Recent progress in the LC-MS/MS analysis of oxidative stress biomarkers

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Abbreviations: 3-NT, 3-nitro-L-tyrosine; 7-KC, 7-ketocholesterol; 8-isoPGF_{2α}, 8-isoprostaglandin F_{2α}; 8-NO₂Gua, 8-nitroguanosine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 8-OHG, 8-hydroxyguanosine; AGE, advanced glycation end product; GSH, glutathione; GSSG, glutathione disulphide; HNE, 4-hydroxy-2-nonenal; HODE, hydroxyoctadecadienoic acid; MDA, malondialdehyde; NO[•], Nitric oxide; ONE, 4-oxo-2-nonenal; OHCh, hydroxycholesterol; OS, oxidative stress; PUFA, polyunsaturated fatty acid; RNS, reactive nitrogen species; ROS, reactive oxygen species

Abstract

The presence of a dynamic and balanced equilibrium between the production of reactive oxygen (ROS) and nitrogen (RNS) species and the in-house antioxidant defense mechanisms is characteristic for a healthy body. During oxidative stress (OS) this balance is switched to an increased production of ROS and RNS, exceeding the capacity of physiological antioxidant systems. This can cause damage to biological molecules, leading to loss of function and even cell death. Nowadays, there is increasing scientific and clinical interest in OS and the associated parameters to measure the degree of OS in biofluids. An increasing number of reports using LC-MS/MS methods for the analysis of OS biomarkers can be found. Since bioanalysis is usually complicated by matrix effects, various types of cleanup procedures are used to effectively separate the biomarkers from the matrix. This is an essential part of the analysis to prepare a reproducible and homogenous solution suitable for injection onto the column. The present review gives a summary of the chromatographic methods used for the determination of OS biomarkers in both urine and plasma, serum and whole blood samples. The first part mainly describes the biological background of the different OS biomarkers, while the second part reports examples of chromatographic methods for the analysis of different metabolites connected with OS in biofluids, covering a period from 2015 till early 2020. The selected examples mainly include LC-MS/MS methods for isoprostanes, oxidized proteins, oxidized lipoproteins and DNA/RNA biomarkers. The last part explains the clinical relevance of this review.

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Introduction

Oxidative stress (OS) is defined as a state of imbalance between excessive oxidant (free) radicals and the insufficient degradation of those radicals by antioxidant systems as an in-house defense mechanism. Consequently, reactive oxygen species (ROS) and / or reactive nitrogen species (RNS) can accumulate inside the body and damage all cellular biomolecules. Therefore, the defense systems should try to prevent uncontrolled ROS accumulation within the cells. These systems include both nonenzymatic molecules (for example glutathione (GSH) and vitamin E) and enzymatic molecules, with superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPX) as the best-known [1]. Under normal circumstances, living organisms control finely regulated systems to maintain low ROS levels, which is beneficial since ROS can act as a second messenger and regulate intracellular signal transduction pathways for the maintenance of physiological functions such as cell growth, proliferation and survival [2,3]. Unfortunately, in case of an imbalance, ROS will be excessively produced in biological systems. This state can be detrimental, since ROS can readily react with lipids, proteins and DNA, leading to metabolic dysregulation, oxidation end products of lipids, DNA and proteins and oxidative damage in cells, tissues or even organs. Such a damage can promote the generation of various secondary reactive species and this can trigger a number of cellular responses, including apoptosis, which often lead to disease development. OS has been implicated in the pathophysiology of a number of chronic disorders including cardiovascular disease [4], diabetes [5] and renal failure [6–8]. Early diagnosis of chronic disorders is important to improve the outcome and prevent or delay complications, indicating a need to reliably and quantitatively measure biomarkers of OS [2,3,9,10].

Reactive oxygen species

Free radicals are independent chemical species with one or more unpaired electrons in their outermost shell. They can be formed when oxygen interacts with certain molecules, and once

formed, they are highly reactive in search for another unpaired electron. When two unpaired electrons react with each other to form a covalent bond, a new nonradical molecule is formed. More often, however, free radicals attack nonradical molecules resulting in a new (secondary) radical molecule, initiating as such a chain reaction. This chain reaction of primary and secondary radicals eventually leads to oxidative damage of several tissues and organs [11]. The most important class of free radicals are those that arise from oxygen, since molecular oxygen has a unique electronic configuration itself. Therefore, the addition of one electron forms superoxide anion ($O_2^{\bullet-}$), which is considered as the 'primary' ROS. This anion can either directly or indirectly, through enzyme- or metal-catalyzed processes, interact with other molecules in order to generate 'secondary' ROS [12]. The predominant source of ROS in all cell types are the mitochondria, since the superoxide anion is mainly generated at the electron transport chain, both in complexes I and III. During energy transduction, a small number of electrons (about 1 – 3%) leak to oxygen, leading to the formation of superoxide, which can be converted to hydrogen peroxide (H_2O_2) by SOD or undergo spontaneous dismutation into water and oxygen [13]. In addition, the superoxide anion can also be formed by the reduction of molecular oxygen by reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), which is located near the cell membrane, and xanthine oxidase, which is distributed throughout the cytoplasm. The previously produced H_2O_2 can generate, via the Fenton reaction, in the presence of transition metals (iron and copper ions), another highly reactive free radical, the hydroxyl radical ($OH^{\bullet}$) [1] (Figure 1).

Reactive nitrogen species

Nitric oxide ($NO^{\bullet}$), a small but reactive radical with one unpaired electron, is generated in biological tissues by three main isoforms of nitric oxide synthases (NOS): epithelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [14]. In mammalian cells, nitric oxide radicals are released via a five-electron oxidative reaction whereby NOS metabolize arginine to citrulline with the formation of

NO[•] (Figure 1) [15]. While NO[•] has a reported half-life of only a few seconds in an aqueous environment, it has a greater stability in an environment with a lower oxygen concentration and at low (e.g., nanomolar) concentrations. NO[•] metabolism is dependent on the presence of cells to react with, since it is known that the rate of NO[•] disappearance in the presence of cells is much more rapid compared to the disappearance in the absence of cells [16]. Additionally, oxygen determines the rate of NO[•] catabolism. NO[•] disappears much more rapidly at 21% oxygen in comparison with only 1% oxygen with the same number of cells. As a consequence, both the synthesis and the metabolism of this radical are determined by the local oxygen concentration of different tissues [17]. NO[•] is an important messenger molecule in the regulation of the blood flow, the endothelium function and neurotransmission, but it can be harmful and cytotoxic when it is produced in excess. NO[•] itself is a weak oxidant; but can react with the superoxide anion to produce a much more reactive molecule, peroxynitrite (ONOO⁻) [18]. ONOO⁻ is at the crossroads of the ROS and NO[•] signaling pathways and can cause protein nitration [1,18,19] (Figure 1). The different subcellular distribution of sources of nitric oxide and superoxide implies that ROS and RNS are distributed differentially throughout the cell [20].

Biomarkers of oxidative stress

The World Health Organization defined a biomarker as any substance, structure, or process that can be measured in the body or its products and influences or predicts the incidence or outcome of disease [21]. Free radicals have a very short half-life (most of them only a few seconds) and their measurement *in vivo* is quite challenging since they are present at very low concentrations. Several oxidation end products are therefore used to assess the redox state, such as end products of lipid peroxidation, DNA damage, or the oxidation or nitration of proteins and amino acids [22]. Although the levels of such oxidative modifications do not measure the ROS levels as such, they are assumed

to be proportional to the ROS levels. The best-known biomarkers for endogenous oxidative damage to lipids, DNA, and proteins are 8-iso prostaglandin $F_{2\alpha}$ (8-isoPGF $_{2\alpha}$), 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 3-nitro-L-tyrosine (3-NT), respectively [14,22].

Lipid peroxidation

Lipid peroxidation is the metabolic process in which free radicals and nonradical species attack lipids, mainly located at the cell membranes. It mostly affects polyunsaturated fatty acids (PUFAs), since they contain double bonds, which are highly reactive with ROS. Arachidonic acid and linoleic acid, for example, are both abundant fatty acids in human cells and are essential for the proper formation and functioning of cell membranes, hormones, the immune system and neuronal transmission [23].

