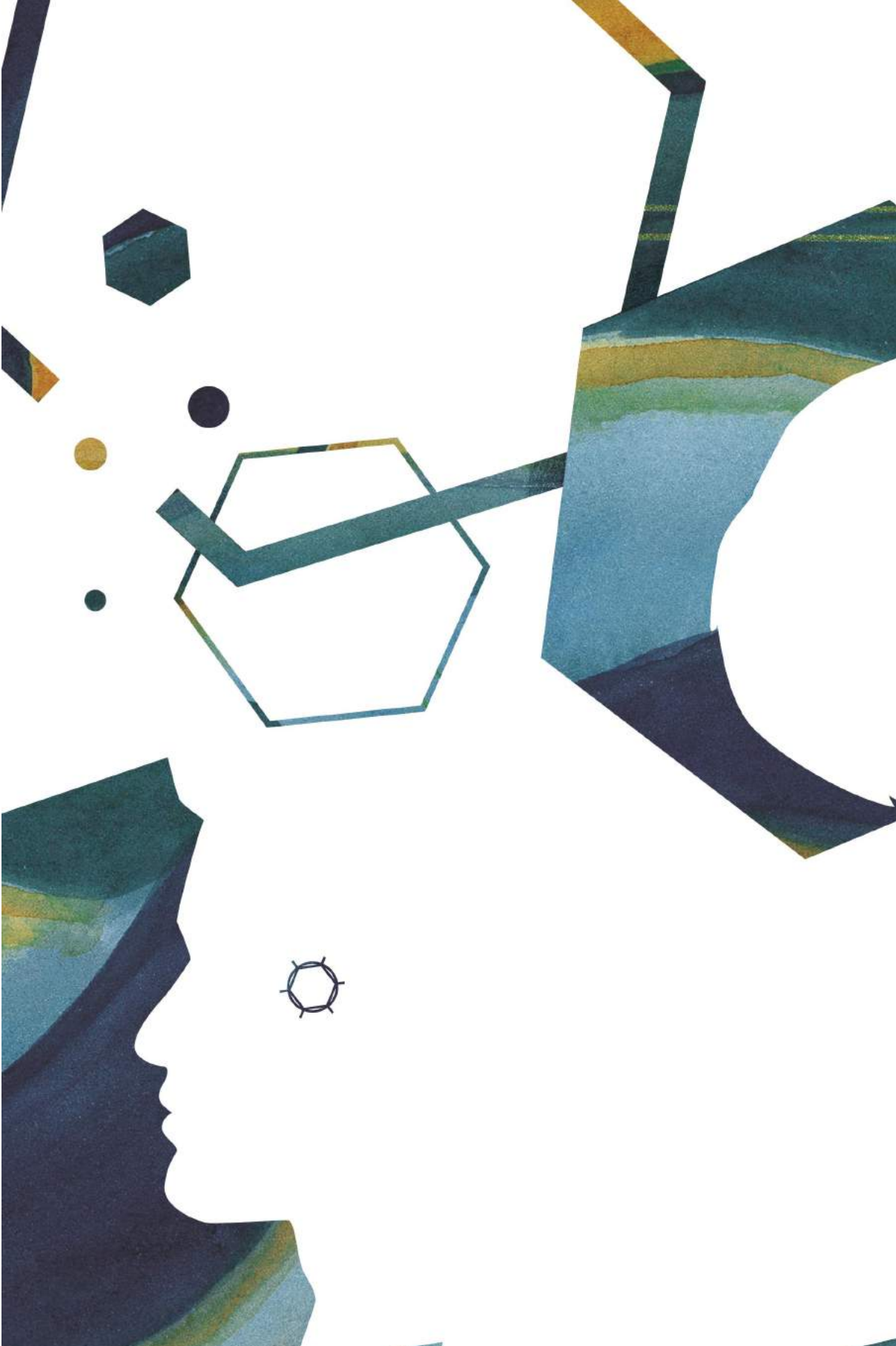
We **hypothesized** that:

1. The skin might be specifically affected in nephropathic cystinosis, potentially due to dermal cystine accumulation, making it a separate novel extra-renal complication, and that the degree of skin involvement might reflect the overall disease severity.
2. Inflammation, caused by widespread tissue cystine crystal accumulation, and mediated by macrophages and phagocytic cells engulfing cystine crystal deposits, could reflect the degree of overall cystine accumulation and hence serve as an indirect novel monitoring modality for cystine-depleting therapy.
3. A kidney progenitor cell niche, and an attempt for a regenerative response is present in cystinosis, however maladaptive, which could be reflected by the high number of undifferentiated cells voided in urine of cystinosis patients, and complementation of *CTNS* via lentiviral vector transduction, holds the potential to rescue the healthy cellular phenotype.
4. The azoospermia observed in male cystinosis patients, which is the sole cause for their infertility, cannot only be explained by an evolving primary hypogonadism, and thus is caused by an additional factor of obstruction or malfunction at the epididymal level.

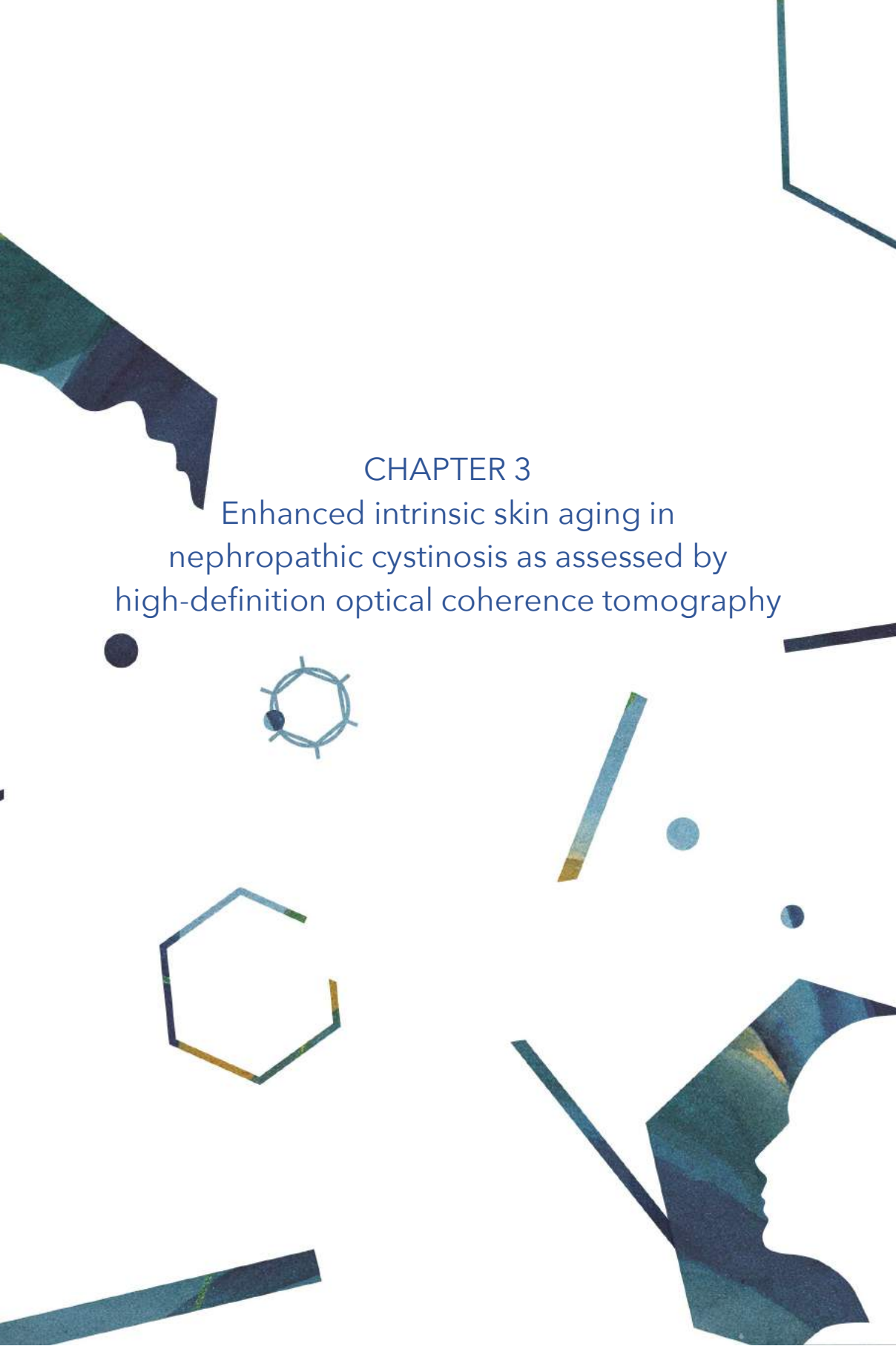
Therefore, we **aimed** to:

1. Explore the distinctive cutaneous features of nephropathic cystinosis, using the currently most advanced and high-end, non-invasive optical imaging technology available in clinical practice (HD-OCT), and to investigate their relation with kidney function and extra-renal complications in order to assess its potential to serve as a non-invasive monitoring tool for disease severity.
2. Investigate the plasma levels of some of the most important biomarkers of macrophage activation in cystinosis patients in a prospective, longitudinal international multicentric study, and to correlate the results with WBC cystine levels and extra-renal complications.
3. Demonstrate the existence of a kidney progenitor cell niche in cystinosis, and to isolate these cells from urine, characterize and assess their potential for differentiation to relevant kidney cell types (renal proximal tubular epithelial cell, podocyte). In addition, we aimed to assess the feasibility of *ex vivo* gene therapy using lentiviral vector technology to rescue the healthy cellular phenotype in these urine-derived kidney progenitor cells.
4. Unravel the mechanism of azoospermia in male cystinosis patients using non-invasive means, via the assessment of biomarkers for epididymal obstruction on scrotal ultrasound, and seminal plasma in adult male cystinosis patients, compared to vasectomy patients and healthy controls.

PART I:
Innovation in monitoring of nephropathic cystinosis



CHAPTER 3
Enhanced intrinsic skin aging in nephropathic cystinosis as assessed by high-definition optical coherence tomography



Chapter based on:

***Veys KRP**, Elmonem MA, Dhaenens F, Van Dyck M, Janssen MMCH, Cornelissen EAM, Hohenfellner K, Reda A, Quatresooz P, van den Heuvel B, Boone MALM, Levtchenko E.*

Enhanced Intrinsic Skin Aging in Nephropathic Cystinosis Assessed by High-Definition Optical Coherence Tomography

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ABSTRACT

Background

Nephropathic cystinosis is a rare inherited multi-systemic lysosomal storage disorder caused by mutations in the lysosomal cystine transporter cystinosin (*CTNS*). Several clinical signs suggest skin involvement to be part of the phenotype. However, the cutaneous manifestations of cystinosis have not yet been investigated in a systematic and quantitative manner.

Objectives

We aimed to investigate the dermatological manifestations of nephropathic cystinosis using high-definition optical coherence tomography (HD-OCT), the currently most advanced non-invasive optical imaging technology, and to explore its correlation with clinical disease severity.

Methods

A cross-sectional case-matched control study was conducted on 18 nephropathic cystinosis patients (11 prior to kidney transplantation), and 54 age- and sex-matched healthy controls.

Results

A significant reduction in epidermal and papillary dermis thickness of non-photoexposed skin was present in cystinosis patients of adolescent and young adult age prior to kidney transplantation. Although epidermal and papillary dermis thinning did not correlate with leucocyte cystine levels or kidney function in patients prior to kidney transplantation, epidermal thinning predicted other extra-renal disease manifestations in patients harboring the common 57kb deletion of *CTNS*. A limited sample size and the absence of a control group of chronic kidney disease and kidney transplant patients, limit further conclusions.

Conclusions

We conclude that enhanced intrinsic skin aging is an inherent phenotypic feature of cystinosis, already apparent in patients of adolescent and young adult age.

3.1 Introduction

Cystinosis (Online Mendelian Inheritance in Man (OMIM) #219800) is a rare autosomal recessive lysosomal storage disease, caused by bi-allelic mutations in the *CTNS* gene leading to malfunctioning of the cystine-proton cotransporter cystinosin [40]. Apart from its kidney disease, which is the first and most severe feature, nephropathic cystinosis is a systemic disorder in which various extra-renal manifestations develop from the second decade of life [47]. Several clinical signs suggest that the skin is involved in the phenotype. This includes the presence of a fair complexion of skin, eyes and hair in mainly Caucasian patients, while progressive coarse facial features and variable degrees of atrophy and telangiectasia can be observed in adult patients [132,134,135,297]. Cystine-crystal loaded macrophages and fibroblasts within the dermis have been demonstrated, while the impairment of melanogenesis leading to hypopigmentation has yet partially substantiated the skin manifestations in cystinosis [132,297,298]. Recently, *in vivo* reflectance confocal microscopy (RCM) was the first non-invasive optical imaging modality applied in cystinosis [134]. However, RCM has a limited penetration depth, slower acquisition time and offers no real-time visualization capabilities, making it less suitable for efficient clinical practice. Moreover, a systematic quantitative study on the dermatological involvement in cystinosis using the most advanced optical imaging technology is currently lacking. High-definition optical coherence tomography (HD-OCT) is a novel, currently most advanced non-invasive optical imaging technique available in clinical practice, offering the advantage of visualization at a higher penetration depth (up to 570 μm), fast image acquisition, real-time visualization and three-dimensional reconstruction capabilities, while keeping a resolution at the cellular level [299]. Hence, HD-OCT permits a profound insight into the epidermal and dermal ultrastructure, including dermal matrix fibers and microvasculature organization [299,300]. In addition, the assessment of the attenuation of light of the epidermis and papillary dermis by use of HD-OCT and appropriate image processing, has shown to be of significant value in the diagnosis of several skin disorders [301–303]. In this study, we hypothesized that dermal ultrastructure might be altered in cystinosis, and cystine crystal accumulation may affect the attenuation of near-infrared light of the papillary dermis. Therefore, we aimed to identify the distinctive cutaneous features of nephropathic cystinosis by means of HD-OCT, and to explore is relationship with clinical disease severity.

3.2 Materials & Methods

3.2.1 Study sample

Eighteen nephropathic cystinosis patients, followed up regularly at the University Hospitals Leuven (UZ Leuven, Belgium) and Radboud university medical center Nijmegen (Radboud umc

Nijmegen, The Netherlands), and 54 age- and sex-matched healthy control subjects were recruited (Table 3.1, Figure 3.1). In order to correlate non-invasive imaging modalities with histopathology, two adult kidney-transplanted nephropathic cystinosis patients were imaged using RCM (VivaScope® 1500, MAVIG, Munich, Germany) and HD-OCT (SkinTell® Agfa Healthcare, Mortsel, Belgium), and two punch skin biopsies (2 mm diameter) were taken per patient at the distal third of the inner side of the right upper arm at the exact same location of imaging. Approval from the local Ethical Committee (Ethische Commissie Onderzoek UZ/KU Leuven, Leuven, Belgium, study number s55514; Centrum Mensgebonden Onderzoek (CMO) Arnhem-Nijmegen, Radboud umc Nijmegen, The Netherlands, study file number 2015-2117, national number NL55449.091.15) and written informed consents were obtained from all participating subjects. Research was conducted in accordance with the last version of the Declaration of Helsinki, the principles of Good Clinical Practice (GCP) and all applicable national and international legislation related to research involving human subjects. Relevant demographic and clinical data were collected from the medical records of the cystinosis patients (Table 3.1).

3.2.2 HD-OCT image acquisition and analysis

In all participants, three HD-OCT images of the skin were taken at the distal third of the ventral side of the right upper arm, all using the same HD-OCT device (Figure 3.2). For each image acquisition, the SkinTell® probe was positioned in a similar angle, and an exact same amount of SkinTell® optical gel (12uL) was applied. Imaging was not performed on sites where ointments or crèmes, skin lesions, eruptions, neoplasia, actinic damage or congenital marks were present. Analysis and further processing of the images were performed by use of specialized software (Analyze 3D / Analyze BCC version V08, and SkinTell® Viewer version 24, Agfa Healthcare, Mortsel, Belgium) provided by the manufacturer. Within each of the three images taken in each subject, three regions of interest (ROI), originating from different quadrants of the image and exhibiting an exact same surface area (pixel delta x: 45 pixels, equal to 135μm; pixel delta Y: 38 pixels, equal to 114μm) were randomly assigned for further analysis (Figure 3.2). In total, this procedure yielded 9 observations per parameter per subject. The predefined criteria to which each ROI must comply, comprise: (1) parallelism between the surface of the skin and the surface of the SkinTell® probe, and the absence of (2) skin appendages (e.g. hair follicles, hair shafts, sweat glands), (3) foreign bodies, and (4) skin furrows in the scanned volume. In the optical coherence tomography (OCT)-signal versus imaging depth plot acquired for each ROI, specific hallmarks were assigned by use of the Analyze 3D / Analyze BCC software, that allow to compute the relevant parameters and tissue optical characteristics used in this study (Table 3.2, 3.3) [299,301–307].

3.2.3 Statistical analysis

Graphpad Prism version 7.0d for Mac OS X (GraphPad Software, La Jolla California USA, www. Graphpad.com) was used for the statistical analysis performed in this study. For each set of 9 observations per parameter that was assessed per subject, the median was calculated. A D'Agostino & Pearson normality test was applied for normality testing of the medians of the parameters of the cystinosis patients and the control subjects. Depending on this distribution, a two-tailed unpaired Student t-test or Mann-Whitney U-test was performed, or a Pearson or Spearman r correlation coefficient was computed for correlation analysis. Data are represented as mean ± standard error of mean (SEM) for normally distributed data, and as median ± 25th and 75th percentiles (inter-quartile range, IQR) for non-normally distributed data, unless otherwise specified.

3.3 Results

3.3.1 Validation of the methodology of HD-OCT image analysis

Our methodology of HD-OCT image analysis using the OCT-signal versus image depth plot, is adapted from Boone M *et al* [300] (Figure 3.2). We validated our methodology by assessing the epidermal and papillary dermis thickness and its relevant optical characteristics (Table 3.2, 3.3) in 33 young-aged (< 24 years of age) and 21 medium-aged (24 - 40 years of age) healthy controls whom were recruited as part of this study (Table 3.4). A significant reduction in epidermal and papillary dermis thickness, and an increase of the intensity at the dermal-epidermal junction was demonstrated in the young-aged versus medium-aged control subjects. These results are in line with the observations of Boone *et al* concerning intrinsic skin aging assessed by HD-OCT, hence validating our adapted methodology for HD-OCT image analysis and the use of these parameters in this study [300].

Table 3.1 Clinical and demographic characteristics of the cystinosis patient cohort

Pt	Sex	Age	Phen	Genetic background	Age at diagnosis	KTx	Age at KTx	Time since KTx
1	M	6.4	INF	57kb del + IVS10-7G>A	1.2	N	*na*	*na*
2	F	6.5	INF	Hom 57kb del	0.9	N	*na*	*na*
3	M	7.6	INF	57kb del + c.926dupG	2.4	N	*na*	*na*
4	F	12.1	INF	57kb del + c.926dupG	0.8	N	*na*	*na*
5	M	12.5	INF	c.681G>A + c.1015G>A	0.9	N	*na*	*na*
6	F	14.0	JUV	57kb del + c.198_218del21	11.3	N	*na*	*na*
7	F	16.0	INF	Hom 57kb del	2.5	N	*na*	*na*
8	F	16.6	INF	57kb del + c.18_21delGACT	1.3	N	*na*	*na*
9	M	16.9	INF	Hom 57kb del	0.8	N	*na*	*na*
10	M	18.0	INF	Hom 57kb del	0.9	N	*na*	*na*
11	M	22.2	INF	Hom 57kb del	1.0	N	*na*	*na*
12	M	24.6	INF	Hom c.926dupG	1.0	Y	8.9	15.7
13	F	25.6	INF	Hom 57kb del	1.7	Y	9.4	16.2
14	M	29.5	INF	57kb del + c.1015G>A	1.0	Y	20	9.6
15	F	30.6	INF	57kb del + c.141-24T>C	3.4	Y	12.9	17.7
16	F	34.0	INF	Hom 57kb del	1.2	Y	12.3	21.7
17	M	38.0	INF	Hom 57kb del	2.4	Y	14.0	24.1
18	F	42.2	INF	57kb del + c.926dupG	2.0	Y	11.6	
							12.3	29.9

Abbreviations: Pt: patient; Phen: cystinosis phenotype; INF: infantile nephropathic cystinosis; JUV: juvenile nephropathic cystinosis; KTx: kidney transplantation; N: no; Y: yes; *na*: not applicable

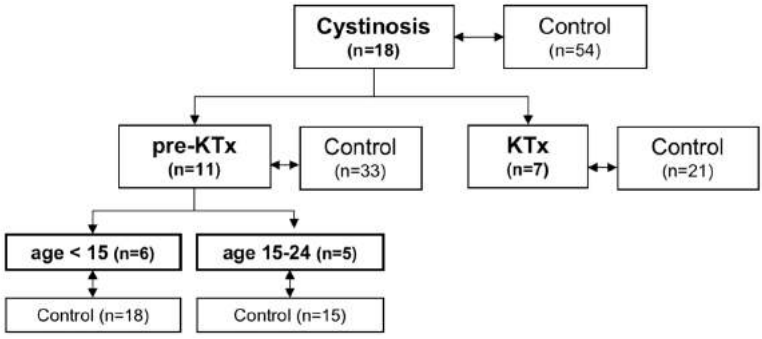

Figure 3.1 Study groups and subgroups

In this cross-sectional study, 18 nephropathic cystinosis patients were compared to 54 age- and sex-matched healthy control subjects, of which 11 patients were prior to kidney transplantation. Within the latter group, 6 patients were below the age of 15 years, while 5 patients were aged 15 to 24 years old. Each cystinosis patient was compared to three age- and sex-matched controls.

Abbreviations: KTx: kidney transplanted, pre-KTx: prior to kidney transplantation.

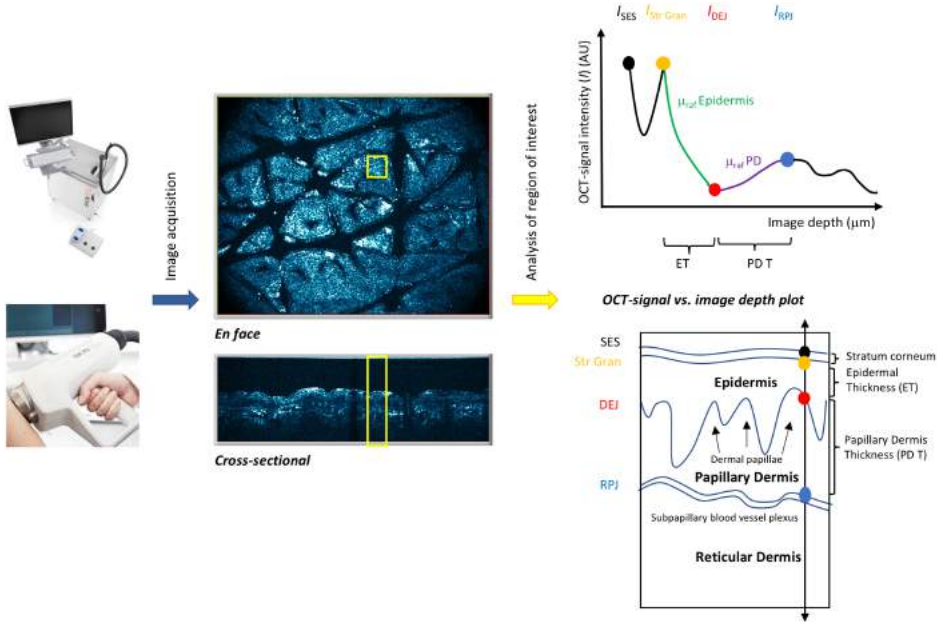

Figure 3.2 HD-OCT image acquisition and analysis workflow

In each of the three HD-OCT images acquired, taken randomly within the distal third of the ventral side of the right upper arm of each subject, three regions of interest (ROI; yellow box) are assigned for analysis (9 observations per parameter per subject) of which one is depicted in this figure (yellow box). In total, 9 ROI's originating from 3 HD-OCT images are analyzed, yielding 9 observations for each parameter per subject. The OCT-signal versus image depth plot is generated for each ROI, and specific hallmarks corresponding to anatomical substrates are assigned, yielding the parameters that are subject of this study. The sketch right below is a schematic representation of a cross-sectional view through the epidermis and dermis of the human skin. Photographs of the SkinTell® device: courtesy of Agfa Healthcare, NV; reproduced with permission.

Abbreviations: see table 3.2 & 3.3.

Table 3.2 (Left) **Established hallmarks in the OCT-signal versus image depth plot of an HD-OCT image and their corresponding anatomical substrates**

Abbreviation	Full description	Definition
SES	Skin Entrance Signal	First peak of the OCT-signal versus image depth plot.
Str. Gran.	Signal of the surface of the stratum granulosum	The first intense signal below the stratum corneum in an HD-OCT skin image, corresponding to the second peak of the OCT-signal versus image depth plot.
DEJ	Dermal-epidermal junction	The first valley in the OCT-signal versus image depth plot, following the skin entrance signal.
RPJ	Reticular-papillary junction	The highest peak in the OCT-signal versus image depth plot, following the dermal-epidermal junction.

Table 3.3 (Right) **Parameters yielded by the analysis of the OCT-signal versus image depth plot acquired from an HD-OCT skin image following the assignment of the established hallmarks**

Abbreviation	Full description	Definition	Unit
ET	Epidermal Thickness (viable epidermis)	Distance between the second peak and the first valley of the OCT-signal versus image depth plot.	µm
PD T	Papillary Dermis Thickness	Distance between the first valley and the highest peak after the valley of the OCT-signal versus image depth plot.	µm
I_{SES}	Intensity of the OCT-signal at the skin entrance	Intensity (magnitude) of the OCT signal at the first peak of the OCT-signal versus image depth plot, corresponding to the surface of the stratum corneum, expressed in arbitrary units (AU).	AU
$I_{Str\ Gran}$	Intensity of the OCT-signal at the surface of the stratum granulosum	Intensity (magnitude) of the OCT signal at the second peak of the OCT-signal versus image depth plot, corresponding to the surface of the stratum granulosum, expressed in arbitrary units (AU).	AU
I_{DEJ}	Intensity of the OCT-signal at the dermal-epidermal junction	Intensity (magnitude) of the OCT signal at the first valley of the OCT-signal versus image depth plot, corresponding to the dermal-epidermal junction (DEJ), expressed in arbitrary units (AU).	AU
I_{RPJ}	Intensity of the OCT-signal at the reticular-papillary junction	Intensity (magnitude) of the OCT signal at the highest peak after the valley of the OCT-signal versus image depth plot, corresponding to the reticular-papillary junction (RPJ), expressed in arbitrary units (AU).	AU
I_{DEJ} / $I_{Str\ Gran}$	Intensity of the dermal-epidermal junction normalized to the intensity of the surface of the stratum granulosum		AU
I_{RPJ} / $I_{Str\ Gran}$	Intensity of the reticular-papillary junction normalized to the intensity of the surface of the stratum granulosum		AU
μ_{raf} Epidermis	Relative attenuation factor of the (viable) epidermis	Direction coefficient of the slope of the log-transformed curve of the (viable) epidermis section of OCT-signal versus image depth plot. Corresponds to the reduced scattering coefficient, indicating the number of scattering events per unit of distance.	μm^{-1}
μ_{raf} PD	Relative attenuation factor of the papillary dermis	Direction coefficient of the slope of the log-transformed curve of the papillary dermis section of OCT-signal versus image depth plot. Corresponds to the reduced scattering coefficient, indicating the number of scattering events per unit of distance.	μm^{-1}

Table 3.4 Validation of our methodology of HD-OCT image analysis based on the assessment of the OCT-signal versus image depth plot

		Young-aged controls	Medium-aged controls	*p*	
n		33	21		
Age (years)	mean (± SEM)	13.4 (± 0.9)	32.4 (± 1.2)		
F:M ratio		15:18	12:9		
Thickness					
Epidermis (μm)	mean (± SEM)	86.3 (± 2.0)	73.7 (± 3.0)	*0.0008*	***
Papillary dermis (μm)	mean (± SEM)	65.9 (± 1.7)	57.1 (± 3.5)	*0.015*	*
OCT-signal Intensity					
$I_{Str\ Gran}$ (AU)	mean (± SEM)	440.9 (± 10.5)	466.6 (± 13.9)	*0.14*	
I_{DEJ} (AU)	mean (± SEM)	71.53 (± 2.6)	89.9 (± 3.1)	*< 0.0001*	***
I_{RPJ} (AU)	mean (± SEM)	162.7 (± 6.0)	181.5 (± 7.8)	*0.06*	
I_{DEJ} / $I_{Str\ Gran}$ (AU)	median (p25; p75)	0.17 (0.14; 0.18)	0.19 (0.18; 0.21)	*0.0001*	***
I_{RPJ} / $I_{Str\ Gran}$ (AU)	median (p25; p75)	0.39 (0.31; 0.43)	0.38 (0.33; 0.42)	*0.99*	
Attenuation					
μ_{raf} Epidermis (μm^{-1})	mean (± SEM)	0,020 (± 0.0003)	0.021 (± 0.0006)	*0.13*	
μ_{raf} Papillary dermis (μm^{-1})	median (p25; p75)	-0.010 (-0.013; -0.009)	-0.011 (-0.014; -0.007)	*0.95*	

A highly significant reduction in epidermal thickness, a significant reduction in papillary dermis thickness and an increase of the intensity of the OCT-signal at the dermal-epidermal junction (including the I_{DEJ} normalized to $I_{Str\ Gran}$, I_{DEJ} / $I_{Str\ Gran}$) is demonstrated in young-aged (<24 years) versus medium-aged (24-40 years) healthy control subjects. These findings are in line with the observations of Boone *et al*, validating our methodology of HD-OCT image analysis, and the use of these parameters for the objectives in this study.
Abbreviations: see table 3.2, 3.3

3.3.2 Epidermis and papillary dermis show thinning in cystinosis patients of adolescent and young adult age prior to kidney transplantation

A highly significant reduction in epidermal thickness (p=0.0074) and papillary dermis thickness (p=0.0062) could be demonstrated in all cystinosis patients compared with their age- and sex-matched controls (Table 3.5, Figure 3.3). In order to exclude possible confounding due to the potential effect of steroid treatment in kidney transplant recipients, a subgroup analysis was performed confined to cystinosis patients prior to kidney transplantation [308–316]. Remarkably, in these patients (15-24 years of age), a significant reduction in epidermal thickness (p=0.0095) and papillary dermis thickness (p=0.0305) was already present (Table 3.6, Figure 3.3).

3.3.3 Epidermal thinning predicts extra-renal manifestations in cystinosis patients harboring the homozygous 57kb deletion

To further explore the significance of epidermal and papillary dermis thinning in non-kidney transplanted cystinosis patients of adolescent age, we correlated the percentage thinning of epidermis and papillary dermis compared to their average of sex- and age-matched controls, with established indicators of disease severity. The latter include the current kidney function, expressed as the estimated glomerular filtration rate (eGFR), and the average WBC cystine levels, the latter being the current gold standard for therapeutic monitoring of cystinosis [47]. Although a linear regression analysis suggested a trend towards a more pronounced epidermal and papillary dermis thinning with a decline in kidney function and a higher average WBC cystine level, no statistical significance was reached (Figure 3.4). Remarkably, in the cystinosis patients prior to kidney transplantation harboring the common homozygous 57kb deletion of *CTNS*, which abolishes all functions of cystinosin, significant thinning of the epidermis predicted the presence of extra-renal manifestations with a maximal positive predictive value (Table 3.7).

Table 3.5 Epidermal and papillary dermis thickness and the tissue optical characteristics of the epidermis and papillary dermis as assessed by HD-OCT in cystinosis patients versus age- and sex-matched controls

		Cystinosis	Control	*p*	
n		18	54		
Age (years)	mean (± SEM)	20.7 (± 2.5)	20.8 (± 1.5)		
F:M ratio		9:9	27:27		
Thickness					
Epidermis (μm)	mean (± SEM)	71.4 (± 2.7)	81.4 (± 1.9)	*0.007*	**
Papillary dermis (μm)	mean (± SEM)	51.5 (± 3.9)	62.3 (± 1.8)	*0.006*	**
OCT-signal Intensity					
$I_{Str\ Gran}$ (AU)	mean (± SEM)	432.6 (± 14.7)	450.9 (± 8.5)	*0.28*	
I_{DEJ} (AU)	median (IQR)	79.0 (68.5; 89.1)	79.9 (68.9; 90.7)	*0.85*	
I_{RPJ} (AU)	mean (± SEM)	160.5 (± 9.4)	170 (± 4.9)	*0.35*	
I_{DEJ} / $I_{Str\ Gran}$ (AU)	median (IQR)	0.20 (0.17; 0.21)	0.18 (0.16; 0.20)	*0.05*	
I_{RPJ} / $I_{Str\ Gran}$ (AU)	median (IQR)	0.35 (0.30; 0.42)	0,39 (0.32; 0.43)	*0.24*	
Attenuation					
μ_{raf} Epidermis (μm^{-1})	median (IQR)	0.02 (0.020; 0.023)	0.02 (0.019; 0.021)	*0.11*	
μ_{raf} Papillary dermis (μm^{-1})	median (IQR)	-0.01 (-0.014; -0.007)	-0.01 (-0.013;-0.008)	*0.68*	

Abbreviations: see table 3.2, 3.3

Table 3.6 Epidermal and papillary dermis thickness in the subgroup of adolescent and young adult cystinosis patients prior to kidney transplantation (aged 15 – 24 years of age) versus age- and sex-matched controls

		Adolescent cystinosis patient prior to KTx	Control	*p*	
n		5	15		
Age (years)	mean (± SEM)	17.9 (± 1.1)	17.8 (± 0.7)		
F:M ratio		2:3	6:9		
Thickness					
Epidermis (μm)	median (IQR)	68.6 (66.4; 74.2)	81.9 (73.1; 97.4)	*0.01*	*
Papillary dermis (μm)	median (IQR)	56.5 (50.9; 57.6)	65.3 (56.5; 74.2)	*0.03*	*

Abbreviations: see table 3.2, 3.3

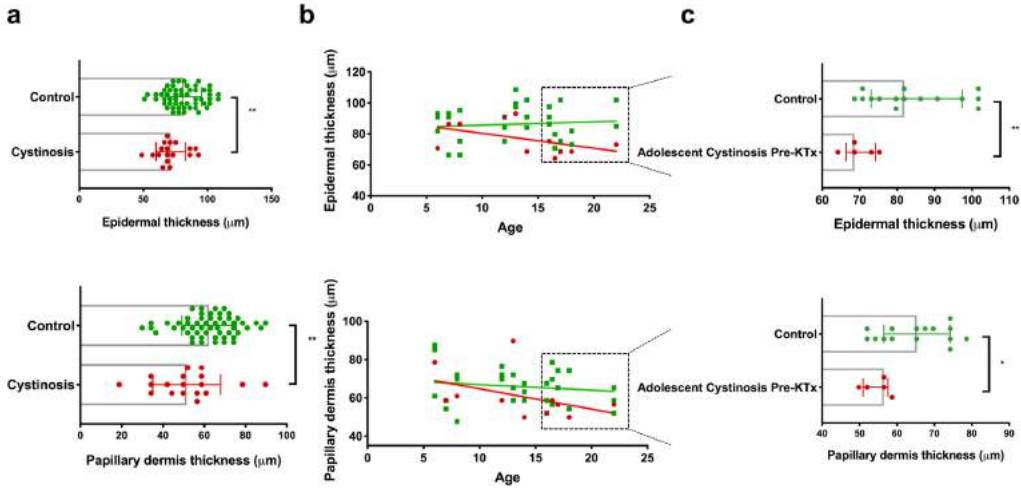

Figure 3.3 Significant reduction of the epidermis and papillary dermis thickness in cystinosis patients of adolescent and young adult age prior to kidney transplantation, compared to age- and sex-matched healthy controls, as assessed by HD-OCT.

Cross-sectional analysis of epidermal (upper graphs) and papillary dermis thickness (lower graphs) in the cystinosis patient cohort (red dots), compared to age- and sex-matched healthy controls (green dots), as assessed by HD-OCT. Each dot represents the median of 9 observations taken for each parameter per subject.

Panel a: A significant thinning of epidermis and papillary dermis is present in cystinosis compared to age- and sex-matched healthy controls. Data presented as mean ± SD.

Panel b, c: Significant epidermal and papillary dermis thinning is apparent from adolescent and young adult age. The full straight line in panel b represents the result of a linear regression performed on the cystinosis cohort (red line) or the control subjects (green line). Data presented as median ± interquartile range.

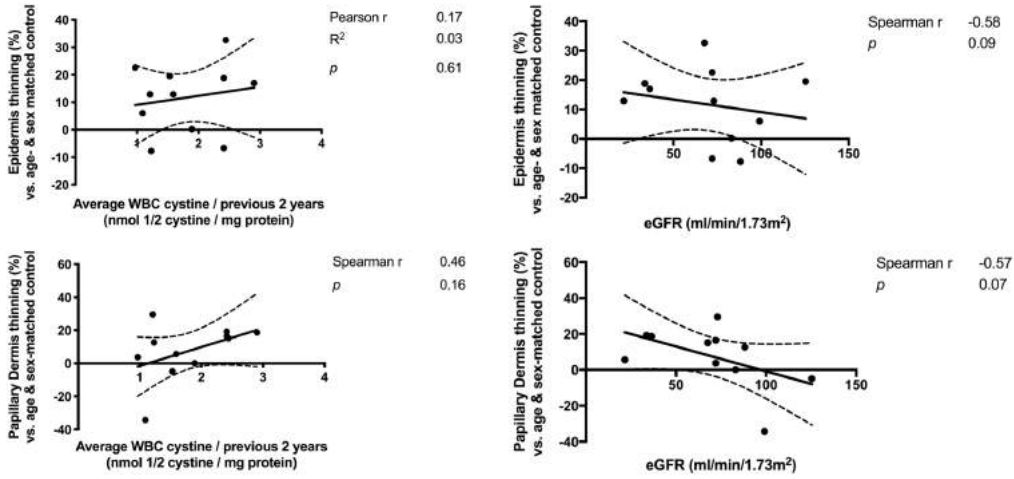

Figure 3.4 Correlation between epidermal and papillary dermis thinning, and parameters of disease severity including kidney function (eGFR) and average WBC cystine levels, does not reach significance in the cystinosis patients prior to kidney transplantation, while a trend can be observed.

Each dot represents the median of the 9 observations per subject. The straight full line represents a linear regression analysis. The dotted lines represent the 95% confidence interval.

Table 3.7 Significant thinning of the epidermis predicts the presence of extra-renal complications in non-kidney transplanted cystinosis patients harboring the common homozygous 57kb deletion of *CTNS*.

Thinning of the epidermis is expressed as the number of standard deviations below the average of the corresponding age- and sex-matched healthy control. The term "extra-renal complications" is expressed as the number of cystinosis extra-renal manifestations present in this patient. Complications that are considered to be extra-renal manifestations of cystinosis in this score, are the following: (1) retinopathy, (2) primary hypothyroidism, (3) diabetes mellitus type 1, (4) swallowing dysfunction and (5) distal myopathy. Age is expressed in years.

Patient	**Age**	**Genetic background**	**Epidermis thinning** (SD)	**Extra-renal complications** (#)
2	6.5	Hom 57kb del	0	1
7	16.0	Hom 57kb del	-2	1
9	16.9	Hom 57kb del	0	0
10	18.0	Hom 57kb del	-2	2
11	22.2	Hom 57kb del	-2	1

3.3.4 Dermal cystine crystal deposition in cystinosis does not alter the attenuation of near-infrared light of the papillary dermis, as assessed by HD-OCT

Non-invasive optical skin imaging using RCM at the ventral side of the upper arm in two kidney-transplanted nephropathic cystinosis patients, showed several highly intense, oval to needle-shaped bright deposits to be present in the papillary dermis, as shown in the representative confocal section images taken below the dermal-epidermal junction (Figure 3.5, panel a). None of these deposits could be identified in the epidermis. HD-OCT imaging at the exact same location showed similar, bright, round- to rectangular-shaped bodies (ranging 3 to 8 μm) in the papillary and reticular dermis, some surrounding the sub-papillary blood vessel plexus, as demonstrated in a representative *en face* HD-OCT image (Figure 3.5, panel b). As evidenced by histology, cystine crystal deposits were shown to be present surrounding the dermal microvasculature in the papillary and reticular dermis (Figure 3.5, panel c & d). These findings are in line with previous reports and suggest that dermal cystine crystal deposition mainly localizes in the papillary dermis, surrounding the subpapillary blood vessel plexus, while the presence of cystine crystal deposits in dermal fibroblasts (Figure 3.5, panel e & f) and the changed conformation of the elastin fibers support the notion of impaired fibroblast function which can underlie signs of premature skin aging [132,134].

Furthermore, we explored whether dermal cystine crystal deposition consistently affects the attenuation of near-infra red light (relative attenuation factor) of the papillary dermis as assessed by HD-OCT. The intensities of the OCT-signal at the dermal-epidermal junction (I_{DEJ}) and reticular-papillary junction (I_{RPJ}), the OCT signal at the RPJ normalized to the OCT signal at the stratum granulosum (I_{RPJ}/ $I_{Str\ Gran}$), nor the relative attenuation factor of the papillary dermis showed a significant difference in cystinosis patients compared to controls (Table 3.4). As a result, in cystinosis, dermal cystine crystal accumulation cannot be specifically quantified by the assessment of the relative attenuation factor of the papillary dermis using HD-OCT.

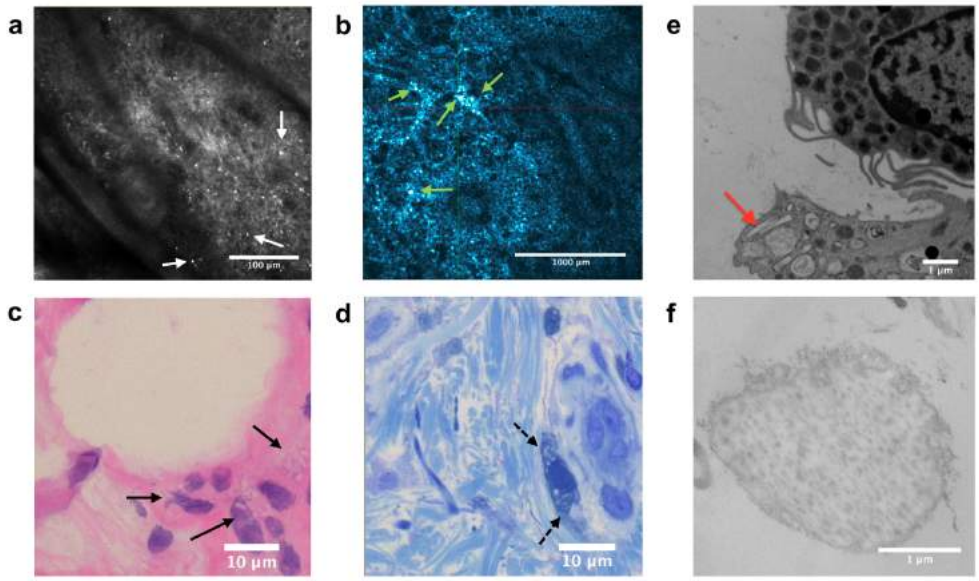

Figure 3.5 Correlation of dermatological findings at the distal third of the ventral side of the right upper arm in a kidney-transplanted nephropathic cystinosis patient (patient n°17), examined by RCM (a) and HD-OCT (b), and its corresponding histological (c, d) and electron microscopy substrate (e, f).

Panel a: Confocal section RCM image of the papillary dermis, demonstrating several intense deposits, very likely to be corresponding to cystine crystal deposits (white full arrows). Scale bar = 100 µm.

Panel b: *En face* HD-OCT image of the papillary dermis, showing intense, round to rectangular shaped deposits surrounding the subpapillary blood vessel plexus (green arrows). Scale bar = 1000 µm.

Panel c: Hematoxylin & eosin staining on frozen punch skin biopsy tissue, showing cystine crystals (black arrows), surrounding the subpapillary blood vessel plexus. Scale bar = 10 µm.

Panel d: Toluidine blue staining on a glutaraldehyde fixed skin tissue specimen. Numerous cystine crystal deposits surrounding the subpapillary blood vessel plexus in the reticular dermis and the papillary vasculature in the papillary dermis in close proximity to histiocytes (black dashed arrows). Scale bar = 10 µm.

Panel e: Electron microscopy image showing cystine crystal deposits (red arrow) in dermal fibroblasts. Electron microscopy magnification 6000x. Scale bar = 1 µm.

Panel f: Electron microcsopy image showing fragmented elastin fibers with irregular contours and elastotic-like osmiophilic inclusions. Scale bar = 1 µm.

3.4 Discussion

In this report, HD-OCT, being a novel and one of the most advanced non-invasive optical imaging techniques currently available in clinical practice, was applied to perform a systematic and quantitative assessment of dermatological manifestations in nephropathic cystinosis.

We validated our methodology of HD-OCT image analysis, adapted from Boone M *et al*, yielding epidermal and papillary dermis thicknesses and tissue optical characteristics relevant for this study [300,302]. By excluding the stratum corneum, the epidermal thickness measurements comprise only the viable epidermis, which corresponds to the approach of Gambilcher *et al* [304,307]. Noteworthy, to our knowledge, HD-OCT has not been applied yet in a pediatric and adolescent population. This underpins the need and importance of our validation while providing reference data for this age group.