Isoprostanes

Since these are some of the best-known biomarkers of OS, several thorough reviews of the biochemistry and utility of F_2 -isoprostanes have already been published [24–26]. F_2 -isoprostanes, also known as prostaglandin-like compounds, are formed *in situ* by free radical oxidation, independent of cyclooxygenase, from mainly arachidonic acid, a PUFA present in phospholipids of cell membranes. After production, they are released by phospholipases. Not only arachidonic acid is responsible for the formation of isoprostanes, docosahexaenoic and eicosapentaenoic acid as well. In contrast, prostaglandins are formed from free arachidonic acid by the action of cyclooxygenases (COX-1 and COX-2)[22]. This type of isoprostanes, the F_2 -isoprostanes, was the first discovered class, and named after the F-type prostane ring, which is analogous to those of the prostaglandins. Depending on the position of the added oxygen molecule, four series of regioisomers are formed (5-, 12-, 8- or 15-series), eventually leading to 32 pairs of enantiomers. When arachidonic acid is oxidized, either *in vitro* or *in vivo*, the 5- and 15-series regioisomers are formed in significantly greater amounts compared to the 8- and 12-series regioisomers. A plausible explanation for this is that the 8- and 12-series regioisomers easily undergo further oxidation. The 5- and 15-series

regioisomers cannot undergo this further oxidation, and therefore, accumulate in tissues and body fluids [24,25]. In addition, F₂-isoprostanes are unaffected by lipid content in diet and thus their measurement in biological fluids as well as exhaled breath condensate (EBC) can provide an estimation of total body production, whereas measurement of F₂-isoprostanes esterified in tissues of interest can provide information to localize and quantify the specific OS. In addition to the F₂-isoprostanes, other classes of isoprostanes can be generated from arachidonic acid as well, which differ from each other with regard to the functional groups on the prostane ring (E₂-isoprostanes, D₂-isoprostanes, A₂-isoprostanes and J₂-isoprostanes). Among these, 8-isoPGF_{2α} and 8-isoPGE₂ are formed abundantly *in vivo*, since they belong to the 15-series regioisomers. Therefore, these two isoprostanes are the most thoroughly investigated [24].

Lipid oxidation derived aldehydes

During the past years, malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) gained the most attention among the aldehydes produced by lipid peroxidation. All three originate from omega-6 PUFAs, such as linoleic and arachidonic acid, and are produced by radical-initiated degradation of these lipids. MDA appears to be the most mutagenic product, considering the existence of MDA-induced DNA interstrand cross-links [27], while HNE and ONE are the most toxic [28].

MDA is an end-product generated *in vivo* by decomposition of arachidonic acid and larger PUFAs, through enzymatic or nonenzymatic processes. MDA can not only occur as free MDA, but it can also exist in conjugated forms by forming covalent bonds with the -SH and -NH₂ groups of proteins and nucleic acids. Once produced, MDA can diffuse into EBC [29] and other biological fluids like saliva [30], blood (plasma or serum) [31] and urine [29,32]. Free MDA, on the one hand, is a minor and variable excretory product, while MDA adducts with guanine can be excreted in urine [33].

HNE and ONE, both cytotoxic and genotoxic, are hydroxyalkenals produced by lipid peroxidation. Both compounds are also formed by the radical-initiated degradation of PUFAs, such as linoleic and arachidonic acids. Once produced in the body, HNE, as well as ONE, undergo phase 1 and phase 2 metabolism. Phase 1 metabolism results in both the oxidation products 4-hydroxy-2-nonenic acid (HNA) and 4-oxo-2-nonenic acid (ONA) and the reduction products 1,4-dihydroxy-2-nonene (DHN) and 4-oxo-2-nonen-1-ol (ONO), respectively [34]. All these metabolites, together with HNE and ONE, have also been shown to undergo phase 2 metabolism, forming Michael-type conjugates with GSH, a reaction mediated by glutathione S-transferase [35]. When the GSH adducts are metabolized by the liver and the kidney, it results in mercapturic acid conjugates (HNE-MA for example), a biomarker usually detectable in urine [34,36]. Aldehydes that escape the cellular metabolism are responsible for the modification of proteins via a non-enzymatic Michael addition. The double bonds of HNE and ONE serve as a site for the Michael addition with mainly the sulfur atom of cysteine, the imidazole nitrogen of histidine and the amine nitrogen of lysine. These adducts may alter the activity of the proteins or cause their degradation by proteasomes [37,38].

Markers of DNA oxidation

8-hydroxy-2'-deoxyguanosine and 8-nitroguanine

Cellular DNA and RNA damage can be caused by ROS generated under different conditions, and this oxidative damage is involved in mutagenesis, carcinogenesis and aging. The oxidative damage is mainly caused by the most reactive free radical, the hydroxyl radical [39,40]. The reaction between the hydroxyl radical and a 2'-deoxyribose or guanine produces C4-OH-adduct radicals, C5-OH-adduct radicals and C8-OH-adduct radicals, respectively. For example, the reaction with 2'-deoxyguanosine leads to the formation of C8-hydroxyguanine (8-OHGua) within its nucleoside form C8-hydroxydeoxyguanosine, 8-OHdG. Keto-enol tautomerism of 8-OHdG favors the oxidized product 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) [41]. Nevertheless, 8-OHdG and 8-oxodG are seen as

the same compound in literature. Additionally, the other nucleobases of DNA react in a similar manner with the radical. Despite that, 8-OHdG is the most abundant DNA lesion since it is relatively easily formed in comparison with the others [41]. Except for DNA damage by ROS, it can also react with RNS, undergoing mainly nitration and deamination of purines. $\text{NO}^\bullet$ is synthesized by either nNOS, iNOS or eNOS. During inflammation, iNOS is highly activated and generates an abundant amount of $\text{NO}^\bullet$. As a consequence, $\text{NO}^\bullet$ can react easily with superoxide to form ONOO^- . ONOO^- can react with different biomolecules, including DNA bases at selected positions. ONOO^- -induced oxidation and nitration of guanine occurs mainly at the C8 position, producing several products such as 8-OHGua and 8-nitroguanine (8- NO_2Gua) [42,43].

Protein oxidation products

3-nitrotyrosine

Tyrosine, a non-essential aromatic amino acid, is mildly hydrophilic due to its benzene ring carrying a hydroxyl group. Therefore, tyrosine is frequently surface exposed in proteins allowing further modification, like nitration, resulting in the formation of 3-NT. Protein tyrosine nitration is associated with nitrosative stress and occurs through the addition of a $-\text{NO}_2$ group in the ortho position of the phenolic hydroxyl group [44]. Both free 3-NT and protein-bound 3-NT can be formed. Free 3-NT represents the turnover of nitrated proteins, since it is a stable post-translational modification. However, not every available tyrosine residue in a protein is available for nitration. Nowadays, several physiological and pathological conditions, like cardiovascular diseases, neurological diseases and renal complications, have been associated with an increased nitration of proteins [45].

Dityrosine and 3-chlorotyrosine

Not only nitrotyrosine can be formed from tyrosine due to oxidative/nitrosative stress, o,o'-dityrosine and 3-chlorotyrosine are stable oxidative modifications of tyrosine as well.

Myeloperoxidase (MPO), a heme peroxidase enzyme, is responsible for the production of 3-chlorotyrosine, since MPO catalyzes the conversion of chloride ions in the presence of hydrogen peroxide to generate HOCl. HOCl, in turn, causes the post-translational modification 3-chlorotyrosine, which is a highly sensitive and specific marker for MPO activity [46]. Additionally, MPO is responsible for the generation of nitrogen dioxide radical, which can make tyrosyl radicals from free tyrosine. Those tyrosyl radicals can oxidize protein tyrosyl residues to form o,o'-dityrosine [47].

Glycoxidation adducts

Protein glycation involves the nonenzymatic attachment of a reducing sugar or sugar derivative to a free amino group on a protein, a reaction also known as the Maillard reaction. Glycation adducts are classified into two groups: early glycation adducts and advanced glycation end products (AGEs). Despite the name, AGEs may be formed in both the early and late stages of the glycation process. Glycoxidation is a term generally used for glycation processes in which oxidation is involved and the AGEs formed thereby have been called glycoxidation products. The AGEs pentosidine, N ϵ -carboxymethyl-lysine (CML) and N ϵ -carboxymethyl-cysteine (CMC) are a few examples [48]. About 90% of CML and pentosidine are bound to proteins in blood. The deleterious effects of AGEs in different tissues are attributed to their chemical, pro-oxidant and inflammatory actions. The biological effects of AGEs are exerted by two different mechanisms: (1) independent of a receptor (damage of protein structure and extracellular matrix metabolism); or (2) involving the AGE receptor (RAGE). AGE-RAGE interaction is able to activate NAD(P)H oxidase, which increases intracellular OS [49]. Therefore, increased levels of AGEs are related to several diseases, like renal disease, diabetes, cardiovascular diseases and neurological diseases [50].