We demonstrated a significant reduction of epidermal and papillary dermis thickness in cystinosis patients starting from adolescent age, while excluding a potential confounding effect of steroid treatment related to kidney transplantation [308–312,314,315,317,318]. Hereby we show in a quantitative manner that skin involvement is part of the phenotype of cystinosis.

Epidermal and dermal thinning are two hallmarks of intrinsic skin aging (ISA), which is further characterized by flattening of the dermal-epidermal junction, hypoproliferation of fibroblasts and reduced biosynthesis and increased degradation of extracellular matrix components [319–322]. Hence, our data points to enhanced intrinsic skin aging being present in cystinosis.

Intrinsic skin aging has been proposed to be related to cellular senescence, which is associated with mitochondrial DNA damage and dysfunction, and cumulative oxidative damage of the keratinocytes and dermal fibroblasts [319,320,322]. As the pathophysiology of cystinosis is characterized by enhanced apoptosis and reactive oxygen species (ROS) production, it is likely that these mechanisms underlie the skin phenotype [21–23,28,323,324]. While melanocytes and keratinocytes have been found to be one of the cell types in which cystinosin-LKG -a specific isoform of cystinosin- is most highly expressed, cystinotic dermal fibroblasts have been demonstrated to be affected by cystine crystal accumulation and show increased signs of apoptosis upon exposure to TNF-alpha, secreted by macrophages that have been activated upon engulfment of cystine crystals [19,134,325]. Moreover, cystinotic cells have impaired autophagic flux resulting in reduced clearance of old mitochondria, and impaired mitochondrial cAMP levels, which altogether results in mitochondrial dysfunction and an increased generation of ROS [39,326–329].

Chronic kidney disease (CKD) is also associated with a phenotype of premature aging, attributed to increased oxidative stress and persistent low-grade inflammation due to uremia and dialysis [330]. Epidermal atrophy is described at several CKD stages, and increases with duration of dialysis

[331–333]. On the other hand, increased IL-1β and IL-18 plasma levels in *in vivo* studies also suggest a certain state of systemic inflammation in cystinosis, induced by the widespread presence of cystine crystals throughout several tissues of the body [25,26]. Although we did not include a control group of chronic kidney disease nor kidney transplant patients, epidermal and papillary dermis thinning did not show a significant correlation with kidney function in our cohort, hence ruling out CKD as the single determining cause of our observations. Moreover, the absence of this correlation demonstrates that the skin manifestations of the cystinosis phenotype seem not to be related only to the severity of the kidney disease. This is also in line with observations of Chiavérini *et al* in the RCM pilot study in cystinosis [134]. Interestingly, while it could be speculated that a more pronounced dermal cystine crystal load could be associated with more pronounced signs of aging of the (papillary) dermis, no significant correlation could be demonstrated between papillary dermis thinning and the average WBC cystine level [19]. However, it remains unclear whether WBC cystine levels adequately reflect cystine accumulation in the cells of epidermis and dermis. On the other hand, the predictive value of epidermal thinning for the presence of other extra-renal organ manifestations in patients with the common *CTNS* 57kb deletion which results in a complete absence of the protein- in contrast to other mutations, suggests that similar mechanisms, which are not necessarily related solely to the transport of cystine by cystinosin, might underlie the nature of the multi-organ disease in cystinosis.

Furthermore, the absence of a significant difference in the relative attenuation factor of the papillary dermis between cystinosis patients and healthy controls, which suggest that the OCT-signal of dermal cystine crystal deposits is non-specific, can be explained by the following factors. First, the presence and intensity of the OCT-signal originating from a cystine crystal deposit depends on its size, shape, and orientation towards the incident near-infrared light beam. Second, HD-OCT shows a slightly lower lateral resolution (3 μm) compared to RCM (1 μm), while the size of dermal cystine crystal deposits ranges from 1 to 10 μm and most crystals tend to show a size of around 3μm, which impedes morphological identification. And third, it is very likely that the number of cystine crystals and their effect size is too low to affect the overall attenuation of a tissue layer, while the low sample size decreases the likelihood that statistical significance can be reached. As a result, we can conclude that RCM serves as a complimentary technique to HD-OCT at the level of identification and quantification of dermal cystine crystal deposits.

Taken together, we conclude that enhanced intrinsic skin aging is a novel phenotypic feature of cystinosis, presenting at adolescent and young adult age, as demonstrated by high-definition optical coherence tomography.

Conflict of Interest disclosure

The authors state no conflict of interest.

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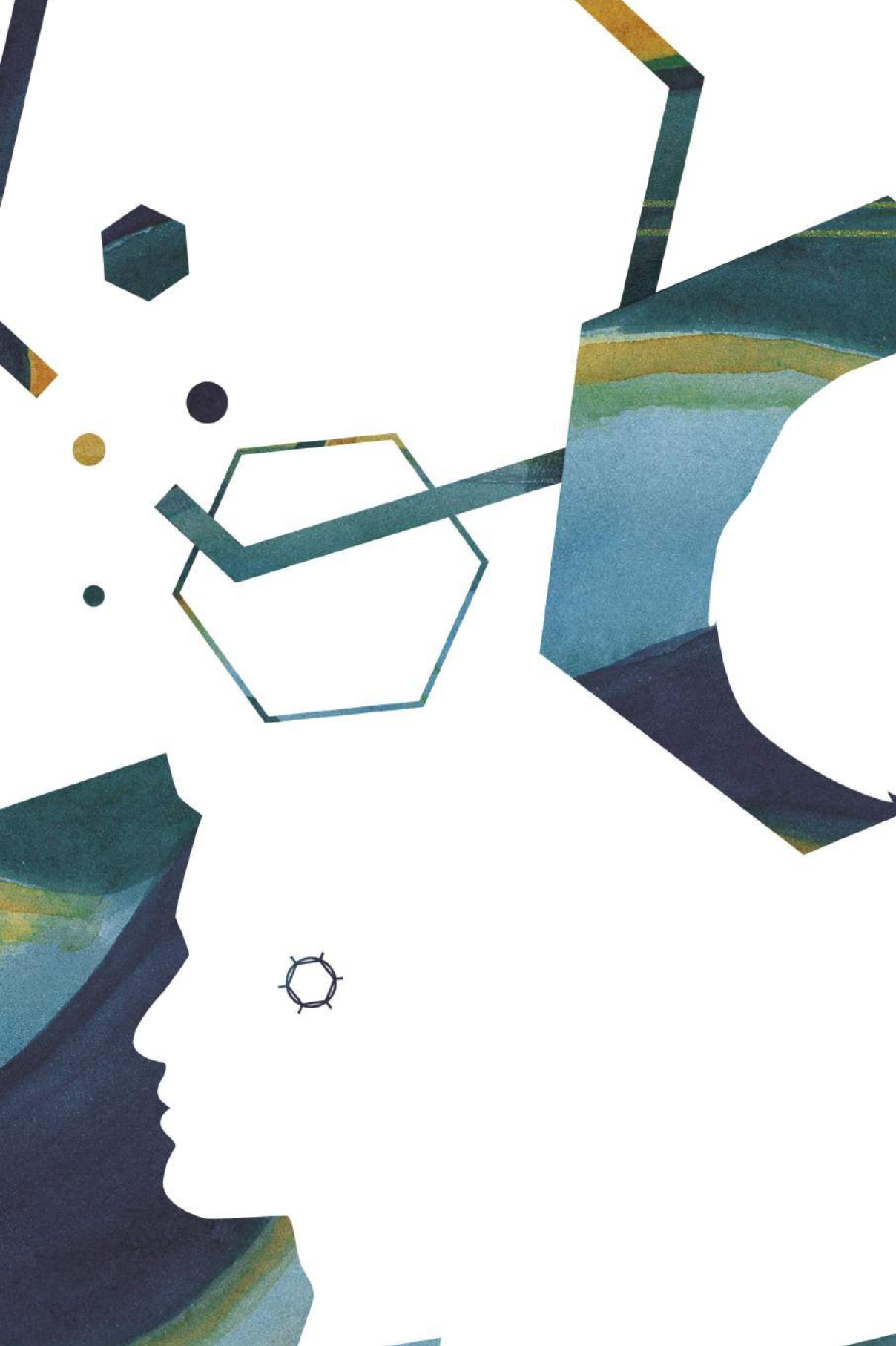

CHAPTER 4
Chitotriosidase: a novel additional biomarker for therapeutic monitoring of nephropathic cystinosis

Chapter based on:

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Biomarkers of macrophage activation for the therapeutic monitoring of nephropathic cystinosis: a prospective multicenter longitudinal study

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Journal of the American Society of Nephrology (JASN)

ABSTRACT

Background and objectives

Nephropathic cystinosis is a hereditary lysosomal storage disorder caused by the dysfunction of the lysosomal co-transporter cystinosin, leading to cystine accumulation and cellular damage in different organs, particularly in the kidney. Cystinosis is treated by the amino thiol cysteamine; however, in order to achieve optimal efficiency, close therapeutic monitoring is recommended. White blood cell (WBC) cystine concentration is the current gold standard for therapeutic monitoring, but the assay is technically demanding and only available in few laboratories in developed nations. Since macrophages are among the most affected cells by cystine accumulation and based on promising pilot data suggesting an important role of inflammation in the pathogenesis of cystinosis, we aimed to explore the potential of biomarkers of macrophage activation for the therapeutic monitoring of cystinosis.

Design, participants and measurements

We conducted a prospective longitudinal study, in which we recruited 61 cystinosis patients receiving cysteamine therapy from three European reference centers: Leuven (Belgium), Nijmegen (The Netherlands) and Traunstein (Germany) from October 2015 to January 2018. During each visit, samples were collected and measured for four biomarkers of macrophage activation (IL-1β, IL-6, IL-18, and chitotriosidase enzyme activity).

Results

Through a multivariate linear regression analysis of the longitudinal data, chitotriosidase enzyme activity and IL-6 resulted as significant independent predictors for WBC cystine levels in cystinosis patients of all ages, while in a ROC analysis chitotriosidase was superior to IL-6 in distinguishing good versus poor (WBC cystine < versus ≥ 2 nmol ½ cystine/mg protein) therapeutic control. Moreover, in patients harboring at least one extra-renal complication, chitotriosidase significantly correlated with the number of extra-renal complications and was superior to WBC cystine levels in predicting the presence of multiple extra-renal complications.

Conclusions

Chitotriosidase enzyme activity may serve as a biomarker for therapeutic monitoring of nephropathic cystinosis.

4.1 Introduction

Nephropathic cystinosis (MIM 219800) is an autosomal recessive lysosomal storage disorder caused by bi-allelic mutations in the *CTNS* gene, which codes for the lysosomal cystine transporter cystinosin. Given *CTNS* is ubiquitously expressed, the absence or malfunction of cystinosin leads to the accumulation and subsequent crystallization of cystine in the lysosomes in all tissues throughout the body [5,40,47]. The earliest and most severely affected organ is the kidney, which manifests with a severe proximal tubular dysfunction (renal Fanconi syndrome) during the first year of life. This is characterized by the defective tubular reabsorption and loss of different solutes in urine resulting in polyuria, failure to thrive and rickets. Later on, glomerular function is affected and end stage kidney disease (ESKD) develops during late childhood or early adolescence [41]. Extra-renal complications (ERC) develop in life mainly affecting the eyes (corneal cystine crystals, keratopathy, retinopathy), endocrine system (primary hypothyroidism, insulin-dependent diabetes mellitus, primary hypogonadism) and neuromuscular system (peripheral myopathy, swallowing dysfunction, central nervous system involvement), among others [47].

Cysteamine is the only available disease-modifying treatment for cystinosis [334,335]. A strict therapeutic monitoring regimen is needed to ensure compliance and efficiency, given that the onset and severity of cystinosis complications are greatly dependent on adherence to optimal dosage [336,337]. Currently, the only available therapeutic monitor for cystine-depleting therapy in cystinosis is the white blood cell (WBC) cystine concentration. Although cystine is the most specific marker for the disease, the WBC cystine assay is not the most practical nor ideal monitoring tool. The common methodologies for cystine measurement (high performance liquid chromatography or liquid chromatography tandem mass spectrometry) [147,338] are technically demanding and expensive, such that the assay is lacking in most developing countries. Moreover, it suffers from inherent impracticalities and difficulties in sampling and storage [25,147,338]. In addition, as neutrophils, the main cystine accumulating cells in blood, have a very short lifespan (<24h) [339], its measurement represents only a very short period of compliance or therapeutic efficiency [25]. WBC cystine levels may not be representative of the total body tissue cystine load, and in case of future treatment modalities such as hematopoietic stem cell-based gene therapy, WBC cystine levels will be obsolete. Hence, the search for alternative biomarkers for therapeutic monitoring which is more practical in use, representative of the long-term therapeutic control and future-treatment proof, is very important [47].

Among cells affected by tissue cystine accumulation, macrophages are particularly amenable. Due to their phagocytic nature, they engulf the debris of dead cells in different tissues including

the cystine crystals inside and around these cells. In addition, lacking the cystine transporter themselves, they are unable to get rid of the accumulated cystine [25]. Moreover, macrophages are long living cells and known to produce considerable amounts of various inflammatory biomarkers in the circulation when activated. Recently, Prencipe *et al*. have reported activation of the inflammasome system in cystinotic cells, with the detection of several cytokines significantly elevated in the plasma of cystinosis patients [340]. Elmonem *et al*. further identified the significant elevation of plasma chitotriosidase activity, an enzyme produced by activated macrophages, in a substantial cohort of cystinosis patients compared with both healthy and pathological renal control groups in a cross sectional analysis [25].

In the current paper, we report the results of the first longitudinal prospective study designed to investigate the potential of four biomarkers of macrophage activation (IL-1β, IL-6, IL-18 and plasma chitotriosidase enzyme activity), as therapeutic monitors for nephropathic cystinosis to compliment or replace the WBC cystine assay.

4.2 Materials and Methods

4.2.1 Patients

A prospective international multicentric study was set up in which sixty-one nephropathic cystinosis patients on cysteamine therapy were sequentially recruited during the period of the study (October 2015 – January 2018) from three European cystinosis reference centers (University Hospitals Leuven, Belgium; Radboud university medical center (umc) Nijmegen, The Netherlands; SüdOstBayern Kliniken Traunstein; Germany). Only patients aged 6 months or older, under cysteamine treatment were eligible for recruitment. At the baseline visit, a detailed medical history was obtained and a multisystem clinical examination was performed. On average, patients were followed up every 3-6 months. At each visit, history and clinical examination were recorded and blood and urine samples were obtained for routine laboratory parameters, including complete blood count, C-reactive protein (CRP), serum creatinine and WBC cystine levels. Elevated WBC count and CRP were used to exclude patients with ongoing infection. Estimated glomerular filtration rate (eGFR) was calculated based on the Schwartz equation for children and the chronic kidney disease epidemiology collaboration (CKD-EPI) equation for adult cystinosis patients. Data on the extra-renal complications (ERC) were obtained from all patients and the status of ERC was expressed as a 5-topic score, based on the five most important and widely recognized complications of in nephropathic cystinosis: (1) primary hypothyroidism, (2) retinopathy, (3) insulin-dependent diabetes mellitus, (4) peripheral myopathy, and (5) swallowing dysfunction (Table 4.1). Patients carrying a homozygous 24-bp

duplication in exon 10 of the *CHIT1* gene (3 patients), or of whom no WBC cystine values nor kidney function could be obtained for the corresponding plasma chitotriosidase activity levels (2 patients) were excluded from further statistical analysis.

Table 4.1 Defining criteria for the diagnosis of the five main established cystinosis extra-renal complications

	Cystinosis extra-renal complication	Diagnostic criteria	Ref
1	Primary hypothyroidism	(1) Biochemical evidence for primary hypothyroidism (increased thyroid stimulating hormone (TSH) > 10 mIU/L combined with a decreased free thyroxin (FT4) level) and/or (2) Treatment with age- & weight appropriate dosage of L-thyroxin	68–70
2	Retinopathy	(1) Clinical signs and symptoms of nyctalopia, impaired color vision, visual field restriction and/or retinal blindness and/or (2) Fundoscopic findings of (peripheral) retinal pigmentary changes (incl. depigmentation and pigmentary mottling) and retinal degeneration, and/or findings of visual field restriction by perimetry	63
3	Insulin dependent diabetes mellitus (Diabetes mellitus type 1)	(1) Biochemical evidence of insulin dependent diabetes mellitus (HbA1c > 6.5%, fasting glucose > 126mg/dl, C-peptide < 0.2 nmol/L) and/or (2) Treatment with appropriate dosages of insulin	72
4	Peripheral myopathy	Clinical signs of significant muscular atrophy of the thenar- and hypothenar musculature, combined with a history of reduced grip strength	80,82,341
5	Swallowing dysfunction	History of difficulties on swallowing solids and/or fluids	84

Defining criteria applied in the retrospective medical record analysis in this cystinosis patient cohort for diagnosing cystinosis extra-renal complications.
Abbreviations: Ref: reference

4.2.2 White blood cell cystine assay

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for WBC cystine evaluation in the three countries of the study as previously described [147]. The concordance of results between the three measuring laboratories was confirmed by the participation in the ERNDIM (European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited Disorders of Metabolism) external quality assurance program for WBC cystine and the successful quantification of eight blinded samples of WBC cystine annually. The average value during the study period was calculated for each patient.

4.2.3 Chitotriosidase enzyme assay

Plasma chitotriosidase activity was assayed based on the fluorometric method developed by Hollak et al with minor modifications as previously described [342,343].

4.2.4 Genetic analyses

The detection of the 24-bp duplication mutation in exon 10 of the *CHIT1* gene was performed as described before [344]. *CTNS* gene analysis was performed in 45 of the 61 patients.

4.2.5 Cytokines assays

The following cytokines were assayed in plasma of each patient for each visit during the study: IL-1β, IL-6 (Quantikine, R&D systems, Minneapolis, MN, USA) and IL-18 (MBL Corporation, Nagoya, Aichi, Japan). Samples were assayed by a sandwich ELISA technique according to manufacturers' protocols.

4.2.6 Ethical approval

This study was approved by the corresponding ethical committees at University Hospitals Leuven, Belgium (Ref. No. s55514(ML9454)), Radboud University Medical Center, Nijmegen, the Netherlands (Ref. No. 2015-2017) and Kliniken Südostbayern, Traunstein, Germany (Ref. No. 2016-013). Informed consents were translated to match the native language of each patient and were signed by all recruited patients or their legal guardians in case of minors.

4.2.7 Statistical analysis

Statistical analysis was performed using Graphpad Prism (version 8.1.0 (221) for Macintosh) and SAS software (version 9.4 of the SAS System for Windows).

D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests were used for normality assessment, and depending on the distribution, parametric or non-parametric tests were applied. In the analysis of the longitudinal data for determining predictors of WBC cystine, linear mixed models were applied (Table 4.3). In the correlation analysis between the proposed biomarkers and the number of extra-renal complications in the subgroup of patients harboring at least one extra-renal complication, a Spearman r correlation coefficient was calculated for each variable assessed (Table 4.5). Receiver Operating Curves (ROC) were created and evaluated using easyROC statistical software [345]. Data were represented as ratios and percentages for categorical variables and as means and standard deviations (SD) or medians and inter-quartile range (IQR) for numerical variables, unless otherwise stated. Statistical significance was based on the 95% confidence intervals (95% CI) or a P value < 0.05 All reported P values are two-sided, unless otherwise stated.

4.3 Results

4.3.1 Baseline demographic, clinical and laboratory characteristics of cystinosis patients

Demographic, clinical and laboratory data on all recruited cystinosis patients are summarized in Table 4.2. Of the 61 cystinosis patients, WBC cystine levels were obtained in 57 patients, due to unavailability of access to the WBC cystine assay at the moment of regular care follow-up visits. Of these 57 patients, 22 were children, 5 were affected with the juvenile cystinosis phenotype,

26 patients harbored at least one extra-renal complication and 30 patients had undergone kidney transplantation before the start of the study. For each cystinosis patient, 2-9 visits were recorded (mean 4.7 ± 2.5), during which samples for macrophage biomarkers were obtained. The average duration of follow-up for all recruited patients was 19.3 ± 6.5 months. None of the patients received kidney graft during the course of the study. Three patients had very low chitotriosidase activity and were confirmed homozygous for the 24-bp duplication of the *CHIT1* gene.

4.3.2 Plasma chitotriosidase enzyme activity as a potential additional therapeutic monitor for cystine-depleting therapy

Forty-two patients showed WBC cystine values, on average, below 2 nmol ½ cystine/mg protein during the study (73.6%) and were considered as sufficiently controlled, while 15 patients showed values above 2 nmol ½ cystine/mg protein (26.3%), corresponding to poor disease control or insufficient adherence to cystine-depleting therapy. Patients with WBC cystine levels below and above 2 nmol ½ cystine/mg protein did not significantly differ in age, gender, clinical phenotype of cystinosis, or the most commonly recognized extra-renal complications of cystinosis (Table 4.2). In addition, in the subgroup of patients without kidney transplantation, no significant difference in eGFR was observed.

Among the four biomarkers of macrophage activation tested, plasma chitotriosidase enzyme activity showed significantly different levels over the categories of WBC cystine levels (< 1, 1-2, 2-3, ≥ 3 nmol ½ cystine/mg protein) when considering the average values over the whole 2-year study period (Figure 4.2, panel a). In addition, a significant linear correlation was demonstrated between the average chitotriosidase activity and WBC cystine levels (Figure 4.2, panel b). Furthermore, in a mixed-effects multivariate regression analysis in all cystinosis patients, chitotriosidase activity resulted as a significant predictor for WBC cystine levels, apart from age and IL-6 (Table 4.3). In a ROC analysis, the performance of chitotriosidase activity and IL-6 concentration was assessed in distinguishing WBC cystine levels below or equal to and above 2 nmol ½ cystine/mg protein, corresponding to good versus poorly controlled cystinosis patients. Herein, chitotriosidase activity resulted superior to IL-6 (Figure 4.2). A cut-off value for chitotriosidase of 150 nmol/ml plasma/h resulted in a corresponding sensitivity of 53% (30% to 75%), specificity of 85% (70% to 93%), and a negative predictive value (NPV) of 83% (68% to 91%) for distinguishing good versus poor therapeutic control (Figure 4.2, Table 4.6).

Table 4.2 Demographic, clinical and laboratory parameters of the cystinosis patient cohort and subgroups stratified according to WBC cystine level < or ≥ 2 nmol ½ cystine/mg protein.

		All cystinosis patients	2-year average WBC cystine level				Difference (95% CI of the difference)	*p*
			<2 nmol 1/2 cystine/mg protein		≥2 nmol 1/2 cystine/mg protein			
Age at recruitment	years (mean ± SD)	22.0 ± 11.2	21.1 ± 11.6		24.4 ± 9.6		3.3 ± 3.4 (-3.4 to 10)	0.32
Age category at recruitment	children/adults (≥ 18) (% children)	39%	18/24	43%	4/11	27%	16% (-8% to 48%)	0.36
	< 12 (%)	19%	10/42	24%	1/14	7%	17% (-0.05% to 46%)	0.26
	12-18 (%)	19%	8/42	19%	3/12	20%	1% (-20% to 31%)	> 0.99
	18-30 (%)	42%	16/42	38%	8/7	53%	15% (-15% to 43%)	0.36
	30-40 (%)	12%	5/42	12%	2/13	13%	1% (-17% to 31%)	> 0.99
	> 40 (%)	7%	3/42	7%	1/14	7%	0% (-14% to 28%)	> 0.99
Gender	male/female (% male)	51%	22/20	52%	7/8	47%	4% (-20% to 29%)	0.77
Phenotype	Infantile/Juvenile (% Infantile)	91%	38/4	90%	14/1	93%	3% (-25% to 18%)	> 0.99
Genetic background	Hom 57kb del/other (% hom 57kb del)	48%	13/19	41%	8/4	67%	38% (3% to 6%)	0.02
Kidney transplantation	Yes/No (% Yes)	53%	19/23	45%	11/4	73%	28% (-5% to 51%)	0.08
eGFR	nKTx (ml/min/1.73m^2)	65.1 ± 30.3	66.3 ± 32.1		58.7 ± 21.2		-7.6 ± 16.8 (-42.4 to 27.2)	0.66
	KTx(ml/min/1.73m^2)	59.3 ± 29.5	69.9 ± 24.9		40.9 ± 28.7		-29 ± 10 (-49.5 to -8.6)	0.007

Table 4.2 (continued) Demographic, clinical and laboratory parameters of the cystinosis patient cohort and subgroups stratified according to WBC cystine level < or ≥ 2 nmol ½ cystine/mg protein.

		All cystinosis patients	2-year average WBC cystine level				Difference (95% CI of the difference)	*p*
			<2 nmol 1/2 cystine/mg protein		≥2 nmol 1/2 cystine/mg protein			
Extra-renal complications	Yes/No (% Yes)	46%	19/23	45%	7/8	47%	2% (-28% to 31%)	> 0.99
	Primary hypothyroidism	28%	10/32	24%	6/9	40%	16% (-11% to 46%)	0.32
	Retinopathy	4%	1/41	2%	1/14	7%	5% (-9% to 32%)	0.46
	Diabetes mellitus type 1	5%	2/40	5%	1/14	7%	2% (-12% to 29%)	> 0.99
	Peripheral myopathy	25%	9/33	21%	5/10	33%	12% (-14 to 42%)	0.49
	Swallowing dysfunction	18%	8/34	19%	2/13	13%	6% (-13% to 36%)	> 0.99
WBC cystine level	nmol ½ cystine/mg protein	1.44 (0.64; 2.3)	1.12 (0.54; 1.47)		3.2 (2.56; 4.85)		2.08 (1.8 to 2.97)	< 0.0001
	< 1 (%)	32%	18/24	43%	*na*	*na*		
	1-2 (%)	42%	24/18	57%	*na*	*na*		
	> 2 (%)	26%	*na*	*na*	15/0	100%		
Disease severity classification	nKTx, ERC- (% Y)	30%	13/29	31%	4/11	27%	4% (-20% to 36%)	> 0.99
	nKTx, ERC+	18%	10/32	24%	0/15	0%	24% (8% to 52%)	0.049
	KTx, ERC-	25%	10/32	24%	4/11	27%	3% (-21% to 33%)	> 0.99
	KTx, ERC+	28%	9/33	21%	7/8	47%	25% (-4% to 53%)	0.09
Chitotriosidase *(Corrected for CHIT)*	nmol/ml plasma/h (median (IQR))	77.11 (49.88; 166.3)	71.33 (43; 128.5)		160 (69.75; 445.3)		88,67 (18.83 to 266.3)	0.006
IL-1ß	(pg/ml) (median (IQR))	0.13 (0.13; 0.19)	0.13 (0.13; 0.19)		0.13 (0.13; 0.18)		0 (-0.01 to 0)	0.6
IL-6	(pg/ml) (median (IQR))	1.68 (1.15; 2.85)	1.56 (1; 2.46)		2.08 (1.68; 4.32)		0.52 (0.09 to 1.72)	0.02
IL-18	(pg/ml) (median (IQR))	717.0 (499.3; 939.3)	705.8 (502.9; 936.5)		747.5 (466.1; 995.9)		41.69 (-174.4 to 281.8)	0.80

Data on the WBC cystine levels were obtained in 57 of all 61 patients; he. Good or poor therapeutic control is assumed here based on WBC cystine levels < or ≥ 2 nmol ½ cystine per mg proteins, respectively. Data on the genetic background of cystinosis was obtained in 45 of the 61 patients in the whole cystinosis cohort. 3 out of 61 patients harboured a 24-bp duplication mutation in exon 10 of the *CHIT1* gene, hence data on chitotriosidase was corrected for patients with this duplication by excluding them.
Abbreviations: *na*: not applicable; CHIT: chitotriosidase genotype.

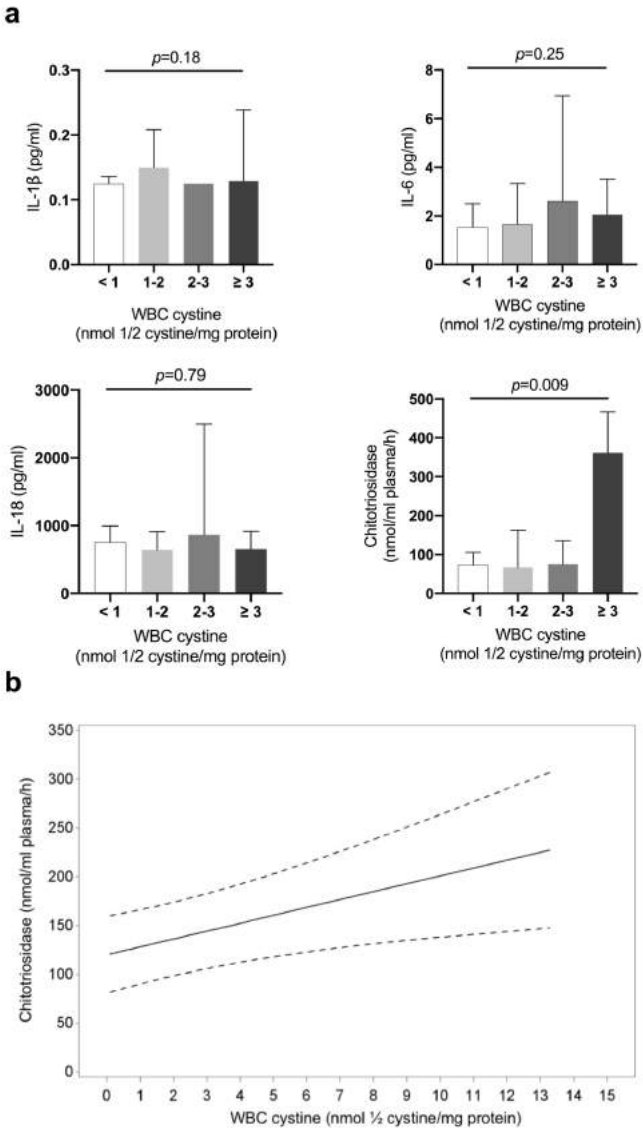

Figure 4.1 Plasma chitotriosidase enzyme activity levels significantly correlate with the 2-year average WBC cystine levels in the whole cystinosis patient cohort.

Panel a: Median values for biomarkers of macrophage activation in cystinosis patients in relation to WBC cystine levels. Biomarker values (IL-1β, IL-6, IL-18 and chitotriosidase) were categorized to WBC cystine values: <1, 1-2, 2-3 and ≥ 3 nmol ½ cystine/ mg protein. Data is presented as median ± IQR. P values were based on Kruskal-Wallis test.

Panel b: Chitotriosidase shows a significant linear correlation with WBC cystine levels in the whole cystinosis patient cohort. Correlation is based on the 2-year average values of chitotriosidase and corresponding WBC cystine levels per subject over the course of the study. The full line represents the estimated linear trend, with the dashed lines representing the 95% CI.

Table 4.3 Mixed-effects multivariable linear regression analysis on the longitudinal data for predictors of WBC cystine level in all cystinosis patients.

Variable	Level	Estimate (95% CI)	*p*
WBC cystine **All cystinosis patients** (n=41)			
Age at recruitment		-0.95 (-1.78;-0.13)	0.02
Gender	F	-0.48 (-1.04;0.07)	0.09
Chitotriosidase		0.005 (0.003;0.007)	<.0001
IL-1β		0.22 (-1.14;1.59)	0.75
IL-6		0.12 (0.05;0.19)	0.0008
IL-18		0.00 (-0.00;0.00)	0.56
Genetic background	Hom 57kb del	0.23 (-0.33;0.79)	0.42

Only IL-6 and plasma chitotriosidase enzyme activity are significant predictors for WBC cystine levels. Data on all variables as predictors of WBC cystine was only available in 41 of the 61 cystinosis patients. *Abbreviations*: CI: confidence interval.

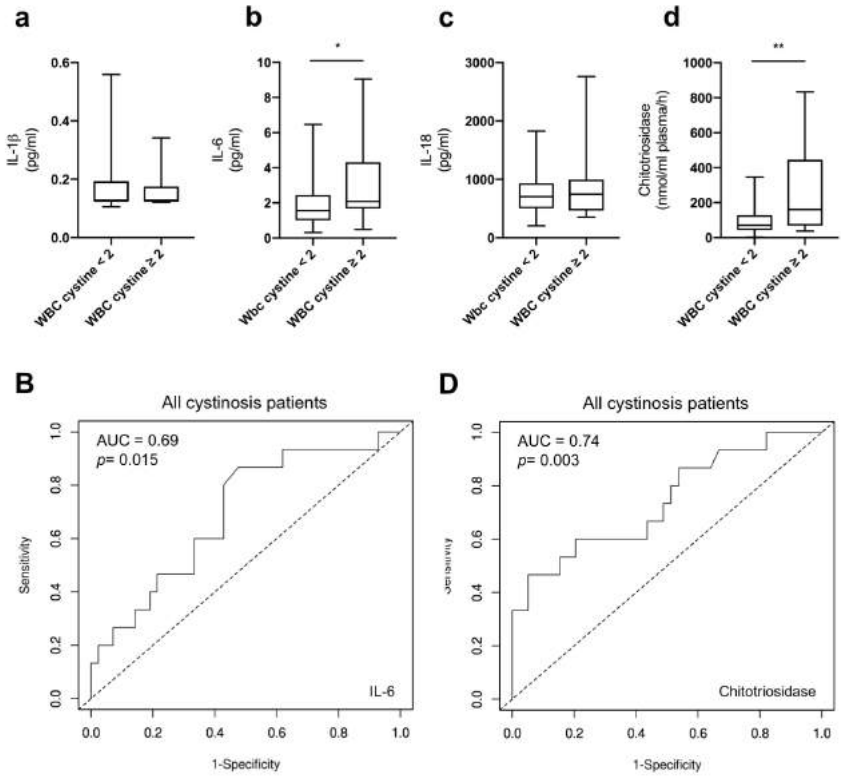

Figure 4.2 Plasma chitotriosidase enzyme activity performs superior to IL-6 of all tested biomarkers of macrophage activation, in distinguishing good vs. poor therapeutic control (WBC cystine < 2 vs. ≥ 2 nmol 1/2 cystine/mg protein).

Panel a-d: Box and whisker plots for IL-1β, IL-6, IL-18 and plasma chitotriosidase enzyme activity levels in all cystinosis patients, according to the 2-year average WBC cystine level (< 2 or ≥ 2 nmol ½ cystine/mg protein).
Panel B & D: Receiver Operating Curve (ROC) analysis of IL-6 and plasma chitotriosidase enzyme activity demonstrating their performance for distinguishing good vs. poor therapeutic control.

4.3.3 Chitotriosidase enzyme activity as a surrogate biomarker for disease extent

Twenty-six of the 61 patients showed at least one extra-renal complication. Figure 4.3 depicts a further characterization of this subgroup concerning the number and type of ERC, while relevant demographic and clinical data are depicted in Table 4.4.

In a correlation analysis of the four proposed markers of macrophage activation, only chitotriosidase activity, apart from WBC cystine levels, yielded a significant correlation with the number of ERCs (Table 4.5).

Upon grouping patients with one versus multiple extra-renal complications, a significant difference was observed in age at recruitment, the status of kidney transplantation, the presence of specific ERCs including primary hypothyroidism and swallowing dysfunction, the average WBC cystine level, and the average chitotriosidase activity level (Table 4.4).

In a ROC analysis, the performance of plasma chitotriosidase enzyme activity for distinguishing the presence of only one versus multiple extra-renal complications was superior (AUC Chitotriosidase 0.83 (95% CI 0.64 – 1.01)) to WBC cystine levels (AUC WBC Cystine: 0.75 (95% CI 0.53 – 0.96)) (Figure 4.4, 4.5; Table 4.6).

When considering a chitotriosidase activity level below or above 250 nmol/ml plasma/h as a cut-off, age, WBC cystine levels, presence and number of ERCs were significantly associated with higher chitotriosidase activity levels (Table S4.1).

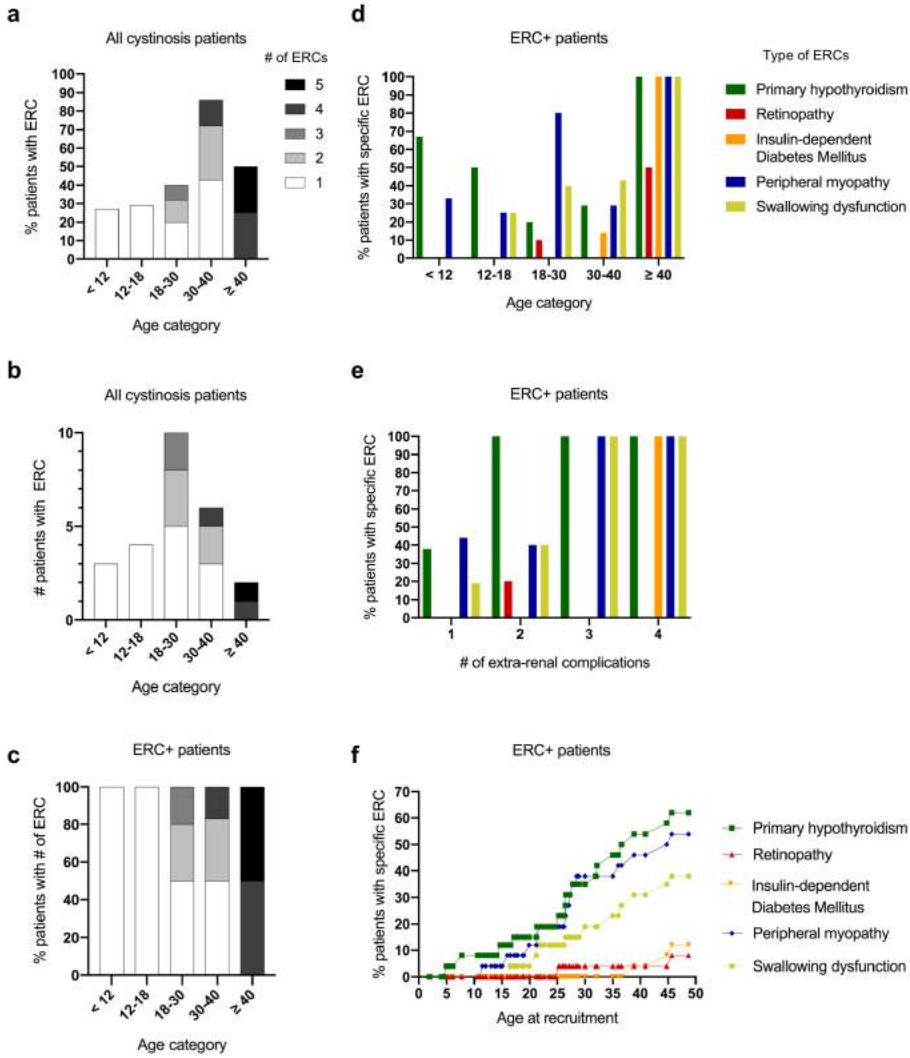

Figure 4.3 Characterization of the subgroup of cystinosis patients harbouring at least one extra-renal complication (ERC).

Panel a: The percentage, or (panel b) absolute number of patients (y-axis) of the cystinosis cohort within a specific age category (x-axis: < 12, 12-18, 18-30, 30-40, ≥ 40) harbouring a certain number of extra-renal complications (1 to 5, according to greyscale of bars).

Panel c: The percentage of patients of the subgroup of ERC+ cystinosis patients within a defined age category harbouring a certain number of extra-renal complications.

Panel d: The percentage of patients of the subgroup of ERC+ patients within a defined age category harbouring a specific extra-renal complication, according to color code (green: primary hypothyrpoidism, red: retinopathy, orange: insulin-dependent diabetes mellitus, blue: peripheral myopathy, yellow: swallowing dysfunction).

Panel e: The percentage of patients of the subgroup of ERC+ patients with a specific extra-renal complication, stratified according to the number of extra-renal complications

Panel f: The percentage of patients of the subgroup of ERC+ patients harbouring a specific extra-renal complication, plotted according to the age at recruitment of the respective subject.

Table 4.4 Demographic, clinical and laboratory parameters of the subgroup of cystinosis patients with extra-renal complications, according to the presence of one (1 ERC) or multiple (> 1 ERC) extra-renal complications.

		All cystinosis patients (n=57)	Extra-renal complication status (n=26)				Difference (95% CI of the difference)	*p*
			1 ERC (n=16)		> 1 ERC (n=10)			
Age at recruitment	years (mean ± SD)	22.0 ± 11.2	21.1 ± 9.2		32.8 ± 8.6		11.8 ± 3.6 (4.3 to 19.2)	0.003
Age category at recruitment	children/adults (≥ 18) (% children)	39%	7/9	44%	0/10	0%	44% (18% to 85%)	0.02
	< 12 (% Yes)	19%	3/13	19%	0/10	0%	19% (-9% to 56%)	0.26
	12-18	19%	4/12	25%	0/10	0%	25% (-3% to 63%)	0.14
	18-30	42%	6/10	38%	5/5	50%	13% (-28% to 49%)	0.69
	30-40	12%	3/13	19%	3/7	30%	11% (-24% to 49%)	0.64
	> 40	7%	0/16	0%	2/8	20%	20% (-9% to 56%)	0.14
Gender	male/female (% male)	51%	10/6	63%	6/4	60%	3% (-35% to 41%)	> 0.99
Phenotype	Infantile/Juvenile (% Infantile)	91%	16/0	100%	10/0	100%	∞ (-0.34 to 0.24)	> 0.99
Genetic background	Hom 57kb del/other (% hom 57kb del)	48%	8/3	73%	5/3	63%	10% (-31% to 53%)	> 0.99
Kidney transplantation	(% Yes)	53%	6/10	38%	10/0	100%	63% (19% to 84%)	0.003
eGFR	All patients		57.7 (36.8; 82.9)		47.8 (13.7; 76.8)		-9.9 (-34.9 to 22.2)	0.44
	nKTx	66.3 (39.8; 86.1)	79.3 (37.4; 84)		*na*			*na*
	KTx	59.2 (39; 82.4)	38.8 (29.8; 81.3)		47.8 (13.7; 76.8)		8.9 (-34.8 to 34)	0.87

Table 4.4 (continued) Demographic, clinical and laboratory parameters of the subgroup of cystinosis patients with extra-renal complications, according to the presence of one (1 ERC) or multiple (> 1 ERC) extra-renal complications.

		All cystinosis patients (n=57)	**Extra-renal complication status** (n=26)				**Difference (95% CI of the difference)**	***p***
			1 ERC (n=16)		**> 1 ERC** (n=10)			
Extra-renal complications	Yes/No (% Yes)	46%	16/0	100%	10/0	100%		
	Primary hypothyroidism	28%	6/10	38%	10/0	100%	63% (19% to 84%)	0.003
	Retinopathy	4%	0/16	0%	2/8	20%	20% (-9% to 56%)	0.14
	Insulin-dependent Diabetes Mellitus	5%	0/16	0%	3/7	30%	30% (-3% to 65%)	0.046
	Peripheral myopathy	25%	7/9	44%	7/3	70%	26% (-17% to 58%)	0.25
	Swallowing dysfunction	18%	3/13	19%	73	70%	51% (7% to 77%)	0.02
WBC cystine level	nmol ½ cystine/mg protein	1.44 (0.64; 2.3)	1.12 (0.54; 1.78)		2.43 (1.38; 4.88)		1.31 (0.06 to 3.53)	0.03
	< 1 (% Yes)	32%	7/9	44%	2/8	20%	24% (-7% to 66%)	0.4
	1-2	42%	7/9	44%	3/7	30%	14% (-20% to 55%)	0.68
	> 2	26%	2/14	13%	5/5	50%	38% (-3% to 69%)	0.07
Disease severity classification	nKTx, ERC- (% Yes)	30%	*na*	*na*	*na*	*na*		
	nKTx, ERC+	18%	10/6	63%	0/10	0%	63% (41% to 106%)	0.003
	KTx, ERC-	25%	*na*	*na*	*na*	*na*		
	KTx, ERC+	28%	6/10	38%	10/0	100%	63% (19% to 83%)	0.003
Chitotriosidase (*Corrected for CHIT)*	nmol/ml plasma/h (median (IQR))	77.11 (49.88; 166.3)	54 (32; 104)		321.8 (78.1; 495.7)		267.8 (36 to 416.5)	0.005
IL-1 ß	(pg/ml) (median (IQR))	0.13 (0.13; 0.19)	0,13 (0,13; 0,19)		0.13 (0.13; 0.19)		0 (-0.03 to 0.03)	0.95
IL-6	(pg/ml) (median (IQR))	1.68 (1.15; 2.85)	1.65 (1.14; 3.16)		2.9 (1.68; 4.71)		1.2 (-0.2 to 2.5)	0.07
IL-18	(pg/ml) (median (IQR))	717.0 (499.3; 939.3)	813.2 ± 485		719 ± 182.9		-94.17 ± 161 (-426.5 to 238.1)	0.56

Data on the genetic background of cystinosis patients prior to kidney transplantation were obtained in 19 of the 29 patients, while in the kidney-transplanted patients data was obtained in 26 of the 32 patients. Data on the WBC cystine levels were obtained in 27 of the 29 patients prior to kidney transplantation, and 30 of the 32 kidney-transplanted patients. 3 out of 61 patients harbored a 24-bp duplication mutation in exon 10 of the *CHIT1* gene. *Abbreviations*: *na*: not applicable; *CHIT*: chitotriosidase genotype.

Table 4.5 Correlation analysis between the proposed biomarkers of macrophage activation and the number of extra-renal complications in the subgroup of patients harboring at least one extra-renal complication.

Variable	Spearman r	95% CI	*p*
# of extra-renal complications ERC+ patients (n=26)			
WBC cystine	0.39	-0.007 to 0.68	0.05
Chitotriosidase	0.55	0.2 to 0.78	0.004
IL-1β	0.058	-0.35 to 0.45	0.78
IL-6	0.31	-0.096 to 0.63	0.12
IL-18	-0.079	-0.46 to 0.33	0.70

Of the biomarkers for macrophage activation studied, apart from WBC cystine, only chitotriosidase is significantly correlated with the number of extra-renal complications in the subgroup of patients harboring at least one extra-renal complication.
The continuous variables studied, concern the 2-year average per subject.
Abbreviations: ERC: extra-renal complications

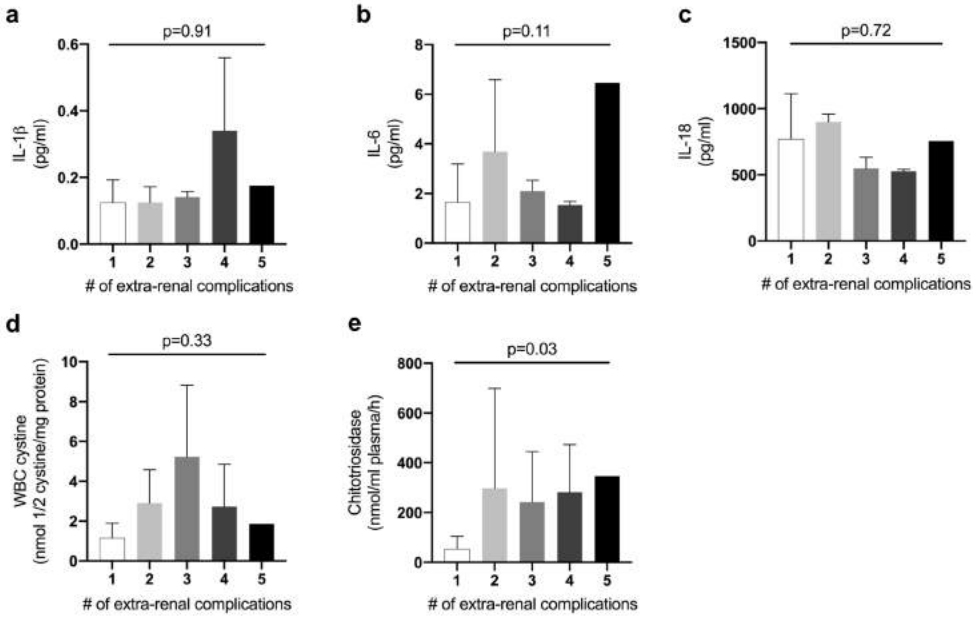

Figure 4.4 Median values for biomarkers of macrophage activation and WBC cystine values in ERC+ patients in relation to the number of extra-renal complications

Biomarker values (a: IL-1β, b: IL-6, c: IL-18, d: chitotriosidase) and WBC cystine levels were categorized according to the number of extra-renal complications. *p* -values were based on Kruskal-Wallis test. Data is presented as median ± IQR.

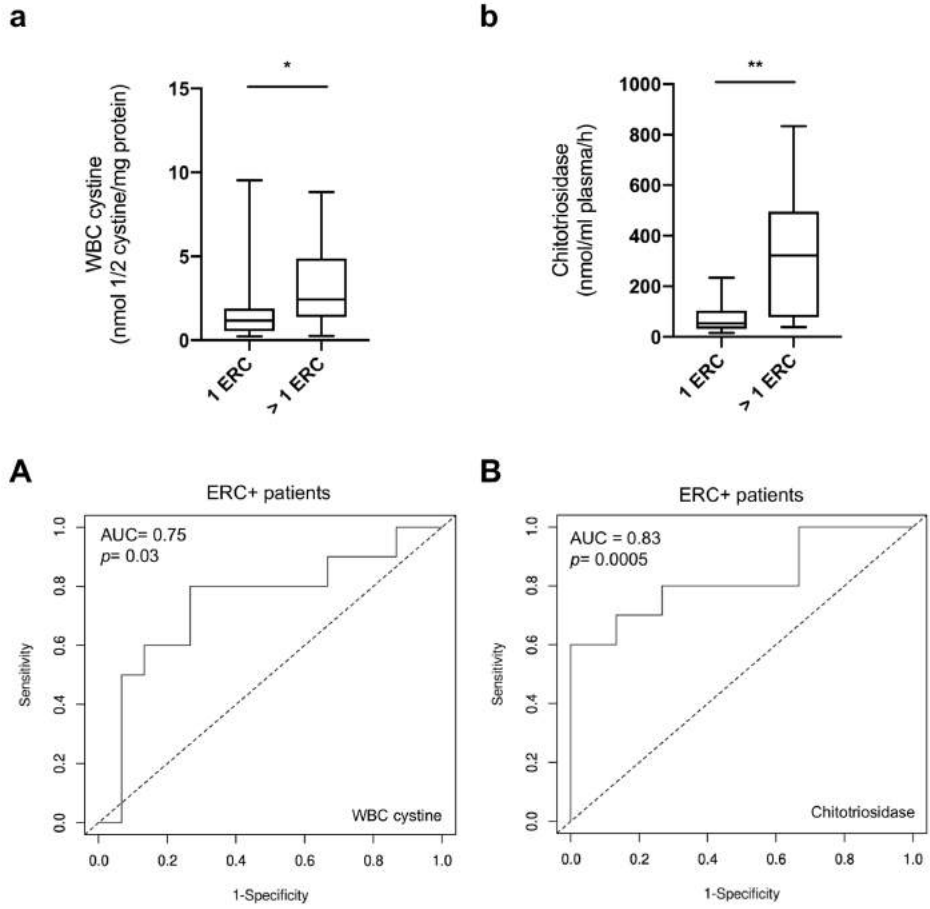

Figure 4.5 Plasma chitotriosidase enzyme activity performs superior to WBC cystine levels of all tested biomarkers of macrophage activation, in distinguishing the presence of one vs. multiple extra-renal complications in patients harboring at least one extra-renal complication.

Panel a, b: Box and whisker plots for the 2-year average WBC cystine level and plasma chitotriosidase enzyme activity levels in patients harboring at least one extra-renal complication, stratified according to the presence of one versus multiple extra-renal complications.

Panel A & B: Receiver Operating Curve (ROC) analysis of 2-year average WBC cystine and plasma chitotriosidase enzyme activity levels demonstrating the superior performance of chitotriosidase for distinguishing the presence of multiple vs. a single extra-renal complication.

Table 4.6 Cut-off levels of chitotriosidase for predicting good vs. poor therapeutic control, and the presence of one vs. multiple extra-renal complications and its corresponding performance measures determined by an ROC analysis.

Biomarker	Population	Cut-off	Sensitivity	Specificity	PPV	NPV	+ LR	- LR
Good vs. poor therapeutic control								
Chitotriosidase (nmol/ml plasma/h)	All patients	150	53% (30% to 75%)	85% (70% to 93%)	57% (33% to 79%)	83% (68% to 91%)	1.24	0.55
Presence of one vs. multiple cystinosis extra-renal complications								
Chitotriosidase (nmol/ml plasma/h)	ERC+ patients	250	60% (31% to 83%)	93% (70% to 100%)	86% (49% to 99%)	78% (55% to 91%)	4.2	0.42

Abbreviations: *na*: not applicable; ERC+: presence of extra-renal complications; PPV: positive predictive value; NPV: negative predictive value; + LR: positive likelihood ratio, -LR: negative likelihood ratio

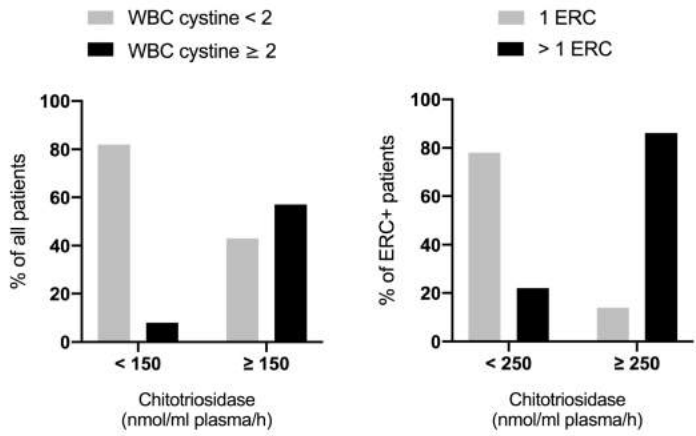

Figure 4.6 Plasma chitotriosidase enzyme activity harbours a high negative predictive value for ruling out poor adherence to cysteamine treatment and a high positive predictive value for identifying patients harboring multiple extra-renal complications

(Left) In the whole cystinosis patient cohort, a plasma chitotriosidase enzyme activity level < 150 nmol/ml plasma/h has a high negative predictive value for ruling out poor compliance to cysteamine treatment, the latter based on high (2-year) average WBC cystine level ≥ 2 nmol ½ cystine/mg protein.

(Right) In cystinosis patients harbouring at least one extra-renal complication, plasma chitotriosidase enzyme activity levels ≥ 250 nmol/ml plasma/h has a high positive predictive value for the presence of multiple extra-renal complications.

4.4 Discussion

In the current chapter, we conducted a 2-year longitudinal study to explore the clinical value of biomarkers of macrophage activation as potential alternative monitors for cystine-depleting therapy in nephropathic cystinosis.

The rational for our study was that WBC cystine measurements are not available in the majority of the countries around the globe and are subject of large variability even in the reference laboratories, while on the other hand, WBC cystine levels might not adequately reflect the whole body cystine burden. Alternatively, interstitial macrophages containing cystine crystals have been demonstrated in various organs, including the skin, gastro-intestinal mucosa, liver, kidney and bone marrow [122,128,132,133,346,347], and macrophage activation has been established as one of the main pathogenic mechanisms of cystinosis [25,26,348]. In addition, substantial evidence also currently indicates a pivotal role for inflammation mediated by macrophages, comprising mainly the macrophage-secreted cytokines IL-6 and TNF-α, the M1/M2 macrophage balance and macrophage-derived monocytes in the progression of CKD towards end-stage renal disease [349–356].

Therefore, it could be hypothesized that widespread progressive cystine crystal accumulation in various tissues could be reflected by inflammatory mediators which are released by macrophages upon exposure to these foreign crystalline moieties. Likewise, the degree of inflammation mediated by macrophages could therefore also reflect the adherence to cystine-depleting therapy [357;358].

Among the four biomarkers for macrophage activation investigated, plasma chitotriosidase enzyme activity was the only promising candidate for clinical use as an alternative or additional monitor for therapeutic monitoring of cystinosis.

Plasma chitotriosidase enzyme activity correlated significantly with WBC cystine values over the longitudinal course of the study, and resulted as a significant predictor for WBC cystine levels in cystinosis patients of all ages (Table 4.3, 4.6; Figure 4.1, 4.2). Via a ROC analysis, a cut-off value for plasma chitotriosidase activity could be established (150 nmol/ml plasma/h) for ruling out poor adherence to cysteamine treatment with a high predictive value (NPV 83%) (Table 4.6; Figure 4.2, 4.6).

In addition, of all biomarkers for macrophage activation tested, chitotriosidase enzyme activity was the only biomarker that demonstrated a significant correlation with the number of extra-renal complications in the subgroup of patients harboring at least one ERC (Table 4.5). Furthermore, in a ROC analysis, chitotriosidase activity yielded a superior performance to WBC cystine levels for identifying patients harboring multiple ERCs for which a cut-off value could be established yielding a high positive predictive value (PPV 86%) (Table 4.6; Figure 4.5, 4.6). Hence, our data

suggest that plasma chitotriosidase enzyme activity can reflect the disease severity and extent, as far as the clinical renal and extra-renal manifestations are mediated by cystine crystal accumulation and its induced inflammation. In this respect, it should also be noted that significantly higher chitotriosidase enzyme activity levels were differentially associated with only some of the extra-renal complications (Table S4.1). While the subgroups of patients harboring at least primary hypothyroidism or peripheral myopathy as an extra-renal complication did not show significantly higher chitotriosidase levels compared to patients without any ERC, chitotriosidase levels did reach significantly (or borderline) higher levels in patients harboring at least retinopathy or insulin-dependent diabetes mellitus respectively (Table S4.1). This finding is in line with the observation of the large cohort-based study on the long-term outcome of cysteamine-treated cystinosis patients by Brodin-Sartorius *et al* that hypothyroidism and peripheral myopathy can still occur at a young age despite early initiation of cysteamine treatment. Hence, these data suggests that cystine accumulation cannot be regarded as the single main pathogenic cause for some of the most frequent and severe extra-renal complications of cystinosis [337].

Initially, chitotriosidase enzyme was suggested as an important factor in the innate immunity against chitin coated pathogens, such as fungi and protozoa, since its natural substrate chitin, is completely absent in mammals. However, its immunomodulatory effects extend far beyond innate immunity [359]. Chitotriosidase was first detected as markedly elevated in the plasma of patients with Gaucher disease, another lysosomal storage disorder characterized by massive accumulations of glucosphingolipids in macrophages[15.] Later, it was found elevated in patients with other lysosomal storage disorders such as Niemann-Pick A/B and C, Gangliosidosis M1 and Krabbe[360,361] as well as, non-lysosomal inflammatory disorders in which macrophages play an important role, such as β-thalassemia, sarcoidosis and multiple sclerosis[362–364]. The enzyme is currently established as a therapeutic monitor for Gaucher disease[365].

In cystinosis, we detected the elevation of the enzyme activity in the plasma of cystinosin knocked-out mice [25,366] and in the homogenates of the cystinosis mutant zebrafish larvae[367] compared to the wild type in each animal model indicating that the phenomenon of macrophage activation and chitotriosidase production is not limited by species boundaries.

The 24-bp duplication in exon 10 of the chitotriosidase gene (*CHIT1*), when homozygously mutated, results in chitotriosidase deficiency. It is relatively common in many ethnic groups including Caucasians, in whom it can be detected in approximately 5% of the general population [368]. In our study, we detected very low activities of the enzyme associated with homozygosity for the 24-bp duplication in three of our recruited patients coinciding with the mutation prevalence. Naturally, the enzyme in those patients cannot be used as a therapeutic monitor for cystinosis

therapy in the future. When suspected, this mutation can be detected with a simple PCR reaction, and was not an obstacle for the clinical use of the enzyme as a biomarker in other diseases[359].
Chitotriosidase activity has many advantages as a biomarker. The enzyme is stable in plasma for a very long time (up to one month at room temperature, more than four months at 4°C and several years at –80 °C) and is also quite stable upon repeated freezing and thawing up to ten cycles [343,369]. Analysis of chitotriosidase enzyme activity is performed through a simple fluorometric technique at a fraction of the time and cost of WBC cystine assay and could be easily available to much less equipped laboratories in developing countries. Furthermore, unlike cystine the enzyme can be assayed in blood spots providing another sample type that has many advantages concerning sample storage and transport[16].
Importantly, our previous cross-sectional study showed a correlation between chitotriosidase enzyme activity and kidney function, with highest levels observed in poorly controlled cystinosis patients in advanced stages of chronic kidney disease (CKD) [25]. Higher chitotriosidase activity levels were also observed in other CKD patients reflecting a general state of inflammation rather than decreased glomerular filtration of the enzyme in view of its large molecular weight [25]. The levels in cystinosis were, however, significantly higher compared with non-cystinotic patients having similar CKD stage, indicating that cystine accumulation acts on top of CKD in these patients [25].
Although promising, our study has several limitations. Cystinosis is a very rare disease with a few thousand patients identified world-wide. While we were able to follow 61 cystinosis patient over a time period of ~2 5 years, this group was still too small for making firm conclusions and establishing clear cut-off values, especially taking into account that the number of patients with poor disease control was less than one fourth of all patients. Nevertheless, even with these low numbers we were able to demonstrate that chitotriosidase enzyme activity levels < 150 nmol/ml/h has a high predictive value for ruling out poor adherence to cysteamine therapy in all cystinosis patients. Although the sensitivity was only ~50%, the specificity was above 80% which is more important to identify cystinosis patients with insufficient disease control as it would have direct therapeutic implications for adjusting cysteamine therapy. Obviously, a similar study in a larger patients' population is required for establishing more reliable cutoff values in the future.
In conclusion, we demonstrate that plasma chitotriosidase enzyme activity can identify cystinosis patients with poor disease control and might be used as an alternative therapeutic monitor in nephropathic cystinosis.

Conflict of Interest disclosure

EL has consultancy agreement with Orphan Europe, Chiesi, Advicenne and Kyowa Kirin.

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Supplemental data

Table S4.1 Factors associated with low (< 250) versus high (≥ 250) plasma chitotriosidase enzyme activity levels in the overall cystinosis patient cohort.

Variable		Chitotriosidase < 250	Chitotriosidase ≥ 250	*p*
Genetic background				
Other	Y/N (% Y)	20/15 (57.14%)	2/6 (25.00%)	0.10
Hom 57kb del	Y/N (% Y)	15/20 (42.86%)	6/2 (75.00%)	
Age at recruitment	n	49	9	0.02
	Mean	19.6	28.8	
	SD	10.29	8.18	
	Median	17.8	26.5	
	IQR	(12.1; 26.5)	(23.6; 28.4)	
	Range	(1.9; 44.8)	(21.3; 45.7)	
Age at KTx	n	20	9	0.69
	Mean	13.5	14.0	
	SD	4.64	4.23	
	Median	12.4	15.0	
	IQR	(10.5; 14.5)	(10.9; 15.6)	
	Range	(8.0; 25.5)	(8.3; 20.0)	
Extra-renal complications	n	48	9	0.01
	Mean	0.5	2.0	
	SD	0.85	1.80	
	Median	0.0	2.0	
	IQR	(0.0; 1.0)	(0.0; 3.0)	
	Range	(0.0; 4.0)	(0.0; 5.0)	
Extra-renal complication score				
0	Y/N (% Y)	29/19 (60.42%)	3/6 (33.33%)	0.001
1	Y/N (% Y)	15/33 (31.25%)	0/9 (0.00%)	
2	Y/N (% Y)	2/46 (4.17%)	3/6 (33.33%)	
3	Y/N (% Y)	1/47 (2.08%)	1/8 (11.11%)	
4	Y/N (% Y)	1/47 (2.08%)	1/8 (11.11%)	
5	Y/N (% Y)	0/48 (0.00%)	1/8 (11.11%)	
WBC cystine (nmol ½ cystine/mg protein)	n	45	9	<.001
	Mean	1.4	4.0	
	SD	1.46	2.17	
	Median	1.2	3.6	
	IQR	(0.6; 1.6)	(2.9; 4.8)	
	Range	(0.2; 9.5)	(1.5; 8.8)	

Comparison of the patient subgroup with a low (< 250) versus high (≥ 250) 2-year average chitotriosidase level on a number of patient characteristics. Age, the presence and number of extra-renal complications, and the WBC cystine level are significantly associated with chitotriosidase levels ≥ 250.
Abbreviations: KTx: kidney transplantation

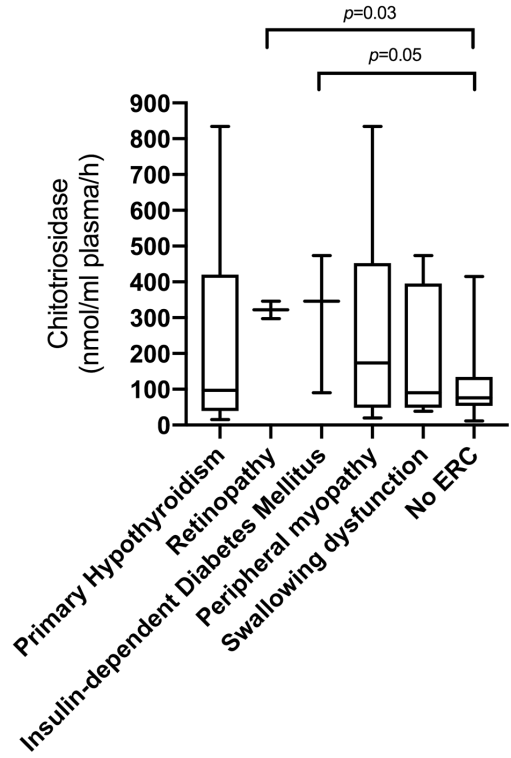
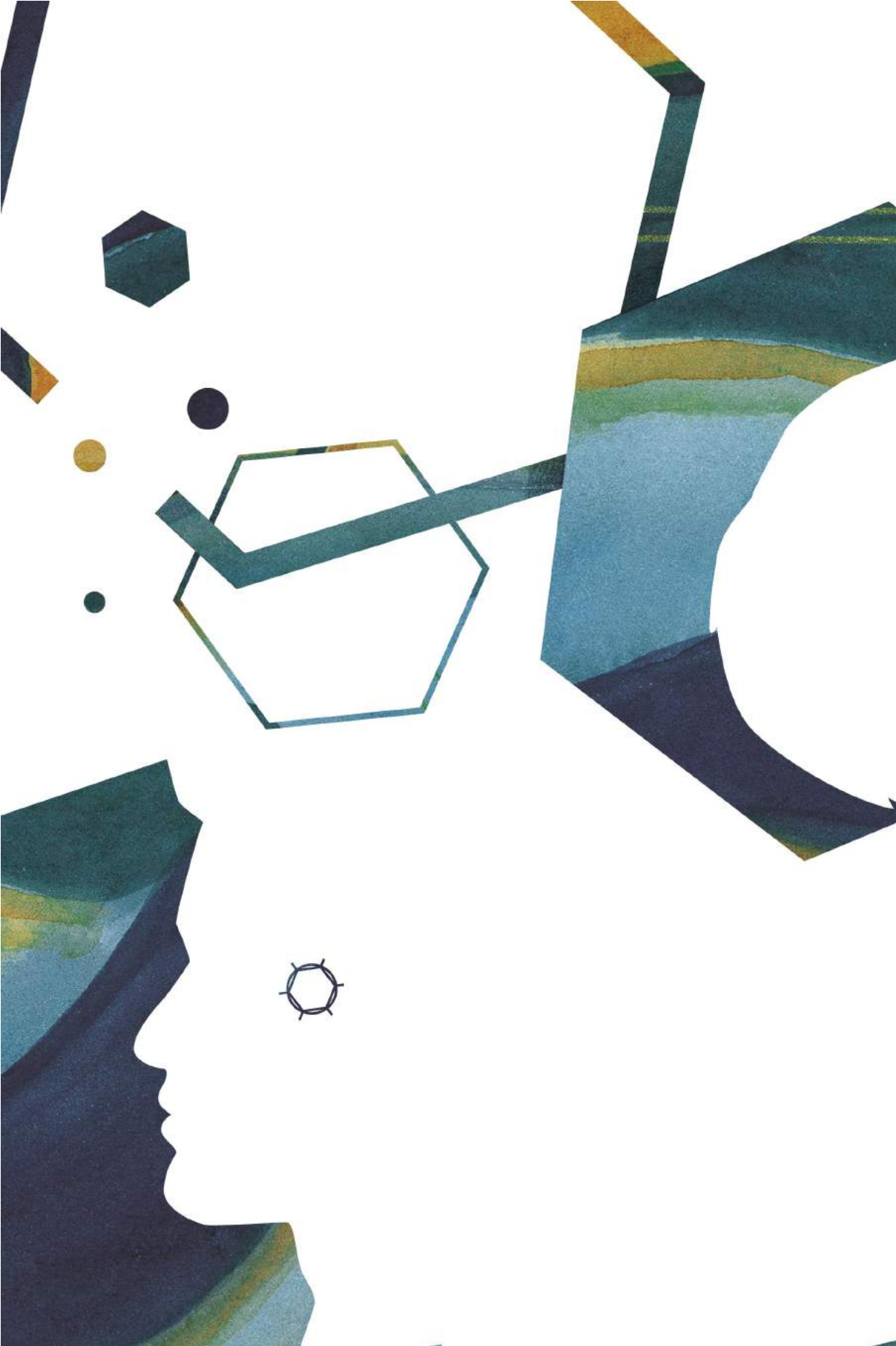


Figure S4.1 High chitotriosidase enzyme activity levels are differentialy associated with patients harboring, amongst potentially others, at least one specific extra-renal complications.

Box and whiskers plot indicating the 2-year average chitotriosidase levels of patients harboring at least the indicated extra-renal complication (e.g. primary hypothyroidism, retinopathy, insulin-dependent diabetes mellitus, peripheral myopathy, swallowing dysfunction), compared to patients harboring no extra-renal complications.
Significantly higher chitotriosidase levels are differentially associated in patients with at least one specific extra-renal complication. While patients harboring at least primary hypothyroidism do not significantly show higher chitotriosidase levels, despite a larger variation in levels, patients harboring at least retinopathy or insulin-dependent diabetes mellitus did show significantly (or borderline significant, respectively) higher chitotriosidase levels.
Abbreviations: ERC: extra-renal complication

PART II:
Innovation in treatment of nephropathic cystinosis



CHAPTER 5
Allogeneic hematopoietic stem cell transplantation transfers wild type cystinosin to nonhematological epithelial cells

Chapter based on:

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Allogeneic HSCT transfers wild type cystinosin to non-hematological epithelial cells in cystinosis: first human report

**contributed equally as first authors; # contributed equally as senior authors*

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ABSTRACT

Background

Cystinosis is an autosomal recessive lysosomal storage disorder characterized by the defective transport of the amino acid cystine out of the lysosome due to a deficiency of cystinosin, the lysosomal cystine transporter. Patients suffer from lysosomal cystine accumulation in various tissues, leading to cellular stress and damage, particularly in the kidney, cornea and other extra-renal tissues. Cysteamine, a cystine-depleting agent, improves survival and delays the progression of disease, but it does not prevent the development of either renal failure or extra-renal complications. Furthermore, the drug has severe adverse effects that significantly reduce patient compliance.

Allogeneic HSCT is currently established as a therapeutic option for many inborn errors of metabolism, where the main pathologic driving factor is an enzyme deficiency. Recent studies in the cystinosis mouse-model suggested that hematopoietic stem cell transplantation (HSCT) could be a curative treatment alternative to cysteamine therapy.

We treated a 16-year-old male suffering from infantile cystinosis and side effects of cysteamine therapy with HSCT.

Results

We were able to demonstrate successful transfer of the wild type cystinosin protein and *CTNS* mRNA to non-hematological epithelial cells in the recipient, as well as a decrease in the tissue cystine-crystal burden.

Conclusions

This is the first report of allogeneic HSCT in a patient with cystinosis, the prototype of lysosomal membrane-transporter disorders.

5.1 Introduction

Cystinosis (MIM219800) is a lysosomal storage disorder (LSD) caused by bi-allelic mutations in the *CTNS* gene (17p13.2) encoding the lysosomal membrane cystine/proton cotransporter cystinosin [40]. Cystinosis is characterized by lysosomal cystine accumulation and crystallization, and manifests clinically with severe polyuria and loss of a diverse range of substances normally reabsorbed in the kidney proximal tubules (renal Fanconi syndrome), leading to end-stage renal disease (ESRD) during childhood or early adolescence. Extra-renal manifestations include photophobia, retinopathy, endocrine dysfunction (hypothyroidism, endocrine pancreatic insufficiency, hypogonadism), peripheral myopathy and central nervous system complications, which mostly develop during the 2nd and 3rd decades of life [40,47]. In cystinosis patients, phagocytic cells, such as blood granulocytes and bone marrow and tissue macrophages, accumulate large amounts of cystine due to their phagocytic nature and their inability to process the phagocytized crystals [340,370]. The only available treatment for cystinosis is the cystine-depleting aminothiol cysteamine. However, this drug does not prevent progressive disease, but merely postpones the development of ESRD and extra-renal complications [47]. Moreover, cysteamine has numerous side effects that severely limit patient compliance [137,371,372].

Allogeneic hematopoietic stem cell transplantation (HSCT) has been established as a successful therapy for not only hereditary hematological disorders, but also for inborn errors of metabolism (IEMs), including many LSDs [373,374]. The first case of a successful bone marrow transplantation in a lysosomal disorder dates back to 1981 in a child affected by Hurler syndrome, or mucopolysaccharidosis type-1 (MPS1) [375]. Since then, thousands of children have received HSCT as a therapeutic modality for LSDs, including other MPS syndromes, metachromatic leukodystrophy, Gaucher, Niemann-Pick A/B, α-mannosidosis and many others [373]. However, the basic concept behind allogeneic HSCT in all these disorders is to replace the production of a deficient enzyme caused by the genetic defect of each LSD. The only IEM, in which HSCT has been established as a therapy for a transporter deficiency, is the peroxisomal disorder adrenoleukodystrophy (ALD). Allogeneic HSCT can reverse demyelination and progression of neurological symptoms when performed early in ALD, but the mechanism of action of HSCT is not clear, and the disease lacks an established animal model [376,377].

The Cherqui group showed that in the cystinosis (*Ctns*$^{-/-}$) mouse model HSCT significantly decreased cystine accumulation in different organs, preserved kidney function, and reversed extra-renal organ damage [32,162,163,378]. They demonstrated the *in-situ* differentiation of stem cells to tissue macrophages within target organs, which transferred cystinosin-bearing lysosomes to adjacent deficient cells via tunneling nanotubes [379]. The paracrine release of cellular microvesicles containing *CTNS* mRNA and protein has also been suggested as another potential

mechanism of action [380]. Strikingly, similar beneficial effects of HSCT have been demonstrated in a mouse model of Dent disease, a non-lysosomal proximal tubular disorder presenting with renal Fanconi syndrome, and caused by the defective function of endosomal chloride-proton exchanger [381]. These animal studies provide the proof of concept for a beneficial effect of HSCT in hereditary diseases, where the defective proteins are endosomal or lysosomal membrane transporters, rather than enzymes. Here, we present a case of an adolescent male with severe infantile cystinosis who underwent HSCT.

5.2 Case

We treated a 16-year-old Caucasian male, who was diagnosed with cystinosis at the age of 2.7 years and started on cysteamine treatment at that time. DNA analysis demonstrated compound heterozygous mutations in the *CTNS* gene: 57-kb deletion & c.926dup (p.Ser310fs*55). At the age of 15, he developed signs of cysteamine toxicity, with cutaneous lesions at the level of lumbar vertebrae and bone lesions (cervical vertebrae, femur and tibia, overgrowth of rib cartilage) due to ongoing renal Fanconi syndrome associated with therapy-resistant copper deficiency [372]. Moreover, he had severe psychological and social problems due to cysteamine-induced halitosis [371]. The patient was subsequently referred to the bone marrow transplantation program at the University Hospitals Leuven. A family conference was held to discuss the potential risks and benefits of HSCT, and both the patient and his parents consented to this treatment option. The procedure was approved by the Institutional Ethical Board at University Hospitals Leuven in December 2012.

At the age of 16, the patient underwent allogeneic HSCT from a fully HLA-matched (10/10) unrelated donor using mobilized peripheral blood stem cells (PBSCs). Cysteamine treatment was discontinued two months before transplantation. Pre-transplant myeloablative conditioning consisted of treosulfan (14g/m^2/day from day -7 to day -5), fludarabine (30 mg/m^2/day from day -7 to day -3), thiotepa (10 mg/kg on day -4) and anti-thymocyte globulin (ATG) (2.5 mg/kg from day -3 to day -1). At the time of transplantation, the patient received 7.88 x10^6 CD34-positive cells/kg, and 166 x10^6 CD3 positive T cells/kg. For post-transplant GVHD prophylaxis, tacrolimus (2x2mg/day, target trough levels 5-10 ng/ml), mycophenolate mofetil (3x915 mg/day), and methotrexate (15 mg/m^2 on day 1 and 10mg/m^2 on days 3, 6 and 11) were administered. Filgrastim (300 µg/day) was also given on days 16, 17 and 19, and neutrophil engraftment was evident on day 22 post-HSCT (day 21: 1,020 neutrophils/mm^3 and day 22: 1,300 neutrophils/mm^3). Full donor chimerism (>95%) was demonstrated in the bone marrow at days 142 and 184 post-HSCT, and in the peripheral blood at days 28, 62, 107, 323, 400 and 462.

The early post-transplant period was complicated by an acute graft-versus-host disease (GvHD, grade III-IV) and adenovirus reactivation presenting with fever and profound diarrhea during the third week post-HSCT. Treatment with cidofovir and systemic corticosteroids (methylprednisolone 2mg/kg/day) was initiated. Response to corticosteroids was satisfactory after 5 days and the dose was tapered gradually. Due to persistently high adenovirus copy numbers and hypogammaglobulinemia, intravenous immunoglobulin (IVIG) was administered at day 36, and later, treatment with ganciclovir was initiated at day 65.

At day 25 post-transplant, the patient developed an altered level of consciousness, speaking and swallowing difficulties, a central facial nerve palsy, diplopia, ataxia, dysmetria, and hyperreflexia. Brain MRI demonstrated central pontine myelinolysis, which was felt to be caused by tacrolimus toxicity. Tacrolimus was therefore replaced with sirolimus. Sirolimus levels were closely monitored and kept within a safe therapeutic range (6-12 ng/ml). Further work-up did not reveal any evidence of infection in the cerebrospinal fluid. Subsequently a more severe pyramidal syndrome developed, for which treatment with levodopa (but not baclofen and diazepam) resulted in a gradual neurological recovery. However, subsequently the patient developed recurrent epileptic seizures and hallucinations associated with the administration of various drugs (calcineurin inhibitors, anti-viral and anti-fungal agents), suggesting a possible disruption of the patient's blood-brain barrier.

Strikingly, within the first few months post-HSCT, the patient's kidney function stabilized and his polyuria resolved (Figure 5.1 a). This suggested that HSCT had been efficacious in reducing his clinical symptoms of cystinosis. Pre-existing low molecular weight proteinuria and glucosuria were initially elevated post-transplant, but then decreased gradually to pre-HSCT levels after six months. The patient's photophobia score also improved over the first 6 months post-transplant from grade 5 to no photophobia. Stomach biopsies taken after transplantation also showed a significant decrease in cystine crystal accumulation (Figure 5.1 b, c). Notably, peripheral white blood cystine levels dropped to the normal range as circulating blood cells differentiated from the healthy donor bone marrow cells. Due to partial graft failure caused by a parvovirus B19 infection, a second HSC infusion from the same donor was administered 15 months after first HSCT, resulting in good reconstitution of the blood. The patient did not receive pre-transplant conditioning or GvHD prophylaxis for the second HSC donation. Full donor chimerism (>95%) was also demonstrated in peripheral blood 140 days following the second donation. However, a severe therapy-resistant acute followed by chronic gastro-intestinal, hepatic, and cutaneous GvHD developed, for which several immunosuppressive agents were used, including prednisolone, azathioprine, cyclosporine, ATG and sirolimus. The severity scores for the liver, GI tract, and skin GvHD were grades 3, 3, and 2, respectively [382]. The kidney function gradually

declined and the patient ultimately required chronic dialysis 18 months following initial HSCT. Unfortunately, 35 months after transplantation the patient developed a severe pneumonia due to a multi-resistant Pseudomonas infection, and succumbed to the disease.

To evaluate the long term levels of donor's mRNA in recipient tissues, we measured the percentage of expression of the wild type (Wt) *CTNS* RNA in renal and liver cells 24 months after HSCT through reverse transcription, followed by next-generation sequencing (NGS) (Figure 5.2 a, b). To obtain renal cells devoid of hematopoietic elements, we isolated tubular epithelial cells from the patient's urine as described previously by our group [24]. Additionally, we measured solid organ (liver) Wt *CTNS* RNA in a liver specimen, which was obtained from a biopsy performed to assess the patient's GvHD. We also used RNA isolated from renal tubular epithelial cells derived from the urine of the same patient prior to the transplantation and a healthy individual as negative and positive controls for the Wt *CTNS* sequencing, respectively.

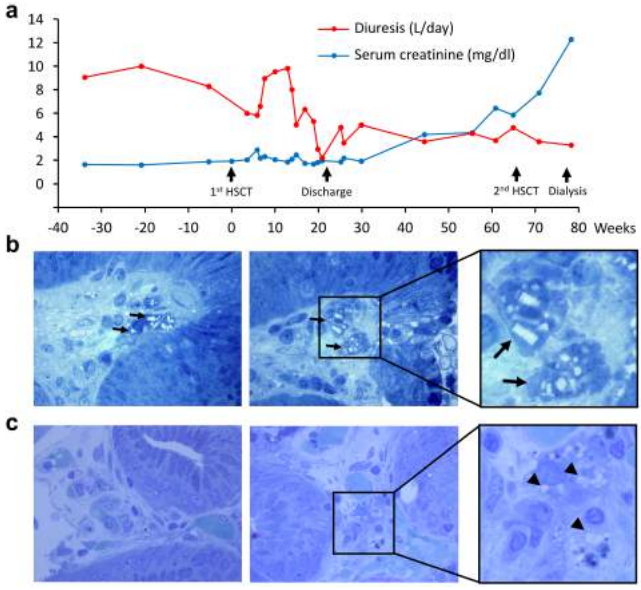

Figure 5.1 Evolution of kidney function and tissue cystine crystal accumulation pre- and post-HSCT.

Panel a: Evolution of diuresis and serum creatinine prior to and following HSCT.

Panel b: Representative images of tissue cystine accumulation in gastric mucosal biopsy before transplantation. Numerous rhomboid and hexagonal cystine crystals are visible inside interstitial macrophages (arrows). The median number of cystine crystals per macrophage was 11.3 (range 7-16).

Panel c: Gastric mucosal biopsy 30 months after transplantation showing macrophages with globular vacuoles (arrowheads). The median number of crystals per macrophage was 7.8 (range 4-13). Tissues were fixed in glutaraldehyde, prepared as 1µm thick sections, and stained with toluidine blue [347]. Images were acquired using a Leica DM2000 LED microscope, Leica DFC290 HD camera and LAS acquisition software (Leica microsystems) at a magnification of 1000x.

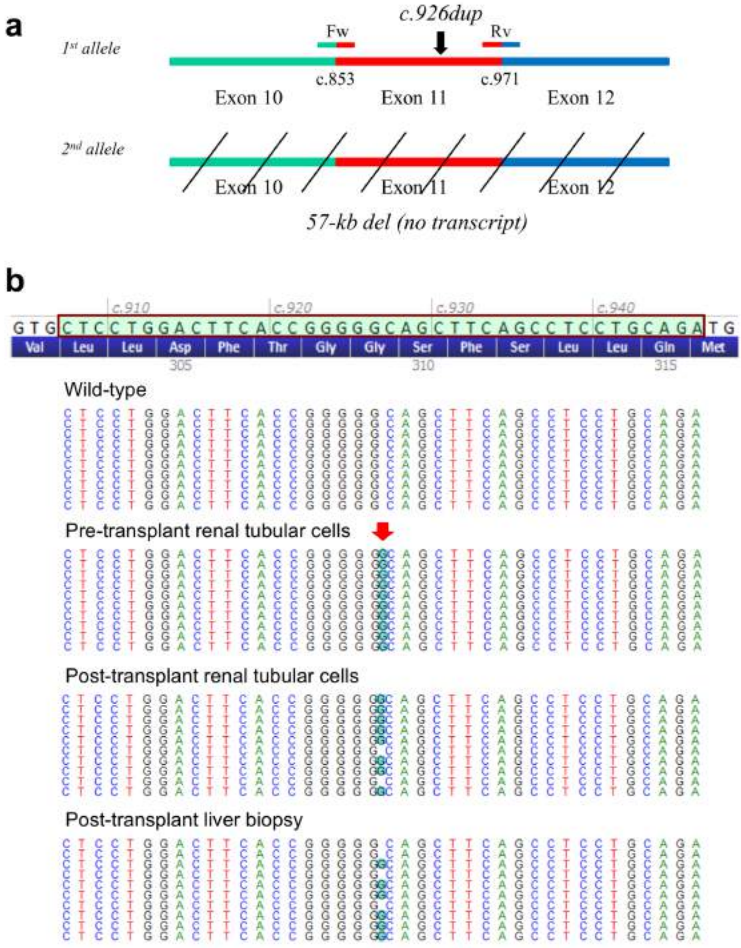

Figure 5.2 Next generation sequencing on urine-derived renal tubular cells prior to and following HSCT, compared to wild-type and liver cells following HSCT.

Panel a: Schematic representation of the primer design and the concept behind determining the percentage of the Wt allele incorporated in the post-transplant samples. The mutated cells are only represented by one allele, as the second allele is lacking a transcript due to the 57-kb deletion.

Panel b: Next generation sequencing (NGS) diagram showing only a representative coverage of 10X for each sample. Actual amplicon coverage by NGS was approximately 100X. RNA isolation for renal tubular cells was performed using the RNeasy plus mini-kit (QIAGEN, Valencia, CA, USA), while for the liver biopsy the TRIzol® reagent (Invitrogen, Waltham, MA, USA) was used. RNA isolated from tubular epithelial cells derived from the pre-transplanted patient and a healthy individual was used as negative and positive controls for the wild type *CTNS* sequence, respectively. cDNA was synthesized with the SuperScript III RT kit (Invitrogen, Waltham, MA, USA). PCR was performed in duplicate for all cDNA samples for the propagation of exons 11 harboring the location of the point mutation of the patient, c.926dup using the primers (Fw: 5' - CCACAGGCCTACATGAACTT- 3', Rv: 5' - TCCACTGGTCGTTGTTGTAG- 3'). NGS was performed using the MiSeq system platform (Illumina, San Diego, CA). The site of the point mutation (c.926dup) is marked by the red arrow.

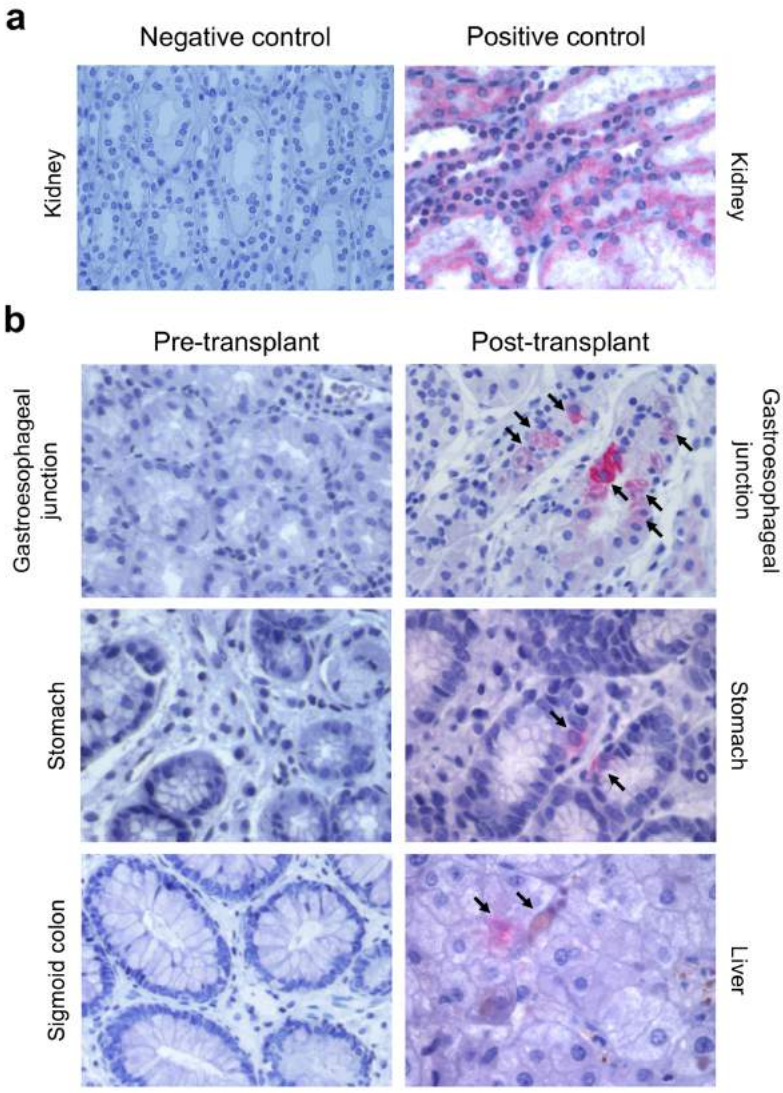

Figure 5.3 Cystinosin-LKG expression prior to and following HSCT.

Panel a: Immunohistochemical staining showing negative and positive controls for the cystinosin-LKG antibody.

Panel b: Immunohistochemical staining for cystinosin-LKG in different tissues of the patient before and 24 months (liver) or 30 months (stomach and gastroesophageal junction) after HSCT showing the expression in some epithelial cells.

Immunostaining was according to the method of Taranta et al,[384] but antigen retrieval was performed at high pH9 (Dako). Images were acquired using a Zeiss AXIO Imager.A1 microscope, AxioCamMRc 5 camera and AxioVision software (Zeiss). All images have a magnification of 400x except stomach mucosa and liver tissue post-transplantation have a magnification of 630x.

As expected, the healthy individual cDNA showed 100% Wt allele, while the pre-transplanted patient cDNA showed 100% mutant allele. Interestingly, the cDNA derived from patient's tubular epithelial cells and liver biopsy 24 months post-HSCT showed incorporation of the Wt allele of 22% and 40%, respectively (Figure 5.2 b). While Wt gene transfer has clearly occurred, the percentage of Wt allele may have been overestimated due to the potentially decreased mutant RNA stability caused by the nonsense-mediated mRNA decay [383].

We further investigated the protein expression of cystinosin by immunohistochemistry in different tissues pre- and post-HSCT using antibodies against cystinosin-LKG, which is an isoform of cystinosin, resulting from an alternative splicing of exon 12 that removes the C-terminal GYDQL motif and replaces it with a string of 38 amino acids containing a ''SSLK'' domain that is strongly conserved among species [384]. At this time, no specific antibodies against conventional cystinosin isoform are available. Pre-transplantation, the patient had no expression of cystinosin-LKG, this is due to one allele lacking the gene promoter needed for mRNA transcription (57-kb del) with the other allele having a frameshift mutation at AA-310 (p.Ser310fs*55), while the antiserum was directed against a cystinosin-LKG-specific sequence spanning amino acids 366–389 [384]. Following HSCT, focal expression of the Wt cystinosin-LKG was evident in non-hematological epithelial cells in several organs, such as the esophagus, stomach, and liver up to 30 months post-transplant (Figure 5.3 a,b).