Oxidized low-density lipoproteins

Low-density lipoprotein is one of the five major groups of lipoproteins which transports all fat molecules around the body in the extracellular fluid, making fats available to body cells. Since cholesterol is insoluble in water, it is carried in a large complex composed of various lipids and proteins, a lipoprotein. Triglycerides and cholesteryl esters are packed in the core of the lipoproteins, and this is surrounded by a phospholipid monolayer. The shell of every low-density lipoprotein contains one molecule of apolipoprotein B-100. More than half of the lipid molecules are PUFA-containing lipids. As a result, upon attack of free radicals, the PUFAs are easily oxidized. The oxidation of a low-density lipoprotein is a complex process, since both the lipids and proteins undergo oxidative changes and form complex products. The formed products depend on a variety of factors. The first factor is the lipid composition of the low-density lipoprotein particle; if a particle is enriched in arachidonic acid, hydroxyeicosatetraenoic acids (HETEs) and hydroperoxyeicosatetraenoic acids (HPETEs) and 5-carbon aldehydes will predominate, whereas if the particle is enriched in linoleic acid, hydroxyoctadecadienoic acids (HODEs), hydroperoxyoctadecadienoic acids (HPODEs), and 9-carbon aldehydes will predominate. The second factor is the duration of oxidation. As the oxidation proceeds, oxidized lipids begin to undergo oxidative fragmentation and disintegration, resulting in oxidized phospholipids, which contain oxidized fatty acids at the sn-2 position. The concentration of circulating oxidized low-density lipoproteins can provide sufficient information for the presence of OS in the body [51–55].

Antioxidants

Glutathione

GSH, one of the major thiol antioxidant and redox buffers of the cell, can be abundantly found in the cytosol, nuclei and mitochondria, even in the millimolar range. GSH is synthesized in the cytosol by glutamate-cysteine ligase and GSH synthetase. GSH is an antioxidant and therefore, it tries to scavenge ROS, which leads to the oxidized form of GSH, glutathione disulphide (GSSG). Since GSSG is

accumulated in the cell under conditions of OS, the ratio GSH/GSSG is a good measure for OS. In general, any condition associated with excessive ROS production will decrease GSH levels or decrease the GSH/GSSG ratio. The oxidation of GSH is reversible as GSSG reductase and NADPH reduce GSSG back to two molecules of GSH [2,56].

Coenzyme Q10

Coenzyme Q10 is a unique lipophilic structure, composed of a benzoquinone ring and an isoprenoid sidechain, which is found in all cell membranes and plasma lipoproteins. The benzoquinone ring is derived from 4-hydroxybenzoic acid, while the 10 isoprenes are derived from mevalonic acid. The sidechain is responsible for the stability of the molecule inside the phospholipid bilayer. In addition, the quinone ring is the functional group that maintains the transfer of electrons from complexes I and II to complex III. Coenzyme Q10 cycles between three different forms: completely oxidized (ubiquinone-10), a semi-oxidized intermediate free radical (semiquinone-10) and a completely reduced form (ubiquinol-10). Except its function in the electron transport chain, coenzyme Q10 has an antioxidant role in the plasma membrane and other intracellular membranes, protecting the phospholipids from peroxidation. When low-density lipoproteins are exposed to OS, ubiquinol-10 is the first antioxidant to be oxidized. Therefore, the ubiquinol-10/ubiquinone-10 ratio may be a sensitive marker for OS in human blood [57–59].

Literature about different sample preparation techniques and possible artifacts

One of the challenges while working with complex biological samples is the sample preparation. A suitable sample preparation is often necessary, since it can reduce the complexity of biological samples, resulting in improved sensitivity and selectivity and improved robustness by reducing the concentration of interfering compounds. Problems due to interfering compounds during the analysis of biological samples by LC-MS/MS are often referred to as 'matrix effects', which is already

thoroughly reviewed [60,61]. Unfortunately, sample preparation techniques in bioanalysis often comprise multistep, time-consuming, and labor-intensive procedures, which can take up to more than half of the total analysis time. Therefore, there is a need for efficient and less time-consuming sample preparations in the bioanalytical methods. Together with a focus on minimizing the use of hazardous chemical compounds for both safety and environmental reasons, the search for new sample preparation techniques and preferably automatic and online applications has started [62].

The most popular sample preparation techniques are the 'dilute and shoot' approach, protein precipitation, liquid-liquid extraction (LLE) and SPE. The applications of solid-phase sorbents, either the traditional sorbents [63] or the more modern sorbents [63–67], are already thoroughly reviewed, since the selection of the right SPE sorbent is essential to retain the right analytes. SPE is a rather time-consuming technique and it is relatively expensive. Modern development of SPE is focused on miniaturization and automation. Techniques such as solid phase microextraction (SPME), online SPE and in-tube SPME are already used in (bio)analysis and several recent reviews describe these different techniques in detail [63,67–70]. Additionally, LLE was also improved over the years, resulting in different novel (micro)extraction techniques, which were reviewed in 2017 with the focus on the analysis of biomarkers in several biological fluids [71]. Niu et al. published a broad overview of sample preparation techniques used for the main biological samples, together with the recent advances and applications of various methods prior to chromatography, spectrometry and electrochemistry analysis [72].

Artifacts caused during sample preparation

Analytical issues are mainly associated with the quantification of oxidative protein modifications. In 2019, a review highlighted the different available methods to detect oxidative modifications, both in amino acids, peptides, and proteins, together with the various artifacts which may be encountered during sample preparation [73]. One of the limitations is the examination of methionine oxidation in a complex matrix, since it is known that methionine oxidation increases with the length of trypsin

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digestion and with the strength of the ionization energy during ESI. Nevertheless, a recent method was published to quantify *in vivo* methionine oxidation circumventing these limitations [74]. Another big issue is the determination of the oxidation of cysteine 34 in plasma albumin since artifactual thiol oxidation of albumin can happen during the sample preparation. Therefore, a recent review described the different approaches and tools for the evaluation of protein cysteine oxidation [75]. It is also known that ubiquitous nitrite, nitrate and an excess of tyrosine could form a 3-NT artifact during sample preparation, especially with acidification, causing an overestimation of 3-NT in complicated biological matrices. Therefore, several (chromatographic) approaches for the analysis of 3-NT are already reviewed [76,77]. On the other hand, several issues can arise when oxysterols are analyzed with LC-MS/MS; (1) isomeric compounds of oxysterols easily gave similar spectra, (2) oxysterols and other sterols are neutral molecules, resulting in poor ionization in the ion source of the MS and (3) autoxidation of cholesterol during sample storage and preparation can happen. To avoid autoxidation, precautions are needed, such as the utilization of antioxidants or performing the sample preparation in an inert atmosphere. Different derivatization methods have been developed over the years to overcome the other problems, which are reviewed in 2013 [78]. The issues with the determination of GSH and GSSG are mentioned later in section 5.5. In addition to protein oxidation, the measurement of F₂-isoprostanes, which was thought to be only generated by free radical-mediated arachidonic acid peroxidation, was also challenged in the late 1990s [79]. Prostaglandin endoperoxide synthase was found to catalyze an enzymatic reaction to F₂-isoprostanes and this synthase is known to be significantly induced during inflammation. Measuring the F₂-isoprostane / prostaglandin F_{2α} ratio can overcome this problem by distinguishing between chemical and enzymatic lipid peroxidation [80].

To avoid artifacts, several reviews summarize the conventional and modern sample preparation techniques for the detection of specific OS biomarkers. In 2018, a review about the quantification of eicosanoids and their metabolites in biological matrices [81] and another review article about the

detection of 8-OHdG were written [82], while in 2019, a review about modern methods of sample preparation for the analysis of oxylipins in biological samples was published [83]. Even more recently, advancements in the analytical quantification of 3-NT were reviewed as well [84].

Strategies to quantify biomarkers in human urine (2015 – 2020)

Urine is a noninvasive matrix sample that can be collected longitudinally and temporally multiple times without affecting the patient. Table 1 summarizes the available research on OS biomarkers utilizing urine as matrix via LC-MS/MS during the period of 2015 – early 2020.

Pretreatment without extraction technique

Only a limited number of articles use simple pretreatment procedures such as the ‘dilute and shoot’ approach or filtration. A newly developed analytical method for the quantification of 8-OHdG, 8-isoPGF_{2α}, 8-NO₂Gua and the mercapturic acid conjugate of HNE (HNE-MA), using sample dilution and centrifugation, was successfully applied in 2015 [85,86]. Additionally, Liu et al. reported in 2015 a UPLC-MS/MS method to quantify both 8-OHdG and 8-hydroxyguanosine (8-OHG) in urine samples of type 2 diabetes patients using only centrifugation as sample pretreatment. A guard column was used to protect the analytical column of the remaining matrix components [87]. In 2017, Sørensen et al. developed another UPLC-MS/MS method to investigate the urinary excretion of the same biomarkers in patients with type 2 diabetes, with the only difference that in this paper the effect of smoking was examined. The sample preparation was the same, but the column used here was 100 mm instead of 50 mm [88]. Another LC-MS/MS method developed by Tranfo et al. was also used for the detection of oxidative damage to nucleic acids, using 8-OHdG, 8-OHG and 8-oxoGua as OS biomarkers, and to reveal possible correlations between these biomarkers and the exposure to benzene. Like the previous mentioned articles, only the ‘dilute and shoot’ approach and centrifugation were used for the sample preparation [89]. Two years later, in 2019, the same

method was used to determine the levels of urinary 8-OHdG, 8-OHG and 8-oxoGua in two groups of gasoline pump attendants occupationally exposed to benzene, including both smokers and non-smokers [90]. On the other hand, Henriksen et al. published an analytical method for the direct analysis of 8-OHdG and 8-OHG in human urine [91] and this method was successfully applied for the determination of these urinary OS biomarkers in obese men with and without hypertension [92]. An analytical method published in 2014 [93] was used by Peña-Bautista et al. in 2019 to determine nine proteins and DNA oxidative damage biomarkers with the 'dilute and shoot' approach in urine samples from patients with an early stage of Alzheimer's disease [94].