5.3 Discussion

There are three major phenotypes for cystinosis. Infantile nephropathic cystinosis is the most severe type, and constitutes over 95% of cases. This disease presents with renal impairment during the first year of life, and development of renal failure by the end of the first decade, along with multi-organ damage. Few cystinosis patients present with juvenile nephropathic cystinosis, which is a milder form of the disease that induces renal injury at a later age. And thirdly, the ocular type, which manifests only with photophobia due to corneal cystine crystal deposition, and spares other organs [40,47].

Our patient presented with typical infantile nephropathic cystinosis; however, he was diagnosed and started cysteamine therapy relatively late, at 2.7 years of age. Cysteamine efficiently decreases cystine content in lysosomes through direct biochemical interaction with cystine. While it delays the natural course of the disease, it unfortunately is not able to prevent many of the pathogenic aspects of cystinosis, including the Fanconi syndrome and the subsequent renal failure [40,47]. Furthermore, our patient experienced significant side effects of cysteamine therapy, including bone involvement and halitosis, which severely impacted him psychosocially.

Allogeneic HSCT has contributed substantially to the improved survival and quality of life in children affected by different IEMs [373]. Moreover, animal models of cystinosis provided the initial proof for the clinical benefits of HSCT. In the study by Syres *et al*,[162] organ-specific cystine content was reduced in all organs tested in cystinotic mice treated with Wt bone marrow. These organs included brain (57% reduction), eye (71%), heart (82%), kidney (70%), liver (95%), muscle (66%) and spleen (87%). Yeagy *et al*,[163] further confirmed the long term protection of HSCT for the mouse kidney up to 15months post-transplant and showed that high-level donor-cell engraftment was essential for efficient therapy. In subsequent studies, HSCT in the cystinotic mouse protected against both eye pathology and hypothyroidism [32,378].

Although our patient showed signs of clinical improvement of cystinosis (decreased polyuria and photophobia), he developed early signs of tacrolimus toxicity starting 4 weeks following the first HSCT. Similar cases with increased chances of nephrotoxicity and neurotoxicity may benefit from a calcineurin-free GvHD prophylaxis, such as the low-dose cyclophosphamide regimen [385]. Following the second HSC donation, our patient developed severe form of GvHD, which did not respond to systemic corticosteroids, calcineurin inhibitors, or anti-metabolites. Moreover, he developed seizures and hallucinations, due presumably to the administration of calcineurin inhibitors and other drugs. These complications combined with his subsequent systemic infection with a multi-drug resistant Pseudomonas led to his clinical deterioration, and ultimate demise. Despite this tragic outcome, this patient demonstrated clear reductions in the cystine crystal loads in mucosal interstitial macrophages in his stomach, as well as transfer of the Wt cystinosin RNA and protein to epithelial cells in multiple organs (GI tract, liver, and kidney), which together are indicative of the potential efficacy of HSCT in the treatment of cystinosis.

5.4 Conclusion

In conclusion, this is the first report describing allogeneic HSCT in a human patient with cystinosis, a lysosomal membrane transporter deficiency. This study broadens the spectrum of genetic diseases that may benefit from HSCT in clinical practice, and validates a novel mechanism of function of HSCs in hereditary metabolic disorders beyond simple compensation for enzyme deficiencies. However, this case history also highlights that the potential benefits of allogeneic HSCT should be carefully balanced against its risks and potential mortality. In our study, the expression of the Wt allele in the recipient's non-hematological epithelial cells with the subsequent decrease in cystine crystal accumulation, resolution of photophobia, and reduction in urinary output demonstrates that the *CTNS* donor-recipient gene and protein transfer does indeed occur, and that it can be functional in humans.

Similar to ALD, and other monogenic diseases, such as different forms of severe combined immune deficiency, thalassemia and MPS, [386,387] the feasibility and efficacy of autologous HSCT following *ex vivo* gene therapy should be explored in cystinosis in future studies to avoid the risks associated with allogeneic HSCT.

Conflict of Interest disclosure

The authors declare no competing financial interests.

Acknowledgments

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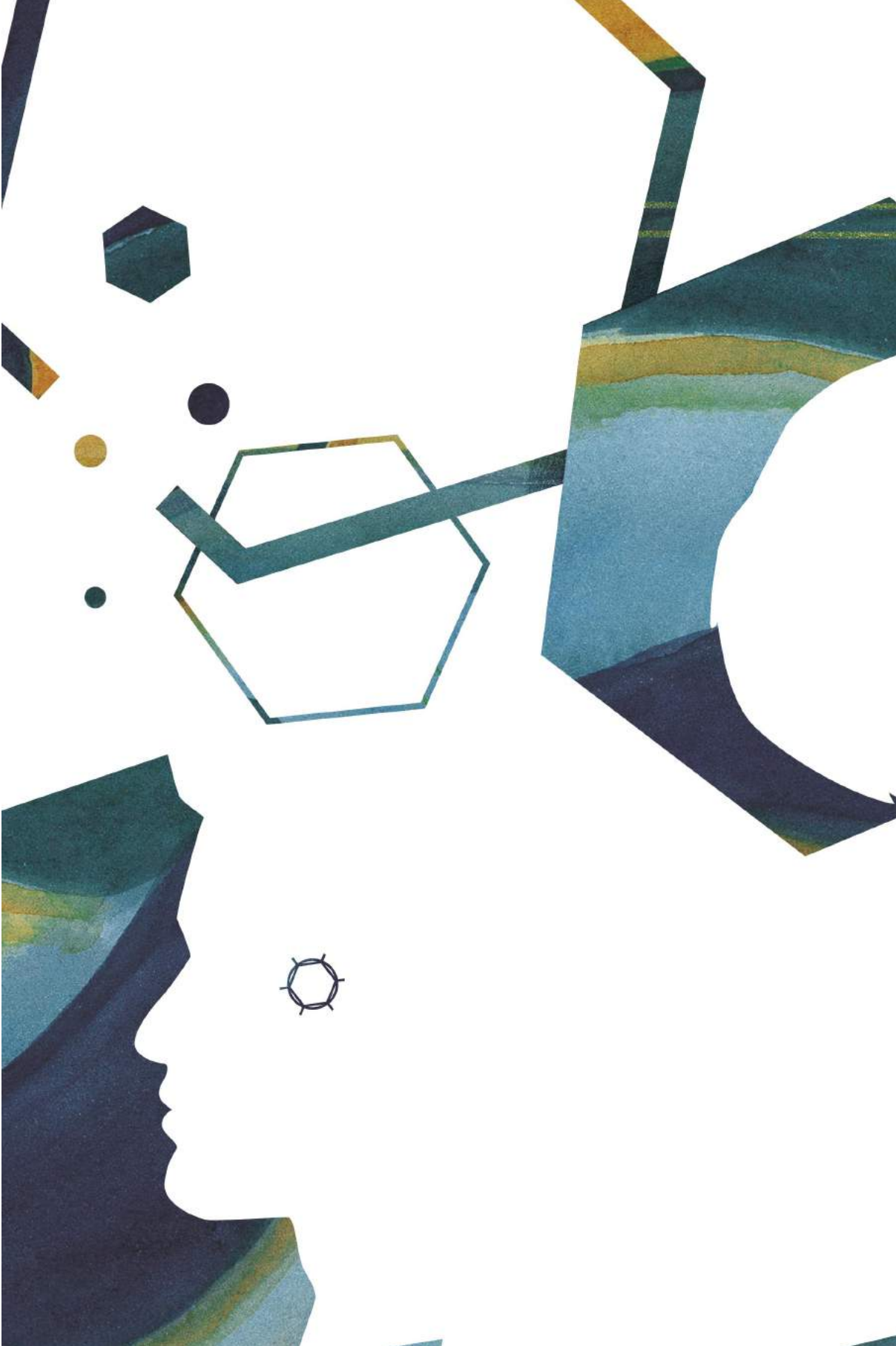

CHAPTER 6
Kidney progenitor cells are present in urine of cystinosis patients and can be genetically rescued via *ex vivo* gene therapy

Chapter based on:

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Kidney progenitor cells are present in urine of cystinosis patients and can be genetically rescued via *ex vivo* gene therapy

Manuscript in preparation for submission

ABSTRACT

Background

Nephropathic cystinosis is a rare inherited multi-system lysosomal storage disorder caused by mutations in the *CTNS* gene. The kidney phenotype in cystinosis initially affects proximal tubules and subsequently podocytes. Both cell types are excessively exfoliated into urine associated with the development of proximal tubular atrophy and focal segmental glomerulosclerosis progressing towards end stage kidney failure. Cysteamine, the only available treatment, does not offer a cure for the kidney disease. We hypothesized that cystinotic kidneys need to undertake a regenerative effort to compensate for ongoing cell losses and explored this hypothesis using kidney tissue and urine of non-transplanted cystinosis patients.

Methods

Fresh midstream urine samples of two nephropathic cystinosis patients were collected in which number of voided undifferentiated cells were quantified compared to healthy controls. Similarly, via incubation in a specific medium, clonal colonies were grown and characterized and underwent a differentiation protocol to a proximal tubular epithelial or podocyte-like cell. Characterization was performed via qPCR, FACS, immunofluorescence and specific functional assays. Complementation of *CTNS* using an integrating lentiviral vector (LV) encoding *CTNS* was performed, and intracellular cystine levels and redistribution of the LAMP1 lysosomal compartment was assessed.

Results

Cystinosis urine-derived kidney progenitor cell clones were established, showing the expression of specific kidney progenitor and mesenchymal cell markers, and the potential to differentiate into a functional proximal tubular epithelial or podocyte-like cell. Complementation of *CTNS* via LV transduction showed significant reductions of intracellular cystine levels and restoration of the altered LAMP1 lysosomal compartment.

Conclusions

We demonstrate that kidney of cystinosis patients contain a kidney progenitor cell niche. These cells can be isolated from urine and be supplemented with the *CTNS* gene resulting in the correction of the phenotype. These findings provide a proof-of-principle of the feasibility of *ex vivo* gene therapy to rescue the healthy cellular phenotype in cystinosis.

6.1 Introduction

Cystinosis (Online Mendelian Inheritance in Man (OMIM) #219800) is a rare autosomal recessive lysosomal storage disease, caused by bi-allelic mutations in the *CTNS* gene leading to the absence or malfunctioning of the cystine-proton cotransporter cystinosin [1].

It is a multisystem disorder in which the kidney is the first and most severely affected organ [5,40]. In the infantile phenotype, the most common and severe form, a generalized proximal tubular dysfunction (renal Fanconi syndrome) develops in infancy followed by progressive glomerular dysfunction leading to end-stage renal disease [47]. The juvenile phenotype is characterized by a less pronounced Fanconi syndrome and kidney function decline [42].

Unfortunately, a cure for the kidney disease in cystinosis is still lacking. While cysteamine, currently the only available disease-modifying treatment, has shown to postpone the onset of end-stage renal disease and increase life expectancy of cystinosis patients, it neither offers a cure for the Fanconi syndrome nor prevents the need for renal replacement therapy [152,337]. In addition, cysteamine comes with significant side-effects hampering compliance and necessitating regular drug monitoring [388]. The lack of improvement of the renal Fanconi syndrome observed in cysteamine-treated cystinosis patients has demonstrated that the profound proximal tubular dysfunction in cystinosis, the first and most severe feature of the disease, is not merely due to cystine accumulation. Indeed, the pathophysiological mechanism linking the absent or defective cystinosin to the renal proximal tubular dysfunction in specific, has not been fully elucidated yet.

Over the past decade, several hypotheses have been put forward, including an increased sensitivity of cystinotic cells to apoptosis, structural alterations and dysfunction of mitochondria, energy imbalance, oxidative stress, endoplasmic reticulum stress and the propensity to cell dedifferentiation [19,21,389–394,22–24,28,31,324,326,327].

More recently, alterations in vesicle trafficking, lysosomal biology and pivotal cell signaling cascades (mTOR signaling) in cystinotic cell lines have indicated that more versatile functions can be allocated to cystinosin [33–35,44,395]. Indeed, morphological alterations of the endosomal compartments comprising the appearance of enlarged and perinuclear clustered early (early endosomal antigen 1, EEA1) and late (LAMP1) endosomes was shown in *CTNS* knock-down experiments in HK-2 cells and cystinosis ciPTECs and via electron microscopy in human kidney biopsy specimens [33]. These observations were consistent with other studies in the cystinotic mouse model [31,38]. Moreover, processing of the cargo endocytosed via the multiligand receptor-mediated endocytosis using megalin, was shown to be delayed both in CTNS KD HK-2 cells and cystinosis ciPTECs, and has been linked with the reduced surface expression of megalin which is associated with a phenotype of dedifferentiation[31]. Importantly, these alterations were only

partially restored upon treatment with cysteamine [33]. In parallel, the accumulation of autophagy substrates (p62, SQSTM1) and of mature autophagosomes in various cystinosis cell lines under normal conditions, have suggested impairment of autophagic flux [38,396]. Together, these observations have underlined the pivotal role of the lysosomal compartment as the final common pathway for the endocytic and autophagic compartment processing.

More recently, the role of transcription factor EB (TFEB), a major regulator of the lysosomal network involved in several aspects of lysosomal biology and homeostasis, has been highlighted in cystinosis by showing that stimulation or overexpression of TFEB was able to lower cystine levels and stimulate delayed endocytic processing, while only prolonged overexpression of TFEB was associated with restoration of the alterations of the lysosomal compartment which were not corrected with cysteamine [34]. Altogether, only very recently, a hypothesis was put forward to explain the link between the loss of function of cystinosin and its role in the lysosomal compartment, with renal proximal tubular epithelial dysfunction, which mainly comprises the generation of mitochondrial reactive oxygen species due to defective autophagic clearance, and subsequent zona occludens-1 mistrafficking, tight-junction disruption and nuclear translocation of ZO-1-associated nucleic acid binding proteins (ZONAB) leading to dedifferentiation via reduced expression of megalin and increased proliferation [38].

Altogether, these observations have identified other key pathophysiological hallmarks linked to the loss of function of cystinosin, apart from lysosomal cystine export, which are crucial in the assessment of the restoration of the healthy cellular phenotype in cystinosis cell models and which may harbor promising molecular targets for drug development.

On the other hand, these insights have underscored the complexity of the pathophysiology of cystinosis and, while they may harbor promising molecular targets for drug development, it has profoundly altered the tenet of cystinosis being a disease of merely lysosomal cystine accumulation, and tempered the presumption for a simple drug-based treatment. From this perspective, innovative strategies for the treatment of cystinosis are rightly needed.

Over the past decade, extensive efforts have been made to establish a hematopoietic stem cell (HSC) based gene therapy in an attempt to provide a cure for cystinosis [162,163,165]. Promising pioneering studies in the cystinosis mouse model by the group of Cherqui et *al* in which allogenic HSC transplantation (HSCT) resulted in significant reduction of cystine content in various relevant tissues and long-term preservation of kidney function, have led to the first phase I clinical trial on stem cell and gene therapy in cystinosis, which was recently approved by the FDA (ClinicalTrials.gov identifier NCT03897361).

However, a recent report on the first allogeneic HSC transplantation in a cystinosis patient has raised concern about the efficacy and potential side-effects related to an HSC-based approach

[397]. Despite the exciting evidence in support of the proposed mechanism by Cherqui and co-workers of the delivery of cystinosin mRNA or protein via tunneling nanotubes to non-hematological cells, the beneficial effect on the kidney phenotype was limited while significant graft-versus-host related morbidity and drug-related toxicity led to a final mournful outcome [397]. Since it is unclear whether the degree of inflammation in the GvHD observed in this case is merely due to the immunogenicity of allogenic HSCs, and to what extent the inflammation induced by the macrophage engulfment of widespread tissue cystine load is a major contributor, careful reconsideration is needed in regard to whether this approach will yield the best therapeutic benefit for cystinosis patients. Therefore, a further search for alternative approaches to establish a cure for the kidney disease in cystinosis is invaluable.

In this respect, the regenerative response of the cystinotic kidney, and the presence of a kidney stem/progenitor cell pool in cystinosis has not been investigated yet. Indeed, maladaptive kidney regeneration has been associated with progressive chronic kidney disease [398–401]. Enhancing a maladaptive regenerative response could be a promising approach to limit tubular or progressive glomerular dysfunction.

Moreover, the possibility of kidney tissue-specific cell therapy may hold a promise for autologous tissue engineering and cell-based gene therapeutic applications in this monogenic disease [34,38,348,402,403].

The actual existence and origin of a kidney stem/progenitor cell pool has been a matter of extensive research and debate for almost two decades [230,245–250,404,405]. Since the kidney has a certain reparative potential, both in cases of tubular and glomerular injury, extensive efforts have been made to identify and characterize the niche of cells responsible for kidney regeneration. The identification of a human kidney stem/progenitor cell pool has mainly been based initially on the expression of putative stem cell surface markers both in attempts to isolate these cells from kidney tissue or urine to proliferate and assess their functionality *in vitro*, as well as in *in vivo* settings on human kidney tissue biopsy stainings of acute and chronic kidney injury [192,214,406,407,217,219,220,224,229,258,263,268]. Later, renal progenitor cells were selected *in vitro* based on other features, including the activity of enzymes specifically over-expressed in stem cells (aldehyde dehydrogenase, ALDH), sphere-forming ability in culture or the expression of other cell-surface markers characteristic for human embryonal kidney [212,224]. Lineage tracing studies in transgenic mice have been applied to overcome the limitations linked to the use of stem cell markers, however the species-specific differences in expression have in part hampered some conclusions [195,277,279,280,292,293].

To summarize, the origin of regeneration of podocytes has been attributed to parietal epithelial cells of the Bowman's capsule and to cells of renin lineage adjacent to the juxtaglomerular

apparatus. On the other hand, tubular regeneration has been linked to a specific phenotype of scattered tubular epithelial cells and segment-specific lineage restricted tubular regeneration [212,217,219,224,271].

However, still regeneration is limited and a maladaptive regenerative response may lead to chronic kidney injury [398–401]. While the presence of a certain type of renal progenitor cells has been claimed to be present in urine of patients with nephrotic syndrome, IgA nephropathy, Henoch-Schönlein nephritis and hemolytic uremic syndrome, characterization was limited, and no *in vivo* data nor information on the functionality and fate of these cells was presented [263]. Recently, we demonstrated a significantly increased loss of podocytes and proximal tubular epithelial cells in urine of nephropathic cystinosis patients [44]. We hypothesized that in compensation for this epithelial cell loss, enhanced kidney regeneration might be present, which could be reflected by the presence of kidney progenitor cells in kidney tissue and urine of cystinosis patients. In this study, we aimed to demonstrate the presence of kidney progenitor cells in renal tissue and urine of cystinosis patients, characterize and assess their potential for differentiation towards mature cells of the nephron epithelium, and their functionality. Furthermore, via lentiviral vector transduction, we aimed to rescue the healthy cellular phenotype in kidney progenitor cells to that observed in healthy individuals hereby providing a proof of principle for the feasibility for *ex vivo* gene therapy in cystinosis.

6.2 Methods

6.2.1 Study participants

For the quantification of cystinotic urine-derived kidney progenitor cells (Cys-uKPC), freshly voided urine samples were obtained from in total 9 nephropathic cystinosis patients and 9 healthy controls (Table 6.1; patient # 1 to patient # 9). For the isolation of Cys-uKPCs, freshly voided urine samples were collected from two non-kidney transplanted (Table 6.1; patient # 7, patient # 10) nephropathic cystinosis patients, both followed regularly at the University Hospitals Leuven (UZ Leuven), Leuven, Belgium. Kidney biopsy specimens were obtained from two cystinosis patients. Approval from the local ethical board of UZ/KU Leuven (Ethische Commissie Onderzoek UZ/KU Leuven) was granted under the study number s54695 and written informed consents were obtained from study participants. Research was conducted in accordance with the last version of the Declaration of Helsinki, the principles of Good Clinical Practice (GCP) and all applicable national and international legislation related to research involving human subjects. Additional relevant demographic and clinical data were collected from the medical records of the participants (Table 6.1).

6.2.2 Kidney biopsy specimens

Kidney biopsy specimens of the native kidneys of one nephropathic cystinosis patient, were retrieved for CD133 and PAX2 stainings.

6.2.3 Quantification of urine-voided undifferentiated, vimentin-positive cells via qPCR

A calibration curve was developed using known amounts of control kidney stem cells, derived from the renal papilla and kindly provided by Prof. B. Bussolati (Università degli Studi di Torino, Turin, Italy). For doing so, control kidney stem cells were sorted by Fluorescence Activated Cell Sorting (FACS) using the BD FACSAria III (BD Biosciences, San Jose, California, USA) and performed by the VIB-KU Leuven FACS Core Facility. Cells were seeded in a 96-well plate containing 4 μl of lysis buffer (0.2% TritonX-100 + RNase inhibitor) yielding a range of number of cells per well from 5 to 500 cells.

Smart-seq2 technology allowed cDNA to be prepared from very low numbers of cells. An adaption of the protocol of Picelli et al was applied up to PCR purification (step 26), and 18 PCR cycles were performed in step 14 [408]. qPCR was performed using the housekeeping gene ß-Actin, and vimentin (*VIM*) as a marker for undifferentiated cells. Following establishment of the calibration curve, freshly voided urine samples were collected from cystinosis patients and healthy subjects (Table 6.1, patient # 1 to patient # 9), centrifuged (200g, 4°C, 5 minutes), and the cell pellet was resuspended in PBS, followed by resuspension in a 1:100 dilution. cDNA was synthesized from the urine-voided cells in the cystinosis patients and controls according to the aforementioned methodology. The cycle threshold (ct) value achieved in the qPCR analysis was plotted in the calibration curve to allow for an estimation of the number of undifferentiated cells voided in urine, which was normalized to urine volume and urine creatinine values.

6.2.4 Isolation of cystinotic uKPCs

Clones of primary cells derived from urine of non-kidney transplanted cystinosis patients, hereafter referred to as cystinotic urine-derived kidney progenitor cells (Cys-uKPCs) were established according to a standardized protocol (Figure S6.1). Based on preliminary data (not shown), isolation of Cys-uKPCs was restricted to two patients who previously showed a high yield of Cys-uKPCs.

Briefly, freshly voided urine samples were centrifuged (300g, 5 minutes, room temperature), the supernatant was removed, and the cell pellet was washed with PBS (Lonza 17-516), followed by re-centrifugation. Depending on the size of the cell pellet, cells were plated in a single or multiple 10 centimeter petridishes and incubated in medium containing a 1:1 ratio of keratinocyte-serum

free medium and progenitor cell medium (1:1 mix), furthermore referred to as uroprogenitor medium, at 37°C in 5% CO_2 [262]. Keratinocyte serum-free medium (Thermo Fisher Scientific 17005-059) was supplemented with 5 ng/ml epidermal growth factor (Sigma-Aldrich E9644), 50 ng/ml bovine pituitary extract (Gibco™ Life Technologies – Thermo Fisher Scientific 13028014), 30 ng/ml cholera toxin (Sigma-Aldrich C8052), 100 U/ml penicillin and 1 mg/ml streptomycin (Westburg DE17-602E). Progenitor cell medium contained ¾ Dulbecco's modified Eagle's medium (Westburg BE12-719F), ¼ Hamm's F12 (Lonza BE12-615F), 10% fetal bovine serum (VWR S181B-500), 0.4 µg/ml hydrocortisone (Sigma-Aldrich H0135), 10^{-10}M cholera toxin, 5 ng/ml insulin (Sigma-Aldrich I5500), 1.8×10^{-4} M adenine (Sigma-Aldrich A2786), 5 µg/ml transferrin (Sigma-Aldrich T8158) plus 2×10^{-9} M 3,39,5-triiodo-L-thyronine (Sigma-Aldrich T0281), 10 ng/ml epidermal growth factor (Sigma-Aldrich E9644) and 1% penicillin-streptomycin (Westburg DE17-602E).

Clonal expansion could be observed starting from 3 to 5 days after the start of incubation and were selected between 9 to 14 days (13 ± 2 days). Each selected clone was plated in a single well of a 24-well plate (P#1). Each time upon reaching 70 – 80% confluence, cells were trypsinized and counted using the BioRad TC20™ automated cell counter (BioRad #145-0101). In passage 2 (P#2), all cells were transferred into a 6 cm petridish, while by the next passage (P#3) 2/3rd of cells were plated in a 10cm petridish. From the 4th passage on a splitting ratio of ¼ was maintained and cells were further proliferated in 10cm petridishes until senescence was reached. Senescence was defined as the inability to form a subconfluent (70 – 80% confluence) monolayer within 14 days of culture. No bacterial contamination was detected in any of the culture samples that were further processed.

6.2.5 Characterization of cystinotic uKPCs via qPCR and flow cytometry

Characterization of these Cys-uKPCs was performed in passage 4 via quantitative polymerase chain reaction (qPCR) for kidney progenitor specific genes Neural Cell Adhesion Molecule 1 (*NCAM1*), CBP/p300 interacting transactivators with glutamic acid (E)/aspartic acid (D)-rich C-terminal domain (*CITED1*), Vimentin (*VIM*) and paired box 2 (*PAX2*). β-Actin was used as a housekeeping gene. Briefly, cell pellets were harvested by trypsinization and RNA was isolated using RNeasy Mini or Micro Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. RNA was further used to generate cDNA using a mix of Oligo (dT) 12-18 Primer (Invitrogen™ Thermo Fisher 18418-012), random primers (Thermo Fisher 48190-011), dNTP mix (100 mM Invitrogen Thermo Fisher 10297018) and SuperScript™ III Reverse Transcriptase (Thermo Fisher 18080-085). The quantitative rt-PCR (qPCR) was executed on a

CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc. Hercules, California), using Platinum™ SYBR™ Green qPCR SuperMix-UDG w/ROX (Thermo Fisher 11744-500), 10 μM of primers and 1 μl of cDNA (5ng/μl). qPCR data was retrieved and processed using the CFX Manager™ software (Bio-Rad).

Upon further proliferation of the Cys-uKPC clonal cell line, subsequent characterization was performed in passage 6 by flow cytometry for mesenchymal cell markers CD73, CD44, CD29, CD90 and CD105, the Human Leucocyte Antigen DR (HLA-DR), and the established kidney progenitor markers CD133 and CD24. An amount of $2x10^5$ cells were incubated with conjugated antibodies (dilution 1:100) for about 40 minutes on crushed ice in dark. Flow cytometry was executed on BD CANTO II (BD Biosciences, San Jose, California, USA) at the VIB-KU Leuven FACS Core Facility. Flow cytometry data were generated and processed using the BD FACSDiva™ software and further analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon, USA).

6.2.6 Differentiation of cystinotic uKPCs to proximal tubular epithelial cells and podocytes

Exploration of the differentiation potential of Cys-uKPCs towards a PTEC and/or podocyte was performed in passage #6 to 7.

Cys-uKPCs were differentiated to proximal tubular epithelial cells (Cys-uKPC - PTEC) by incubation in PTEC medium (DMEM F12, Insulin 5 μg/ml, Thyroxin 5 μg/ml, Selenium 5 ng/ml, Hydrocortisone 36 ng/ml, EGF 10 ng/ml, Tri-iodothyronine 40 pg/ml, 10% FBS, 1% penicillin-streptomycin) for 7 days. Expression of PTEC-specific markers Aquaporin-1 (*AQP1*), P-gp or multidrug resistance protein 1 (*MDR1*) (*ABCB1*), and cubilin (*CUBN*) was demonstrated by PCR, and changes in cellular morphology during differentiation were monitored by light microscopy.

Cys-uKPCs were differentiated to podocytes (Cys-uKPC - Podo) by incubation in VRAD medium (DMEM-F12, 10% FBS, 100 nM vitamin D3, 100 μM all-trans retinoic acid) for 7 days [217]. Expression of podocyte-specific markers nephrin (*NPHS1*), synaptopodin (*SYNPO*), podocalyxin (*PODXL*), and CD2-associated protein (*CD2AP*) was demonstrated by qPCR and immunofluorescence staining, and changes in cellular morphology during differentiation were monitored by light microscopy.

A clone that showed a sufficient proliferation capacity to allow a full characterization via qPCR (P#4) and flow cytometry (P#6), expression of kidney progenitor genes *CITED1*, *NCAM1*, *VIM* and *PAX2* and demonstrated the potential to show significant upregulation of PTEC- and/or podocyte-specific genes following the respective differentiation protocol, was considered as an established cystinotic uKPC clone.

6.2.7 Functional assessment of cystinotic uKPC-derived PTECs and podocytes

P-glycoprotein calcein assay

In order to explore the functionality of Cys-uKPC derived PTECs, the activity of P-glycoprotein (P-gp) was assessed in a spectrophotometric assay using Calcein-AM (Molecular probes Invitrogen – Life Technologies, C1430) as a substrate for P-gp, and PSC-833 as a P-gp inhibitor (Valspodar; Tocris Bioscience, 4042).

Briefly, 75.000 cells of Cys-uKPC, Cys-uKPC – PTEC, wild-type ciPTEC and cystinotic ciPTEC cell lines were seeded per well in triplicates in 24-well plates, allowing for the assessment of a P-gp inhibited, P-gp non-inhibited, and vehicle control condition. Cells were differentiated for 7 (primary cells) to 10 (conditionally immortalized cell lines) at 37°C, 5% CO_2 via incubation in PTEC medium. The conditions destined for P-gp inhibition, were pre-incubated with PSC-833 (2 μM in DMSO) for 30 minutes at 37°C in the dark, while the non-inhibited and control conditions were pre-incubated with DMSO. Hereafter, both inhibited and non-inhibited conditions were incubated with Calcein-AM (1 μM) for 60 minutes at 37°C in the dark. P-gp transport activity was stopped by washing twice in ice-cold HBSS/HEPES and cells were lysed via incubation in 0.1% Triton in warm HBSS/HEPES for 20 minutes at 37°C in dark. The lysed cells were scraped and lysates were transferred to a flat bottom transparent 96-well plate (GBO Greia Bio-One, 655090). Serial dilution series were prepared of the lysates of the conditions in which the highest concentration of Calcein was expected in the lysate. Fluorescence of the lysates was determined at ex485/em535 nm using the POLARstar® system (BMG LABTECH), in kind collaboration with the SWITCH Laboratory, VIB-KU Leuven.

Albumin endocytosis assay

The functionality of cystinotic uKPC-derived podocytes was assessed by analyzing the endocytic uptake capabilities of albumin in an endocytosis assay.

Briefly, 80.000 cells of Cys-uKPC, Cys-uKPC – Podo, wild-type ciPodocyte and cystinotic ciPodocyte cell lines were seeded per well in triplicate on 13mm sterile glass cover slips in 24-well plates destined for incubation at 37°C and 4°C, the latter for allowing the assessment of the temperature dependency of albumin endocytosis. The wild type ciPodocyte and cystinotic ciPodocyte cell lines were differentiated via incubation in Podocyte medium for 10 days (conditionally immortalized control cell lines), while the cystinotic-uKPC derived Podocyte was obtained via incubation of Cys-uKPC in VRAD medium for 7 days (primary cells), and undifferentiated Cys-uKPCs were kept on uroprogenitor medium, all at 37°C, 5% CO_2. Following differentiation, serum starvation was applied via incubation in serum- and supplement-free medium (DMEM-F12; Lonza, Breda, The Netherlands) during 2 hours at 37°C. Hereafter,

incubation with complete medium supplemented with 100 μg/ml of Alexa Fluor® 555-conjugated BSA (Life Technologies, A-34786) was performed for 60 minutes in parallel at 37°C and at 4°C. The experiment was terminated by washing five times with ice-cold PBS, and fixation of cells with 4% paraformaldehyde for 15 minutes at RT. Cover slips were mounted on glass slides (Menzel Gläser SuperFrost® Plus, Thermo Scientific, J1810AMNZ) using fluorescence mounting medium (Dako S3023) supplemented with DAPI (1:1000). Uptake of the labeled BSA was analyzed by fluorescence microscopy using the Nikon Eclipse Ci microscope (Nikon Corporation, Tokyo, Japan). Quantification of the labeled BSA uptake was analyzed using Fiji/ImageJ software, and expressed as the Cell Corrected Integrated Density. Correct interpretation of actual BSA uptake by the individual cells was ensured by visualization of the alignment of the cell plasma membranes via enhancement of image brightness and contrast in Fiji/ImageJ.

Calcium influx assay

Furthermore, the functionality of cystinotic uKPC-derived podocytes was assessed by analyzing the calcium influx via the Transient receptor potential cation channel subfamily C member 6 (TRPC6) in a Fura calcium imaging assay [409]. Cystinotic-uKPCs were seeded on sterile glass cover slips in 12-well plates and differentiated to podocytes by incubation in VRAD medium for 7 days. Following differentiation, the Cys-uKPC derived Podocytes were incubated with 2 μM Fura-2 acetoxymethyl ester (Invitrogen™ F1201) for 20 minutes at 37°C. Standard imaging solutions consisted of 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4 by NaOH). Perfusion of the bath solutions was based on gravity via a multi-barreled pipette tip with a single outlet of 0.8 mm diameter. Intracellular Ca^{2+} concentration was determined based on the ratio of fluorescence detected upon alternating excitation at 340 and 380 nm, using a Lambda XL illuminator (Sutter instruments, Novato, USA) and an Orca Flash 4.0 camera (Hamamatsu Photonics Belgium, Mont-Saint-Guibert, Belgium) on a Nikon Eclipse Ti fluorescence microscope (Nikon Benelux, Brussels, Belgium). The imaging data were recorded and analyzed using NIS-elements software (Nikon) and IgorPro 6.2 (WaveMetrics, USA). 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Sigma-Aldrich 06754), a TRPC6 agonist, was applied (150 mM) in order to stimulate calcium influx and assess the response rate of the cell type studied [410]. Cells showing an increase in calcium amplitude larger than 50 nM and a slope increase more than 3-fold the standard deviation, were considered as responders to OAG stimulation. Only cells that responded to the positive control, ionomycin, at the end of the experiment were taken into account. All experiments were performed on at least 3 independent cover slips. Undifferentiated Cys-uKPCs were also assessed, while cystinotic ciPodocytes, incubated for 10-14 days at 37°C, were used as a positive control.

6.2.8 Lentiviral vector design and transduction experiments

Self-inactivating (SIN) lentiviral vector (LV) constructs, encoding either eGFP or CTNS (full length cDNA; tagged with a C-terminal 3HA tag) as a transgene, together with a puromycin antibiotic selection cassette driven from a human elongation factor-1 alpha (EF-1 α) promotor, were developed by the Leuven Viral Vector Core (LVVC) (Figure S6.2), referred to as LV_CTNS-3HA and LV_eGFP respectively. Validation of the LV constructs was performed in 293T cells and cystinotic ci control cell lines (fibroblasts and podocytes) corroborating CTNS protein expression via immunofluorescence staining and Western Blot (data not shown).

One cystinotic uKPC clonal cell line was transduced in a 1/3 serial dilution series with LV_CTNS-3HA and LV_eGFP as vehicle control. We selected a condition with a high vector concentration (vector titer: 1173333 pg p24/ml) and a low vector concentration (vector titer: 1610 pg p24/ml) referred to as Cys-uKPC LV_CTNS-3HA$_{high}$ and Cys-uKPC LV_CTNS-3HA$_{low}$. Antibiotic selection (puromycin 1 μg/ml) allowed to select a polyclonal cell line stably expressing CTNS-3HA. Following proliferation, transduced cystinotic uKPCs were harvested for isolation of RNA, assessment of intracellular cystine levels, and immunofluorescence staining for LAMP1, HA and eGFP.

6.2.9 Cystine measurements

For each condition in which intracellular cystine levels needed to be tested, 10^6 cells were grown in 10cm petridishes in triplicate. Cells were washed with PBS, 200 µl of ice-cold N-ethylmaleimide (NEM) was added in order to block free thiol groups, followed by collecting of the cells by scraping. One hundred µl of 12% sulfosalicylic acid (SSA) was added for protein precipitation to occur, vortexed for 30 seconds for homogenization, and centrifuged (10 min, 13.000 rpm, 4°C). The cystine-containing supernatants were isolated for cystine assay and kept immediately at –80 °C, while the protein pellet was incubated with 300 µl of 0.1 M NaOH (Sigma-Aldrich) overnight, and then transferred to –80 °C until day of analysis.

Cystine concentrations were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Total protein concentrations were determined on the precipitated protein pellets by a BCA assay. Cystine concentrations were expressed as nmol ½ cystine/mg protein.

6.2.10 Immunofluorescence staining and microscopy

Cells were seeded on sterile glass 13 mm or 18 mm diameter cover slips in a 24-well plate or 12-well plate, at a cell density of 20.000 to 40.000 cells/well respectively (Sarstedt, Germany). Cells were fixed 24 hours after seeding, using 4% paraformaldehyde for 15 minutes at RT, and subsequently kept on PBS at 4°C until further processing. Cells were permeabilized by

incubation with freshly prepared 0.1% Triton X-100 (Sigma-Aldrich) in PBS on ice (4°C) for 5 to 10 minutes, followed by blocking in a filtered (0.2 μm Whatman™ filter) blocking buffer containing 2% FBS, 2% BSA and 0.2% gelatin in PBS. Primary antibodies (Table S3), dissolved in blocking buffer at desired concentrations, were introduced and incubation occurred at 4°C overnight, followed by incubation of the secondary antibodies, diluted at specific concentrations, for 45 to 60 minutes in a dark humidity chamber at 4°C. Mounting was performed using fluorescence mounting medium (FluorSave™, Calbiochem® - Millipore), in which 6-diamidino-2-phenylindole (DAPI; Dako, S3023) was diluted 1:1000, on glass slides (Menzel Gläser SuperFrost® Plus, Thermo Scientific, J1810AMNZ).

Immunofluorescence microscopy was performed using the Nikon Eclipse Ci microscope (Nikon Corporation, Tokyo, Japan), while confocal fluorescence microscopy images were recorded on the Zeiss® LSM 880 – Airyscan (Carl Zeiss Microscopy GmbH, Germany) (Cell and Tissue Imaging cluster / Cell Imaging Core (CIC), supported by Hercules AKUL/15/37_GOH1816N and FWO G.0929.15 to Pieter Vanden Berghe, KU Leuven). Microscopy images were processed and analyzed using Zeiss ZEN imaging software and Fiji/ImageJ. Analysis and quantification of the cellular distribution of the LAMP1 lysosomal compartment was performed using Fiji/ImageJ software. Localization of LAMP1 -positive structures within the 25th percentile of the area between the nuclear rim and the plasma membrane proximal to the nucleus, was defined as perinuclear. Conversely, localization within the outer 75th percentile, distal to the nucleus, was defined as peripheral.

6.2.11 Immunohistochemistry

PAX2 and CD133 co-stainings were performed on kidney biopsy specimens retrieved from two nephropathic cystinosis patients using antibodies and respective dilutions as indicated in Table S6.3.

6.2.12 Statistical analysis

Graphpad Prism (version 8.1.0 (221) for Mac OS X) (GraphPad Software, La Jolla California USA, www. Graphpad.com) was used for the statistical analysis performed in this study.

D'Agostino & Pearson normality test was applied in order to check for normality of the distribution of the medians of the parameters of the cystinosis patients and the control subjects. Depending on this distribution, a two-tailed unpaired Student t-test or Mann-Whitney U-test was performed. Data are represented as mean ± standard deviation (SD) for normally distributed data, and as median ± 25th and 75th percentiles (inter-quartile range, IQR) for non-Guassian distributed data, unless otherwise specified.

6.3 Results

6.3.1 Nephropathic cystinosis is characterized by a urinary loss of undifferentiated, vimentin-positive cells

The total number of cells and the number of undifferentiated (vimentin-positive) cells freshly voided in urine in non-kidney transplanted nephropathic cystinosis patients with different genetic backgrounds (Table 6.1, patient # 1 to # 9; aged 10.3 ± 4.36 years) and 9 pediatric healthy controls, were determined, normalized to urine volume and urine creatinine (Table 6.2, Figure 6.1). A 260-fold higher total number of cells voided in urine (Mann-Whitney, *p*=0.001), as well as a significant higher excretion of undifferentiated vimentin-positive cells was demonstrated (Mann-Whitney, *p*=0.002) in urine of nephropathic cystinosis patients (Figure 6.1, Table 6.2). In contrast, no loss of undifferentiated cells was observed in the healthy control subjects. No correlation could be demonstrated between the number of undifferentiated cells voided in urine and proteinuria (Spearman r -0.38, *p*=0.32), kidney function (eGFR; Spearman r -0.03, *p*=0.96) nor the actual WBC cystine level (Spearman r 0.57, *p*=0.12) (Table 6.1).

6.3.2 Kidney progenitor cells expressing specific kidney progenitor markers reminiscent of early nephrogenesis, are present in urine of nephropathic cystinosis patients

We hypothesized that the higher number of undifferentiated *VIM*-positive cells reflects ongoing kidney regeneration in nephropathic cystinosis. Thus, we set out to characterize the urine-voided undifferentiated cells in detail for two cystinosis patients (patient # 7 & # 10) (Table 6.1) and developed clones at three consecutive 3-monthly timepoints spanning a period of 9 months, through incubation of urine-derived samples in uroprogenitor medium. The total number of clonal colonies growing upon incubation of the urine-derived samples ranged from 0.6 to 1.2 colonies/ml urine volume for patient #10 and #7, respectively (Table 6.3). The difference reached a borderline significance (*p*=0.5) (Table 6.3). Notably, the patient showing the lower number of clonal colonies per urine volume, exhibited a significantly higher degree of proteinuria (protein/creatinine ratio patient#7 1.84 ± 0.08 g/g creatinine, patient#10 3.11 ± 0.59 g/g creatinine, *p*=0.02), worse kidney function (eGFR patient#7 78 ± 10, patient #10 42 ± 5, *p*=0.006) and a more poor compliance (difference of WBC cystine level 0.94 ± 0.24 nmol ½ cystine/mg protein, 95% CI of difference 0.28 to 1.6, *p*=0.02) (Table 6.3).

Table 6.1 Demographic and clinical characteristics of the cystinosis patients included in the study at the time of enrollment, and correlation with the amount of urine-voided undifferentiated vimentin-positive cells.

Pt	Age (year)	Sex	Genetic background	eGFR (ml/min/1.73m^2)	Urine protein/creatinine (g/g creatinine)	WBC cystine (nmol ½ cystine/mg protein)	Vimentin-positive cells (cells/ml urine/mmol creatinine)
1	9	F	57kb del + c.926dupG	72	3.19	0.76	0.11
2	4	F	Hom 57kb del	> 90	5.01	1.7	0.06
3	15	M	Hom 57kb del	> 90	2.83	1.56	0.008
4	12	F	c.198_218del + c.926dupG	47	9.25	1.86	0.01
5	11	F	57kb del + c.926dupG	> 90	1.7	0.72	0
6	5	M	57kb del + c.926dupG	> 90	5.06	1.04	0
7	14	F	57kb del + c.198_218del	85	2.37	2.24	0.38
8	16	F	Hom 57kb del	27	0.87	2.88	0.06
9	7	M	57kb del + IVS 10-7G>A	> 90	0.43	2.32	2.11
10	16	M	Hom 57kb del	46	2.94	3.14	*nas*

Abbreviations: *nas:* not assessed

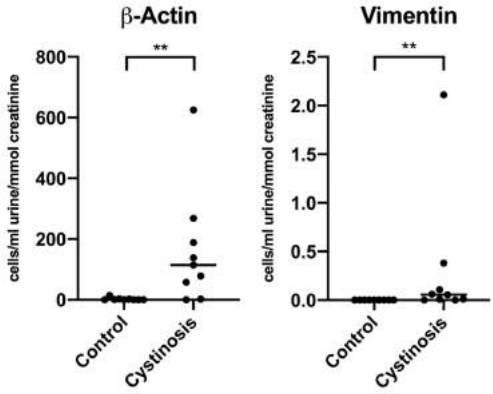

Figure 6.1 Quantification of undifferentiated, vimentin-positive cells in urine of cystinosis patients versus healthy controls

The total amount of cells voided in urine of cystinosis patients is significantly higher compared to healthy controls (left). Importantly, the amount of undifferentiated, vimentin-positive urine-voided cells also reaches significantly higher levels in cystinosis compared to controls (right).

Table 6.2 Quantification of urine-voided cells in cystinosis versus healthy controls.

	Control (n=9)	Cystinosis (n=9)	Difference (95% CI of difference)	*p*
β-Actin	0.44 (0.02 - 2.66)	114.5 (30.28 – 228.2)	114.0 (2.6 to 253.9)	0.001
Vimentin	0.00 (0.00 - 0.00)	0.06 (0.004 – 0.24)	0.06 (0.0 to 0.38)	0.002

Both the total number of cells voided in urine (first row), and the number of undiffentiated, vimentin-expressing cells (second row) were assessed in cystinosis patients and healthy controls. Data is presented as median number of cells/ml urine/mmol creatinine (IQR).

Finally, based on their gene expression profile, potential of proliferation and differentiation, a total of 6 clones of primary cells (n=6), hereafter coined as cystinotic urine-derived kidney progenitor cells (Cys-uKPCs), were established from these two cystinosis patients (5 clones for patient #7 and 1 clone for patient #10; Table 6.3).

The average number of established Cys-uKPCs per harvesting time point ranged from 0.3 ± 0.6 to 1.7 ± 2.1. For all 6 established Cys-uKPCs, the average total days and passages in culture before reaching senescence was 85 ± 20 days and 11 ± 3 passages, respectively. In the Cys-uKPC clone exhibiting the highest proliferation potential, the majority of cells showed a spindle-shaped to rhombic morphology and a characteristic pattern of organization and expansion (Figure 6.2, panel a). The lowest cell doubling times observed per Cys-uKPC ranged from 20.87 to 47.01 hours. The total amount of cells yielded per Cys-uKPC when reaching senescence ranged from 1.39×10^8 to 1.46×10^{12} (Figure 6.2, panel b).

All Cys-uKPC clones showed expression of the kidney progenitor genes *NCAM1*, *CITED1*, *VIM* and *PAX2* as demonstrated by qPCR in P#4 (Table 6.4, Ct-values). For *NCAM1* and *VIM*, expression levels (relative fold expression; RFE) were similar (*VIM*: average RFE 0.99 ± 0.59) or close (*NCAM1*: average RFE 0.38 ± 0.30) to those observed in human fetal kidney (hFK) tissue (Figure 6.2, panel c). For none of the Cys-uKPC clones a significant correlation could be demonstrated between the cell doubling time at the passage of characterization (P#4), and the relative fold expression for any of the kidney progenitor genes *NCAM1*, *CITED1*, *VIM* and *PAX2* compared to human fetal kidney (data not shown).

Furthermore, all of the Cys-uKPC clones showed expression of the cell surface markers CD73, CD44, CD29 and CD24, characteristic for mesenchymal stem cells (Table 6.5a), while in only one Cys-uKPC clone (Cys-uKPC clone # 1) a substantial subpopulation showed expression of CD133 (Figure 6.2 panel d, Table 6.6b). None of the Cys-uKPCs expressed HLA-DR.

Taken together these data demonstrate that kidney progenitor cells, expressing specific kidney progenitor markers reminiscent of early nephrogenesis, are present in urine of nephropathic cystinosis patients.

Table 6.3 Yield of establishment of cystinotic kidney progenitor clonal cell lines (Cys-uKPCs) from urine in two nephropathic cystinosis patients over three consecutive harvesting timepoints.

	Patient # 7				Patient # 10				Difference (95% CI of difference)	*p*
Sample	**1**	**2**	**3**	mean ± SD	**1**	**2**	**3**	mean ± SD		
Age (years)	13	13	14		16	16	16		2.7 ± 0.3 (1.7 to 3.6)	0.001
Urine volume (ml)	59	99	88	82 ± 21	56	109	120	95 ± 34	13 ± 23 (-51 to 77)	0.6
Total # of clonal colonies	49	138	99	95 ± 45	22	71	86	60 ± 33	-36 ± 32 (-125 to 54)	0.33
Total # of clonal colonies/ urine volume (ml)	0.8	1.4	1.1	1.1 ± 0.3	0.4	0.7	0.7	0.6 ± 0.2	-0.5 ± 0.2 (-1.1 to 0.002)	0.05
# of clonal colonies selected	19	9	18	15 ± 6	11	9	18	13 ± 5	-2.67 ± 4.19 (-14.3 to 8.97)	0.56
average day of selection of colonies	13	9	12	11 ± 2	13	9	12	11 ± 2	0 ± 1.7 (-4.72 to 4.72)	> 0.99
# of clonal colonies reaching P#6 (%*)	9	3	5	6 ± 3 (36 ± 10%)	2	2	3	2 ± 1 (19 ± 3%)	-3.33 ± 1.8 (-8.32 to 1.65)	0.14
# of clonal colonies reaching P#10 (%*)	4	0	1	2 ± 2 (8 ± 11%)	1	0	1	1 ± 1 (5 ± 5%)	-1.0 ± 1.25 (-4.5 to 2.5)	0.47
# of established Cys-uKPCs (%*)	4	0	1	1.7 ± 2.1 (8.9 ± 10.9%)	0	1	0	0.3 ± 0.6 (3.7 ± 6.7%)	-1.3 ± 1.2 (-4.8 to 2.1)	0.35
Protein/creatinine ratio (g/g creatinine)	1.76	1.89	1.89	1.84 ± 0.08	2.94	2.62	3.76	3.11 ± 0.59	1.26 ± 0.34 (0.31 to 2.21)	0.02
eGFR (ml/min/1.73m^2)	88	78	68	78 ± 10	46	45	36	42 ± 5	-36 ± 7 (-54 to -17)	0.006
Serum creatinine (mg/dl)	0.8	0.91	1.05	0.92 ± 0.13	1.31	1.36	1.71	1.46 ± 0.22	0.54 ± 0.15 (0.14 to 0.94)	0.02
WBC cystine (nmol ½ cystine/mg protein)	2.45	1.86	1.91	2.07 ± 0.33	3.14	2.73	3.18	3.02 ± 0.25	0.94 ± 0.24 (0.28 to 1.6)	0.02

Abbreviations: *nas:* not assessed; P#: passage number; #: number
Data presented as mean ± SD; *: percentage expressed in function of the number of selected clonal colonies.

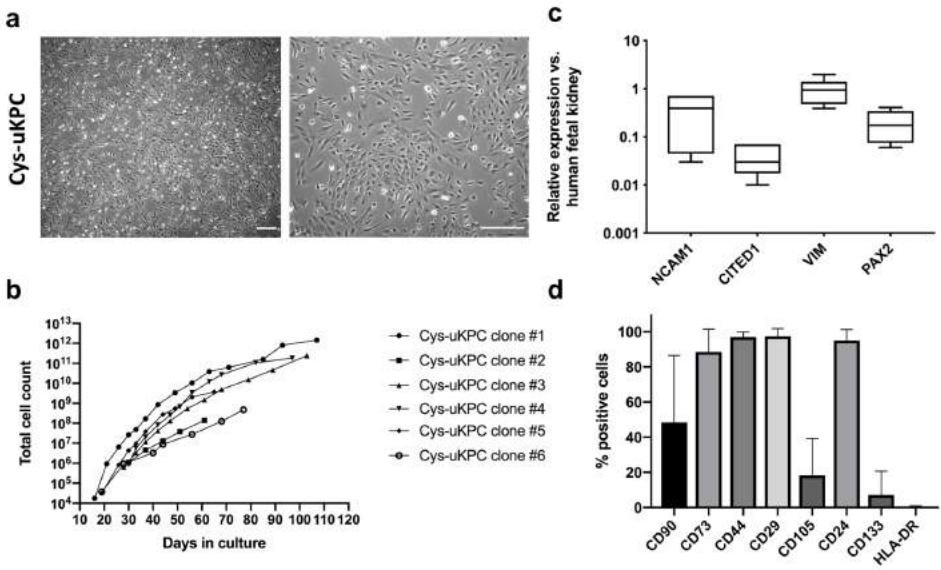

Figure 6.2 Characterization of the established cystinotic urine-derived kidney progenitor clones (n=6)

Panel a: Phase-contrast microscopy of a representative clone (Cys-uKPC clone # 1) showing a spindle-shaped to rhombic cellular morphology and a characteristic pattern of *in vitro* expansion and organization.

Panel b: *In vitro* proliferation capacity versus total days in culture before reaching senescence of the established Cys-uKPCs.

Panel c: Box plot representing the expression of kidney progenitor specific genes in the Cys-uKPCs (n=6), relative to human fetal kidney tissue.

Panel d: Expression of mesenchymal stem cell markers (CD90, CD73, CD44, CD29, CD105) and presumed specific kidney progenitor cell surface markers (CD24, CD133) in Cys-uKPCs (n=4).

Table 6.4 Expression of kidney progenitor genes in the established cystinotic urine-derived kidney progenitor clones (n=6) at passage 4.

	NCAM1	***CITED1***	***VIM***	***PAX2***
Ct	26.11 ± 2.61	30.55 ± 0.81	18.46 ± 0.64	24.7 ± 1.12
Δ Ct	9.13 ± 2.77	13.58 ± 1.06	1.49 ± 1.04	7.98 ± 1.08
Relative fold expression vs. hFK	0.38 ± 0.30	0.04 ± 0.03	0.99 ± 0.59	0.20 ± 0.14

Abbreviations: hFK: human fetal kidney
Data presented as mean ± SD for all cystinotic uKPCs (n=6).

Table 6.5a Expression of cell surface markers in the established cystinotic urine-derived kidney progenitor clones (n=4*) at passage 6.

	CD90	**CD73**	**CD44**	**CD29**	**CD105**	**CD24**	**CD133**	**HLA-DR**
% positive cells	48.3 ± 38.2	88.6 ± 13	97.1 ± 2.8	97.5 ± 4.3	18.3 ± 20.9	95.1 ± 6.2	7.2 ± 13.4	0.6 ± 0.4

Data presented as mean ± SD for all cystinotic uKPCs (n=4*).
* Data was obtained in only 4 of the 6 Cys-uKPC clones.

Table 6.5b Expression of cell surface markers in the individual established cystinotic urine-derived kidney progenitor clones (n=4*) at passage 6.

	CD90	**CD73**	**CD44**	**CD29**	**CD105**	**CD24**	**CD133**	**HLA-DR**
Cys-uKPC clone # 1	62.5	99.9	98.2	91.0	47.8	99.8	27.3	0.9
Cys-uKPC clone # 3	35.1	83.2	93.4	99.6	4.3	95.2	0.6	0.5
Cys-uKPC clone # 4	3.1	72.7	96.6	99.3	2.6	86.3	0.1	0.1
Cys-uKPC clone # 5	92.6	98.4	99.9	100	18.4	99.3	0.7	0.8

Data presented as mean ± SD of the percentage positive cells in the individual cystinotic uKPC clones (n=4*)
* Data was obtained in only 4 of the 6 Cys-uKPC clones.

6.3.3 A kidney progenitor cell niche is present *in vivo* in nephropathic cystinosis

In a next step, we tried to corroborate our findings in kidney biopsy specimens.

Immunofluorescence co-stainings for CD133 and PAX2 in (native) kidney biopsy specimens of one nephropathic cystinosis patient showed the focal presence of cells co-expressing CD133 and PAX2 scattered throughout the proximal tubular epithelium as well as in the parietal epithelium of the Bowman's capsule (Figure 6.3, panel g, h). In contrast, no expression of either markers was present in kidney tissue retrieved from a patient with minimal changes disease nephrotic syndrome (Figure 6.3, panel f). These results further add up to the evidence in support of our hypothesis of enhanced ongoing kidney regeneration in cystinosis, and the presence of an *in vivo* kidney progenitor cell niche in cystinosis kidney biopsies.

Figure 6.3 A kidney progenitor cell niche co-expressing CD133 and PAX2 is present in situ in cystinotic kidney (next page)

Panel a & b: Immunohistochemistry (a) and immunofluorescence staining (b) for *PAX2* in healthy fetal kidney tissue (28 weeks gestational age) demonstrates a profoundly strong presence of PAX2 at the superficial nephrogenic rim, in contrast to regions of differentiating kidney.
Scale bar panel a: 200 μm; scale bar panel b: 50 μm.

Panel c & d: Immunohistochemistry for CD133 of an adult transplant kidney in a 13-year old recipient, demonstrating cytoplasmic positivity in epithelial cells of the Bowman's capsule and tubular epithelial cells, of which in some the apical border is strongly positive.
Scale bar panel c: 25 μm; scale bar panel d: 50 μm.

Panel e: Immunofluorescence staining for CD133 in the context of acute tubular injury, showing positivity at the apical border of tubular epithelial cells.
Scale bar: 50 μm.

Panel f: Immunofluorescence co-staining for CD133 and PAX2 in a kidney tissue specimen of minimal changes disease-type nephrotic syndrome shows no signs of positivity for neither markers.
Scale bar: 50 μm.

Panel g & h: Immunofluorescence co-staining for CD133 and PAX2 in kidney tissue specimens of one nephropathic cystinosis patient, showing focal co-expression of CD133 and PAX2 in cells scattered throughout the tubular epithelium (full white arrows in panel g, h) and the parietal epithelial cells of the Bowman's capsule (dashed white arrows in panel g).
Scale bar panel g & h: 50 μm.

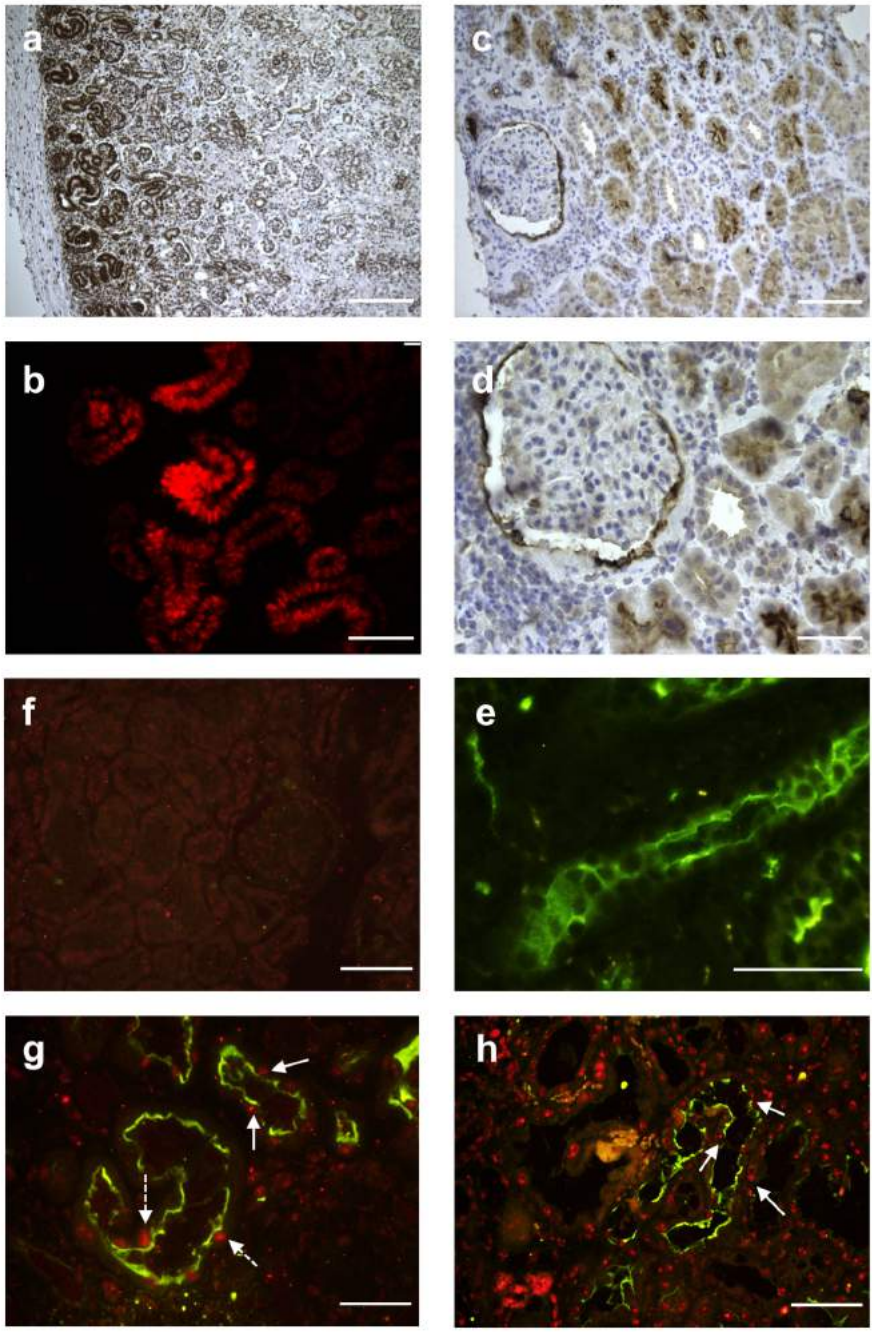

Figure 6.3 A kidney progenitor cell niche co-expressing CD133 and PAX2 is present in situ in cystinotic kidneys

6.3.4 Cystinotic urine-derived kidney progenitor cells show differentiation potential towards a functional proximal tubular epithelial cell or podocyte

Cys-uKPC derived podocytes

Three of the 6 established Cys-uKPC clones (Cys-uKPC clone #1, 5, 6) showed the potential to differentiate towards a podocyte-like phenotype, of which the results of a representative clone (Cys-uKPC clone # 1) is depicted in Figure 6.4. During differentiation, cellular morphology altered in a substantial amount of cells, more specifically the evolution towards large, binucleated arborized cells showing multiple cellular protrusions resembling podocyte foot processes (Figure 6.4, panel a). Podocyte differentiation was confirmed at the level of gene expression, via the upregulation of expression of podocyte-specific genes *SYNPO*, *PODXL* and *CD2AP* relative to the baseline expression prior to differentiation and normalized to the housekeeping gene β-Actin in the Cys-KPC-derived podocytes (Figure 6.4, panel b). In addition, an increased expression of synaptopodin and podocalyxin was demonstrated in the Cys-uKPC – Podocytes by immunofluorescence staining (Figure 6.4, panel c).

The functionality of the podocytes derived from the three Cys-uKPC clones showing a podocyte fate (Cys-uKPC – Podo), was assessed in an albumin endocytosis assay, and calcium uptake assay. In the podocytes derived from the representative Cys-uKPC – Podo clone (Cys-uKPC clone # 1), a significant 3.4-fold increase (Mann Whitney, p=0.003) in albumin uptake was demonstrated in comparison to the undifferentiated progenitor (Cys-uKPC) (Figure 6.4, panel d-j). More specifically, no uptake was observed in the undifferentiated Cys-uKPC (Figure 6.4, panel g) and the amount of albumin uptake in the Cys-uKPC – Podo was in line with that of to the conditionally immortalized cystinosis podocyte (Figure 6.4, panel e vs. 6.4, panel i). The temperature-dependence of the process of albumin endocytosis was demonstrated by the absence of albumin uptake at 4°C (Figure 6.4, panel f, h, j). Furthermore, the activity of the Transient Receptor Potential Cation Channel, subfamily C, member 6 (TRPC6) was assessed in the Cys-uKPC – Podocytes in a calcium uptake assay, using Fura-2 acetoxymethyl ester (Fura-2 AM) as a membrane permeant ratiometric Ca^{2+} selective fluorescent indicator. Upon perfusion with 1-oleoyl-2-acetyl-sn-glycerol (OAG)-containing buffer, the induced changes in $[Ca^{2+}]_I$ were detected via Fura-2 AM by changes in fluorescence, while only cells showing a response to ionomycin were considered as true responders (Figure 6.4, panel k, l). In this assay, Cys-uKPC – Podocytes showed a 1.7-fold higher number of responders compared to the undifferentiated Cys-uKPCs, reaching levels comparable to differentiated conditionally immortalized Podocytes (OAG responders in Cys-uKPC vs. Cys-uKPC – Podo vs. ciPodocyte^{WT} (mean ± SD): 37.86 ± 27.52 % vs. 64.33 ± 21.83% vs. 71.29 ± 20.77 %).

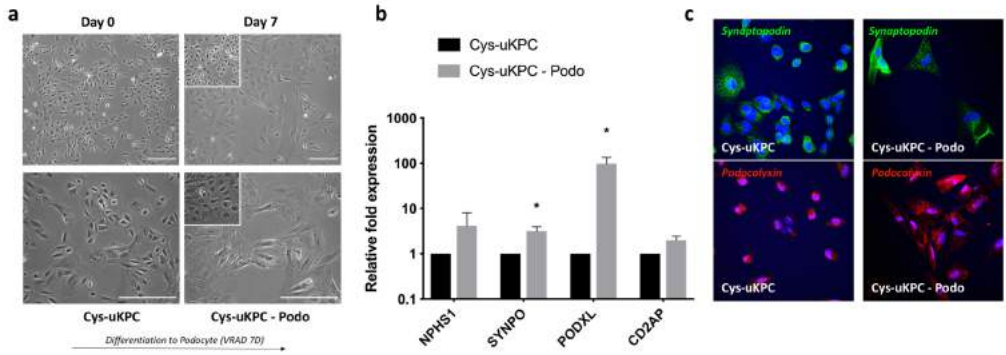

Figure 6.4 Differentiation potential of cystinotic urine-derived kidney progenitor cells towards a functional podocyte.

Panel a: Phase contrast microscopy demonstrating cellular morphological changes during differentiation from a cystinotic urine-derived kidney progenitor cell (day 0, left column) towards a podocyte (day 7, right column) at magnification 100x (upper row) and 200x (lower row). Cys-uKPCs become larger and show an arborized morphology with protrusions resembling podocyte foot processes. The small subfigures in the left top corner of the microscopy pictures in the right column, depict the evolution of cellular morphology of Cys-uKPCs not undergoing the differentiation protocol at the same timepoint. Scale bar (all subpanels): 250 μm.

Panel b: Cys-uKPCs show upregulation of podocyte-specific genes *NPHS1*, *SYNPO*, *PODXL* and *CD2AP* during differentiation towards a podocyte.

Panel c: Immunofluorescence staining for podocyte-specific proteins synaptopodin and podocalyxin showing increased protein expression and typical distribution in the Cys-uKPC-derived podocytes.

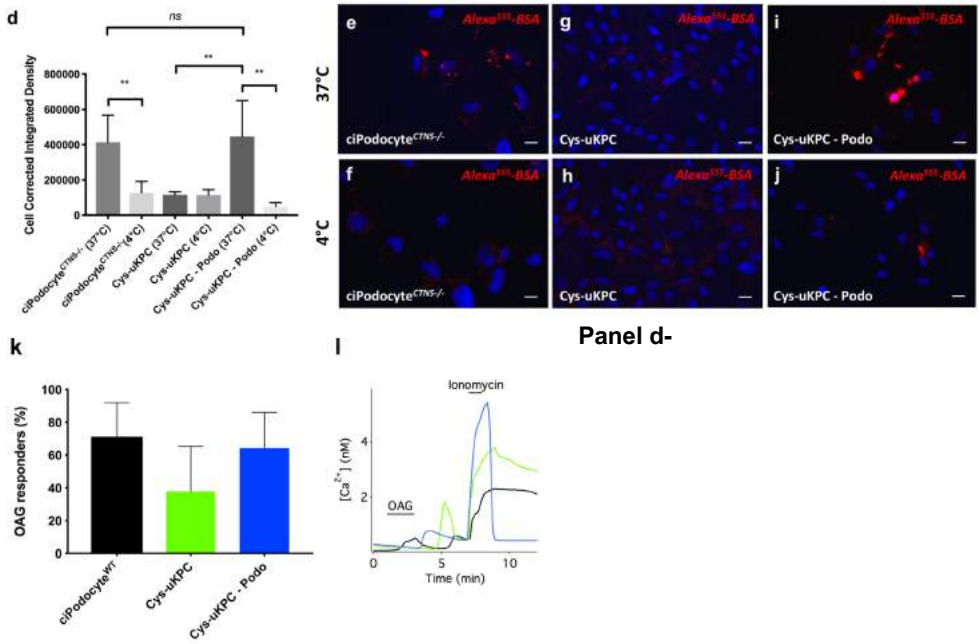

Figure 6.4 (continued) Differentiation potential of cystinotic urine-derived kidney progenitor cells towards a functional podocyte.

Panel d-j: Cys-uKPC-derived podocytes (panel d, i) show the capability of albumin endocytosis similar to differentiated conditionally immortalized podocytes (panel d, e), in contrast to undifferentiated Cys-uKPCs (panel d, g). The temperature dependency of albumin endocytosis was demonstrated in all cell lines (Panel e vs. f, g vs. h, i vs. j). (Cell corrected integrated density Cys-uKPC vs. Cys-uKPC - Podo: 118172 AU vs. 402791 AU; actual difference: 284619 AU; 95% CI of difference: 211091 to 454293; p=0.003; Cell corrected integrated density ciPodocyte$^{CTNS-/-}$ vs. Cys-uKPC – Podo: 442469 AU vs. 402791 AU; actual difference: -39678 AU; 95% CI of difference: -216228 to 269379, $p > 0.99$).

Panel k, l: Cys-uKPC-derived podocytes (blue line) show an increased response of calcium influx to OAG in a Fura calcium influx assay compared to undifferentiated Cys-uKPCs (green line), resembling the response of ciPodocytes (black line). This demonstrates the functionality and major contribution of TRPC6 in the calcium entry of Cys-uKPC – Podocytes.

Cys-uKPC derived PTECs

Four of the 6 established Cys-uKPC clones (Cys-uKPC clone # 2, 3, 4, 5) differentiated towards a proximal tubular cell-like phenotype, of which the results of a representative clone (Cys-uKPC clone # 4) are depicted in Figure 6.5. During differentiation, the majority of the Cys-uKPC cells showed changes in cell morphology, comprising cellular enlargement and the acquisition of an elongated, spindle- to somewhat cobblestone-like shape, providing a more uniform appearance to the cell population (Figure 6.5, panel a). At the level of gene expression, a significant upregulation of PTEC-specific genes *ABCB1* (6-fold increase, one-sample t-test, p=0.0001) and *CUBN* (117-fold, one-sample t-test, p=0.03) compared to the expression prior to differentiation (relative fold expression versus Cys-uKPC at P#4) was demonstrated in the Cys-uKPC-derived PTECs (Cys-uKPC – PTEC) via qPCR (Figure 6.5, panel b). In addition, expression of *AQP1*, a highly specific marker for proximal tubular epithelial cells, was exclusively restricted to the Cys-uKPC – PTEC, in contrast to the undifferentiated Cys-uKPC (Figure 6.5, panel c).

Furthermore, in a calcein-AM efflux assay, the activity of the P-glycoprotein (*ABCB1*) of this Cys-uKPC -derived PTEC was explored, and compared to a urine-derived conditionally immortalized proximal tubular epithelial cell (ciPTEC$^{CTNS-/-}$) isolated from a cystinosis patient harboring a homozygous 57kb deletion in the *CTNS* gene. The cell lysates of the Cys-uKPC-derived PTECs that were incubated with a P-gp inhibitor (PSC-833) showed a 1.5-fold significant increase in fluorescence intensity due to the intracellular calcein accumulation, compared to the Cys-uKPC-derived PTECs that were not incubated with a P-gp inhibitor (unpaired t-test, p=0.0005, difference between means of fluorescence intensity: 10419 ± 1022 AU, 95% CI of difference: 7583 to 13256). In contrast, the cell lysates of the undifferentiated Cys-uKPC did not show a significant different fluorescence intensity between the conditions with or without the presence of a P-gp inhibitor (difference between means of fluorescence intensity: 7328 ± 3529 AU, 95% CI of difference: -2471 to 17127, p=0.1065). Finally, the P-gp efflux pump in the Cys-uKPC – PTEC (34.67%) yielded a similar degree of activity (difference of P-gp transport activity: 6.06 ± 5.58 %, 95% confidence interval: -9.42 to 21.54%) as observed in the ciPTEC$^{CTNS-/-}$ (28.60%) (Figure 6.5, panel d).

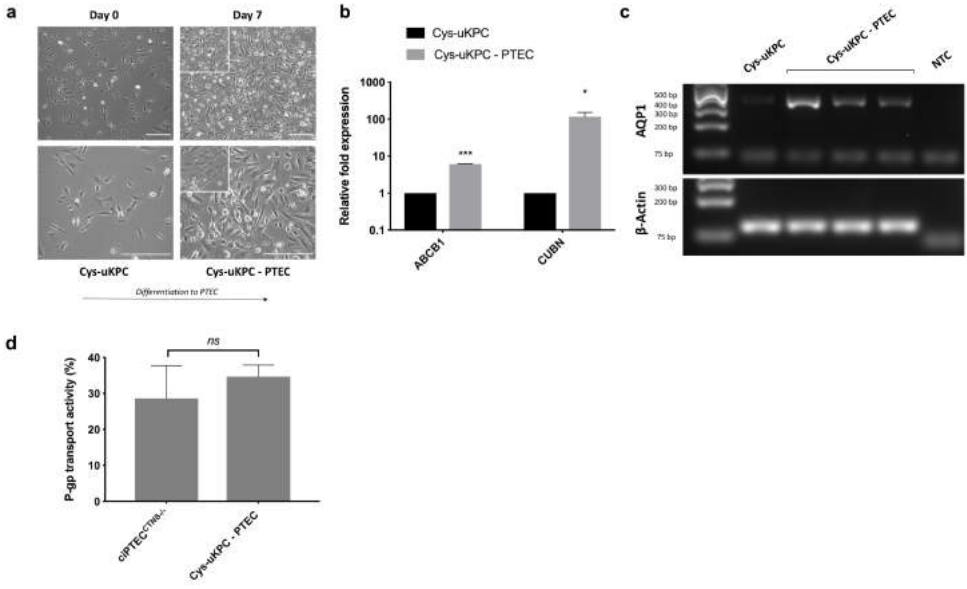

Figure 6.5 Differentiation potential of cystinotic urine-derived kidney progenitor cells towards a functional proximal tubular epithelial cell

Panel a: Phase contrast microscopy demonstrating cellular morphological changes during differentiation from a cystinotic urine-derived kidney progenitor cell (day 0, left column) towards a proximal tubular epithelial cell (day 7, right column) at magnification 10x (upper row) and 20x (lower row). Cys-uKPCs become more elongated, spindle-like cells. The small subfigures in the left top corner of the microscopy pictures in the right column, depict the evolution of cellular morphology of Cys-uKPCs not undergoing the differentiation protocol at the same timepoint. Scale bar (all subpanels): 250 μm.

Panel b, c: Cys-uKPCs show upregulation of PTEC-specific genes *ABCB1* and *CUBN*, and de novo expression of the highly specific PTEC-marker *AQP1* following the differentiation protocol towards a proximal tubular epithelial cell.

Panel d: Cys-uKPC-derived PTECs show a similar activity of P-glycoprotein (MDR-1) compared to cystinotic ciPTECs as assessed in a Calcein assay. In contrast, Cys-uKPCs did not show significant signs of a functional P-glycoprotein being present (data not shown).
The P-gp efflux pump in the Cys-uKPC – PTEC (34.67%) yielded a similar degree of activity (difference of P-gp transport activity: 6.06 ± 5.58 %, 95% confidence interval: -9.42 to 21.54%) as observed in the ciPTEC$^{CTNS-/-}$ (28.60%).

6.3.5 Ex vivo gene therapy rescues the cellular phenotype in cystinotic urine-derived kidney progenitor cells

In order to explore the feasibility of *CTNS* complementation in an *ex vivo* gene therapy setting with successful rescue of the cystinotic phenotype, the Cys-uKPC clone exhibiting the highest proliferating capacity (Cys-uKPC clone # 1) was transduced with a SIN-lentiviral vector containing 3HA-tagged *CTNS* cDNA driven by the human EF-1 α promotor at a high and low viral vector concentration. Transduced cells were selected using puromycin, resulting in polyclonal cell lines stably expressing CTNS-3HA, Cys-uKPC LV_CTNS-3HA$_{low}$ and Cys-uKPC LV_CTNS-3HA$_{high}$. A significant reduction in cystine level was demonstrated in the Cys-uKPC in which *CTNS* was complemented (Cys-uKPC LV_CTNS-3HA$_{low}$) compared to the non-transduced Cys-uKPC (Cys-uKPC LV_CTNS-3HA$_{low}$ cystine level: 1.46 ± 0.15 nmol ½ cystine/mg protein; Cys-uKPC cystine level: 3.62 ± 0.57 nmol ½ cystine/mg protein; difference between means: -2.15 ± 0.34; 95% CI of difference: -3.09 to -1.21; p=0.003) and the eGFP-transduced Cys-uKPC; the latter was considered as a vehicle control (Cys-uKPC LV_eGFP$_{low}$ cystine level: 2.83 ± 0.21; difference between means of Cys-uKPC LV_CTNS-3HA$_{low}$ and Cys-uKPC LV_eGFP$_{low}$ cystine level: 1.37 ± 0.15; 95% CI of difference: 0.95 to 1.79) (Figure 6.6 a). The non-transduced Cys-uKPC and the vehicle control did not show significant different cystine levels. Importantly, alongside the notable decrease in cystine levels, a significant 1.6-fold reduction of the perinuclear clustering of the LAMP1 lysosomal compartment was demonstrated in the Cys-uKPC LV_CTNS-3HA$_{low}$ compared to the non-transduced Cys-uKPC (Figure 6.6, panel b, c). In contrast, no redistribution of the LAMP1 lysosomal compartment was detected in the Cys-uKPC LV_CTNS-3HA$_{high}$ condition compared to the non-transduced Cys-uKPC, neither so in the vehicle control (Cys-uKPC LV_eGFP$_{low}$) (Figure 6.6 b, c).

Figure 6.6 Complementation of *CTNS* via lentiviral vector transduction in cystinotic urine-derived kidney progenitor cells rescues the healthy cellular phenotype in cystinotic urine-derived kidney progenitor cells

Panel a: Lentiviral vector transduction in Cys-uKPCs significantly reduces intracellular cystine levels (Cys-uKPC LV_CTNS-3HA$_{low}$) compared to a vehicle control (Cys-uKPC LV_eGFP$_{low}$).

Panel b, c: Lentiviral vector transduction in Cys-uKPCs restores the distribution of the LAMP1 lysosomal compartment from an enlarged perinuclear clustered localization (Cys-uKPC) towards a healthy phenotype of a more homogenous distribution (Cys-uKPC LV_CTNS-3HA$_{low}$), in contrast to a vehicle control (Cys-uKPC LV_eGFP$_{low}$). Neonatal kidney stem progenitor cells (nKSPCs) were used as healthy controls. Importantly, at high transduction efficiencies (> 95%), the restoration of this phenotype could not be observed (Cys-uKPC LV_CTNS-3HA$_{high}$). Quantification of the LAMP1 lysosomal distribution in these described conditions confirmed the significant alteration towards a healthy phenotype.(LAMP1 lysosomal compartment localization in perinuclear area in Cys-uKPC LV_CTNS-3HA$_{low}$ compared to the non-transduced Cys-uKPC: 36.21 ± 6.25% versus 57.43 ± 7.29% respectively; difference between means: -21.22 ± 5.54; 95% CI of difference: -36.61 to -5.823; p=0.02). Perinuclear and peripheral distribution were defined as localization within (< p25) or outside (> p25) the 25th percentile of the distance between nuclear rim and plasma membrane, respectively.

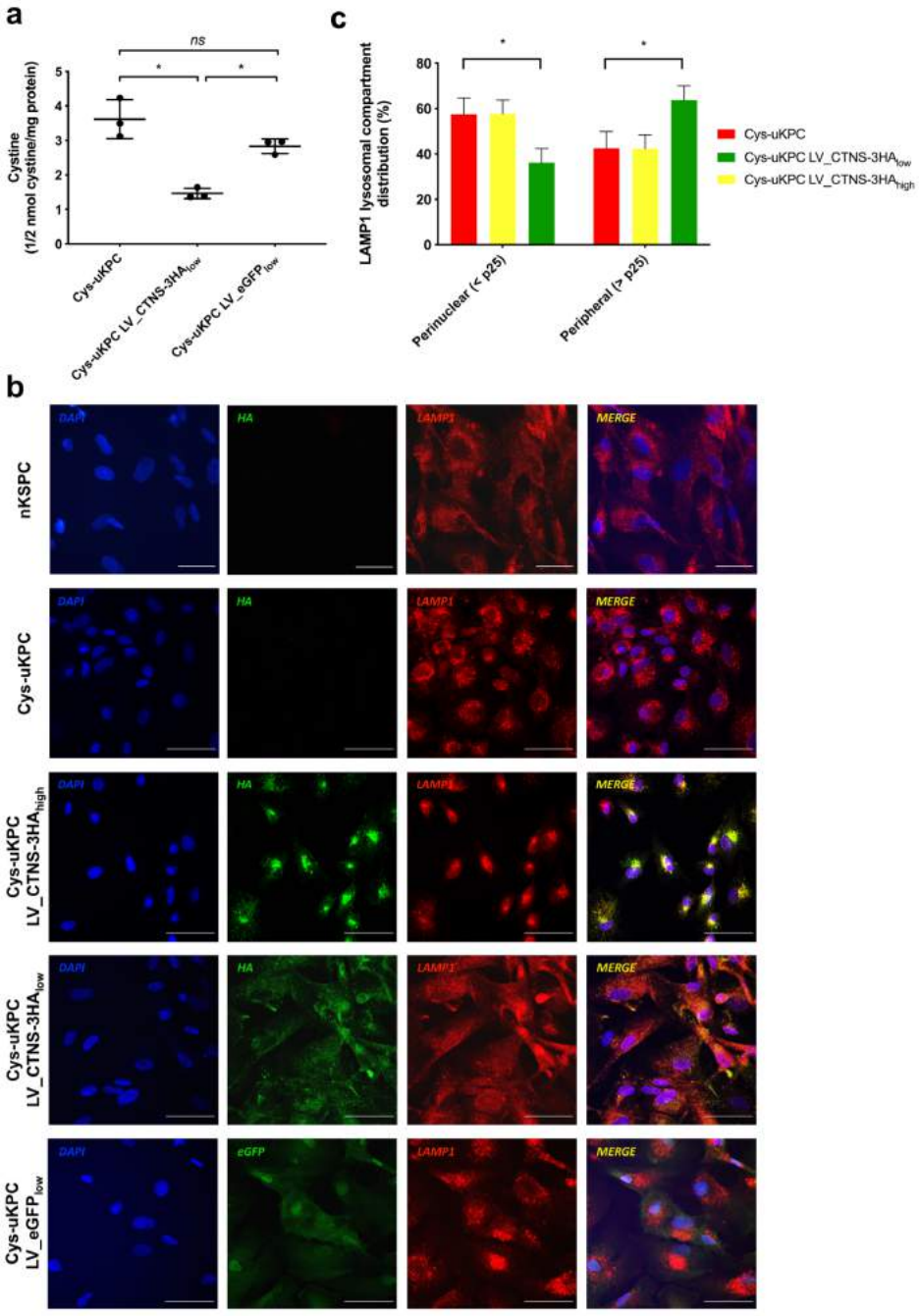

Figure 6.6 Complementation of *CTNS* via lentiviral vector transduction in cystinotic urine-derived kidney progenitor cells rescues the healthy cellular phenotype in cystinotic urine-derived kidney progenitor cells

6.4 Discussion

The presence of kidney progenitor cells in urine in cystinosis patients led us to hypothesize that this may reflect ongoing kidney regeneration, albeit maladaptive. These kidney progenitor cells could serve as a novel source for disease modelling, drug screening, tissue engineering and autologous cells for cell therapeutic approaches.

In this study, we have demonstrated the presence of undifferentiated cells in the urine of nephropathic cystinosis patients and showed that these can be isolated, quantified and cultured *in vitro* for a substantial number of passages. We have shown that clonal colonies derived from these cells express several specific markers reminiscent of the early stages of nephrogenesis, and carry the intrinsic potential to differentiate towards a functional podocyte or proximal tubular epithelial-like cell. These features allow the described clones to be referred to as kidney progenitor cells (Cys-uKPCs). Strikingly, we have provided evidence of the *in vivo* existence of a kidney progenitor cell niche, and showed this niche to be enriched in a cystinosis patient. Moreover, we have demonstrated the feasibility of rescue of the healthy cellular phenotype upon complementation with *CTNS* via lentiviral vector transduction in these cystinotic urine-derived kidney progenitor cells.

Therefore, our study provides a novel prospective for disease modelling and a proof of principle for the feasibility of *ex vivo* gene therapy and a potential innovative source of autologous cell-based gene therapy to treat kidney disease in cystinosis.

The actual existence of a stem/progenitor cell niche to be present in the human adult kidney has been a matter of debate for more than a decade [250,411,412]. Multiple studies have attempted to demonstrate the presence of a stem cell niche in adult human kidney, characterized and described their *in vitro* properties, and their potential in *in vivo* animal models of acute and chronic injury [212–214,217,258,260,263,264,407].

Here, we describe the presence of undifferentiated, *VIM*-expressing cells in urine of cystinosis patients, while none of these cells were present in healthy aged-matched controls. For the purpose of quantifying urine-voided cells, we developed a novel method utilizing single-cell RNA extraction and amplification technology. This allowed to establish a calibration curve of Ct-values for a housekeeping gene and a specific marker for undifferentiated cells (vimentin) for known numbers of characterized control kidney stem cells derived from the renal papilla, starting from very low numbers of cells [258,408]. To the best of our knowledge, a similar method has not been described yet. Since none of these cells were shown to be present in the urine of healthy control subjects, we hypothesized that the loss of undifferentiated cells in urine might reflect the attempt for kidney regeneration in cystinosis kidneys. Indeed, in cystinosis patients we demonstrated that the urine is a source of undifferentiated cells which show the capacity to self-renew, proliferate

exponentially *in vitro*, are clonogenic, and differentiate towards specific cells of the nephron epithelium.

In contrast to the majority of the previous studies on kidney progenitor cells which are tissue-derived, the kidney progenitor cells we describe are derived from urine, which is an elegant, non-invasive source [213,214,217,224,225,258,260,262]. To our knowledge, only one paper has shown the presence of a renal progenitor cell type (u-RPC) in urine of patients with kidney disease [263]. However, this study was limited to patients harboring a glomerular disease, no correlation was provided with human *in vivo* findings, the u-RPCs described were not quantified and characterization was limited [263]. Moreover, no data was provided in this study supporting the functionality of the u-RPC-derived tubular cells and podocytes.

The established Cys-uKPC clonal cell lines presented in this work, showed a substantial *in vitro* proliferative, clonogenic and differentiating potential. Remarkably, these properties were neither restricted to the clones co-expressing CD24 and CD133, nor to those presenting the five cell-surface markers identifying mesenchymal stem cells (CD90, CD73, CD44, CD29 and CD105) [214,217,225]. While CD24 and CD133 have been proposed to specifically identify kidney stem/progenitor cells in case they are co-expressed [214,225,258], in our study CD24 CD133 co-expression was only present in one of the 6 established Cys-uKPC clones. In contrast, Cys-uKPC clones were characterized based on the expression of a panel of several kidney progenitor markers, including *CITED1* which is highly specific to the nephron progenitor cells of the cap mesenchyme during nephrogenesis [203,413], and *NCAM1* of which reactivation of expression has shown to define a population of kidney epithelial cells with clonogenic and stem/progenitor properties [212,213]. Moreover, the shortest doubling times and highest proliferative potential was observed in the Cys-uKPC clone with the highest level of co-expression of all nephron progenitor markers *NCAM1*, *CITED1*, *VIM* and *PAX2* when compared to human fetal kidney tissue.

While no correlation was demonstrated between the number of undifferentiated (*VIM*-positive) cells voided in urine, and kidney function, proteinuria or WBC cystine levels, the total number of clonal colonies was lower in the older cystinosis patient (16 years of age vs. 13 years of age) that showed a higher degree of proteinuria, lower kidney function and higher WBC cystine levels. Therefore, it could be speculated that the enhanced attempt for kidney regeneration and subsequent loss of undifferentiated cells in urine, precedes a major progressive decline in kidney function (GFR). If true, this suggests a specific time window may exist during which kidney progenitor cells are voided more abundantly in urine and higher yields of more potent cells could be harvested. Of note, the discrepancy between the findings involving the number of undifferentiated cells in urine versus the number of growing clonal colonies *in vitro* might be explained by the fact that not all *VIM*-positive cells show all properties kidney progenitor cells.

Various hypotheses have been proposed for defining the *in vivo* origin of regenerating cells of the kidney, depending on their potency and fate [213,214,217,224,258,407]. Studies on human adult kidney tissue-derived stem/progenitor cells have demonstrated that regeneration of podocytes could be fueled from parietal epithelial cells of the Bowman's capsule [214,217]. Murine lineage tracing studies of renin-producing cells and models of podocyte injury have suggested cells of renin lineage, residing in the juxtaglomerular apparatus, to be the source of regenerated podocytes [277–280]. On the other hand, scattered cells throughout the tubular epithelium have been attributed as the source for tubular regeneration [224,407]. By demonstrating the presence of cells co-expressing CD133 and PAX2 scattered throughout the tubular epithelium and the parietal epithelium of the Bowman's capsule in a nephropathic cystinosis patient, our results support both theorems on podocyte and tubular regeneration, and our hypothesis regarding the attempt towards an enhanced kidney regeneration in cystinosis (Figure 6.3).

Despite the controversy on the use and specificity of stem cell markers for the identification of kidney stem/progenitor cells in pathological settings, and the role of cell culture conditions and relevance *in vitro* observations compared to *in vivo* findings, remarkably the kidney tissue specimen of a minimal changes disease nephrotic syndrome did not show signs of CD133 nor PAX2 positivity [250].

Furthermore, the upregulation of PTEC- or podocyte-specific markers in Cys-uKPCs upon differentiation, combined with the observed functionality in a P-gp assay or albumin endocytosis and Fura calcium assay respectively, demonstrated the potential of differentiation of our established Cys-uKPC clones.

In Cys-uKPC-derived PTECs, characterized by upregulation of PTEC-specific genes *ABCB1*, *CUBN* and de novo expression of *AQP1*, we selected to assess the function of P-glycoprotein, also known as MDR-1 (encoded by *ABCB1*). P-glycoprotein, an ATP-dependent exporter of cationic and amphiphatic components located at the apical membrane of proximal tubular epithelial cells, has been reported not to be affected in cystinosis urine-derived ciPTECs [414]. While in the Cys-uKPC-derived PTECs and cystinotic ciPTEC a significantly increased intracellular accumulation of Calcein was observed upon incubation with a P-gp inhibitor (PSC833), no significant alterations of Calcein accumulation could be demonstrated in the Cys-uKPC clone prior to differentiation (data not shown). This observation rules out the possibility for a functional P-glycoprotein to be present in the Cys-uKPC prior to differentiation and, conversely, demonstrates functionality of Cys-uKPC-derived PTECs. Moreover, P-gp transport activity in Cys-uKPC-PTECs paralleled that of cystinotic ciPTECs (Figure 6.5, panel d).