Liquid-liquid extraction and protein precipitation

To our knowledge, no research group developed a new method using LLE as sample preparation technique. Nevertheless, an article published in 2018 used the LLE principle for the determination of eicosanoids in urine samples, in order to study the effect of smoking cessation. They used a previously optimized and validated LC-MS/MS method [95], and decided to add two additional analytes, namely the prostaglandins tetranor-prostaglandin D-metabolite and prostaglandin $F_{2\alpha}$ to establish a more comprehensive eicosanoid profile [96]. On the other hand, He et al. developed a method for the simultaneous investigation of 8-OHdG and 6-sulfatoxymelatonin excretion into urine using protein precipitation as sample preparation technique [97]. 6-sulfatoxymelatonin is a major metabolite of melatonin and it is believed that melatonin helps with the repair of DNA damage by ROS, hence it may affect the urinary excretion of 8-OHdG [98].

Solid phase extraction

Since 8-OHdG, 8-isoPGF_{2 α} and 3-NT are the best-known OS biomarkers, several analytical methods were developed to determine one of these three biomarkers, using SPE as the sample preparation method. The quantification of 3-NT was reported by Li et al. [99,100]; while two other analytical methods were developed for the determination of 8-isoPGF_{2 α} [101,102]. In 2016, Guo et al. developed an offline SPE-UPLC-MS/MS method for the quantification of 8-OHdG in urine samples

and it was successfully applied to compare the urinary levels in patients with colorectal cancer with those of healthy controls, both in 2016 [103] and in 2018 [104]. Recently, the same research group developed an analytical method for the measurement of 8-OHG in human urine, and again, this method was successfully applied for the determination of the RNA OS biomarker in urine of both patients with colorectal cancer and healthy volunteers [105].

Turnu et al. investigated the free-radical-mediated DNA damage during cardiac surgery, using two different analytical methods, where the method for the quantification of 8-OHdG was self-developed [106]. The measurement of 8-OHdG only required dilution of the urine sample and a total run time of 14 minutes, while the determination of 8-isoPGF_{2α} was performed by modifying a previously described LC-MS/MS method [107]. SPE was chosen as sample preparation method and the total run time was 40 minutes. Additionally, two other studies examined both the oxidative damage biomarker 8-isoPGF_{2α} and the inflammation biomarker prostaglandin E₂ metabolite, with distinct analytical methods adapted from Yan et al. (2007) [108] and Neale and Dean (2008) [109], respectively. Since both biomarkers are elevated in cigarette smokers, they were interested in the longitudinal stability of these biomarkers [110] and the effects of smoking cessation on these biomarkers [111]. The same applies to the study of Kant et al., where 8-OHdG, 8-isoPGF_{2α} as well as (5'R)- and (5'S)-8,5'-cyclo-2'-deoxyadenosine were examined in the urine of diabetes patients. An analytical method was applied for 8-OHdG and the adenosines, while another one was used for the determination of 8-isoPGF_{2α} [112], both developed methods were based on previously published LC-MS/MS methods [113,114].

In contrast, a single SPE-LC-MS/MS method to facilitate the simultaneous quantification of biomarkers for oxidative DNA damage, inflammation and lipid peroxidation, namely 8-OHdG, 8-isoPGF_{2α}, 8-NO₂Gua and HNE-MA was developed for the first time in 2016 [36]. Two years later, in

2018, exactly the same method was used, together with another analytical method for the determination of bisphenol A, to examine the interactive effects of bisphenol A and OS on fetal reproductive indices [115]. On the other hand, Ren et al. developed a method to simultaneously determine five parabens, bisphenol A, triclosan and 8-OHdG in human urine, which is the first analytical study for the determination of these compounds together [116]. Both DNA and RNA OS biomarkers, 8-OHdG and 8-OHG respectively, are determined in human urine samples in an analytical method developed by Cervinkova et al. [117]. In addition, Rodríguez-Gonzalo et al. were the first to report a method for the measurement of both DNA- and RNA-derived urinary nucleosides [118]. Zhao et al. reported the first HPLC method for the analysis of 8-OHdG, 8-OHG and 8-isoPGF_{2α}, while using the same purification step [119]. In 2018, an aptamer-based online magnetic SPE method for the determination of 8-OHdG in human urine was published, using Fe₃O₄-aptamer magnetic nanoparticles [120]. The aptamer for 8-OHdG was already reported in 2009 [121]. Depending on the analytes of interest, sometimes SPE is used together with another sample preparation technique in order to ensure that every analyte is detected. In 2018, Martinez and Kannan developed and validated a LC-MS/MS method for the simultaneous determination of seven different urinary biomarkers of oxidative damage to lipids, proteins and DNA, using SPE preceded by a derivatization step with 2,4-dinitrophenylhydrazine [122]. Despite the fact that several studies have already determined these biomarkers together, this research is innovative as the previous studies all used multiple analytical techniques [123–125].

Kuligowski et al. developed a UPLC-MS/MS method to investigate isoprostanes, isofurans, neuroprostanes and neurofurans in urine samples of preterm infants < 32 weeks of gestation within the frame of two randomized, controlled, and blinded clinical trials [126]. In addition, a new method for the determination of both neuroprostanes and dihomio-isoprostanes in epileptic patients was developed as well. The SPE technique had originally been published in another paper from the same

group [127], but in this research minor modifications were made [128]. A few years later, this group used the same analytical method for the quantification of neuroprostanes and dihomio-isoprostanes in the urine of elite triathletes after two weeks of moderate-altitude training [129].

Modern advances in sample preparation

Several reported analytical methods used an automated online SPE system, which consists of an extraction column and a 10-port switching valve, in order to clean up the sample before it goes to the analytical column. This technique was used for the determination of 8-OHdG and cotinine [130], oxidative and nitrosative stress biomarkers (8-OHdG, 8-isoPGF_{2α}, 8-NO₂Gua, HNE and HNE-MA) [131] and guanine-derived OS biomarkers, together with MDA [132], where the quantification of MDA was based on a previously published article [133]. Additionally, Chao et al. developed an analytical method for the simultaneous detection of 3-NT and 3-nitro-4-hydroxyphenylacetic acid in human urine [134], together with the measurement of oxidative (8-OHdG, 8-OHG) and methylated (N7-methylguanine, N3-methyladenine) DNA lesions. The latter methods had already been published before [135–137]. On the other hand, Zhang et al. developed an online SPE RAM method for the quantification of monohydroxybutenylmercapturic acid, N-acetyl-S-(3,4-dihydroxybutyl) cysteine, as metabolites of 1,3-butadiene, an environmental pollutant found in tobacco smoke, and 8-OHdG, as oxidative damage biomarker [138].

Nowadays, the focus is not only on the automation of the SPE technique but also on its miniaturization. A few papers, using in-tube online SPME, have already appeared in the field of OS biomarkers in human urine samples. The research group of Kataoka et al. published most of these articles. In 2015, they developed a method to measure urinary 8-isoPGF_{2α} [139], while in 2016 another in-tube SPME method was reported for the quantification of urinary 8-OHdG [140]. Additionally, two years later, in 2018, the compounds 8-isoPGF_{2α} and 8-OHdG and 3-NT were determined by a newly developed method [141]. In 2019, another research group optimized and

validated an LC/MS protocol for the simultaneous determination of 8-OHdG, 3-hydroxyphenanthrene and 1-hydroxypyrene after in-tube SPME [142].

Oxidative stress biomarkers quantified in human plasma, serum and whole blood (2015 – 2020)

Human whole blood, plasma and serum are commonly used matrices in bioanalysis. Both plasma and serum are derived from whole blood. Serum is obtained from blood after coagulation, while the anticoagulants EDTA or heparin are added to a blood sample before the removal of blood cells to obtain plasma. As already reviewed, the use of the wrong matrix can lead to improper diagnosis and results [143]. However, another study revealed that the metabolic profiles from either plasma or serum can be analyzed, as long as the same blood sample is used [144]. Both the time and the temperature conditions between the sample collection and sample processing or storage are crucial variables, which can affect the stability of proteins or enzymes in the sample. Therefore, blood samples should typically be processed and stored within two - four hours after collection [145,146].