Functionality of Cys-uKPC-derived podocytes were assessed in an albumin endocytosis and TRPC6 calcium assay. Several knock-out animal models and genetic mutations in nephrotic

syndrome patients have underpinned the crucial role of endocytosis in podocyte biology, including its key importance in maintaining the glomerular filtration barrier and cytoskeletal dynamics [415,416]. By demonstrating endocytic uptake capabilities identical to differentiated wild type podocytes, Cys-uKPC - Podocytes show to have acquired an essential prerequisite for achieving proper podocyte homeostasis.

TRPC6, a receptor-activated non-selective Ca^{2+} permeable cation channel is one of the main transporters responsible for calcium influx in podocytes and an important component for maintaining the integrity of the slit pore diaphragm [417]. It is expressed in the podocyte foot processes and shows interactions with essential slit diaphragm proteins nephrin and podocin [417–419]. The association of TRPC6 gain-of-function mutations and altered expression levels in the development of various proteinuric diseases, ranging from minimal changes disease to focal segmental glomerulosclerosis (FSGS), membranous and diabetic nephropathy, have underscored the importance of this channel and Ca^{2+} dynamics in podocyte function [420–423]. An increased OAG-induced response of Ca^{2+}-entry upon differentiation of Cys-uKPCs to podocytes reaching levels similar to wild type ciPodocytes, indicates a substantial contribution of TRPC6 in Ca^{2+} entry in Cys-uKPC-derived podocytes, and illustrates its differentiated status. Taken together, being valid read-outs for podocyte function, the acquisition of albumin endocytotic uptake capabilities and TRPC6-mediated Ca^{2+} influx demonstrate the maturation of podocyte key features during the differentiation of Cys-uKPC to Cys-uKPC - Podocytes.

Given our cystinotic uKPCs showed the mutually exclusive potential to differentiate towards a proximal tubular- or podocyte-like cell, our data are in favour of the hypothesis that the kidney progenitor cell niche is a transient phenotype of proximal tubular epithelial cells and/or podocytes undergoing dedifferentiation, which can be redifferentiated to a predestined fate [250].

Obviously, for the development of future treatment strategies, gene therapeutic approaches should be aimed at restoring all functions of the protein of interest. During the last decade, the pathophysiology of cystinosis has shown to encompass various alterations in cellular biochemistry, trafficking processes and signaling pathways, hereby expanding the significance of cystinosin function far beyond merely cystine transport [19,21,30,31,33–36,324,394]. As introduced, cystine level reduction does not suffice as a sole parameter for restoring the healthy cellular phenotype in a cystinosis cell. The perinuclear clustering of enlarged LAMP1-positive endolysosomal structures, that has been described in both PTECs derived from kidneys of the C57BL/6 mice$^{Ctns-/-}$, and urine-derived ciPTECs from cystinosis patients, is a pathophysiological hallmark of cystinosis that can serve as a read-out for restoration of the healthy endolysosomal trafficking and lysosomal biology [30,31,33,34,39]. Upon complementation of *CTNS* using a lentiviral vector construct and EF-1α as a promotor, and while working at low vector concentrations, we

demonstrated a shift of the LAMP1 lysosomal compartment from a clustered perinuclear localization to a more homogenous widespread cellular distribution. Importantly, this redistribution coincided with significant lower cystine levels, hereby restoring the two main cellular pathophysiological hallmarks of cystinosis. Until now, only in the context of lentiviral vector-induced overexpression of TFEB, the master regulator of lysosomal biology, a significant reduction in the number and size of lysosomes has been reported in cystinosis urine-derived ciPTECs [34]. Remarkably, electron microscopy-based observations occurred only one week after transduction and six days following a significant reduction in cystine levels, while potential changes in cellular distribution were not described.

In addition, our data also indicate that upon complementation, *CTNS* expression levels should be maintained within a specific range, since overexpression was clearly associated with a perturbed lysosomal distribution and signs of cellular toxicity (Figure 6.6, panel b). In our experience, rescue of the distribution of the LAMP1 lysosomal compartment was only achieved at low transduction efficiencies in the transduction experiments using our LV construct. This result might be related to the properties of the EF-1α promotor in our LV construct, compared to the endogenous *CTNS* promotor. On the other hand, the longer duration of culture needed to recover antibiotic selection in the conditions of low versus high transduction efficiency, and the subsequent development towards a more homogenous monoclonal population are potential confounding factors that could contribute to our observations. Both the number of copy integrations of *CTNS* in the genome and the properties of the promotor driving the expression of the transgene are critical for obtaining expression levels that may come close to resembling the physiological situation. Based on preliminary experiments demonstrating a lower but more long-term stable expression levels of an *eGFP* transgene compared to other non-human promotors including cytomegalovirus (CMV) and silencing-prone spleen focus forming virus (SFFV), we previously selected EF-1α as a promotor for *CTNS*. In any future lentiviral vector-based gene therapeutic approach, various promoters for *CTNS* including the endogenous *CTNS* promoter [424,425].

The potential future applications of the Cys-uKPCs described in this study, range from serving as a platform for disease modelling and drug screening, to kidney bio-engineering applications and a potential source for future autologous stem cell-based therapy.

In contrast to tissue-derived approaches, which most commonly involve (repeated) kidney biopsies, the derivation of cell lines from urine constitutes an elegant non-invasive, more cost-effective, and virtually unlimited source in cystinosis patients [426]. Cells can be harvested easily and on multiple time points, without the need for specific processing steps including enzymatic

digestion, or specific culture substrates. Being primary cells showing a substantial *in vitro* expansion capacity, immortalization is irrelevant.

Compared to directed differentiation approaches from induced pluripotent stem cells (iPSC) to specific kidney cell types, the use of urine-derived kidney progenitor cells avoids potential epigenetic alterations which might occur during iPSC reprogramming, chromosomal aberrations during prolonged *in vitro* expansion, and expensive laborious expansion and differentiation protocols [427].

Genome-wide expression analysis and DNA-methylation studies have shown that the extent of iPSC reprogramming, the remaining epigenetic memory and culture conditions may affect the potential of self-renewal, the differentiation capacity [428–430] and immunogenicity of iPSC-derived cell lines [431,432]. To date, whether autologous iPSC-derived cells will be tolerated by the host, remains to be elucidated [433]. In addition, autoimmune destruction of iPSC-derived cells has also been suggested, for which certain means of immunoprotection would be needed in case of translation to a clinical setting [434]. Therefore, tissue-derived stem/progenitor cells should still be regarded as an appealing source for tissue regeneration and personalized cell-based therapeutic applications.

Opportunities for the development of a personalized platform for disease modeling and drug screening, add up to the advantages of urine as an autologous source for kidney progenitor cells in inherited kidney diseases [263]. While clear read-outs of the cystinosis phenotype in cystinosis proximal tubular epithelial cells and podocyte cell lines are readily available, the cystinosis-specific features of 3D-culture systems including organoids and still have to be defined [33,44]. Hence, cell-based platforms remain an attractive platform for studying mechanism of disease at the cellular level.

Finally, proceeding to a cystinosis animal model harboring a relevant kidney phenotype would constitute the next step towards further exploring their direct *in vivo* potential [367]. Ideally, several routes of administration would need to be considered (subcapsular, parenchymal, intra-arterial), to elucidate whether engraftment and replenishment of the kidney stem/progenitor cell niche or progenitor-derived differentiated cell types, is feasible and correlates with improvement of kidney function. Given that hematopoietic stem cells (HSC) [163,165,254,255,435] have shown not to contribute significantly to the generation of nephron epithelium structures , and the effect of HSCT on kidney function by providing Cystinosin mRNA or protein via a paracrine (tunneling nanotubes) effect is limited [379,397], it could be speculated that replenishing or stimulating the nephron progenitor cell niche in the kidney constitutes a more attractive therapeutic approach to cure the kidney phenotype in cystinosis. In addition, while MSCs are currently the most studied cell types for cell-based therapy in kidney disease and disregarding any controversy about their potential in the

currently few ongoing clinical trials, most of the modes of action of MSCs are linked to their secretome and immunomodulatory properties. This leaves MSCs less well suited for a long-term curative approaches to chronic kidney disease [436–438].

In this respect, apart from providing a potential source for cell-based gene therapy, our study also suggests that the maladaptive regenerative response per se could be a target for the development of future therapeutic strategies [276,290,439]. Pharmacological enhancement of cellular processes determining proximal tubular cell and podocyte regeneration could be a promising mode of action [276]. For example, CXC chemokine ligand 12 (CXCL12), a chemokine involved in stem cell homing and activation, has been attributed a renoprotective effect in terms of enhancing podocyte regeneration upon CXCL12 blockade. Hence, CXCL12 is involved in a podocyte-progenitor feedback mechanism, which balances podocyte regeneration versus glomerular scar formation and could therefore hold a potential to enhance repair of glomerular function [290]. Finally, we could also speculate that complementation of *CTNS* in the kidney progenitor cell niche *in situ* might be sufficient to rescue the dysfunctional regenerative response. Therefore, kidney-targeted direct genetic repair, such as the development of AAV-based vectors, could be a promising approach [425,440].

Future perspectives on the kidney progenitor cell niche should envisage the further exploration of the *in vitro* capabilities of Cys-uKPCs, to develop organoid structures, and the *in vivo* potential for engraftment, differentiation and improvement of kidney function in an animal model. Obviously, in order to proceed to a pre-clinical setting, the isolation and culturing process of Cys-uKPCs should be transformed to a xeno-free procedure, and further excessive characterization and assessment for genetic aberrations of the Cys-uKPCs and *CTNS* complemented progenitor cells would be mandatory.

Taken together, we demonstrate that patients with cystinosis have a kidney niche of progenitor cells. These cells can be isolated from urine and differentiated towards a functional proximal tubular cells or podocytes. Moreover, we demonstrated that rescue of the healthy cellular phenotype via an *ex vivo* gene therapeutic approach is feasible, hereby providing a novel platform for disease modeling, drug screening and another step towards kidney tissue bio-engineering and potentially autologous cell-based gene therapy.

Conflict of Interest disclosure

The authors declare no competing financial interests.

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Supplemental data

Table S6.1 Overview of mesenchymal stem cell markers assessed in the characterization of cystinotic uKPCs

Gene	Protein	Alias	Expression	Function	Reference	
Mesenchymal stem cell						
NT5E	CD73		- hAK: cortical stroma - Undifferentiated MSC - Other: differentiated lymphocytes, T-lymphocytes	Membrane-bound, GPI-linked, ecto-5'-nucleotidase: catalyzes conversion of purine 5'-mononucleotides to nucleosides - One of the primary molecules to identify MSCs by the ISCT position statement Involved in: - BM-MSC interactions: MSC migration - Protection against ischemia-reperfusion injury in proximal tubule - Other: modulation of adaptive immunity	Misumi Y *et al.*, 1990 Colgan SP *et al.*, 2006 Bauerle JD *et al.*, 2011 Jian R *et al.*, 2011 Sung SJ *et al.*, 2017 Mushahary D *et al.*, 2018	441–446
THY1	CD90	Thy-1 cell surface antigen	- hFK: predominantly renal tubular epithelium, not in nephrogenic zone (± 25% of HFK cells) - hAK: visceral epithelial cells of glomerulus (hgl-MSCs) - Undifferentiated MSC - Other: human CD34+ BM-derived HS cells, endothelial, smooth muscle cells, nervous tissue (neuronal and glial cells), lymphoid tissue (not in mature human T-cells)	Membrane-bound, GPI-linked, N-glycosylated glycoprotein with single V-like Igg domain - Cell-adhesion protein - One of the primary molecules to identify MSCs by the ISCT position statement Involved in: - Cell-cell and cell-matrix interactions between inflammatory mediators of immune response - Assists in inflammation and repairing damaged tissue by synthesis of growth factors, cytokines and ECM components - Growth and differentiation of stem cells - Anti-Thy1.1 glomerulonephritis is characterized by aberrant tubular angiogenesis, glomerular injury and tubulointerstistial fibrosis in rat	Metsuyanim S *et al.*, 2009 Bruno S *et al.*, 2009 Kisselbach L *et al.*, 2009 Cina DP *et al.*, 2011 Mushahary D *et al.*, 2018	259,266,446–448
ENG	CD105	Endoglin	- hAK: endothelial cells in all arteries, tubular capillaries and glomerular endothelial cells - Undifferentiated MSC	Type 1 membrane glycoprotein, subunit of TGF-β1 receptor complex - One of the primary molecules to identify MSCs by the ISCT position statement Involved in: - Modulating response to TGF-β1, TGF-β3, Activin-A, BMP-2, BMP-7 - Cytoskeletal organization, cell morphology and migration - Angiogenesis: tumor growth, survival, metastasis of cancer cells - Maintenance, self-renewal and chemoresistance in renal cancer stem cells	Saroufim A *et al.*, 2014 Hu J *et al.*, 2017 Mushahary D *et al.*, 2018	446,449,450

Table S6.1 (continued) Overview of mesenchymal stem cell markers assessed in the characterization of cystinotic uKPCs

Gene	Protein	Alias	Expression	Function	Reference	
Mesenchymal stem cell (continued)						
CD44	CD44	- HCAM: Homing Cell Adhesion Molecule - Pgp-1: phagocytic glycoprotein-1	- hFK: intraglomerular, cortical and medullary interstitial cells - hAK: activated glomerular parietal epithelial cells - Other: urothelial basal cells	Cell surface adhesion receptor for hyaluronic acid; Interacts with ligands: osteopontin, fibronectin, collagens, selectins, MMPs Involved in: - Nephrogenesis: subset of progenitor/stem cells in early phase of kidney development and podocyte and/or interstitial cell differentiation - Regeneration: activated glomerular parietal epithelial cells	Desai S *et al.*, 2000 Fatima H *et al.*, 2012 Froes BP *et al.*, 2017	451–453
ITGB1	CD29	ITGB1: Integrin β-1	- hFK: in vitro hFK epithelial progenitor cells - hAK: glomerulus (podocytes, mesangial and endothelial cells), tubular epithelium (proximal and distal tubular epithelial cells, collecting duct cells)	Cell surface receptor; associated with Integrin α 1 and 2 to form integrin complexes, which function as collagen receptors Involved in: - Cell adhesion, migration, proliferation and apoptosis; maintenance of normal tissue architecture - Nephrogenesis: metanephric induction, nephron differentiation, branching morphogenesis, collecting duct morphogenesis - Increased expression in glomerulonephritis and CKD, and acute tubular injury	Kreidberg JA *et al.*, 2000 Loo D *et al.*, 2008 Matthew S *et al.*, 2012 Pozzi A *et al.*, 2013 Iervolino A *et al.*, 2018	454–457
ANPEP	CD13	- Alanyl aminopeptidase (AAP) - Aminopeptidase N (AP-N)	- hAK: renal proximal and distal tubular cells - Other: GIT tract: small intestinal & renal microvillar membrane	Microvillar membrane/ brush border bound enzyme in small intestinal tract and renal tubular cells Involved in: - Final digestion of peptides from protein hydrolysis - Unclear function in kidney	Mantle D, 1991 Van der Hauwaert C *et al.*, 2013	458,459
HLA-ABC	HLA-ABC	Human LeucocyteAntigen	- hAK: glomeruli, renal tubular epithelium	MHC class I cell surface receptor	Hart DN *et al.*, 1981 Müller CA *et al.*, 1989	460,461
HLA-DRA	HLA-DR	Human Leucocyte Antigen – antigen D Related	- hAK: glomerular and peritubular capillaries: renal microvascular endothelial cells (RMEC), epithelium of Bowman's capsule, interstitial cells, endothelial cells, proximal tubular epithelial cells - Antigen-presenting cells (macrophages, B-cells, dendritic cells)	MHC class II cell surface receptor HLA-DR and its ligand constitute to ligand for T-cell receptors Involved in: - Immune stimulation: presentation of peptide antigens to immune system, for eliciting or suppressing T-helper cell responses - Decline of HLA-DR+ glomerular cells in mesangioproliferative glomerulonephritis and membraneproliferative glomerulonephritis	Scott H *et al.*, 1981 Müller CA *et al.*, 1989 Muczynski KA *et al.*, 2003	461–463

Table S6.1 (continued) Overview of mesenchymal stem cell markers assessed in the characterization of cystinotic uKPCs

Gene	Protein	Alias	Expression	Function	Reference
Mesenchymal stem cell (continued)					
VIM	VIM	Vimentin	- hFK: undifferentiated cells of MM; visceral epithelial cells of glomeruli - hAK: parietal epithelial cells of Bowman's capsule, interstitial cells, tubular cells: co-localization with CD133+ CD24+ cells - MSCs (various types)	Type III intermediate filament cytoskeletal protein - Major cytoskeletal component of mesenchymal cells Involved in: - Supporting position of organelles, maintaining cell shape, cytoplasmic integrity - Response to injury: Upregulation following kidney injury - MET & EMT: expressed upon EMT and before MET	Holthöfer H *et al.*, 1984 Bussolati B *et al.*, 2005 Ye Y *et al.*, 2011 Lindgren D *et al.*, 2011 Smeets B *et al.*, 2013 192,219, 224,258, 464

Table S6.2 Primer list

Target gene	GeneID	Protein	Forward primer (5'-3')	Reverse primer (5'-3')
ACTB	60	β-Actin	AAGAGCTACGAGCTGCCTGA	GACTCCATGCCCAGGAAGG
CTNS	1497	cystinosin	CCACAGGCCTACATGAACTT	TCCACTGGTCGTTGTTGTAG
Kidney stem/progenitor cell markers				
NCAM1	4684	Neural cell adhesion molecule 1	GTCCTGCTCCTGGTGGTTGT	TGACCGCAATGCACATGAA
CITED1	4435	Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 1	AGGATGCCAACCAAGAGATG	TGGTTCCATTTGAGGCTACC
VIM	7431	vimentin	ACACCCTGCAATCTTTCAGACA	GATTCCACTTTGCGTTCAAGGT
PAX2	5076	paired box 2	AACGACAGAACCCGACTATG	ATCCCACTGGGTCATTGGAG
SIX2	10736	SIX homebox 2	CTCAAGGCACACTACATCGAG	GTTGTGGCTGTTAGAATTGGA
Proximal tubular epithelial cell markers				
CD13	290	Alanyl aminopeptidase	CACACACCGTTCCTGGATCTCCTCT	GCTCCAACAGGCGAAGGTCACT
ABCB1	5243	ATP binding cassette subfamily B member 1; MDR-1; P-gp	AGCTTAACACCCGACTTACAGA	ACCTCTTCAGCTACTGCTCCAGCT
LRP2	4036	megalin	ACAATGCATCCCCAACTCCT	AGGCCCCATTGTCACAAGTA
CUBN	8029	cubulin	CCCCTTCTTGGGAAGTTCTG	CCACGTGGGTGAAGTTGATT
AQP1	358	Aquaporin 1	ATCGAGATCATCGGGACCCTCCA	TGTCGTCGGCATCCAGGTCATA
Podocyte markers				
NPHS1	4868	nephrin	CGCAGGAGGAGGTGTCTTATTC	CGGGTTCCAGAGTGTCCAAG
SYNPO	11346	synaptopodin	AGCCCAAGGTGACCCCGAAT	CCCTGTCACGAGGTGCTGGC
PODXL	5420	podocalyxin	CTTGAGACACAGACACAGAG	CCGTATGCCGCACTTATC
CD2AP	23607	CD2 associated protein	AGGCTGGTGGAGTGGAAC	CAGAGAAGGTATAGGTGAAGTAGG
WT1	7490	Wilm's tumor protein 1	GGACAGAAGGGCAGAGCAACCA	GTCTCAGATGCCGACCGTACAA

Table S6.3 Antibodies list

Name	Target	Manufacturer	Catalog #	Dilution
Immunofluorescence staining				
Primary antibodies				
rabbit anti-aquaporin 1	AQP1	Merck-Millipore®	AB 2219	1:100
mouse anti-synaptopodin	SYNPO	Progen Biotechnik	65294	1:100
rabbit anti-podocalyxin (C-terminal)	PODXL	Abcam®	Ab154305	1:100
mouse anti-HA.11 epitope tag	HA.11	BioLegend®	901515	1:1000
rabbit anti-LAMP-1	LAMP-1	Cell Signaling	#9091	1:200
Secondary antibodies				
donkey anti-mouse IgG H&L Alexa Fluor® 555		Abcam®	Ab150106	1:300
goat anti-rabbit IgG Alexa Fluor® 546		Invitrogen™ Thermo Fisher	A11035	1:400
Immunohistochemistry				
Primary antibodies				
rabbit anti-PAX2	PAX2	Abcam®	Ab79389	1:250
mouse anti-CD133	CD133	Thermo Fisher	MA1-219	1:100
Secondary antibodies				
goat anti-rabbit IgG (H+L) Alexa Fluor® 546		Invitrogen™	A11035	1:400-1:500
goat anti-mouse IgG H&L Alexa Fluor® 488		Abcam®	Ab150113	1:300-1:500
Flow cytometry				
FITC-conjugated mouse anti-human CD34	CD34	BD Pharmingen™	555821	1:100
PE-conjugated mouse anti-human CD14	CD14	R&D Systems®	FAB3832P	1:100
FITC-conjugated mouse anti-human CD90	CD90	Milli-Mark™	FCMAB211F	1:100
PE-conjugated mouse anti-human CD45	CD45	R&D Systems®	FAB1430P	1:100
PE-conjugated mouse anti-human CD73	CD73	BD Pharmingen™	550257	1:100
FITC-conjugated mouse anti-human CD29	CD29	Acris / OriGene	SM1578F	1:100
PE-conjugated mouse anti-human CD44	CD44	BD Pharmingen™	550989	1:100
PE-conjugated mouse anti-human CD105	CD105	R&D Systems®	FAB10971P	1:100
FITC-conjugated mouse anti-human CD24	CD24	BD Pharmingen™	555427	1:100
PE-conjugated mouse anti-human CD133	CD133	MACS Miltenyi Biotec	130-080-801	1:100
APC-conjugated mouse anti-human CD13	CD13	eBioscience™	17-0138-42	1:100
FITC-conjugated mouse anti-human HLA-ABC	HLA-ABC	BD Pharmingen™	555552	1:100
PE-conjugated mouse anti-human HLA-DR	HLA-DR	BD Pharmingen™	555561	1:100

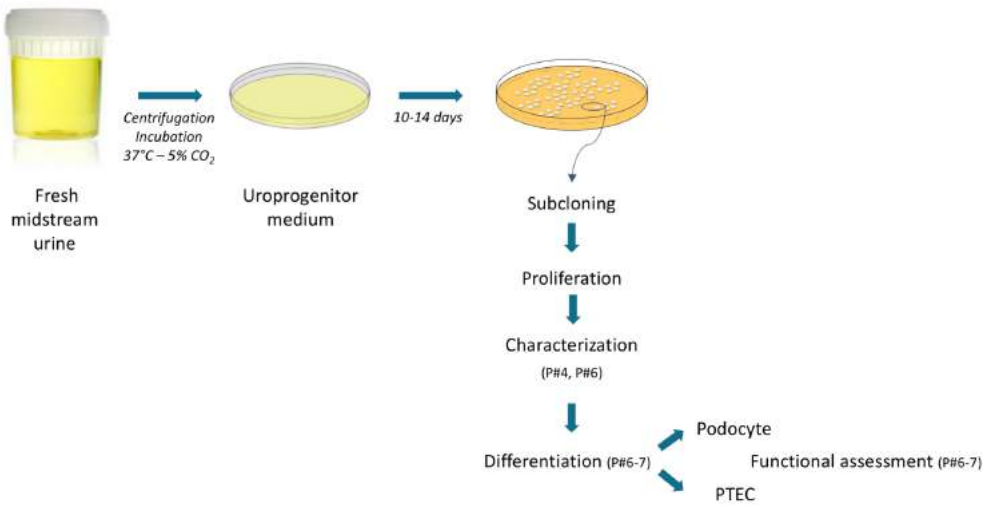


Figure S6.1 Schematic representation of the standardized protocol for isolation and establishment of cystinotic urine-derived kidney progenitor cells

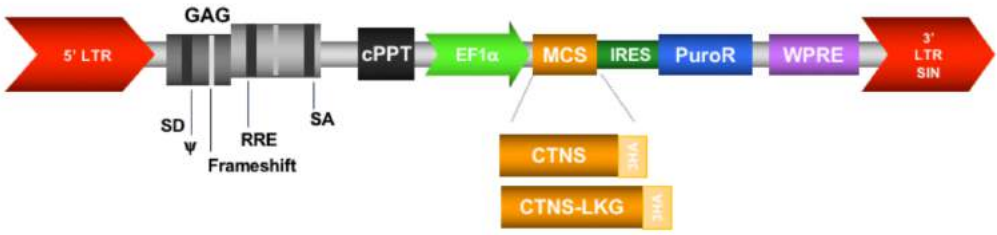
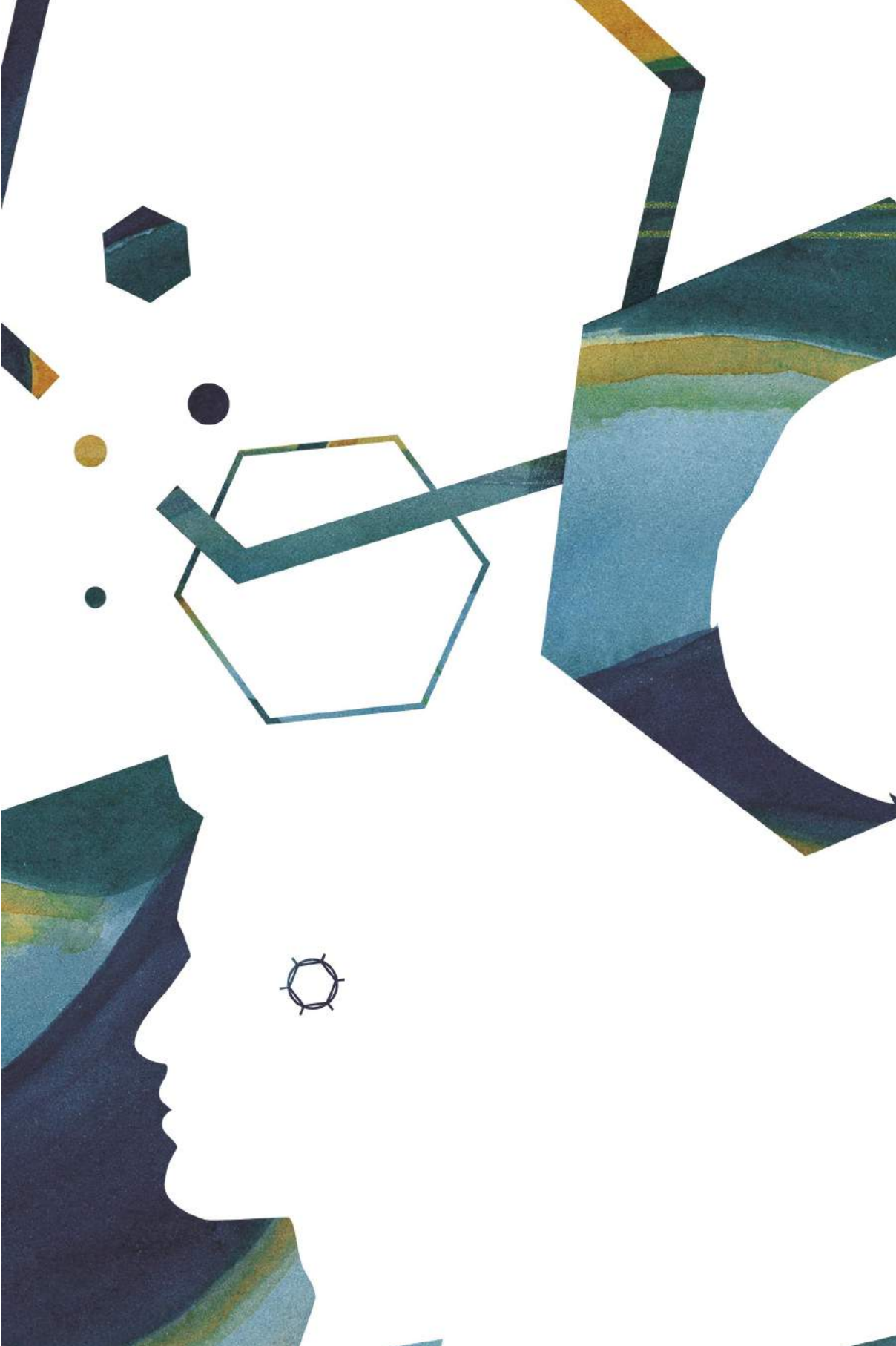


Figure S6.2 Graphical representation of the SIN lentiviral vector constructs used in the transduction experiments for complementation of CTNS

Several self-inactivating (SIN) lentiviral vector (LV) construct, using a human elongation factor-1 alpha (EF-1 α) promotor, a puromycin resistance antibiotic selection cassette (PuroR), and containing *eGFP* or *CTNS* or *CTNS-LKG* (full length cDNA; tagged with a 3HA tag) as a transgene, was developed through collaboration with the Leuven Viral Vector Core (LVVC). The vector was designed on a central polypurine tract (cPPT) structure.

Abbreviations: LTR: long tandem repeat; SD: splice donor site; psy: packaging signal; RRE: Rev responsive element; IRES: internal ribosomal entry site; WPRE: Woodchuck Hepatitis Virus Posttrancriptional Regulatory Element



CHAPTER 7
First successful conception induced by a male cystinosis patient

Chapter based on:

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First successful conception induced by a male cystinosis patient

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ABSTRACT

Background

Cystinosis is a rare autosomal recessive lysosomal storage disease characterized by multi-organ cystine accumulation, leading to renal failure and extra-renal organ dysfunction. Azoospermia of unknown origin is the main cause of infertility in all male cystinosis patients. Although spermatogenesis has shown to be intact at the testicular level in some patients, no male cystinosis patient has been reported yet to have successfully induced conception.

Case

We present the first successful conception ever reported, induced by a 27-year old male renal transplant infantile nephropathic cystinosis patient through percutaneous epididymal sperm aspiration (PESA) followed by intracytoplasmic sperm injection (ICSI). After 36 weeks and six days of an uncomplicated pregnancy, a dichorial diamniotic (DCDA) twin was born with an appropriate weight for gestational age and in an apparently healthy status. Moreover, we demonstrate that sperm of epididymal origin in selected male cystinosis patients can be viable for inducing successful conception.

Conclusions

Our observation opens a new perspective in life for many male cystinosis patients whom nowadays have become adults, by showing that despite azoospermia fathering a child can be realized. In addition, our findings raise questions about the possibility of sperm cryopreservation at a young age in these patients.

7.1 Introduction

Cystinosis (OMIM #219800) is a rare, autosomal recessive lysosomal storage disease, caused by mutations in the *CTNS* gene encoding cystinosin, a lysosomal proton-cystine cotransporter [1] It is characterized by lysosomal cystine accumulation and crystal formation in all organs and tissues [1,40]. The disease initially affects the kidneys, however during the first decades of life, various endocrine organs can also be affected [68,71,72] In addition to primary hypothyroidism, growth retardation and pancreatic insufficiency, hypergonadotropic hypogonadism has been reported as a frequent finding in male cystinosis patients [75]. As of yet, in contrast to a few female patients that have given birth, no male cystinosis patient is known to have induced pregnancy. More recently, it was shown that this infertility in male cystinosis patients is due to azoospermia of yet unknown origin [76].

Treatment with the cystine-depleting drug cysteamine delays the onset of end-stage renal disease and prevents multi-organ damage [149,337]. While first described in 1976, the use of cysteamine in cystinosis patients only became widespread in the early 90ies with the availability of the commercial preparation of cysteamine bitartrate (Cystagon®) [6,465]. Currently, a growing population of cystinosis patients who were treated with cysteamine starting from infancy or early childhood, is reaching young adulthood. This prolonged life expectancy raises novel quality of life issues such as male infertility. Here, for the first time ever, we report a successful conception induced by epididymal sperm of a male cystinosis patient through assisted reproductive technology (ART).

7.2 Case

A 27-year old male nephropathic cystinosis patient and his wife were consulted at the fertility clinic for realizing their wish of having children.

The patient was formerly diagnosed with nephropathic cystinosis at the age of 4 years when he presented with renal Fanconi syndrome and photophobia. Slit lamp examination revealed the presence of corneal cystine crystals. The diagnosis of cystinosis was confirmed by genetic analysis of the *CTNS* gene showing a compound heterozygous mutation (the common 57kb deletion on one allele, and a point mutation in the lariat branch site of intron 4: c.141-24T>C on the other allele). The latter mutation results in skipping of exon 5 during transcription [466].The leucocyte cystine level at the moment of diagnosis was 7.8 nmol ½ cystine/mg protein. Ever since the initiation of treatment, the total dose of cysteamine has been between 1.3 to 1.9 g/m^2 per day. End stage renal disease developed by the age of 10 years requiring renal replacement therapy in the form of peritoneal dialysis. Deceased donor kidney transplantation followed at the

age of 11. The immunosuppressive regimen consisted of ciclosporin A, azathioprine and low-dose prednisone. Both growth and pubertal development were delayed. At the age of 15, the Tanner stages of puberty were A1P2G2. Height was 153.5 cm, which is below -2 standard deviations (SD), and bone age lagged behind 2 years in comparison to calendar age. Recombinant human growth hormone (rhGH) therapy was initiated after kidney transplantation and resulted in a catch-up growth and a final height of 176 cm. At the age of 18 years, the patient reached a full sexual maturation with Tanner stage 5 and a final bilateral testes volume of 18 ml with a normal consistence. In addition, thyroid function tests always remained within the normal range. Until now, no signs of renal transplant rejection have ever occurred and current eGFR is 110 ml/min/1.73m^2.

A previous fertility status evaluation at the age of 21 years showed normal levels of FSH and LH, testosterone and inbibin B (Table 7.1, first column). However, no sperm was present in the ejaculate obtained by masturbation, which led to the diagnosis of azoospermia.

Following the consultation at the fertility clinic, the pituitary-testicular axis hormonal status was re-assessed (Table 7.1, last column). Because of proven azoospermia, and in order to avoid complications related to testicular sperm extraction (TESE), a percutaneous epididymal sperm aspiration (PESA) was performed. The funniculus was anesthetized with prilocain hydrochloride (Citanest®) and 0.25ml of epididymal fluid was aspirated on the left side, and 0.5ml on the right side. This procedure revealed the bilateral presence of mature spermatozoa. In total, from the left epididymis 0.2x10^6 spermatozoa were harvested (0.80x10^6/ml); the right epididymis yielded 2.5x10^6 spermatozoa (5.0x10^6/ml). In both sides 1% of the spermatozoa showed progressive motility, and 8% showed non-progressive motility. The sperm was mixed with Test Yolk Buffer (Irvine Scientific, USA) and cryopreserved in abidance of an intracytoplasmatic sperm injection (ICSI) procedure.

In the first ICSI attempt two oocytes (metaphase II) were obtained, of which one was fertilized. In the second attempt, six out of seven oocytes were fertilized. Single embryo transfer did not result into pregnancy and cryopreservation of the remaining embryos was not possible due to insufficient quality.

A new PESA procedure was performed before the third ICSI attempt. Fifty thousand spermatozoa were obtained from the right epididymis (aspirated total epididymal fluid: 0.5ml; concentration of spermatozoa: 0.10x10^6/ml), with 12% progressive and 14% non-progressive motility. The semen was again cryopreserved. In the third attempt, eight oocytes could be obtained of which six were fertilized. Double embryo transfer followed on day three. One embryo was suitable for cryopreservation on day five (blastocyst). Two weeks later, a pregnancy test resulted positive, and at seven weeks of gestation an intact dichorial diamniotic (DCDA) twin was

identified by ultrasound. Pregnancy was monitored in a regional hospital and at a gestational age of 36 weeks and six days the DCDA twin was born via an uncomplicated caesarean section. Both newborns were apparently healthy with respective birth weights of 2910 g (son) and 2884 g (daughter). Subsequently, DNA analysis of the cord blood showed the heterozygous c.141-24T>C mutation in the *CTNS* gene in both offspring,
confirming the paternity of our cystinosis patient.

Table 7.1 Hormonal levels of the pituitary-testicular axis in the reported male cystinosis patient during (early) adulthood

Results are reported from the first fertility assessment at the age of 21 years (first column; reported as patient 2 in Besouw *et al* 2010), until the assessment at the age of 27 years prior to the first percutaneous epididymal aspiration (PESA) procedure (last column). *Hormonal levels which are above the upper limit of normal. Based on these results, no primary hypogonadism can be observed. The ejaculate showed azoospermia at the age of 21 and 27 years (data not shown).

		Reference value						
Age	Years		21	22	22	23	26	27
FSH	U/L	1.5-11.0	7.79	5.5	6.3	7.4	8.3	8.4
Inhibin B	pg/L	150-400	210					174
LH	U/L	1.4-8.5	7.44	4.6	7.4	10.0*	7.4	7.3
Testosterone	nmol/L	11-45	16	9.5	21.6	30.3	15	17
Creatinine	umol/L	60-110	83	96	93	100	109	115
WBC cystine	nmol ½ cystine/mg protein	< 1	0,96	2,9	2,42	1,42	2,14	0,78
Cysteamine dose					1050mg qid			

7.3 Discussion

Over the last few decades, the advent and progress in cystine-depleting treatment and renal replacement therapy (RRT) have significantly improved the prognosis of cystinosis patients [40,149,337]. As a result of earlier diagnosis, rapid initiation of adequate cysteamine therapy and improved therapeutic monitoring, the life expectancy of cystinosis patients has been greatly extended, allowing them nowadays to survive into adulthood. This favorable evolution has created a new perspective, raising specific adult-related issues. Hence, being one of the essential human needs and major determinants of quality of life, having children has become a matter of concern to adult cystinosis patients and their families.

Here, for the first time, we report a successful conception induced by a male cystinosis patient through ART with PESA followed by ICSI. The paternity of our patient could be confirmed as both offspring inherited the rare heterozygous mutation in the *CTNS* gene, which has only been described in the index case and his sister thus far [466]. In addition to the previous report of Besouw

et al which has shown intact spermatogenesis at the testicular level, we demonstrate for the first time that sperm of epididymal origin can be viable in selected male cystinosis patients, and can be used for inducing conception successfully [76].

To this date, the exact pathophysiology of the primary hypogonadism and azoospermia observed in patients with cystinosis is not yet fully understood. In a first organized report on reproductive function in male infantile nephropathic cystinosis patients after renal transplantation published in 1993, hypergonadotropic hypogonadism was shown to be present in the majority of patients (70%) [75]. This finding could neither be explained by the effect of the previous chronic renal insufficiency, nor by renal transplantation and immunosuppressive agents used in the established regimens. In a more recent study, the pituitary-testicular axis remained within normal limits in a subset of male patients although azoospermia was present in all of them [76]. Surprisingly, a testicular biopsy in one of these patients showed an intact spermatogenesis (Johnsen score 8-9) [76,467]. As azoospermia was also present in patients treated with cysteamine starting from early age, it remained uncertain whether cysteamine had no therapeutic effect on the pathophysiology of azoospermia, or the drug itself would be involved in causing infertility. In addition, as azoospermia was documented in some patients in the presence of a normal renal function (eGFR), altered renal function cannot be regarded as a major determinant of fertility status [76].

Some hypothetical causes for this azoospermia in nephropathic cystinosis patients with a normal pituitary-testicular axis and renal function, can be suggested. First, as spermatogenesis at the testicular level has shown to be intact, it could be hypothesized that epididymal sperm maturation is altered. Epididymal sperm maturation is a process in which spermatozoa acquire their motility and fertilizing capacity during transit from caput to cauda epididymis [468]. This is a crucial step in the establishment of male fertility. Herein, the composition and changing biochemical properties (water, sodium, potassium, bicarbonate, pH, calcium, osmolality) of the epididymal luminal fluid play a vital role [468–471]. The renal proximal tubule and the epididymis share the same embryonic precursor -the intermediate mesoderm-, both are highly metabolically active and serve a shared goal of maintaining electrolyte and acid-base homeostasis through similar transepithelial transport processes. Therefore, although -in contrast to the renal proximal tubule- the sensitivity of epididymal tubular epithelial cells to cystinosin dysfunction is unknown, one could speculate that epididymal transepithelial electrolyte transport in cystinosis is impaired. Indeed, altered acidification of the epididymal luminal fluid milieu has been recognized as a cause of infertility [469,470].

Second, *in vivo* and *in vitro* data on cysteamine, being the only available disease-modifying drug, suggest an indirect local as well as a systemic endocrine effect, the latter through actions of

ghrelin. Via inhibition of somatostatin secretion, cysteamine increases plasma ghrelin levels [472]. Ghrelin has shown to exert a central inhibitory effect on FSH and LH secretion, as well as a local inhibitory effect on Leydig cell proliferation and testosterone secretion [473]. In addition, cysteamine can potentially induce alterations of posttranslational modifications in epididymal sperm [474]. Furthermore, a spermicide effect of cysteamine has been documented by a moderate reversible inhibition of human acrosin, a protease which is released from the acrosome of the spermatozoa in the acrosome reaction, which is crucial to the penetration of the zona pellucida [475]. However, whether cysteamine penetrates the blood-testes barrier and which concentrations can be reached in the human sperm, is currently unknown.

Cystine crystal deposits and signs of increased fibrosis have been previously demonstrated in testes in an infantile nephropathic cystinosis patient under cysteamine therapy [75]. However, in this case, the patient had clear signs of primary hypogonadism, sufficient to explain his infertility. As several studies demonstrated recently that cystinosin is involved in numerous cellular processes, one can argue whether merely the lysosomal cystine accumulation should be considered as the only pathophysiological mechanism involved in infertility [31,33,34,44,476]. In this regard it is of note that the cystinosin-LKG isoform, of which its localization is not limited to the lysosomal membrane, has the highest expression in the testes compared to other organs [477]. Obviously, further research is needed in order to unravel the underlying mechanism of the azoospermia observed in male cystinosis patients. In the meanwhile, ART can help these patients to realize their wish of having children.

As an important subset of male cystinosis patients have been described to evolve into a primary hypogonadism as early as the second decade of life, sperm cryopreservation in pubertal male cystinosis patients could be considered to maximize their chances on fathering a child. PESA offers the advantage of avoiding complications related to sperm retrieval techniques at the testicular level. Moreover, the success rate of a surgical sperm retrieval technique, like PESA, followed by ICSI, is non-inferior in comparison to the regular *in vitro* fertilization (IVF) with ejaculated semen [478]. It remains uncertain however, whether the ejaculate of pubertal boys with cystinosis will contain sperm cells that can be used for cryopreservation at all, since this has never been tested before. Obviously, the optimal timing at which cryopreservation would have to be performed will raise ethical dilemmas, and may also verge on the borders of current scientific and practical feasibility. Because of the vulnerability of this age group, the potential emotional impact of fertility preservation and the ethical concerns, psychological counseling should be a regular part of a multidisciplinary team, experienced in guiding patients through the cryopreservation and ART procedures. Ultimately, the most important ethical justification for

considering fertility preservation techniques in cystinosis patients is to serve their best interests and universal needs in life.

Taken together, this first successful conception induced by a male cystinosis patient, may be considered as a milestone and the dawn of a new era for the entire cystinosis community.

Conflict of Interest disclosure

The authors state no conflict of interest.