Table 2 summarizes the available research on OS biomarkers utilizing either plasma, serum or whole blood samples as matrix via newly developed LC-MS/MS methods during the period of 2015 – early 2020.

Lipid peroxidation

Lipid peroxidation biomarkers are becoming increasingly popular as OS biomarkers to determine in blood derived biofluids, especially in plasma. Before 2015, lipid peroxidation biomarkers, mainly F₂-isoprostanes, were mostly detected by GC-MS/MS [147,148], although it requires derivatization, and only a few LC papers were published [80,149–151]. These papers were mostly characterized by LLE methods, such as the analysis of plasma F₂-isoprostane class IV isomers in pregnant women [149,152–154]. On the other hand, the modified LC-MS/MS methods of Fam et al. (2002) [155] and Coolen et al. (2005) [156], which both apply SPE as sample preparation technique, were used in 2018

to determine F₂-isoprostanes and F₄-isoprostanes as markers of intracranial aneurysm development [157]. In contrast, Cháfer-Pericás et al. developed in 2015 the first UPLC-MS/MS method for the determination of large numbers of F₂-isoprostanes and other total peroxidation byproducts in newborn serum samples, using small sample volumes and SPE as sample preparation method [158]. This UPLC-MS/MS approach was successfully applied for the determination of lipid peroxidation biomarkers in cord serum [159] and was used to develop another UPLC-MS/MS method for the quantification of lipid peroxidation biomarkers in newborn plasma samples [160]. A method for the simultaneous quantification of 20 isoprostanes from different PUFAs was published in 2016 and used for the investigation of human plasma and cerebrospinal fluid as well as mice biofluids and tissues [161]. In the same year, Black et al. developed two different LC-MS/MS methods for the measurement of 8-OHdG and the total concentration of 8-isoPGF_{2α} in human plasma, both with a different 96-well SPE as sample preparation method [162]. One year later, this method was used for the measurement of OS in individuals with depression and anxiety disorders [163,164]. In 2017, the first validated UPLC-MS/MS method for the simultaneous quantification of 28 lipid peroxidation biomarkers in newborn plasma samples was reported, using only 100 µL of plasma. This method applied the SPE method of the previously mentioned Cháfer-Pericás et al. papers [165]. One year later, a new method was described with a total run time of 12 min [166], allowing the parallel quantification of 27 isoprostanes and 8 isofurans derived from six different PUFAs in human plasma, using a previously published SPE method [167]. On the other hand, Aszyk et al. published in the same year the first analytical paper describing the separation of four regioisomers (F₂-isoprostanes) in biological material within less than 8 minutes, using weighted linear regression and LLE as sample preparation technique [168]. In 2020, a reliable analytical method combining micro-extraction by packed sorbent (MEPS) and UPLC-MS/MS to determine 8-isoPGF_{2α}, 8-isoPGE₂ and prostaglandin E₂ in dried blood spots was developed [169]. MEPS is a miniaturized and sophisticated version of SPE, using a syringe and a cartridge packed with few milligrams of sorbent stationary phase. Compared to SPE, it is faster and despite a lower amount of sample is used, it achieves the same sensitivity [170].

This paper reported a fast, easy and semi-automated offline MEPS procedure for the determination of these lipid peroxidation biomarkers. It guaranteed an effective sample cleanup in less than 10 minutes with only 50 μ L sample, and the cartridges can be used up to 100 times [169].

The measurement of MDA, a lipid oxidation derived aldehyde, in whole blood, plasma and serum samples is challenging. Therefore, the use of derivatization, before a protein precipitation step, is often required. Since existing methods all have some limitations with the detection of MDA [171–173], Sobsey et al. developed a new method with a simple sample preparation, using 3-nitrophenylhydrazine as derivatization reagent [174].

DNA and RNA oxidation markers

In addition to lipid peroxidation biomarkers, DNA and RNA oxidative damage markers are likely to be examined as well. Seven DNA catabolites are evaluated in a newly developed UPLC-MS/MS method to determine the levels of these markers in the plasma of triathletes before and after intensive elite training for two weeks [175]. Since these markers are not all the same, they need different pretreatments. After protein precipitation, a previously published SPE method for 8-OHdG [176] was modified in order to use the same cartridge for the extraction of 8-azidoadenosine, 8-phenylthioadenosine, 8-OHdG, 8-OHG and 8-oxoGua. On the other hand, the previously published SPE method for the determination of cGMP [177] was adapted in such a way that cGMP, 8-NO₂-cGMP, 8-NO₂Gua and 8-NO₂Gua could be extracted. Recently, Alamil et al. validated for the first time a UPLC-MS/MS method for the simultaneous quantification of nine exocyclic DNA adducts induced by eight main exogenous and endogenous aldehydes, namely formaldehyde, acetaldehyde, acrolein, crotonaldehyde, MDA, HNE, glyoxal and methylglyoxal. This method is applicable for real human samples (e.g., plasma) and it is possible to extend the method to the quantification of other

adducts, such as those generated from aldehydes obtained from both environmental and OS pathways [178].

Oxidized low-density lipoproteins

Except arachidonic acid, linoleic acid and cholesterol can be oxidized as well to HODE and hydroxycholesterol (OHCh), respectively. Therefore, a LC-MS/MS method was reported for the quantification of HODE, OHCh and isoprostanes in plasma and erythrocytes of healthy human subjects [179], which has recently been used for the determination of HODE in plasma of epilepsy patients [180]. Besides OHCh, 7-ketocholesterol (7-KC) and cholestane-3 β ,5 α ,6 β -triol may be assumed to be the best biomarkers among the oxysterols and in addition, these two compounds are Niemann-Pick C1 specific biomarkers as well. Therefore, an analytical method was developed to quantify both compounds in human plasma, using a derivatization step [181]. This method was successfully applied to further investigate the Niemann-Pick C1 and C2 disease [182,183], but recently, also to examine the role of these compounds in the presence of OS in patients with type 1 and type 2 diabetes, where it seems that both 7-KC and cholestane-3 β ,5 α ,6 β -triol may be used as sensitive biomarkers for monitoring OS in diabetes patients [184]. On the other hand, several oxysterols (24-OHCh, 25-OHCh, 27-OHCh, 7 α -OHCh, 7 β -OHCh and 7-KC) were examined in plasma of patients with abdominal aortic aneurysm, using two previously described UPLC-MS/MS methods [185]. Two different extraction methods were used for the quantification of free oxysterols (7-KC and 27-OHCh) and the quantification of the total oxysterols. The used sample preparation techniques were protein precipitation based on Helmschrodt et al. [186] and a saponification step followed by SPE based on Narayanaswamy et al. [187] and Mukhopadhyay et al. [188], respectively.

In 2015, Yamada et al. published a method for the comprehensive quantification of eicosanoids and related compounds with high speed continuous ionization polarity switching and validated the method in mouse tissue [189]. Three years later, this method has been used to evaluate the

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differences in serum eicosanoids in healthy, enteritis and colorectal cancer individuals [190]. In contrast, Suhr et al. reported a newly developed LC-MS/MS method for the determination of seven eicosanoids in human plasma, using ferromagnetic particle enhanced deproteinization as a sample preparation technique and online SPE [191]. Using ferromagnetic particles is a rapid procedure since no additional filtration or centrifugation is necessary. Eicosanoids have a broad variability of physico-chemical properties, and since these particles only bind to denatured proteins, this is a promising approach for eicosanoids [192]. For the determination of both prostaglandins and eicosanoids in human plasma, Miller et al. combined two published methods [193,194] into one method, resulting in a rapid and sensitive method using UPLC-MS/MS for the simultaneous determination of eicosanoids and prostaglandin metabolites of arachidonic acid. SPE extraction was used to prepare the sample for injection, whereafter the eicosanoids were measured. A second injection was performed of the same extracted sample, which allowed the separation and assessment of the prostaglandin metabolites [195]. More papers concerning the analysis of eicosanoids in biological fluids can be found in the review of Chhonker et al., published in 2018 [81].