Acknowledgements

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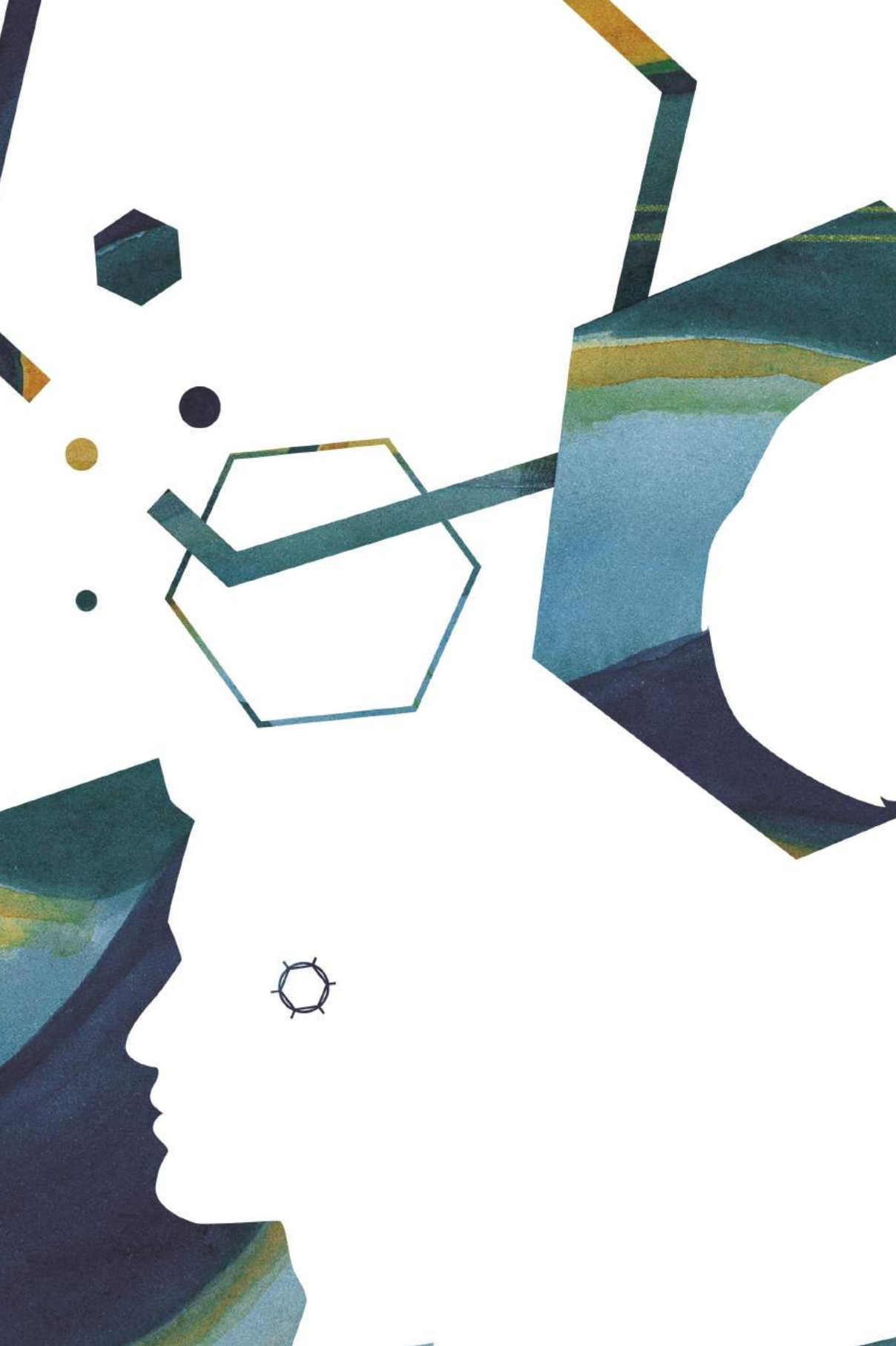


CHAPTER 8
Unravelling the mechanism of azoospermia in nephropathic cystinosis

Manuscript in preparation

ABSTRACT

Background

Nephropathic cystinosis is a rare inheritable multisystem disorder, caused by mutations in *CTNS* and characterized by widespread cystine accumulation in all tissues. Apart from the kidney, which is the first and most severely affected organ, primary hypogonadism in male cystinosis patients is a known extra-renal complication. However, azoospermia has been described in absence of signs of hypogonadism. Recently, viable spermatozoa could be retrieved from the epididymal level and induce a succesful conception via ICSI. Therefore, we hypothesized that on top of an evolving gonadal failure, an obstructive factor beyond the epididymal level contributes to the azoospermia observed in cystinosis. We aimed to demonstrate signs of obstruction of the genital tract by non-invasive means, using the assessment of markers on seminal plasma and soft biomarkers for obstruction on scrotal ultrasound.

Design, participants and methodology

A retrospective analysis was performed on cystinosis patients who had undergone testicular biopsy and epididymal sperm aspiration procedures.
A prospective cohort study was set up to identify signs of epididymal obstruction via the assessment of seminal plasma biomarkers (ECM1, NAG) and markers of obstruction via scrotal ultrasound in 9 cystinosis patients, 9 patients post-vasectomy and 7 healthy controls.

Results

In a retrospective analysis, we demonstrated ongoing spermatogenesis until the phase of late spermatids at the testicular level in 3 infantile type cystinosis patients, in presence of azoospermia. In addition, viable spermatozoa could be retrieved at the epididymal level in two other azoospermic infantile type cystinosis patients. In a prospective study, lower levels of ECM1 and NAG in seminal plasma were present while a significantly increased craniocaudal diameter of the caput epididymis normalized to the testicular volume, was demonstrated in infantile type cystinosis patients.

Conclusions

In infantile type male nephropathic cystinosis patients, seminal plasma and scrotal ultrasound biomarkers indicate signs of obstruction beyond the epididymal level which, on top of an evolving gonadal failure, contribute to the development of azoospermia.

8.1 Introduction

Nephropathic cystinosis (MIM 219800) is a rare, inheritable multi-systemic lysosomal storage disorder caused by bi-allelic mutations in the *CTNS* gene, which codes for the lysosomal cystine transporter cystinosin [1]. While kidney is the first and most severely affected, other organ systems get affected resulting mainly in ophthalmic, endocrine and neuromuscular complications [47]. The currently only available disease-modifying treatment cysteamine has – if initiated timely- shown to delay the progression of chronic kidney disease and reduce the number of extra-renal complications, hereby improving life expectancy [149,152,337]. However, it does not prevent end-stage renal disease, nor the development of all extra-renal complications observed in cystinosis. While female cystinosis patients are known to be fertile, male cystinosis patients suffer from fertility issues and no spontaneous induced successful pregnancy has been reported yet in a male cystinosis patient [479]. In the early nineties, primary hypogonadism was reported in a substantial proportion (70%) of adult male cystinosis patients [75]. Of note, in this study, the patient population descended from an era before the widespread availability of cysteamine bitartrate (Cystagon®). However, it was argued later that the infertility observed in male cystinosis patients is due to an azoospermia. In this descriptive cohort study by Besouw *et al*, azoospermia was present in all of the infantile type cystinosis patients studied, despite normal sexual hormonal levels of the pituitary-testicular axis and normal testicular volumes, and regardless the kidney transplantation status or age of initiation of cysteamine treatment [76]. Hence, it was concluded that gonadal failure is not the only factor contributing to infertility and that, conversely, the azoospermia observed in infantile type cystinosis patients is not merely due to a (severe) primary hypogonadism.

Recently we reported the first successful pregnancy induced by a male infantile type nephropathic cystinosis patient via percutaneous epididymal sperm aspiration (PESA) followed by intracytoplasmic sperm injection (ICSI). Strikingly, in this patient, despite azoospermia being present, viable spermatozoa could be retrieved at the epididymal level that were able to induce a successful conception, resulting in the birth of a healthy twin [480].

Therefore, we hypothesized that, on top of an evolving gonadal failure, an obstruction or dysfunction at the epididymal level might underlie the azoospermia observed in male nephropathic cystinosis patients. Unfortunately, the C57BL/6 *Ctns*$^{-/-}$ mouse model shows no fertility issues, making it unsuitable for studying the infertility observed in male cystinosis patients [481].

In this study we aimed to further unravel the cause of azoospermia in male nephropathic cystinosis patients in terms of demonstrating or excluding a potential obstructive factor via innovative non-invasive means by the assessment of biomarkers for obstruction on seminal

plasma and scrotal ultrasound. Indeed, recently, a two-marker screening algorithm using two seminal plasma biomarkers (including extracellular matrix protein 1, ECM1 and testicular expressed 101, TEX101) has been reported for identifying the cause of azoospermia as obstructive versus non-obstructive in a non-invasive manner [482,483]. In addition, neutral alpha-glucosidase is an established biomarker on seminal plasma specific to epididymal function or obstruction beyond epididymal level [484]. Also, the caput epididymis diameter has been put forward as a useful soft biomarker and predictor for obstruction of the genital tract in men with azoospermia [485]. Hence, in this study, in a first retrospective analysis, we focused on the spermatogenesis at the testicular and epididymal level of male cystinosis patients having azoospermia. Next, in a prospective cohort study, we investigated the presence of markers for obstruction at the epididymal level in seminal plasma and scrotal ultrasound.

8.2 Materials and Methods

8.2.1 Patients

In a retrospective analysis, male cystinosis patients who had undergone a testicular biopsy or percutaneous epididymal sperm aspiration (PESA) as part of regular care, were recruited. The corresponding Johnsen score of the testicular biopsies, the presence or absence of viable epididymal sperm, the sexual hormonal levels, kidney function, and semen analysis results were obtained from the medical records [467].

For a prospective part of this study, 9 male adult cystinosis patients (average age 34 ± 7.4 years) followed regularly at the University Hospitals Leuven (UZ Leuven, Leuven, Belgium), or the Radboud university medical center Nijmegen (Radboudumc, Nijmegen, The Netherlands), 9 patients post-vasectomy (average age 41.8 ± 7.9 years; average days after vasectomy: 56 ± 26 years) and 7 healthy fertile controls (average age 33.2 ± 6.6 years) were recruited. A history and clinical examination, scrotal ultrasound and blood sampling for sexual hormonal levels (serum LH, FSH, testosterone, Inhibin B) and kidney function (eGFR) were performed. In addition, a sperm sample was produced by the participating subjects via masturbation after at least two days of abstinence, and analyzed.

8.2.2 Histopathological studies

Formalin-fixed and paraffin-embedded testicular biopsy specimens obtained from three different infantile-type cystinosis patients, were stained with Periodic-Acid Schiff (PAS) for evaluating spermatogenesis by applying Johnsen's score. This is a 10-point scoring system where 1

represents tubules showing no spermatogenesis, while 10 represents tubules showing full spermatogenesis.

8.2.3 Biomarkers for obstruction in seminal plasma and scrotal ultrasound

On the semen sample the following parameters were assessed according to the WHO guidelines: volume, viscosity, pH, concentration, mobility and morphology [486]. Seminal plasma was isolated by centrifugation of the semen sample (300g, 10 minutes, 20°C), aliquoted and immediately frozen and kept at -80°C until further processing.

Extracellular matrix protein 1 (ECM1) was assessed on seminal plasma via a solid phase sandwich enzyme immunoassay (ELISA) according to the manufacturer's protocol (Sino Biological Inc., SEK10362).

Neutral Alpha-Glucosidase was assessed on seminal plasma via an enzymatic spectrophotometric assay, according to the manufacturer's protocol (EpiScreen Plus ™ FertiPro NV, Beernem, Belgium).

Scrotal ultrasound examinations were performed by radiologists experienced in urological imaging in both UZ Leuven and Radboudumc Nijmegen, with the patient in supine position. The following parameters were assessed according to an earlier described methodology: diameter of the caput (craniocaudal), corpus (anteroposterior) and cauda (craniocaudal) epididymis expressed in mm, bilateral testicular volumes expressed in cm^3, and the presence of epididymal cysts and sperm granuloma [485]. Testicular volumes were calculated by the following formula: radius long axis x radius short axis a x radius short axis b x 4/3 x π. The average caput epididymis craniocaudal diameter normalized to testicular volume (mm/cm^3) was obtained by the mean of the measurements concerning the right and left testis, each normalized to the respective testicular volume determined by ultrasound.

8.2.4 Ethical approval

Approval from the local ethical board of UZ/KU Leuven (Ethische Commissie Onderzoek UZ/KU Leuven) and Radboudumc Nijmegen (Centrum Mensgebonden Onderzoek, CMO Arnhem – Nijmegen) was granted under the study number s61017 and NL65400-091-18 file 2018-4249 respectively. Written informed consents were obtained from study participants. Research was conducted in accordance with the last version of the Declaration of Helsinki, the principles of Good Clinical Practice (GCP) and all applicable national and international legislation related to research involving human subjects.

8.2.5 Statistical analysis

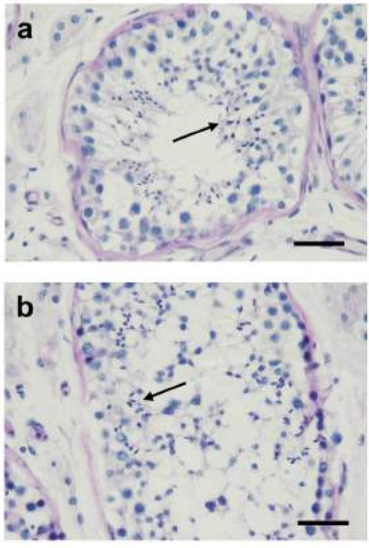
Graphpad Prism (version 8.1.0 (221) for Mac OS X) (GraphPad Software, La Jolla California USA, www. Graphpad.com) was used for the statistical analysis performed in this study.
A D'Agostino & Pearson normality test was applied for assessing the distribution of the data as being normally (Gaussian) distributed or not, and whether variances were equal. Depending on this result, parametric or non-parametric tests were performed. Data are represented as mean ± standard deviation (SD) for normally distributed data, unless otherwise specified.

8.3 Results

8.3.1 Spermatogenesis is intact at the testicular level and viable spermatozoa are present at epididymal level in cystinosis, despite azoospermia

In five infantile-type male cystinosis patients, either at the testicular level (3 of 5 patients), or the epididymal level (2 of 5 patients), intact spermatogenesis and viable spermatozoa could be demonstrated respectively, despite showing azoospermia (4 of 5 patients; one patient was not able to deliver a semen sample), (Figure 8.1). The spermatogenesis at the testicular level showed the presence of mature spermatozoa in all three patients studied, as demonstrated by the quantitative histological grading system Johnsen score (Table 8.1, Figure 8.1). One of the two patients of whom viable spermatozoa were retrieved from the epididymal level (patient # 4) concerns the same patient that was reported having induced a successful conception via ICSI [480].

Figure 8.1 Histopathological evaluation of testicular biopsies of azoospermic infantile cystinosis patients showing intact spermatogenesis.



Periodic-Acid Schiff (PAS) staining for formalin-fixed paraffin-embedded testicular biopsies from three different infantile cystinosis patients.
Panel a patient 1, age 16 years
Panel b patient 3, age 28 years
Panel c patient 5, age 33 years.

Arrows indicate the presence of elongated spermatids, indicative for the final stages of testicular spermatogenesis. Scale bar = 50 μm.

Table 8.1 Demographic and clinical characteristics of cystinosis patients included in the retrospective part of the study

Pt	Age (year)	Type	Age at initiation cysteamine	KTx	eGFR (ml/min/1.73m²)	LH (IU/L)	FSH (IU/L)	Testosterone (nmol/L)	Inhibin B (ng/L)	Testis volume (ml)	Semen analysis	Testicular sperm	Johnsen score	Epididymal sperm
					> 90	1.7 – 8.6	1.2 – 7.7	9 – 38	105 - 439					
1	16	INF	3	N	39	4.6	5.9	18	*nav*	*nav*	*nav*	Y	8-9	*na*
2	25	INF	*nav*	Y	*nav*	16.5	28	*nav*	*nav*	*nav*	Azoospermia	*na*	*na*	Y
3	28	INF	1.5	Y	50	16	19	11.4	91	18	Azoospermia	Y	7-8	*na*
4	29	INF	4	Y	> 90	12	16	22	127	18	Azoospermia	*na*	*na*	Y
5	33	INF	18	Y	60	9.6	7.3	22.2	96	18	Azoospermia	Y	8-9	*na*

Abbreviations: Pt: patient; KTx: kidney transplantation status; INF: infantile; *na:* not applicable; *nav*: not available; eGFR is expressed in ml/min/1.73m^2

8.3.2 Seminal plasma and scrotal ultrasound show signs of epididymal obstruction

Given the signs of ongoing spermatogenesis at the testicular level or viable spermatozoa at the epididymal level in spite of azoospermia being present, signs indicative for obstruction at the epididymal level were assessed on seminal plasma and scrotal ultrasound. Analysis for ECM1 on seminal plasma of cystinosis patients, post-vasectomy patients and healthy controls, showed a significantly reduced level of ECM1 in infantile type cystinosis patients compared to controls (median ECM1 in cystinosis, infantile phenotype: 6.69 μg/ml; controls: 43.85 μg/ml; difference between medians: -37.19 μg/ml; 95% CI of difference: 23.5 to 81.06 μg/ml; p=0.005) (Figure 8.2, panel a). In addition, post-vasectomy patients showed significantly lower levels of ECM1 compared to controls (median ECM1 in vasectomy: 5.91 μg/ml; actual difference between medians: 37.93 μg/ml; 95% CI of difference: 31.47 to 79.94 μg/ml; p=0.0002). Notably, infantile type cystinosis patients did not show significant different ECM1 levels compared to vasectomy patients (p=0.46) (Figure 8.2, panel a). Next, neutral alpha-glucosidase enzyme activity was assessed on seminal plasma. While a trend could be observed towards lower NAG activity levels per ml ejaculate in infantile and juvenile type cystinosis patients compared to controls, no significance could be reached (infantile type cystinosis patients median NAG activity: 15.35 mIU/ml, controls: 31.8; p=0.5) (Figure 8.2, panel b, left). Post-vasectomy patients did show significant lower NAG activity levels compared to controls, confirming the accurate performance of the test (vasectomy patients median NAG activity: 4.15 mIU/ml, controls: 31.80 mIU/ml, p=0.003) (Figure 8.2, panel b, left). However, when considering the NAG enzyme activity levels per ejaculate, the infantile type cystinosis patients yielded results within the range of post-vasectomy patients (infantile type cystinosis patients median NAG activity/ejaculate: 13.40 mIU/ejaculate (9.9 – 34.8), post-vasectomy patients median NAG activity/ejaculate 6.7 mIU/ejaculate (5.57 – 15.38)) and below the WHO lower reference limit of the normal range (20 mIU/ejaculate) (Figure 8.2, panel b, right). For both ECM1 and NAG activity in seminal plasma, the results for the juvenile type cystinosis patients were within the same range of the infantile type cystinosis patients (Figure 8.2, panel b and c). In addition, the caput epididymis craniocaudal diameter normalized to testicular volume resulted significantly higher in infantile type cystinosis patients compared to healthy fertile controls (cystinosis: 1.61 ± 0.59 mm/cm^3; controls: 0.5 ± 0.18 mm/cm^3; mean difference: -1.12 ± 0.18; 95% CI of difference: -1.49 to -0.75 mm/cm^3; p< 0.0001) (Figure 8.2, panel a). In contrast, one patient with the ocular phenotype who showed normospermia, showed similar results to control participants, although statistics could not be performed due to the lack of other ocular cystinosis patients. Meanwhile, patients with the juvenile phenotype, who showed oligospermia, showed also intermediary results between control participants and infantile cystinosis patients (Table 8.2, Figure 8.2, panel c).

Table 8.2 Demographic and clinical characteristics of cystinosis patients included in the prospective part of the study

Pt	Age (year)	Type	Age at initiation cysteamine	KTx	eGFR	LH (IU/L)	FSH (IU/L)	Testosterone (ng/dL)	Inhibin B (ng/L)	Testis volume (Le – Ri) (ml)	Days abstin	Semen volume (ml)	Semen pH	Sperm concentration ($x10^6$/ml)	Spermatocyte morphology (% normal)
					> 90	1.7 – 8.6	1.2 – 7.7	5.0 – 20.0	105 - 439	≧ 18	≧ 2	2 - 5-	≧ 7.2	≧ 20	≧ 4
1	23	INF	1.5	N	57	7.4	6.7	7	115	12 - 10	2	0.5	7.8	6	1
2	29	INF	0.5	Y	28	13	9.3	6	na	17.5 – 15	2	1	7.5	Azoospermia	*na*
3	31	INF	1.5	Y	15	41	56	4	53	14 – 12	7	2.3	7.3	Azoospermia	*na*
4	32	INF	1	Y	20	106	132	4.4	< 10	4 – 4	7	1.5	6.7	Azoospermia	*na*
5	39	INF	3	Y	31	47	140	6	< 10	6 – 8	2	1.5	8.3	Azoospermia	*na*
6	39	INF	1.5	Y	20	33.2	49.3	7.5	30	6 – 6	3	0.6	7	Azoospermia	*na*
7	29	JUV	10	N	84	12	4	8	138	15 – 15	1	0.9	7.8	6.4	5
8	35	JUV	12	N	62	8.2	2.6	10.3	234	25 – 20	3	1.8	7.7	15.9	4
9	48	OC	na	na	90	7.8	2.4	7.1	345	20 – 20	2	4.0	7.2	71.6	14

Abbreviations: Pt: patient; KTx: kidney transplantation status; OC: ocular; JUV: juvenile; INF: infantile; Le: left; Ri: right; *na*: not applicable; abst: abstinence; eGFR is expressed in ml/min/1.73m^2.

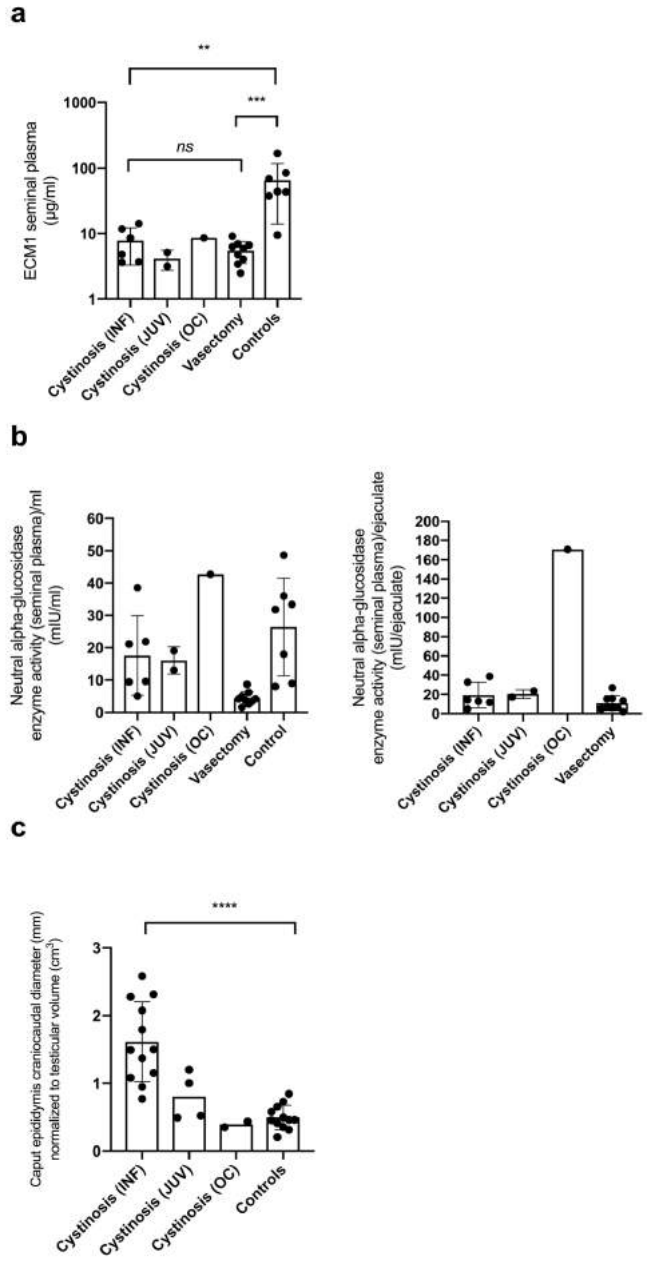


Figure 8.2 Non-invasive assessment of signs for genital tract obstruction at the epididymal level in cystinosis

Assessment of biomarkers of epididymal obstruction via markers on seminal plasma via ECM1 (panel a) and NAG (panel b), and via scrotal ultrasound (panel c).

8.4 Discussion

In this study, we aimed to further explore the origin of the azoospermia observed in male nephropathic cystinosis patients.

Recently we demonstrated in an azoospermic adult male cystinosis patient that viable mature spermatozoa could be retrieved at the epididymal level via PESA, which, followed by ICSI resulted in a successful conception and birth of healthy twins [480]. In the current paper, we first aimed to further corroborate this observation by reviewing testicular biopsies and percutaneous epididymal sperm aspiration procedures performed in male cystinosis patients for regular care purposes. The ascertainment of the presence of viable spermatozoa at epididymal level in another patient, and of ongoing spermatogenesis at testicular level in three other patients (Table 8.1) is in line with our previous report and indicative of a potential obstruction or malfunction at the epididymal level.

Based on these important observations and in support of the already available evidence, we aimed to further appoint and explore a potential obstructive cause for the azoospermia in male cystinosis patients.

The origin of azoospermia is of key importance to the approach and management of male patients suffering infertility [487,488]. While insufficient evidence is available to exclusively link obstructive and non-obstructive causes with specific sperm retrieval techniques, approaches for achieving sperm are essentially different in both cases [489]. Insight into the underlying pathophysiology enables physicians to apply the most efficient techniques that will result in the highest yield of sperm retrieval while minimizing iatrogenic morbidity. In non-obstructive causes for azoospermia, which is due to gonadal failure, retrieval of spermatozoa for further assisted reproductive techniques (ART) is almost exclusively possible at the testicular level via testicular sperm aspiration (TESA) or (microsurgical) testicular sperm extraction ((m)TESE). In contrast, obstructive causes for azoospermia necessitate a fundamentally different approach: depending on the site and cause of obstruction, several potentially successful options are available for retrieval of viable spermatozoa, ranging from approaches focused at the vasal (microsurgical or percutaneous vasal aspiration, MVSA, PVSA), epididymal (PESA), or testicular level (TESA or (m)TESE). The procedures needed in the two aforementioned possibilities show important differences at the technical level and their associated risks and complications [488,490]. TESE is a more laborious, technically demanding procedure, which needs more profound anesthetical support and can involve complications ranging from hematoma and scar formation to permanent devascularization of the testis in case of multiple biopsies. Moreover, multiple testicular procedures have shown to be associated with a significant risk of developing severe

hypogonadism [491–493]. Hence, procedures at the testicular level can further compromise the already jeopardized testicular function in cystinosis, hereby reducing the success rate of any future needed intervention [493].

In contrast, PESA can be performed more quickly and easily under local anesthesia, is associated with less complications, and is effective in terms of fertilization and pregnancy rates [489,494]. In addition, frozen-thawn epididymal sperm has shown satisfying fertilization and pregnancy rates, which makes it an appealing approach for developing a cryopreservation strategy at an early age in young adult cystinosis patients [495–498]. Obviously, the yield of sperm retrieval, fertilization rates and clinical pregnancy is higher in sperm retrieval techniques which are performed in the context of obstructive versus non-obstructive causes of azoospermia [489,499,500]. Given the pivotal importance of carefully considering the most appropriate procedure, and the already precarious setting of male cystinosis patients being highly at risk of developing gonadal failure, further evidence in support of an obstructive factor contributing to azoospermia could be highly valued. In order to avoid any potential harm to the testicular function of male cystinosis patients recruited in this study, we aimed to utilize non-invasive means for demonstrating a potential obstructive cause of azoospermia via the assessment of markers of obstruction on seminal plasma, and soft biomarkers for obstruction at the epididymal level via scrotal ultrasound.

Recent efforts for establishing non-invasive strategies aimed at better selection of patients requiring invasive fertility procedures, have yielded a two-marker screening algorithm using two seminal plasma biomarkers (including extracellular matrix protein 1, ECM1) for identifying the cause of azoospermia as obstructive versus non-obstructive [482,483]. While this screening protocol has not been implemented yet fully in clinical practice for the assessment of azoospermia, the pre-clinical evaluation of these markers has yielded promising data [501]. On the other hand, neutral alpha-glucosidase enzyme activity has been established for some decades already as a specific epididymal enzyme marker and has been applied in the clinical work-up of patients with azoospermia[484].

ECM1 is a secreted, ubiquitous extracellular matrix glycoprotein responsible for the maintenance of the structural integrity of tissue, and of which a function in angiogenesis, enchondral bone formation and tumor biology has been described [502]. In the male reproductive system, ECM1 is highly expressed at protein level in the cytoplasm of squamous epithelia and glandular cells of the epididymis, and to a lower extent by the seminal vesicles while it is completely absent in the testes and prostate [482,503]. In fact, the epididymis harbors the highest protein expression of ECM1 in the whole human body.

ECM1 was for the first time associated with male fertility in the context of the seminal plasma proteome project, which was aimed at identifying biomarkers for the diagnostic work-up of azoospermia [504,505]. ECM1 was one of the 18 final biomarker candidates that were selected after initially more than 2000 proteins in seminal plasma of men with normospermia, non-obstructive azoospermia and post-vasectomy patients were identified via tandem mass spectrometry, followed by verification using a selected reaction monitoring assay (SRM) [504–506]. In the investigations that followed, levels of ECM1 were shown to be relatively high in normospermia (± 40 μg/ml) and non-obstructive azoospermia (± 20 μg/ml), while it was notably decreased in obstructive azoospermia or post-vasectomy patients (± 1 μg/ml) [482]. Therefore, ECM1 levels in seminal plasma could serve as a non-invasive biomarker indicative for obstruction in case of azoospermia. In the two-step algorithmic approach presented by Drabovich and coworkers, low ECM1 concentrations were presented as indicative for obstructive azoospermia [482]. However, the exact function of ECM1 in the epididymis and in the seminal plasma, still needs to be elucidated.

While little is known about extracellular matrix protein 1 (ECM1) in male fertility, and its function in the epididymis in specific, neutral alpha-glucosidase is an established and well-studied specific marker for epididymal function.

The neutral fraction of alfa-glucosidase activity is specifically related to the epididymis [507]. More precisely, most of the enzyme activity is confined to the caput and corpus epididymis [508].

In males with azoospermia due to a bilateral obstruction distally to the epididymis, seminal plasma contains very low neutral alpha-glucosidase enzyme activity levels [509–514]. However, in non-azoospermic males, a functional deficiency of the epididymis is also associated with low NAG levels and in patients with low testosterone levels, also low levels of NAG are observed [515,516]. Despite the current controversy of the use of NAG in the clinical assessment of azoospermia, it is still found to be valuable specifically in the suspicion of epididymal pathology associated with functional or structural alterations [484].

Furthermore, recently, the caput epididymis diameter has been put forward as a useful soft biomarker and predictive for obstruction of the genital tract in men with azoospermia [485]. Noteworthy, in this study a significant correlation was demonstrated between the mean caput epididymis diameter and the mean testicular volume [485].

Here, in our study, we applied both the assessment of the seminal markers ECM1 and NAG, and soft biomarkers for epididymal obstruction via scrotal ultrasound, being non-invasive, innovative means in order to elucidate the cause of azoospermia in cystinosis.

We have shown a significantly reduced amount of ECM1 in seminal plasma in infantile type cystinosis patients, compared to healthy controls (Figure 8.2, panel a). Vasectomy patients also

showed a highly significant difference. Though the cystinosis patients did not show ECM1 levels below the cut-off of 2.3 μg/ml as proposed by Drabovich and coworkers, in our experience also vasectomy patients did not show, on average, ECM1 levels below this cut-off (Figure 8.2, panel a). These differences might be attributed to inter-laboratory variability due to different assays used, and optimization processes. Remarkably, the patient with the ocular phenotype also showed a relatively low level of ECM1, however since this subgroup concerns only one patient, no statistical analysis could be performed on this group. On the other hand, at this stage, it could be speculated that a low level of ECM1 indicates a malfunction of the epididymis which could be present irrespective of an obstruction at the epididymal level. Hence, further investigations on the effect of cystinosis on epididymal cells are still required.

Furthermore, neutral alpha-glucosidase enzyme activity (/ml) in seminal plasma yielded, on average, lower levels in the infantile type cystinosis patients, compared to controls though significance could not be reached (Figure 8.2, panel b). However, when considering the neutral-alpha glucosidase enzyme activity levels per ejaculate, on average the levels of the infantile and juvenile type cystinosis patients together showed levels comparable to vasectomy patients, and lower than the WHO lower reference limit (20 mIU/ejaculate). The rationale for considering the relative concentration to ejaculate volume in contrast to the absolute concentrations, is that the semen volume in infantile cystinosis patients was markedly reduced to normal controls, which could affect the interpretation of results.

Furthermore, we demonstrated a significantly increased craniocaudal diameter of the caput epididymis normalized to testicular volume, in infantile type male cystinosis patients compared to healthy controls (Figure 8.2, panel c). The cystinosis patients with the ocular phenotype, who has a normospermia, showed similar results to controls, while the patients with the juvenile phenotype who have oligospermia, showed intermediary results. Remarkably, these observations thus coincide with the age of onset and severity of the kidney phenotype, comprising the severity of the renal Fanconi syndrome and the progression of chronic kidney disease.

Taken together, these observations made via the assessment of seminal plasma biomarkers and soft biomarkers for obstruction via scrotal ultrasound show alterations that suggest an obstruction at the epididymal level in nephropathic cystinosis patients, which provide an explanation for the azoospermia observed.

In our study, the cystinosis patients with the juvenile phenotype showed an oligospermia, while infantile patients were previously known to be azoospermic. Remarkably, here we can also report one infantile-type cystinosis patient having an oligospermia. However, this 23-year old patient, despite being homozygous for the common 57kb deletion of *CTNS*, also has other signs of an

unexplained mild phenotype including a well-preserved kidney function (eGFR 57 ml/min/1.73m^2), a distinctly mild renal Fanconi syndrome and low WBC cystine levels (average 1.0 to 1.5 nmol ½ cystine/mg protein) in spite of a poor compliance with cysteamine therapy. Therefore, this patient should be considered as having an atypical infantile phenotype which could be hypothesized to be due to unknown disease modifiers [517].

Nevertheless, the presence of an oligo- versus azoospermia in this cystinosis patient cohort and their corresponding caput epididymis ultrasound measurements, seem to coincide with the degree of the kidney involvement. In fact, it could be hypothesized that any epididymal malfunction that might occur in cystinosis could parallel the degree of renal proximal tubular dysfunction, which could provide a cause for genital tract obstruction. Several arguments can support this hypothesis. First, both epididymis and the renal proximal tubule share the same embryonic precursor, show important structural homology and, being highly metabolically active, are required to maintain electrolyte and acid-base homeostasis through several transepithelial transport processes [518–521]. In cystinosis, renal proximal tubular epithelial damage is associated with increased shedding of exfoliated damaged cells and cellular debris in urine [44,402,522]. The increased shedding of cells in renal tubuli can eventually lead to the formation of tubular casts and might cause tubular obstruction [522]. In this respect, if the epididymal function would parallel the pathophysiology of the renal proximal tubule, we could speculate that a patient harboring a more pronounced Fanconi syndrome could also show an increased risk for developing azoospermia. For now, we cannot rule out epididymal malfunction to be the cause for the low ECM1 levels in seminal plasma in cystinosis patients since this could be due to altered expression and/or secretion of ECM1 in the epididymal epithelium. However, since the ultrasound results showed clear differences between controls and infantile cystinosis patients, it is more likely for an obstruction to be present beyond epididymal level than epididymal malfunction that causes azoospermia in infantile type nephropathic cystinosis patients.

In addition, our data still leave the opportunity for azoospermia to be a more progressive phenomenon, making it possible that a specific time-window could exist during which oligospermia can be observed and spermatozoa can be retrieved non-invasively.

To fully elucidate the obstructive factor contributing to azoospermia, transcriptomics on a *CTNS* knock-out or knock-down human epididymal cell line could reveal important clues on underlying pathophysiological mechanisms at the epididymal level in cystinosis.

We conclude that we have obtained additional indications via seminal plasma and scrotal ultrasound biomarkers in support of a malfunction or obstructive factor at the epididymal level contributing to the azoospermia observed in nephropathic cystinosis patients. Hence, the infertility in male cystinosis patients is not merely due to an evolving primary gonadal failure.
Therefore, if taken into consideration, sperm retrieval techniques in the context of ART procedures should be directed at the epididymis (e.g. via PESA), hereby avoiding invasive techniques at testicular level that could further compromise gonadal function. Cryopreservation of epididymal sperm seems an appealing and feasible strategy for fertility preservation that should be considered in in young adult cystinosis patients.
In addition, for the first time we also demonstrated that male juvenile cystinosis patients could be fertile, albeit subfertile, having an oligospermia. Our suggestion of a potential link between the severity of the renal Fanconi syndrome and degree of epididymal malfunction could further be explored *in vitro* by use of *CTNS* knock-down experiments in human epididymal cell lines.

Conflict of Interest disclosure

The authors state no conflict of interest.

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CHAPTER 9
GENERAL DISCUSSION & FUTURE PERSPECTIVES

In this thesis, we aimed to provide answers to some of the most important contemporary challenges faced in the current clinical management and research areas of nephropathic cystinosis.

The introduction of cysteamine and the advent and progress of renal replacement therapy have significantly improved the life-expectancy and outcome of cystinosis patients [149,151,152,155,337,523]. After more than two decades, the heritage of the widespread available cystine-depleting therapy has become apparent in the delayed progression of chronic kidney disease, the need for renal replacement therapy at later age, improved growth and reduced incidence of extra-renal manifestations. Hence, while cysteamine has allowed patients to survive into adulthood, treating physicians have witnessed the evolution of a once fatal disease, to a treatable chronic metabolic disorder.

On the other hand, large cohort-based studies have meanwhile unveiled the limits of cystine-depleting therapy. In parallel, further progress in unravelling the pathophysiology of cystinosis has deepened insight in what seems to be a much more complicated disorder then merely the widespread lysosomal accumulation of the di-amino acid cystine. The elucidation of the involvement of cystinosin in vesicle trafficking, lysosomal biogenesis, mTOR signaling and autophagy and the role of inflammation is a silent incitement to the cystinosis research field for exploring new horizons when aiming for improvement in the management of cystinosis [25,28,30,34,36,38,44,348]. Indeed, though cysteamine has proven to be a substantial disease-modifying agent, it imposes a nonnegligible burden on cystinosis patients due to a narrow therapeutic window, and options for a cure of the disease are still lacking.

Therefore, obviously the research field is faced with the general challenges of providing a better treatment for the kidney disease and the invalidating extra-renal complications, but also with finding solutions for the side-effects and limitations of the current cysteamine treatment. Indeed, though cysteamine has the ability to slow down the progression of CKD, it offers no cure for the renal Fanconi syndrome, and some extra-renal complications can still occur at a young age despite early and adequate cysteamine treatment [40,76,337]. Also, the currently only available modality for monitoring cysteamine treatment, measuring WBC cystine content, which is the cornerstone of the treatment of cystinosis, is limited and imperfect. Moreover, no means are available for assessing the long-term compliance, nor to assess the severity of the affected patients.

Likewise, apart from the research field, also clinicians treating cystinosis patients are confronted with challenges in a more than ever diverse patient population: from the young, often - in high-resource countries - well-treated paediatric patients, to adolescents struggling with the reality of their illness and adults faced with all issues of grown-up life. Of the latter, some are hopeful while

other rather anxious about a future which is still an unridden path. At this stage, the phenotype of cysteamine-treated adult cystinosis patients is still evolving while some of the clinical findings become more pronounced. One of these clinical manifestations which become more apparent at adolescent and adult age are specific cutaneous features. To date, the involvement of the skin in cystinosis was reported in a limited way [132], while recently it was proposed to harbor an important and useful feature: the skin could be approached to assess body cystine accumulation, and thus response to cystine-depleting therapy [134]. However, a systematic, quantitative cohort study using the most advanced, non-invasive optical imaging technology currently available, was not undertaken yet. Furthermore, one of the most universal needs in human life, the ability to have children, and the recently observed issue of male infertility in cystinosis, have also become a sincere concern in the young adult cystinosis patient population [76,481]. Being one of the main determinants of quality of life, treating physicians cannot remain untouched when faced with cystinosis patients struggling with this issue. In this context, this thesis was aimed to provide answers to some of these important challenges in the current management of cystinosis.

9.1 Innovation in monitoring of nephropathic cystinosis: biomarker development

9.1.1 Enhanced intrinsic skin aging in nephropathic cystinosis as assessed by high-definition optical coherence tomography

In **chapter 3**, we aimed to utilize a high-end, novel, non-invasive optical imaging technology in order to define the distinctive dermatological features of cystinosis and to explore its potential in monitoring disease severity.

To this end, high-definition optical coherence tomography (HD-OCT) was applied to assess characteristics of the dermal ultrastructure and its specific optical characteristics.

Recently, the assessment of tissue optical characteristics was shown to be of added value in the non-invasive diagnosis of various skin disorders, ranging from basal cell carcinoma to melanocytic skin lesions [303,524–527].

One of the first added values of our research concerns the validation of our newly developed methodology, for the assessment of epidermal and papillary dermis thickness. This comprises the first data available on non-invasive skin measurements performed in a paediatric to adolescent and young adult population via HD-OCT. The epidermal and papillary dermis thinning of non-sunexposed skin areas in nephropathic cystinosis patients of adolescent to young adult age, as observed via HD-OCT, has substantiated in a quantitative manner at least part of the clinical phenotype of skin involvement in cystinosis. These signs suggest that an enhanced intrinsic skin aging is present in cystinosis, which becomes apparent from adolescent age, prior to kidney transplantation.

Furthermore, in our cohort study in patients harboring the common homozygous 57kb deletion of *CTNS*, the presence of significant epidermal thinning, compared to age- and sex-matched controls, predicted the presence of extra-renal complications with a maximal positive predictive value. The homozygous 57kb deletion of *CTNS* obviously abolishes all functions of cystinosin and this genotype has been associated with a more severe phenotype in terms of number of extra-renal complications and mortality [152]. Hence, based on these results, we hypothesized that in this subpopulation the observed skin phenotype might be most pronounced, and hence also reflect the extent of the disease. Therefore, it could be suggested that signs of enhanced intrinsic skin aging might serve as a non-invasive diagnostic tool to assess the disease severity status. To what extent these signs can be of use in the longitudinal follow-up of patients, needs to be further explored in a large prospective study. In any case, for the first time, we demonstrated in a quantitative manner the specific involvement of the skin in the cystinosis phenotype.

9.1.2 Chitotriosidase: a novel additional biomarker for therapeutic monitoring of nephropathic cystinosis

As aforementioned, the only modality available for monitoring the treatment of cystinosis is the WBC cystine level assay. However, both the implementation of the WBC cystine level assay in all relevant medical centers involved in the regular care of cystinosis patients around the globe, and its applicability in the daily clinical practice is hampered by important technical, practical and procedural issues [25]. The technology needed (HPLC, LC-MS/MS) is expensive and the sample processing requires expertise, highly skilled operators, extensive optimization and strict standardized operating procedures. More than ever, implementation in an efficient and cost-effective manner within existing healthcare systems is mandatory. Sampling to processing time windows need to be respected, which requires flexibility and attention of both patients and health care providers. Importantly, due to the short half-life of white blood cells and of cysteamine, WBC cystine levels merely reflect short-term adherence to cysteamine therapy [25].

Apart from longitudinal data of WBC cystine levels, and obvious clinical signs and laboratory follow-up data, no other genuine means are at hands for the clinicians neither to assess the long-term effectiveness and adherence to cystine-depleting therapy, nor to appraise the extent of disease severity.

Moreover, most of the studies reporting on the outcome of cystinosis patients on cystine-depleting therapy, mostly used the use of cysteamine and duration of cysteamine treatment as parameters to compare outcome, while the WBC cystine level was only used in a newly developed compliance score (composite compliance score) [155]. Moreover, once aspired future therapies including hematopoietic stem cell gene therapy, could find its way into clinical practice, the WBC cystine level assay will no longer be of any value [165]. These arguments underpin the

need for innovation and development of alternative strategies for the monitoring of cystine-depleting therapy.

The establishment of a biomarker which could replace or provide a substantial added value to the WBC cystine assay harbors the potential to significantly improve the outcome of cystinosis patients worldwide. This was the main incentive for our cystinosis biomarker project as described in **chapter 4**. Here, we aimed to explore the utility of markers of macrophage activation for the monitoring of cystine-depleting therapy in nephropathic cystinosis. Since macrophages are the main cells responsible for clearing cell debris and deposits of inappropriate substances, including cystine crystal depositions, in all tissues throughout the body, this part of the innate immune system can be considered as one of the first systems affected by cystinosis, and a potential key player in the pathophysiology of cystinosis. Markers of macrophage activation have been successfully established as a diagnostic screening tool and for the monitoring of enzyme-replacement therapy in Gaucher's disease [342,528].

In cystinosis, the activation of macrophages upon exposure to and engulfment of cystine crystals has recently been robustly demonstrated in *in vitro* experiments [25]. The degree of activation could therefore constitute an indirect parameter for the body tissue cystine crystal deposition. Indeed, in an international multicentric prospective cohort study, we demonstrated that plasma chitotriosidase enzyme activity levels correlate significantly with WBC cystine levels, and is a significant independent predictor for WBC cystine levels in cystinosis patients of all ages. We could establish a cut-off level for plasma chitotriosidase enzyme activity that can distinguish adequate versus poorly controlled cystinosis patients on cysteamine therapy, with a reasonably high negative predictive value. Since chitotriosidase enzyme activity can be assessed on dried blood spots, and given the high predictive value for ruling out less well controlled patients, this feature of chitotriosidase could be particularly useful for the monitoring of cystinosis patients in remote areas and developing countries where the WBC cystine assay is not available. Obviously within the context of the industrialized countries, it might be a useful parameter for the same purposes, in addition to WBC cystine levels. In this way, it can overcome the shortcomings of the WBC cystine assay which is merely an indicator for short-term compliance.

Furthermore, chitotriosidase enzyme activity levels were significantly higher in patients with multiple extra-renal complications and showed to be superior to WBC cystine levels for identifying this subgroup of severely affected patients with a high positive predictive value. This observation indicates that chitotriosidase enzyme activity does reflect, to some extent, the degree of tissue cystine accumulation for as far the complications involved are caused by the cystine accumulation and its related inflammation. Indeed, in our study, only some of the major cystinosis extra-renal complications showed to be specifically associated with increased

chitotriosidase activity levels (retinopathy, insulin-dependent diabetes mellitus). In this respect, our observations are in line with the large cohort studies on long-term cysteamine treatment, in which it was shown that some of the major extra-renal complications still occur at a young age despite early and adequate cysteamine treatment (primary hypothyroidism, peripheral myopathy) [54]. The observation that age is, albeit independently, significantly associated with increased chitotriosidase levels, is in further support of the concept that cystine accumulation is a life-long progressive and cumulative phenomenon which can be reflected by markers of macrophage activation. Hence, in this respect, our data suggest that chitotriosidase could serve as a surrogate biomarker for disease severity. In addition, our observation also highlights the potential importance of inflammation in the pathophysiology of cystinosis. There are several indications for a substantial detrimental contribution of cystine-crystal induced chronic inflammation and the role macrophages in the progression of chronic kidney disease [25,26]. In contrast, to what extent inflammation is involved in the development of specific extra-renal complications, still needs to be elucidated.