ROS can also lead to oxidative modification of low-density lipoproteins, resulting in carbonylated apolipoproteins and oxidized lipoproteins, which are largely composed of phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. Phosphatidylcholine is the most abundant phospholipid in mammalian cells. Therefore, low-density lipoproteins oxidation primarily results in the oxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC). This results in the generation of several oxidation products, such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine (PONPC) as well as 1-palmitoyl-(2-glutaroyl)-*sn*-glycero-3-phosphocholine (GPC) and 1-palmitoyl-2-(5,6-epoxy-isoprostane)-*sn*-glycero-3-phosphocholine (PEIPC), which are the most prominent formed oxidation products [54,196]. In a recently published article, a LC-MS/MS method was described for the

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measurement of an extensive panel of oxidized phosphatidylcholines in human plasma, including POVPC, PONPC, PGPC. This method was used to investigate the possibly altered plasma concentrations of these phosphatidylcholines in patients with chronic kidney disease and chronic kidney disease with co-morbid periodontitis compared to healthy controls [197]. The extraction of the lipids was performed by the methyl-*tert*-butyl ether (MTBE) method as previously reported [198], and the analytical method was a modification of a previous article, published in 2017 by the same research group. They developed an analytical method for the detection of POVPC in serum and at the same time, they investigated the hypothesis that POVPC could be a modifiable marker of Alzheimer's disease that relates to cognitive performance [199]. The previously mentioned oxidized phospholipids had modifications at the *sn*-2 position of the glycerol backbone, while, in contrast, plasmalogens are a unique class of membrane glycerophospholipids containing an alkenyl group including a vinyl-ether bond at the *sn*-1 position and a PUFA at the *sn*-2 position [200]. Plasmalogens are divided into two dominant head groups, ethanolamine and choline. Recently, Ikuta et al. developed an analytical method for the analysis of both ethanolamine plasmalogen and choline plasmalogen of serum lipoproteins in healthy controls and in patients with non-alcoholic steatohepatitis [201]. Before analysis, the serum samples were subjected to gel filtration chromatography as previously reported [202]. The developed UPLC-MS/MS method was based on a previous publication [203], where they determined serum plasmalogens in order to find an association with metabolic disorders.

Protein oxidation products

In 2011, a LC-MS/MS method was developed to analyze stable end products of protein oxidation, such as *meta*-tyrosine, *ortho*-tyrosine, *o,o'*-dityrosine, 3-chlorotyrosine and 3-NT, in human plasma by the use of protein precipitation followed by SPE extraction [204]. Later on, this method was successfully applied in several papers (1) to determine these oxidative modifications in patients with interstitial lung disease [205], (2) to assess the role of OS in mediating adverse outcomes of the

metabolic syndrome, like cardiovascular autonomic neuropathy, and to examine the effect of a structured lifestyle for 24 weeks in patients with the metabolic syndrome [206], (3) and to test the hypothesis that 3-chlorotyrosine can predict the severity of both chronic kidney disease and the associated coronary artery disease [207]. In the meantime, a fast and reliable LC-MS/MS method, using a simple mixed-mode SPE approach, was developed and validated to quantify 3-NT in human plasma [208]. Recently, another LC-MS/MS method was developed for the simultaneous determination of 3-NT, 3-chlorotyrosine and 3-bromotyrosine in plasma of colorectal cancer patients, using protein precipitation as sample preparation technique [209].

Antioxidants

The simultaneous measurement of GSH and GSSG levels is a useful indicator of OS, however, GSH levels in plasma are variable which leads to inconclusive results [10]. Differences in these concentrations have been suggested to be a result of an inadequate sample processing technique, since the half-life of GSH at room temperature ranges from 5 to 20 minutes. GSH undergoes auto-oxidation during every step of the sample preparation. Despite several (recent [210]) attempts have been made to prevent the oxidation, such as derivatization [210,211] and acidification [212], this leads to new problems, which were reviewed in 2017 [213]. Acidification with perchloric acid or trichloroacetic acid also works as a deproteinization step and, additionally, acetonitrile or methanol were also suggested for protein precipitation. Nevertheless, this step can introduce errors as well, such as the interference of trichloroacetic acid with the chromatographic separation and the depletion of GSH by co-precipitation with proteins [214,215]. In 2018, ultrafiltration was suggested as an alternative sample preparation technique, in particular for plasma samples [216]. One year later, in 2019, a study was conducted to investigate how the sample preparation procedures affect the level of GSH/GSSG in human plasma [217]. At the same time, a protocol for reliable measurement of GSH and GSSG in plasma by UPLC-MS/MS was developed [217], which was based on a previously published method [218]. The tripeptide GSH consists of γ -glutamate, glycine and

cysteine. Ophthalmic acid, a GSH analog which is released into the systemic circulation by the liver, can be formed when the cysteine is replaced by 2-aminobutyrate. Metabolomics studies in mice, which had acetaminophen induced acute liver failure, have identified ophthalmic acid as a marker for OS and hepatic GSH depletion [219]. With that in mind, a study was performed to determine whether the human serum concentrations of ophthalmic acid were detectable in patients with acute liver failure due to acetaminophen, and whether ophthalmic acid could be an appropriate biomarker for this disease [220], with the help of a previously described LC-MS/MS method [221].

On the other hand, methods to extract coenzyme Q10 from human plasma have varied from SPE [222] to LLE with hexane [223] to protein precipitation using isopropanol [224,225]. Another recently published method for the quantification of both reduced and oxidized coenzyme Q10 was developed, using extraction of human plasma in isopropanol and direct injection of the extract [226].

Human serum albumin adducts and methionine residues

During this five-year period, other possible OS markers were evaluated in human plasma, serum or whole blood as well. The human serum albumin adducts, and methionine residues are two of these examples. Cys34 of human serum albumin is the only free thiol and therefore, a nucleophilic hotspot that accounts for about 80% of the antioxidant capacity of serum. Cys34-SH is oxidized to Cys34-SOH, which can undergo further oxidation to Cys34-SO₂H or Cys34-SO₃H or it can interact with low-molecular-weight thiols, most likely free cysteine [227]. The relative amounts of oxidized and cysteinylated Cys34 have been related to the redox state of the serum and therefore, to the global OS. Studies have shown that a marked increase in cysteinylation of Cys34 in human plasma occurs in chronic liver and kidney diseases and diabetes mellitus [228]. Grigoryan et al. developed an untargeted analytical method for quantifying Cys34 adducts in tryptic digests of human serum/plasma [229]. This method was used to measure the Cys34 adducts of human serum albumin

in patients with chronic lung or heart disease and healthy controls, which has shown that these adducts indeed differ between healthy persons and patients [230].

On the other hand, other research has shown that methionine residues in serum albumin (Met-111 and Met-147) are highly oxidized to methionine sulfoxide in patients with diabetes and renal failure, and in healthy smokers compared to non-smoker controls. To measure this, they developed a novel label-free LC-MS/MS method to quantify methionine oxidation in proteins present in the human peripheral circulation [231]. A few years later, in 2020, they used this method to quantify oxidized and non-oxidized Met-147 of human serum albumin. They determined whether the oxidation status of this residue reflects the OS induced during disease pathophysiology and whether this might represent a useful biomarker of OS, which they have shown in diabetes patients since it reflects glycemic fluctuations and hypoglycemia [232].

The clinical relevance of this review

Several recent (systematic) reviews have appeared about the available and evaluated biomarkers and their respective levels in different diseases, such as in chronic obstructive pulmonary disease [233], cancer [234], idiopathic pulmonary fibrosis [235], early stage ADPKD [7], Alzheimer's disease [236] and diabetes [237]. In the online supporting information, data about the reported reference values of several biomarkers can be found, together with some levels observed in different diseases.

The two previous chapters in this review summarized newly published LC-MS/MS methods (ranging from 2015 – 2020) for the quantification of several OS biomarkers. As aforementioned, most of these methods are already used to measure OS biomarker levels in certain diseases. Table 3 presents a summary of the biochemical section of this review (the attack site of ROS / RNS and their terminal

metabolites) together with the behavior of these metabolites in several diseases, with crosslinks to the methods listed in Tables 1 and 2.

The focus of this table is on the three most important processes of OS, being lipid (per)oxidation, DNA and RNA oxidation and protein oxidation. As can be seen from the table, lipid oxidation metabolites are examined in both urine and plasma/serum samples, while DNA and RNA oxidative metabolites and protein oxidation metabolites are mainly investigated in urine samples and plasma/serum samples, respectively. For the purpose of this review, the most powered study with an important difference in level of OS biomarker between controls and patients in each category is presented briefly.

Lipid peroxidation

Stroke is one of the leading causes of mortality and morbidity worldwide. A number of cardiovascular risk factors and several diseases, including obesity and diabetes mellitus, are known causes of stroke. Despite evidence supports that OS is associated with atherosclerosis and cardiovascular diseases, the relationship between OS and the risk of incident stroke had yet to be explored in 2015. Therefore, urinary 8-iso-PGF_{2α}, HNE-MA, 8-OHdG and 8-NO₂Gua were examined in 131 individuals (106 non-hemorrhagic and 25 hemorrhagic) and 131 age- and sex-matched controls, which were selected among participants who were free of stroke events at baseline and during a median follow-up period of 13.8 years [131].