Some caveats have to be pointed out when interpreting the results of our study. Since the treatment of cystinosis patients and general awareness of the disease has profoundly improved during the last two decades, the adult cystinosis patient patients in our cohort carry the heritage of an era in medicine at which the management of cystinosis is not gratuitously comparable to current regular care practice [5]. Also, immunosuppressive agents frequently used in regimens for kidney transplant recipients such as tacrolimus, mycophenolate mofetil and prednisolone, have been reported to affect the release of proinflammatory cytokines by macrophages [529–532]. Due to the low number of kidney transplant patients in this cohort study, and the relatively high number of different regiments used, we were not able to correct for a potential confounding in the data of kidney transplanted cystinosis patients. Of note, the kidney transplanted cystinosis patients generally showed higher chitotriosidase enzyme activity levels, compared to the non-kidney transplanted cystinosis patients. Furthermore, about 5% of the general population are homozygous for the 24-bp duplication mutation in exon 10 of the *CHIT* gene, which makes the biomarker unsuitable of use in a similar proportion of the cystinosis population. Finally, improved consensus on the definition and clinical diagnostic criteria of some of the extra-renal manifestations in cystinosis, the implementation of harmonized guidelines in cystinosis reference centers and establishment of a wide collaborative network for an extensive international cystinosis registry, such as the Rare Disease Cohort – European Cystinosis Cohort (RaDiCo-ECYSCO) initiative, could further support the organization and management of multicentric observational studies, and improve the accuracy and quality of the data retrieved [141,145,533].

9.2 Innovation in treatment of nephropathic cystinosis

9.2.1 Allogeneic hematopoietic stem cell transplantation transfers cystinosin to nonhematological epithelial cells in cystinosis

Despite the progress that has been made in the clinical management and understanding of the disease, a curative treatment for cystinosis is still lacking.

Over the past two decades long-term follow-up data have revealed that cystine depletion does not suffice in the treatment of cystinosis patients. Meanwhile, the further unravelling of the pathophysiology in all its complexity has partially explained these clinical findings and tempered the assumption for success of drug-based treatment strategy.

Since cystinosis is a metabolic disorder and given the success in other lysosomal storage disorders, treatment strategies aimed at the replenishment of the absent or dysfunctional protein via an allogeneic hematopoietic stem cell transplantation, seemed an alluring and promising approach [162]. However, the basic concept underlying the successful allogeneic HSCTs in other metabolic disorders is fundamentally different from the context of cystinosis [534–536]. The replenishment of a deficient or defective enzyme might not simply be compared to the widespread intracellular introduction of a properly functioning, complex protein, in a specific cellular organelle, at amounts which are comparable to tissue-specific endogenous expression levels.

Nevertheless, now about a decade ago, the first impressive results of bone-marrow derived stem cell transplantation in the cystinosis mouse model by the group of Stephanie Cherqui, in which significant reductions of tissue cystine content in various relevant organs and the preservation of kidney function was shown, constituted a strong incentive to pursue this highly promising strategy [162]. The potential of HSCT to preserve kidney function for long-term in the cystinosis mouse model was later confirmed in a longer follow-up study (7 to 15 months post-HSCT) [163]. Shortly thereafter, the concept for an autologous *ex vivo* genetically modified HSCT was proven to be successful by showing significant reductions in tissue cystine content in all relevant organs including kidney by 8 months post-HSCT and stabilization of kidney function in the cystinosis mouse model [165]. In the same study, a potential mode of action explaining these findings was presented. A previously undescribed transfer of functional cystinosin from *CTNS* expressing cells to *CTNS* deficient cells via tunneling nanotubes was presented in *in vitro* and *in vivo* experiments [165,379]. In a co-culture system, transfer of eGFP-tagged cystinosin from cystinotic fibroblasts, transduced with a lentiviral CTNS-eGFP construct, to adjacent cystinotic DsRed fibroblasts was demonstrated by the appearance of green vesicles in the DsRed cells [165]. Remarkably, this method of cross correction also seemed to be present *in vivo*: in an autologous HSCT experiment

in the cystinosis mouse model using DsRed Ctns$^{-/-}$ HSCs, transduced with a lentiviral vector construct containing eGFP-CTNS as a transgene, green vesicles co-localizing with LAMP2 appeared in non-color host tissue cells, which is consistent with the transfer of eGFP-tagged cystinosin protein from the transduced HSC DsRed cells to host tissue cells and the correct localization of cystinosin in the lysosomal compartment [165]. Another pioneering study described the paracrine effect of bone-marrow derived mesenchymal stem cells on cystine accumulation in co-cultured cystinotic fibroblasts and PTECs via microvesicles containing cystinosin and *CTNS* mRNA [380,537]. Of note, the latter mechanism has meanwhile been described extensively in various biological fields [537].

In this historical setting and perspective, the first human allogeneic HSCT in cystinosis was performed in our hospital and is described in **chapter 5**.

In this 16-year old boy, the cysteamine induced toxicity and multifocal bone lesions due to a severe renal Fanconi syndrome and therapy-resistant copper deficiency was the motive for the consideration of an allogeneic HSCT.

A fully matched (10/10) unrelated donor allogeneic HSCT ensued and full donor chimerism was shown following the HSCT at multiple time points in bone marrow and peripheral blood. Though the patient ultimately underwent the most cumbersome outcome, he provided invaluable insight to the scientific world far beyond the cystinosis research field. First, we were able to demonstrate a clear reduction in cystine crystal load in mucosal macrophages of the stomach, and the transfer of cystinosin mRNA or protein to nonhematological epithelial cells in the kidney and various sites of the gastro-intestinal tract, enabling the confirmation of findings of the group of Cherqui in a human *in vivo* setting. Clinically, a significant improvement in the severe polyuria was observed from 12 weeks after HSCT while maintaining kidney function, followed later by reduction of low molecular weight proteinuria and glucosuria. While it has been demonstrated that the actual contribution of bone-marrow derived cells to kidney repair is very limited in terms of engraftment and integration in kidney tissue, delivery of cystinosin mRNA or protein via transfer using tunneling nanotubes, might exert a significant contribution [254,435]. Strikingly, also the complaints of photophobia disappeared with corneal cystine crystal scores reaching an all-time low level, which is in line with a more recent report of the Cherqui group on the therapeutic effect of HSCT in the cystinosis mouse on corneal pathology [164]. Taken together, these findings indicate the potential efficacy of HSCT in the treatment of cystinosis. However, the morbidity and fatal outcome in this case should encourage to a careful reflection and consideration. The profoundly severe central nervous system complications (central pontine myelinolysis, drug-associated recurrent epileptic seizures and hallucinations) observed already in the first weeks following

HSCT, and the serious degree of the graft-versus-host disease following the second HSC donation, are of an exceptional nature.

The episode of central pontine myelinolysis / posterior reversible encephalopathy (PRES) syndrome which occurred only a couple of weeks after engraftment of the initial HSCT, could not be attributed to a CNS infection or cerebrovascular event. Since early CNS complications following HSCT, including PRES, have been associated with calcineurin inhibitors, this severe complication was presumed to be related to tacrolimus [538]. Later, the drug-associated recurrent epileptic seizures and hallucinations unmasked a significantly increased sensitivity of the CNS to neurotropic drugs. It could be speculated that alterations at the blood-brain-barrier might have a role in the development of these highly extraordinary side effects [88]. The severity of the GvHD of the liver following the second HSC donation, presenting with severe cholestasis and ultimately liver failure, is quite remarkable since the unrelated donor was fully matched (10/10) and the GvHD was resistant to multiple immunosuppressive agents (steroids, mycophenolate mofetil, azathioprine, anti-thymocyte globulin). Ultimately, therapy using molecular adsorbent recirculating system (MARS) of the liver was applied. Not accidentally, the liver is characterized by a significant cystine tissue content in cystinosis patients [358,539]. Therefore, it could be hypothesized that the inflammation related to the clearance of the widespread cystine crystal depositions by the infiltrating donor-derived macrophages, has significantly contributed to the severity of the GvHD of the liver. Importantly, GvHD and CNS complications have been reported as significant inter-related factors for increased mortality [540]. Finally, the extensive immunosuppressive regimen that was applied has ultimately led to the enhanced susceptibility to severe opportunistic infections, which was the cause of the demise of the patient. Altogether, the highly complicated course following HSCT in this first described case in cystinosis yet, should encourage to carefully reassess all potential causative factors involved in the morbidity and mortality of this patient, to revise the concept of HSCT in cystinosis in general, and, if considered to be undertaken in a patient, balance the risks versus benefits of this radical treatment. Also, given the possibility for a differential effect at the organs involved in cystinosis, there is a likely possibility that HSCT will not provide a full cure for cystinosis and thus, if proven successful, the disease will evolve into a new medical entity for which still additional treatments will be necessary. Also, in our case, while a full donor chimerism was shown in the bone marrow and peripheral blood at multiple time points following HSCT, the level of expression of cystinosin in the various organs tested was only moderate, which is in stark contrast with the high level of engraftment of wild-type cystinosin expression that was required for preservation of kidney function in the study of Cherqui [162,163,165]. Therefore, it could be questioned whether, even if

successful, this rather low level of wild-type cystinosin expression would be sufficient for the correction of the cystinotic phenotype.

Undoubtly, as observed in other lysosomal storage disorders, several factors could affect the final outcome after HSCT, including the underlying genotype, the age at HSCT, the efficacy of the donor-derived cells to clear cystine-crystal deposition and to transfer cystinosin to the host cells, and obviously, transplant-related morbidity and mortality, including the response to neuro- and nephrotoxic drugs [534].

9.2.2 Kidney progenitor cells can be derived from urine of cystinosis patients and corrected with ex vivo gene therapy

Because there is no guarantee that HSCT could offer an enduring significant improvement in cystinosis, and due to the lack of any other disease-modifying treatment, there is still a need for innovation in the treatment of cystinosis. In this respect, especially the kidney disease, which is the first and most severe feature in cystinosis and for which still no specific curative treatment is available yet, should be focused on.

In **chapter 6**, we aimed to explore whether a kidney stem/progenitor niche resides in cystinosis kidney that could be used as a tool to enhance kidney repair or develop strategies for kidney regeneration. If a kidney stem/progenitor cell pool would be present, ideally this should be harvested in a non-invasive way, for example via the urine. First, we demonstrated that excessively high amounts of undifferentiated cells are being voided in the urine of cystinosis patients prior to kidney transplantation, when compared to healthy controls. Upon culturing urine in a specific medium, clonal colonies are grown of which some, upon selection and further proliferation, demonstrate the expression of a set of genes reminiscent of an early phase in nephrogenesis, including *CITED1*, *PAX2, VIM* and *NCAM1*. Of these, *CITED1* is highly specific for the nephron progenitor cells of the early cap mesenchyme, while *VIM* is a mesenchymal marker. Both are indicative for these cells to be in a stage prior to mesenchymal-to-epithelial transition. Notably, not all of these clonal colonies co-expressed the presumed kidney stem progenitor markers CD24 and CD133 [217,248,263,407]. Some of these clonal colonies showed the potential to proliferate and self-renew substantially, and to differentiate to either a proximal tubular-like cell (PTEC) or podocyte-like cell as demonstrated by the upregulation of expression of specific PTEC- or podocyte genes and/or protein. Therefore, clonal colonies showing the expression of kidney progenitor genes, the capacity to self-renew and to differentiate to specific kidney cells, were allocated as cystinosis urine-derived kidney progenitor cells (Cys-uKPCs). Furthermore, the functionality of Cys-uKPC derived PTECs and podocytes was demonstrated in

comparison to their undifferentiated counterpart, in support of the data of upregulation of PTEC- and/or podocyte-specific markers. Moreover, we could demonstrate the presence of CD133+ PAX2+ coexpressing cells scattered throughout the renal tubular epithelium and Bowman's capsule in a cystinosis patient, which suggests the *in vivo* presence of a kidney progenitor cell niche in cystinosis. To date, the isolation of urine-derived kidney progenitor cells has been described in congenital, steroid-sensitive and steroid-resistant nephrotic syndrome, Henoch-Shönlein purpura and Lupus nephritis [263]. However, they have merely been characterized based on the expression of CD133 and CD24, the functionality of the differentiated cells was not assessed and no human *in vivo* data (kidney biopsy stainings) demonstrating the presence of this niche *in situ*, was presented [263].

In our study, we aimed to restore the healthy cellular phenotype in these cystinosis uKPCs by complementation of *CTNS* via lentiviral vector transduction; this in order to provide a proof of principle of the feasibility of *ex vivo* gene therapy in cystinosis. Upon transduction with our *CTNS* bearing lentiviral vector construct in a low transduction efficiency range, we demonstrated a highly significant reduction of cystine levels and rearrangement of the LAMP1 lysosomal compartment from a clustered perinuclear localization to a more homogenous distribution. Being key pathophysiological hallmarks, significant reductions in cystine levels and redistribution of LAMP1 bearing lysosomes strongly suggest a restoration towards a healthy cellular phenotype in these cystinosis cells. The non-invasive nature of the source for these cells holds an important benefit. Likewise, in contrast to iPSCs, kidney progenitors offer the advantage of avoiding multiple, laborious, time-consuming and expensive differentiation protocols and avoids potential epigenetic alterations.

As a result, these cystinosis uKPCs could serve as a promising platform for studying disease biology, drug screening, and further development of bio-engineering and kidney regeneration strategies. The potential for derivation of kidney organoids or tubuloids from these primary cells should be further explored, as well as their potential for enhancing kidney repair in a cystinosis animal model.

9.2.3 Unravelling the mechanism of azoospermia in male nephropathic cystinosis

Finally, as aforementioned, the cystinosis patient population faces multiple issues when reaching adulthood and efforts should be made to address some of the most universal needs in life.

In **chapter 7 and 8** we demonstrated that the issue of male infertility in cystinosis has not remained unanswered. We provided a sincerely hopeful message to the cystinosis patient community that fathering a child is possible, despite the azoospermia, if the issue is addressed

timely. We have presented promising data that suggests a role for the epididymis in the problem of azoospermia in male cystinosis patients. We applied scrotal ultrasound for the assessment of epididymal morphology, and analyzed seminal plasma biomarkers in order to discover signs suggestive for epididymal obstruction; both innovative measures in the assessment of azoospermia [482,485]. Currently we are undertaking the next steps needed to further clarify our observations via *in vitro* experiments and further human *in vivo* investigations. Finally, our data also suggest that cryopreservation in young adult male cystinosis patients should be carefully taken into consideration. If so, the techniques applied should be as minimally invasive as possible, and therefore, given our findings, be aimed preferentially at the epididymal level.

Future perspectives

Innovation in therapeutic monitoring of nephropathic cystinosis

A large cohort, long-term prospective study is needed to validate our findings and further explore the potential of chitotriosidase as a biomarker of cystinosis. It cannot be excluded that, as multi-omic (genomic, transcriptomic, metabolomic) approaches are emerging, other potential biomarkers for cystinosis in blood or urine might be discovered. The further elucidation of the extra-renal manifestations observed in cystinosis and the development of specific diagnostic criteria, the establishment of a thorough international cystinosis registry through extensive collaboration networks, and the set-up of biobanks will be crucial for the success of the identification and elucidation of new biomarkers for cystinosis. We intend to initiate this large cohort study in the near future, and aim to involve the current Rare Diseases Cohort – European Cystinosis Cohort (RaDiCo – ECYSCO, Paris, France) registry partners and to call upon the National Institute of Health (NIH, Washington, USA) for establishing firm collaborations.

In parallel, a longitudinal follow-up study to examine the evolution of the dermatological manifestations of cystinosis over time using HD-OCT could be considered to investigate its value in disease monitoring.

Innovation in treatment of nephropathic cystinosis

The potential future applications for the kidney progenitor cell niche which we identified in cystinosis patients, are manifold ranging from a platform for studying disease biology and drug screening, to a potential source for personalized patient-tailored kidney regeneration and bio-engineering strategies.

Obviously, the model should be further fine-tuned by investigating additional altered cell biological processes observed in the pathophysiology of cystinosis on the cellular level. Together

with the restoration of the cellular phenotype which we observed upon complementation of *CTNS* via lentiviral vector transduction, this two-dimensional cell model could serve as a drug screening platform.

Next, the potential of the urine-derived kidney progenitor cells to form three dimensional structures or organoids should be investigated as these might further provide insight in the biology cystinosis on the cell organizational level.

Furthermore, the lentiviral vector construct could further be optimized to achieve the endogenous *CTNS* expression levels by testing the endogenous *CTNS* promotors.

Finally, the *in vivo* potential for kidney regeneration of these Cys-uKPCs should be explored in a cystinosis rodent model with a pronounced kidney phenotype. Various administration routes should be considered, including the renal artery, intraparenchymal and subcapsular injection.

The mechanism of azoospermia in nephropathic cystinosis, and the potential involvement of the epididymis in the disease biology herein, will be further explored *in vitro* via transcriptomic analysis of human epididymal cell lines in which *CTNS* has been knocked down.

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SUMMARY

Cystinosis is a rare, inheritable metabolic disorder caused by mutations in the gene *CTNS* which encodes a cystine transporter, cystinosin, located in the membrane of the lysosome, a important cellular organelle responsible for mainly the breakdown of various biomolecules. Due to the loss of function of this transporter, cystine a di-amino acid consisting of two cysteine molecules, accumulates in the lysosomes followed by cystine crystal formation which is the pathognomonic hallmark of the disease. Cystinosis mainly affects the kidney, more specifically the cells of the renal proximal tubule which is the most metabolically active part of the nephron, the functional part of the kidney, involved in reabsorbing many of solutes that are freely filtered through the glomerulus, the filter part of the nephron. Later, the disease also affects the podocytes, the cells that form an important part of the glomerular filtration barrier. Clinically, the disease therefore is characterized by the loss of many solutes in urine, leading to failure to thrive, polyuria and polydipsia. Chronic kidney disease develops, which gradually evolves towards end-stage renal disease if it is left untreated. Since the transporter, cystinosin, is present in all cells of the body, many other organs apart from the kidney, can also get affected, including mainly the eyes, endocrine glands, muscles and central nervous system.

Fortunately, a highly specific, disease-modifying treatment is available. Cysteamine is an aminothiol which is able to cleave the disulphide bond of cystine, enabling the lysosome to be depleted of cystine. Since its widespread availability, cysteamine has shown to postpone the onset of end-stage renal disease, improve growth and decrease the incidence of extra-renal complications. However, cysteamine has significant side-effects, and to ensure effectivity drug monitoring is necessary. The current only available modality for monitoring cysteamine treatment, concerns an assay in which the cystine level in white blood cells is assessed. However, this WBC cystine assay suffers important practical and technical limitations, hampering its availability and further potential improvements in outcome of cysteamine-treated cystinosis patients. Other alternative or additional modalities for monitoring of cysteamine treatment would be highly valued in the clinical management of cystinosis.

Nevertheless, cysteamine treatment has significantly improved the life expectancy of cystinosis patients, allowing them to survive into adulthood. This changing spectrum of patients has, in part, changed the face of the disease while some clinical manifestations come to the fore. Some specific dermatological manifestations observed in cystinosis are more pronounced in the (young) adult cystinosis population. Also, specific issues related to adult life, including the wish for having children, has become a relevant topic for the adult cystinosis patient population. The infertility in male cystinosis patients which recently has been appointed to an azoospermia of yet unknown origin, has turned this topic into a real concern for the cystinosis patient community.

Furthermore, despite the progress that has been realized in the management of cystinosis due to the advent of renal replacement therapy and cysteamine treatment, a cure for cystinosis is lacking.

Hematopoietic stem cell transplantation has been an alluring approach for providing this cure in cystinosis, given the recent promising data in the cystinosis mouse model. However, it remains uncertain whether this future treatment strategy will be sufficient to provide a cure for the kidney disease and all extra-renal complications.

In this thesis, we aimed to address some of these challenges faced in the management of cystinosis.

In **chapter 3**, we utilized the currently most advanced non-invasive optical imaging technology available in clinical practice (HD-OCT) to identify distinctive cutaneous features of cystinosis in a quantitative manner. We demonstrated that signs of an enhanced intrinsic skin aging are present in cystinosis patients, which are apparent from adolescent to young adult age, prior to kidney transplantation. Moreover, in cystinosis patients whom the *CTNS* gene is absent due to a large deletion (hom 57kb del), significant thinning of the epidermis predicted the presence of (other) extra-renal complications, which suggests that in these patients, the degree of skin involvement reflects the overall disease severity. Hereby, for the first time, we could demonstrate in a quantitative manner the involvement of the skin in the phenotype of cystinosis.

In **chapter 4**, we aimed to explore the potential of biomarkers of macrophage activation in blood plasma to serve as an alternative or additional modality for the monitoring of cysteamine therapy. We demonstrated that blood plasma chitotriosidase activity, an enzyme secreted by macrophages upon exposure and engulfment of cystine crystals, significantly predicts white blood cell cystine levels. Furthermore, we could establish a cut-off value for chitotriosidase enzyme activity (150 nmol/ml plasma/h) distinguishing good versus poor therapeutic control with cysteamine treatment (in terms of WBC cystine levels < or ≥ 2 nmol ½ cystine/mg protein respectively), that harbors a high negative predictive value for ruling out patients with a poor compliance. Moreover, chitotriosidase enzyme activity levels were superior to WBC cystine levels in predicting the presence of multiple extra-renal complications in patients having at least one extra-renal complication. A cut-off value for chitotriosidase enzyme activity at 250 nmol/ml plasma/h showed a high positive predictive value for identifying patients with multiple extra-renal complications. A large, multicentric international prospective study is needed to validate and corroborate our findings.

In **chapter 5**, we described the first case of allogeneic hematopoietic stem cell transplantation in a cystinosis patient. Despite convincing evidence in support of the proposed mechanism of the group of Stephanie Cherqui of transfer of *cystinosin* mRNA or protein from the graft

hematopoietic stem cells to epithelial cells of various organs, the limited therapeutic effect on the renal and extra-renal complications, significant morbidity and mournful outcome of the patient should encourage to reflection and careful reconsideration of the concept of HSCT.
Therefore, other innovative strategies for the treatment of cystinosis, and the kidney phenotype in specific, should be explored.
In **chapter 6**, we described for the first time, the presence of kidney progenitor cells in the urine of cystinosis patients. Some of these cells show the potential to differentiate into a functional proximal tubular epithelial – or podocyte-like cell *in vitro*. Moreover, we demonstrated that, by delivering the *CTNS* gene using viral vector technology, the main cellular hallmarks of cystinosis, comprising cystine accumulation and altered distribution of the lysosomal compartment, could be rescued towards the healthy phenotype. These cells offer many advantages in terms of the method of harvesting, the avoidance of the need for immortalization, cost-effectiveness and utility in future applications. Currently, these urine-derived kidney progenitor cells constitute a novel platform for studying disease biology, new drug development and future kidney bio-engineering. Obviously, the potential of these cells should be further explored *in vitro*, e.g. organoid formation, and in a cystinosis disease model *in vivo*.
In **chapter 7** and **8**, we addressed one of the concerns of the young adult cystinosis patient population and one of the most universal human needs in life, the wish of having children. We described the first case of a successful conception induced by a male, infantile-type nephropathic cystinosis patient via assisted reproductive techniques. This case holds a promising message to the cystinosis patient community that fathering a child is feasible, if considered timely. Furthermore, we aimed to further unravel the origin of the azoospermia observed in male cystinosis patients. Indeed, in male cystinosis patients, previous studies have suggested that, on top of an evolving primary hypogonadism with increasing age, the azoospermia can only be explained by additional pathogenic factors involved at, presumably, the epididymal level.
Therefore, we have initiated a multicentric study in which we aimed to further define the origin of azoospermia in cystinosis patients, via non-invasive means. In this currently still ongoing project, we have found indications for an obstruction or malfunction from the epididymal level onwards. We proposed that cryopreservation could be a useful strategy to be considered at young adult age in order to preserve their fertility potential. If considered, techniques for harvesting sperm cells should be as minimally invasive as possible, aimed preferentially at the epididymal level.
In conclusion, in this thesis, we have addressed some of the important challenges faced in the current therapeutic monitoring and treatment of nephropathic cystinosis.

SAMENVATTING

Cystinose is een zeldzame, erfelijke stofwisselingsziekte die wordt veroorzaakt door mutaties in het gen *CTNS*. Dit gen codeert voor een transporteiwit, cystinosine, die cystine uit het lysosoom transporteert. Het lysosoom is een belangrijk onderdeel van de cel en voornamelijk verantwoordelijk voor de afbraak van verschillende moleculen. Door de mutaties in het gen *CTNS*, zal cystinosine ofwel afwezig zijn ofwel niet functioneel, waardoor cystine zich stapelt in de lysosomen, en aanleiding geeft tot de vorming van cystine kristallen, het unieke kenmerk van deze aandoening.

Cystinose tast voornamelijk de nier aan, en meer specifiek de cellen van de proximale tubulus, een van de onderdelen van de nier met een zeer actieve stofwisseling. Hierdoor gaan vele stofjes, die onder normale omstandigheden worden gereabsorbeerd, terug verloren in de urine. Zuigelingen en jonge kinderen gaan hierdoor grote hoeveelheden plassen en slecht groeien. Later wordt ook de eigenlijke 'filter' van de nier, de glomerulus, aangetast, en meer specifiek de podocyten. Geleidelijk ontwikkelt zich een chronische nieraandoening waarbij uiteindelijk de nierfunctie vervangen dient te worden; dit onder vorm van dialyse en uiteindelijk een niertransplantatie. Aangezien alle cellen van het lichaam zijn aangetast, manifesteert de ziekte zich ook in meerdere organen, o.a. de ogen, de hormonale klieren, de spieren en het centraal zenuwstelsel.

Gelukkige is er een zeer specifieke behandeling beschikbaar die het verloop van de ziekte in een belangrijke mate kan wijzigen. Cysteamine is een molecule die cystine kan afbreken, waarbij deze afbraakproducten het lysosoom kunnen verlaten. Sinds de wijdverspreide beschikbaarheid van cysteamine kon worden aangetoond dat deze behandeling de progressie van het chronisch nierlijden kan afremmen, de groei van kinderen kan verbeteren, en de ontwikkeling van complicaties in andere organen kan verminderen. Echter, cysteamine heeft ook belangrijke nevenwerkingen, en om de effectiviteit van het geneesmiddel te verzekeren is regelmatige monitoring noodzakelijk. Op dit moment is er slechts één methode beschikbaar om de goede inname van cysteamine te controleren, namelijk de bepaling van het gehalte cystine in de witte bloedcellen. Deze test is echter niet optimaal en heeft enkele belangrijke technische en praktische tekortkomingen, die zijn wereldwijde beschikbaarheid en mogelijke verbeteringen in de prognose van cystinosepatiënten, beperken. Andere bijkomende technieken om de behandeling met cysteamine beter op te volgen, zouden erg gewaardeerd zijn in de behandeling van patiënten met cystinose.

Onomstotelijk heeft cysteamine reeds aangetoond de levensverwachting van patiënten met cystinose in een belangrijke mate te bevorderen. Wat ooit een levensbedreigende stofwisselingsziekte was bij kinderen, is vandaag een chronische behandelbare aandoening van ook volwassenen tot op middelbare leeftijd.

Deze evolutie in het spectrum van patiënten heeft sommige klinische manifestaties meer naar de voorgrond gebracht. Sommige specifieke tekenen van de huid zijn meer uitgesproken op (jong)volwassen leeftijd, terwijl ook sommige existentiële bekommernissen van het volwassen leven, zoals kinderwens, bijzonder relevant geworden zijn voor de volwassen cystinose populatie.

Ondanks de vooruitgang die werd geboekt in de behandeling van cystinose dankzij nierfunctievervangende therapie en cysteamine, blijft een genezende therapie nog op zich wachten. Dankzij recente beloftevolle resultaten van hematopoïetische stamceltransplantatie in het muismodel van cystinose, heeft deze behandelingsaanpak bijzonder veel allure verworven. Op vandaag blijft het echter voorlopig onduidelijk of deze behandelingsstrategie een effectieve genezing zal aanbieden voor het nierlijden en alle andere complicaties van cystinose.

Dit proefschrift heeft zich gericht op deze hedendaagse uitdagingen waarmee zowel clinici als de onderzoekswereld geconfronteerd worden in de behandeling van cystinose.

In **hoofdstuk 3**, hebben we de huidig meest geavanceerde, dermatologische niet-invasieve optische beeldvormingstechniek aangewend (HD-OCT) om specifieke huidafwijkingen bij cystinose op een kwantitatieve manier de identificeren. We hebben aangetoond dat er tekenen zijn die een versnelde intrinsieke huidveroudering suggereren bij patiënten met cystinose vanaf adolescente leeftijd, voorafgaand aan niertransplantatie. In patiënten bij wie het *CTNS* gen afwezig is o.w.v. een grote deletie (hom 57kb del), voorspelde een significante verdunning van de opperhuid de aanwezigheid van andere complicaties. Dit suggereert dat, in deze patiënten, de graad van huidaantasting een indruk kan geven over de mate van ziekte-ernst. Met dit onderzoek konden we voor de eerste maal, op een kwantitatieve wijze aantonen dat de huid betrokken is in het fenotype van cystinose.

In **hoofdstuk 4** hebben we het potentieel onderzocht van markers van macrofaagactivatie in bloedplasma, om dienst te doen als een alternatieve methode om de behandeling met cysteamine te monitoren. We hebben aangetoond dat de activiteit van chitotriosidase, een enzyme dat vrijgegeven wordt door macrofagen wanneer deze blootgesteld worden aan cystine kristallen en deze opruimen, op significante wijze het cystinegehalte in witte bloedcellen voorspelt. We konden een afkapwaarde definiëren van de activiteit van chitotriosidase (150 nmol/ml plasma/h) om een onderscheid te maken tussen een goede en zwakke mate van therapietrouw met cysteamine (WBC cystine gehalte respectievelijk < of ≥ 2 nmol ½ cystine/mg eiwit). Deze afkapwaarde vertoonde een grote negatief predictieve waarde om een zwakke therapietrouw uit te sluiten. Chitotriosidase activiteit was tevens superieur aan cystinegehalte in witte bloedcellen om de aanwezigheid van meerdere (> 1) complicaties te identificeren in patiënten die minstens reeds één complicatie vertonen. Een afkapwaarde van chitotriosidase

van 250 nmol/ml plasma/h vertoonde een sterk positief predictieve waarde om patiënten met meerdere extra-renale complicaties de identificeren. Een grote, internationaal multicentrische langetermijnsstudie is nodig om onze bevindingen te valideren.

In **hoofdstuk 5** hebben de eerste casus beschreven van een allogene hematopoietische stamceltransplantatie in een patiënt met cystinose. Ondanks overtuigende aanwijzingen ter ondersteuning van de hypothese van de groep van Stephanie Cherqui, in verband met transfer van cystinosine mRNA en/of eiwit van de hematopoietische stamcellen van de donor naar epitheelcellen van verschillende organen van de recipiënt, dient het beperkte therapeutische effect op het nierlijden en de complicaties van cystinose, de belangrijke morbiditeit en uiteindelijk mortaliteit er ons sterk op aan te dringen tot reflectie en een uitgebreide heroverweging van het concept van hematopoietische stamceltransplantatie.

Daarnaast dienen andere innovatieve strategiëen voor de behandeling van cystinose, en in bijzonder het nierfenotype, verder onderzocht te worden.

In **hoofdstuk 6** hebben we voor de eerste maal de aanwezigheid van voorlopercellen van nierweefsel in de urine van patiënten met cystinose aangetoond. Sommige van deze cellen vertonen het potentieel om in laboratoriumomstandigheden te differentiëren naar een cel, gelijkend op een proximale tubulus cel of een podocyt, met een zekere functionaliteit. We hebben ook aangetoond dat, door het *CTNS* gen in deze cellen terug in te brengen door middel van een virale vector, de belangrijkste cellulaire kenmerken van cystinose, waaronder de stapeling van cystine, en de gewijzigde distributie van de lysosomen, konden hersteld worden in de richting van het gezonde fenotype. Deze voorlopercellen van nierweefsel (kidney progenitor cells), bieden vele voordelen, waaronder de methode van isolatie, het vermijden van de nood aan immortalisatie van cellen, kosten-effectiviteit en het veelvoud aan potentiële toepassingen. Deze voorlopercellen van nierweefsel vormen vandaag een nieuw platform om de celbiologie van de ziekte te bestuderen, nieuwe geneesmiddelen te ontwikkelen, en de toekomstige ontwikkeling van kunstnieren. Uiteraard dient het potentieel van deze cellen verder onderzocht worden in het laboratorium (*in vitro*), en in een diermodel van cystinose (*in vivo*).

In **hoofdstuk 7** en **8**, hebben we een van de belangrijkste bekommernissen van jongvolwassen patiënten met cystinose, en een van de meest universele noden van de mens, behandeld: de kinderwens. We hebben de eerste casus beschreven van een succesvolle bevruchting door een mannelijke patiënt met het infantiele type cystinose, via proefbuisbabytechnieken. Deze casus bevat de beloftevolle boodschap dat, als mannelijke patiënt met cystinose,het verwekken van kind mogelijk is, indien dit tijdig wordt overwogen. Verder hebben we geprobeerd om de oorzaak van de afwezigheid van zaadcellen in de zaadlozing bij mannelijke cystinosepatiënten (zgn. ‘azoospermie’), te ontrafelen.

Voorgaande studies hebben immers gesuggereerd dat, naast een zich ontwikkelend primair hypogonadisme met toenemende leeftijd, de azoospermie enkel kan verklaard worden door bijkomende factoren die zich vermoedelijk op het niveau van de epididymis bevinden.
In deze context hebben we een multicentrische studie opgestart, waarmee we verder de oorsprong van azoospermie bij mannen met cystinose willen ontrafelen, aan de hand van niet-invasieve onderzoeken. In dit project hebben we aanwijzingen gevonden voor een obstructie of dysfunctie vanaf het niveau van de epididymis. We hebben ook gesuggereerd dat het invriezen van sperma (zgn. 'cryopreservatie') op jonge leeftijd een zinvolle strategie kan zijn om het vruchtbaarheidspotentieel te bewaren. Indien dit overwogen zou worden, is het uiteraard aangewezen om zo minimaal invasieve technieken aan te wenden, die voornamelijk gericht zijn op de epididymis.
We besluiten dat we in deze thesis enkele van de belangrijkste hedendaagse uitdagingen in de monitoring en behandeling van nefropathische cystinose hebben onderzocht en besproken.

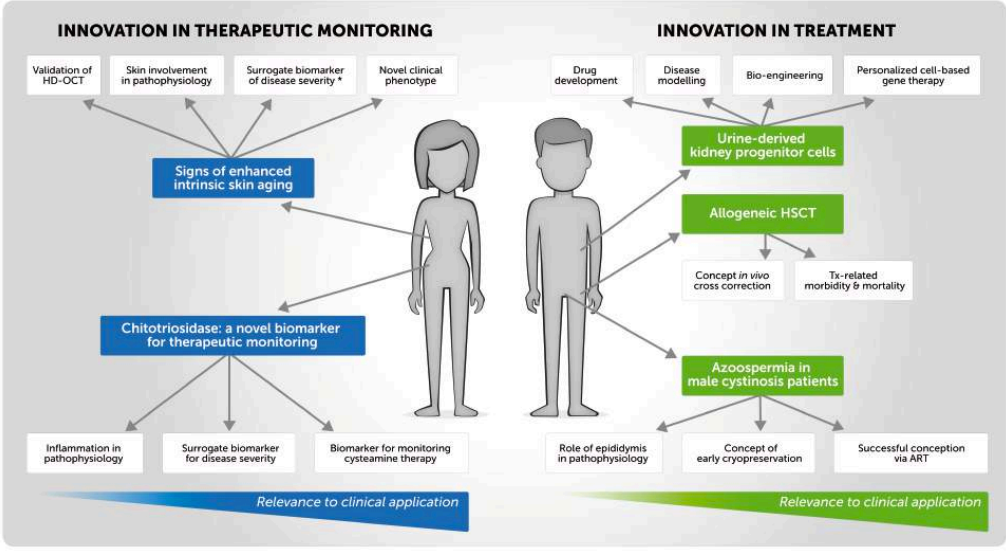

Figure 9.1 Overview of the main contributions of the work presented in this thesis to the cystinosis research field and their relevance to clinical application

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At the moment of a PhD defense, the organ point of a 4-year long project, truth must be honored and thus the fact must be acknowledged that this thesis would never have been realized without the help and support of a significant number of supervisors, collaborators, colleagues, patients and volunteers, family and friends.

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Personal contribution statement

The author of this thesis was responsible for, and involved in the design of all studies described, the management and the practical execution of experiments, the collection and management of all data generated, the data analysis and drafting of the manuscripts.
The figures depicted in this thesis are inspired by multiple published figures and prepared by Daisy Hombroeckx.

CURRICULUM VITAE

PERSONAL DETAILS

full name	Koenraad Robert Paul Veys
date of birth	°21st march 1986 - Leuven
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EDUCATIONAL QUALIFICATION

1998 - 2004	Secondary Education – Lyceum OLV Vlaanderen Kortrijk *Summa cum laude 30.06.2004* *Laureate of Secondary Education Zwevegem 02.07.2004*
2004 - 2007	**Academic Bachelor in Medicine** – KU Leuven campus Kortrijk (KULAK) *Summa cum laude 03.07.2007*
2007 - 2011	**Master in Medicine** – KU Leuven *Summa cum laude 30.06.2011* iFOM – FAIMER Examination; *17.12.2010, Leuven* (International Foundations of Medicine) *"Certificate of Degree of Excellence"*
2011 - 2012	**Master in Specialized Medicine** – KU Leuven Specialization: Internal Medicine
2012 - 2019	**Master in Specialized Medicine** – KU Leuven Specialization: Paediatrics **Paediatric Residency Program** - UZ Leuven *Certificate of academization – University Program Physician Specialist Faculty of Medicine KU Leuven 31.07.2015*
2015 - 2019	**PhD project** "Innovation in monitoring and treatment of nephropathic cystinosis" Promotor: Prof. Dr. E. Levtchenko; Co-promotor: Prof. Lambertus van den Heuvel, Rik Gijsbers, Fanny Oliveira Arcolino Funded by Research Foundation Flanders (Fonds Wetenschappelijk Onderzoek (F.W.O.) Vlaanderen

EXTRACURRICULAR CERTIFICATES & DIPLOMAS

2012	NLS – Neonatal Life Support – Provider course; *Aartselaar, Antwerp*
2013	EPLS – European Paediatric Life Support; *Leuven*
2013	APLS – Advanced Paediatric Life Support; *Oostmalle, Antwerp*
2015-2017	IPNA – ESPN – International Pediatric Nephrology Association – European Society for Paediatric Nephrology Junior for Master Classes; *2015 – 2016 Leuven; Glasgow 2017* *"Diploma of completed 3-year cycle IPNA-ESPN Master in Paediatric Nephrology"*
2018	GCP Certificate – European Forum Good Clinical Practice (EF-GCP); *Leuven*
2018	GOSH – ICH Continuing Medical Education (CME) in Nephrology Week: Paediatric Nephrology & Urology; *Great Ormond Street Hospital – Institute for Child Health, London, UK*

SKILLS

2015-2018 KU Leuven & VIB courses – CLT Writing Skills in Biomedical Sciences; Creative Thinking; Business Psychology; Basic Statistics; Statistics in Prism; Conflict Management.

SCIENTIFIC EXPERIENCE

2005 Scientific research clerkship – Student Researcher
"RYR1 mutations in leak Ryanodine receptors and their correlation with Malignant Hyperthermia (MH) and Central Core Disease (CCD); *16.08.2005 – 11.09.2005; Laboratory of Physiology, Interdisciplinary Research Centre (IRC), KULAK*
Supervisors: Patrick Desmet, Prof. Dr. Z. Debyser

SCIENTIFIC OUTPUT

Publications in peer-reviewed journals

2016 Veys KR, Besouw MT, Pinxten AM, Dyck MV, Casteels I, Levtchenko EN. Cystinosis: a new perspective. Acta Clin Belg 2016; 71: 131-7.

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Elmonem MA*, Veys K*, Oliveira Arcolino F, Van Dyck M, Benedetti MC, Diomedi-Camassei F, De Hertogh G, van den Heuvel LP, Renard M, Levtchenko E. Allogeneic HSCT transfers wild-type cystinosin to nonhematological epithelial cells in cystinosis: First human report. Am J Transplant. 2018; 18:2823-2828. * shared first authorship

Van Rijssel AE, Knuijt S, Veys K, Levtchenko EN, Janssen MCH. Swallowing dysfunction in patients with nephropathic cystinosis. Mol Genet Metab. 2019

2019 Veys KRP, Elmonem MA, Dhaenens F, Van Dyck M, Janssen MCH, Cornelissen EAM, Hohenfellner K, Reda A, Quatresooz P, van den Heuvel B, Boone MALM, Levtchenko E. Enhanced intrinsic skin aging in nephropathic cystinosis assessed by High-Definition Optical Coherence Tomography. Journal Invest Dermatol., *in press*

Papers in review

2019 Veys KRP*, Elmonem MA*, Van Dyck M, Janssen MCH, Cornelissen EA, Hohenfellner K, Prencipe G, Emma F, van den Heuvel LP, Levtchenko E. Macrophage activation biomarkers for the therapeutic monitoring of nephropathic cystinosis: a prospective multicenter longitudinal study. * shared first authorship

Papers in preparation

Veys KRP, Arcolino FA, Elmonem MA, Reda A, Janssen MCH, Cornelissen EA, van den Heuvel LP, Levtchenko E. *Ex vivo* gene therapy rescues cystinotic phenotype in urine-derived kidney progenitor cells of nephropathic cystinosis patients: a tool for personalized medicine.

Reda A, Veys KRP, Kadam P, Camps C, Taranta A, Rega LR, Cyr, D, Emma F, van den Heuvel F, Goossens E*, Levtchenko E*.
Unravelling the origin of azoospermia in male cystinosis patients.

Presentations International Congresses

2016 9th International Cystinosis Conference 2016 – Valencia, Spain
Oral presentation: *"High Definition Optical Coherence Tomography (HD-OCT) non-invasive skin imaging in therapeutic monitoring of nephropathic cystinosis"*

2017 European Society for Paediatric Nephrology (ESPN) – 50th Annual Meeting – Glasgow, UK
Poster presentation: *"First successful conception induced by a male cystinosis patient"*

2018 European Cystinosis Conference & E-Rare International Rare Diseases Research Consortium (IRDiRC) Meeting – Berlin, Germany
Oral presentation: *"Alternative biomarkers for therapeutic monitoring of nephropathic cystinosis"*
Poster presentation: *"Enhanced intrinsic skin aging in nephropathic cystinosis as assessed by High-Definition Optical Coherence Tomography"*

European Society for Paediatric Nephrology (ESPN) – 51st Annual Meeting – Analya, Turkey
Oral presentation: *"Macrophage activation biomarkers for the therapeutic monitoring of nephropathic cystinosis: a prospective multicenter longitudinal study"*
Oral presentation: *"Cysteamine improves tubular reabsorption of LMW compounds in cystinotic zebrafish, but has no effect on defective megalin expression"*
Poster presentation: *"Enhanced intrinsic skin aging in nephropathic cystinosis as assessed by High-Definition Optical Coherence Tomography"*

2019 5th International Dublin Cystinosis Workshop – Dublin, Ireland
Oral presentation: "Male Fertility in Cystinosis
Oral presentation: "Allogeneic HSCT transfers wildtype cystinosin to nonhematological cells in cystinosis: first human report
Poster presentation: "Chitotriosidase: a novel alternative biomarker for therapeutic monitoring in nephropathic cystinosis"

56th European Renal Association – European Dialysis and Transplantation Association (ERA-EDTA) International Congress Budapest, Hungary (13th – 16th June 2019)
Oral Presentation: *"Chitotriosidase: a novel alternative biomarker for monitoring of nephropathic cystinosis* – Awarded with *Travel Grant & Best Oral Presentation* of session 'Kidney disease from childhood to adult'

Presentations National Congresses

2016 Cystinosegroep Nederland: patiëntendag – Amerongen, Nederland
Oral presentation: *"Non-invasive skin imaging for therapeutic monitoring of nephropathic cystinosis"*

2017 Belgische Vereniging Kindergeneeskunde (BVK) – Antwerp, Belgium
E-Poster presentation: *"Urine derived renal progenitor cells of cystinosis patients: the key to kidney regeneration ?"*

2018 Belgian Society for Stem Cell Research (BeSSCR) Annual Meeting – *26.10.18* Leuven, Belgium
Poster presentation: *"Urine-derived kidney progenitor cells in nephropathic cystinosis: the key to kidney regeneration ?"*

Cystinosegroep Nederland: patiëntendag – *10.11.18* Leersum, Nederland
Oral presentation: *"Chitotriosidase: a novel alternative biomarker for therapeutic monitoring of nephropathic cystinosis ?"*

	2019	47th annual congress of Belgian Association of Pediatrics (Belgische Vereniging voor Kindergeneeskunde, BVK) (21st of march, Brussels) Oral presentation: *"Chitotriosidase: a novel alternative biomarker for therapeutic monitoring of nephropathic cystinosis ?"*
AWARDS	2015	FWO PhD Candidate Fellowship – Research Foundation Flanders (Fonds Wetenschapellijk Onderzoek Vlaanderen, F.W.O.), grant number 11Y5216N.
	2019	56th ERA-EDTA International Congress Budapest, Hungary (13-16th June 2019) * Travel Grant * Best Oral Presentation (top 10 best abstracts) 'Chitotriosidase: a novel alternative biomarker for monitoring of nephropathic cystinosis in session 'Kidney disease, from childhood to adult'; *abstract percentile:* ***91.48/100***
OTHER ACTIVITIES	2017	Involved in Rare Disease Cohorts – European Cystinosis Cohort (RaDiCo-ECYSCO) European Cystinosis registry initiative
	2019	IPNA – ESPN Membership