The urine samples were thawed and centrifuged. Before the supernatant was filtered (0.22 μm), it was diluted with 1 mM ammonium acetate. Hereafter, the sample was purified by automatic online SPE, using an Oasis HLB extraction column. The purified sample was automatically transferred into an analytical column and separated with a mobile phase gradient ranging from 65% solvent A (1 mM ammonium acetate in 95% methanol) to 99.5% solvent A. Solvent B consisted of 1 mM ammonium acetate in 5% methanol (Table 1) [131].

A new insight into the relationship between OS and the risk of incident stroke was found. Urinary 8-iso-PGF_{2α} was elevated in the participants with stroke compared to controls. Additionally, the levels of urinary 8-iso-PGF_{2α} are correlated with the risk for developing incident stroke. In contrast, urinary levels of HNE-MA, 8-OHdG and 8-NO₂Gua were not significantly different between cases and controls and they were not correlated with risk of incident stroke. In conclusion, urinary 8-iso-PGF_{2α} is a significant biomarker of OS for prediction of the incident stroke risk, independent of conventional cardiovascular risk factors [131].

DNA and RNA oxidation biomarkers

It is already known that urinary 8-OHdG levels are elevated in diabetes patients with complications; however, the evidence on the association between urinary 8-OHG and diabetes complications was relatively scarce in 2016. Therefore, Liu et al. analyzed the levels of urinary 8-OHdG and 8-OHG in patients with diabetes and healthy controls [87]. In total 1316 persons were recruited; 633 patients with type 2 diabetes of which 267 had complications such as retinopathy, nephropathy, and other vascular complications and 683 age- and sex-matched healthy controls. To measure the urinary DNA and RNA markers, the urine samples were thawed, heated and centrifuged before injection into a UPLC-MS/MS system, using an Acquity UPLC BEH C18 column and a suitable VanGuard precolumn (Table 1). The mobile phase was 0.1% (v/v) formic acid (A) and 100% methanol (B), applied in a gradient elution.

They found that the urinary levels of both 8-OHdG and 8-OHG were significantly higher in the diabetes group (with and without complications) compared to the control group. Looking at the concentration changes of both urinary markers, there was a clear and significant increasing trend for 8-OHG between diabetes patients with complications compared to those without complications. A significant difference in 8-OHG was also found between patients with macrovascular complications and those without. The present study demonstrated significantly higher levels of oxidatively generated damage to DNA and RNA in patients with type 2 diabetes compared with healthy controls

subjects. The authors suggest a role for 8-OHdG and 8-OHG in the molecular mechanism contributing to the complications of type 2 diabetes. Further studies should be initiated to evaluate the potential clinical applications of 8-OHG as a biomarker in diabetes that could be used for risk stratification, progressive course, selection of appropriate therapeutic intervention, and the monitoring of response to therapy. The findings not only provide further insights into the pathophysiological mechanisms responsible for the progression of complications in diabetes but also indicate that 8-OHG has more advantages than 8-OHdG in clinical applications [87].

Oxidized low-density lipoproteins

A focal dilation of more than 50 percent of the normal diameter of an aorta is called an abdominal aortic aneurysm (AAA). Although the histopathological features are well documented, not much is known about the cellular and molecular mechanisms underlying this pathogenesis. Endoplasmic reticulum stress is a critical factor in developing atherosclerosis, heart failure or other cardiovascular diseases. It is known that the oxysterol 7-KC accumulates in atherosclerotic lesions and that it induces endoplasmic reticulum stress in vascular cells. In addition, oxysterols are also involved in the pathophysiology of cardiovascular diseases, and therefore, the assumption is that the oxysterol levels might be altered in plasma of AAA patients as well. Levels of free 7-KC and other oxysterols (24-OHCh, 25-OHCh, 27-OHCh, 7 α -OHCh and 7 β -OHCh) were measured in the plasma of 94 AAA patients and 47 healthy controls [185]. Two extraction methods were developed and validated for (1) the quantification of 7-KC and 27-OHCh, using protein precipitation [186] and (2) the quantification of total oxysterols introducing a saponification step, followed by SPE [187,188] (Table 2).

The plasma levels of 7-KC were significantly higher in patients with AAA compared to controls. In contrast, the plasma levels of 24-OHCh, 27-OHCh and 7 α -OHCh were decreased in patients with

AAA, while 7 β -OHCh levels were similar between both groups. The plasma levels of 25-OHCh were undetectable. More interestingly, a positive correlation between 7-KC plasma levels and the vascular expression of endoplasmic reticulum stress markers was found. These findings indicate a strong association between an enhanced expression of endoplasmic reticulum stress markers, OS and 7-KC in AAA [185].

Concluding remarks

Over the years, analyzing OS biomarkers with LC-MS/MS methods has become more and more interesting. As mass spectrometers become more sensitive, there are also more possibilities to measure biomarkers in biofluids, since several biomarkers are only found in low concentrations. Given the often low concentration of biomarkers, a sample preparation technique able to isolate and concentrate the target analytes, is usually required. It is noteworthy that there is an evolution towards automated miniaturized sample preparation techniques, preferably directly coupled to the chromatographic device. The shift towards the use of online fully automated extraction LC-MS/MS will most likely continue in the near future.

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Conflict of interest

The authors have declared no conflict of interest.

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Figure legends

Figure 1: Schematic representation of the link between oxidative stress, antioxidants and lipid peroxidation.

$O_2^{\bullet-}$: superoxide, SOD: superoxide dismutase, CAT: catalase, H_2O_2 : hydrogen peroxide, GSH: glutathione, GSSG: oxidized glutathione, NADPH: reduced nicotinamide adenine dinucleotide phosphate, $NADP^+$: nicotinamide adenine dinucleotide phosphate, $OH^{\bullet}$: hydroxyl radical, eNOS: endothelial nitric oxide synthetase, $ONOO^{\bullet}$: peroxynitrite, PUFA: polyunsaturated fatty acid, $NO^{\bullet}$: nitric oxide; MPO: myeloperoxidase.

Table 1: Newly developed methods quantifying oxidative stress biomarkers in human urine using LC-MS/MS (2015-2020)

Oxidative stress biomarkers	Matrix volume	Analytical column	Analysis time	Application	Publication year	Ref.
8-OHdG, 8-OHG	Not specified	Acquity BEH C18 (50 mm x 2.1 mm; 1.7 μ m)	3 min	To analyze the levels of urinary 8-OHdG and 8-OHG in patients with type 2 diabetes with and without complications (n = 267; n = 366) and healthy controls (n = 683)	2015	[87]
3-NT	250 μ L	Ultra PFPP (150 mm x 2.1 mm; 3 μ m)	7 min	To investigate the pathology of 3-NT and its role as a biomarker of OS in clinical settings (no patients included)	2015	[99]
Isoprostanes, isofurans, neuroprostanes, neurofurans	600 μ L	Kinetex C18 (100 mm x 2.1 mm; 1.7 μ m)	8 min	To investigate possible correlations between lipid peroxidation byproducts and free radical-associated conditions (healthy infants and infants with bronchopulmonary dysplasia, examined from postnatal day 1 - 28)	2015	[126]
8-isoPGF _{2α} , HNE, HNE-MA, 8-OHdG, 8-NO ₂ Gua	20 μ L	Gemini-NX-C18 (50 mm x 2.0 mm; 3 μ m)	20 min	To explore the relationship between OS and the risk for incident stroke (131 patients without stroke and 131 patients with stroke)	2015	[131]
3-NT, NHPA	3 mL	Inertsil ODS-3 C18 (250 mm x 2.1 mm; 5 μ m)	30 min	To investigate the relationship between oxidative and nitrosative stress (method is applied to urine samples from 65 healthy volunteers)	2015	[134]
8-isoPGF _{2α}	100 μ L	Zorbax Eclipse XBD-8 (250 mm x 2.0 mm; 4 μ m)	15 min	To determine 8-isoPGF _{2α} in urine samples and analyze the effects of OS and antioxidant intake (number of volunteers not mentioned)	2015	[139]
MHBMA, DHBMA, 8-OHdG	500 μ L	Supelco RP C18 (75 mm x 3.0 mm; 3 μ m)	20 min	To investigate the correlation between 1,3-butadiene exposure and oxidative damage (59 non-smokers and 233 smokers)	2015	[138]
F ₄ -neuroprostanes and F ₂ -dihomoisoprostanes	Not specified	Acquity BEH C18 (50 mm x 2.1 mm; 1.7 μ m)	10 min	To evaluate the neuroprostanes and dihomoisoprostanes in epileptic patients (n = 15) in comparison with controls (n = 15)	2015	[128]

8-OHdG, 8-NO ₂ Gua, 8-isoPGF _{2α} , HNE-MA	100 μL	Gemini-NX-C18 (100 mm x 2.1 mm; 3 μm)	15 min	The development of a LC-MS/MS method for the simultaneous determination of several OS biomarkers (no patients included)	2016	[36]
8-OHdG	500 μL	Acquity BEH C18 (100 mm x 2.1 mm; 1.7 μm)	5 min	To assess the association with oxidative DNA damage and the risk of colorectal cancer (84 patients and 142 healthy controls)	2016	[103]
8-OHdG, R-cdA, S-cdA	1 mL	Zorbax SB-Aq (150 mm x 2.1 mm; 3.5 μm)	26 min	To investigate the levels of OS biomarkers in patients with prediabetes (n = 47) and type 2 diabetes (n = 43) in comparison with healthy volunteers (n = 27)	2016	[112]
8-isoPGF _{2α}	2 mL	Zorbax Eclipse (50 mm x 2.1 mm; 5 μm)	10 min			

Table 1 continued: Newly developed methods quantifying oxidative stress biomarkers in human urine using LC-MS/MS (2015-2020)

Oxidative stress biomarkers	Matrix volume	Analytical column	Analysis time	Application	Publication year	Ref.
8-OHG, 8-OHdG, 2dG	Not specified	Kinetex XB-C18 (50 mm x 3.0 mm; 2.6 μm)	6.5 min	To evaluate a possible correlation between smoking and the levels of several nucleotides (21 healthy non-smokers and 21 healthy smokers)	2016	[118]
Parabens, bisphenol A, triclosan, 8-OHdG	4 mL	Atlantis C18 (150 mm x 2.1 mm; 5 μm)	18 min	The development of a method for the simultaneous determination of parabens, bisphenol A, triclosan and 8-OHdG (no patients included)	2016	[116]
8-isoPGF _{2α}	Not specified	Acquity BEH C18 (150 mm x 2.1 mm; 1.7 μm)	15 min	To quantify urinary 8-isoPGF _{2α} for the assessment of OS and establish a reference interval for pediatric populations (n = 136)	2016	[101]
8-OHdG, cotinine	20 μL	Agilent Eclipse Plus C18 (100 mm x 4.6 mm; 3.5 μm)	12 min	To investigate the relationship between OS levels, cotinine levels and total NNAL concentrations in urine of humans exposed to cigarette smoke (40 healthy non-smokers and 40 healthy smokers)	2016	[130]
8-OHdG	100 μL	Zorbax XBD-8 (150 mm x 4.6 mm; 3.5 μm)	4 min	To assess the effects of OS by smoking and antioxidant	2016	[140]

		mm; 5 μ m)		intake by green tea or alcohol drinking (3 healthy non-smokers and smokers)		
8-OHdG, 8-OHG	110 μ L	Acquity BEH C18 Shield (100 mm x 2.1 mm; 1.7 μ m)	20 min	To study the effect of smoking in patients with type 2 diabetes (462 daily smokers, 1341 former smokers and 918 never smokers)	2017	[88]
8-OHdG, 8-OHG, 8-oxoGua	Not specified	Synergi Polar RP C18 (150 mm x 4.6 mm; 4 μ m)	25 min	To associate the exposure to benzene and smoking with oxidative damage to nucleic acids (n = 131)	2017	[89]
3-NT	250 μ L	Pentafluorophenyl column (150 mm x 2.1 mm; 3 μ m)	7 min	Integration of miniaturized SPE for the detection of 3-NT in human urine samples (no patients included)	2017	[100]
8-OHdG, 8-OHG	500 μ L	Meteoric Core C18 BIO (50 mm x 4.6 mm; 2.7 μ m)	3.2 min	To optimize and validate a rapid UHPLC-MS/MS method (10 healthy volunteers and 30 cancer patients)	2017	[117]
8-isoPGF _{2α}	200 μ L	XBridge C18 (100 mm x 2.1 mm; 3.5 μ m)	40 min	To examine the association between urinary levels of 8-isoPGF _{2α} and 8-OHdG and to evaluate 8-OHdG levels in patients with coronary disease (n = 52) and in control subjects with cardiovascular risk factors without overt cardiovascular disease (n = 40)	2017	[106]
8-OHdG		Kinetex F5 (100 mm x 2.1 mm; 2.6 μ m)	14 min			
8-OHdG, 8-OHG, 8-iso-PGF _{2α}	1 mL	Poroshell 120 SB-C18 (150 mm x 3.0 mm; 2.7 μ m)	15 min	To determine OS biomarkers in human urine using only one method (40 smokers and 20 non-smokers)	2017	[119]

Table 1 continued: Newly developed methods quantifying oxidative stress biomarkers in human urine using LC-MS/MS (2015-2020)

Oxidative stress biomarkers	Matrix volume	Analytical column	Analysis time	Application	Publication year	Ref.
6-sulfatoxymelatonin, 8-OHdG	397.5 μ L	Synergi Hydro-RP (50 mm x 1.0 mm; 4 μ m)	8 min	To measure the urinary concentrations of 6-sulfatoxymelatonin and 8-OHdG simultaneously in human urine samples (5 volunteers)	2018	[97]

MDA, 8-OHdG, o,o'-dityrosine, 8-isoPGF _{2α} , 15-PGF _{2α} , 8,15-PGF _{2α} , 11-PGF _{2α}	500 μL	Zorbax Aq (150 mm x 2.1 mm; 3.5 μm)	30 min	The development of a method for the analysis of seven biomarkers of OS, based on derivatization and SPE (no patients included)	2018	[122]
8-isoPGF _{2α} , 8-OHdG, 3-NT	100 μL	Inertsil CX (250 mm x 2.1 mm; 5 μm)	20 min	To assess the effect of OS in non-smokers and smokers (3 healthy non-smokers and 3 healthy smokers)	2018	[141]
8-isoPGF _{2α}	200 μL	Xbridge BEH C18 (50 mm x 1.0 mm; 2.5 μm)	12 min	To establish a longitudinal stability test of eicosanoid biomarkers of oxidative damage and inflammation in smokers (clinical trial of 1250 randomized patients)	2019	[110]
PGE-M	400 μL	Kinetex C18 (50 mm x 3.0 mm; 2.6 μm)	12.5 min			
8-oxoGua, 8-OHdG, 8-OHG, MDA	20 μL	Luna PFP (150 mm x 2.0 mm; 5 μm)	17 min	To assess the ability of the OS biomarkers to biomonitor OS <i>in vivo</i> (60 healthy non-smokers, 68 healthy smokers, 43 COPD non-/ex- smokers, 44 COPD smokers and 100 patients with MV)	2019	[132]
8-OHdG	Not specified	Symmetry C8 (150 mm x 2.1 mm; 3.5 μm)	11 min	The development of an online magnetic SPE-HPLC-MS method for the detection of 8-OHdG (7 lung cancer patient and 7 healthy volunteers)	2019	[120]
8-OHdG, OH-PAHs	Not specified	Symmetry C8 (150 mm x 2.1 mm; 3.5 μm)	24 min	To study the health risk of exposure to PAHs (no patients included)	2019	[142]
8-OHG	200 μL	Acquity BEH C18 (100 mm x 2.1 mm; 1.7 μm)	5 min	The development of a novel method for the detection of 8-OHG (65 colorectal cancer patients and 76 healthy volunteers)	2020	[105]
8-isoPGF _{2α}	400 μL	Acquity BEH C18 (150 mm x 2.1 mm; 1.8 μm)	11 min	The development of a SPE-UPLC-MS/MS method for the measurement of free and total 8-isoPGF _{2α} using an automatic liquid handling platform (30 non-smokers and 30 smokers)	2020	[102]

8-hydroxy-2'-deoxyguanosine or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), 3-nitro-L-tyrosine (3-NT), 8-nitroguanine (8-NO₂Gua), 8-iso-prostaglandin F_{2α} (8-isoPGF_{2α}), malondialdehyde (MDA), 11β-prostaglandin F_{2α} (11-PGF_{2α}), 2-deoxyguanosine (2dG), monohydroxybutenylmercapturic acid (MHBMA), N-acetyl-S-(3, 4-dihydroxybutyl) cysteine (DHBMA), Prostaglandin E₂ metabolite, (PGE-M), 4-hydroxy-2-nonenal (HNE), 3-nitro-4-hydroxyphenylacetic acid (NHPA), (5'R)- and (5'S)-8,5'-cyclo-2'-deoxyadenosines (R-cdA and S-cdA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronide conjugate (NNAL), chronic obstructive pulmonary disease (COPD), mechanical ventilation (MV), monohydroxy polycyclic aromatic hydrocarbons (OH-PAHs)

Table 2: Newly developed methods quantifying oxidative stress biomarkers in human plasma, serum or whole blood using LC-MS/MS (2015-2020)

Oxidative stress biomarkers	Matrix volume	Analytical column	Analysis time	Application	Publication year	Ref.
8-OHdG, 8-OHG, 8-OHGua, cGMP, 8-NO ₂ -cGMP, 8-NO ₂ Guo, 8-NO ₂ Gua	1 mL (plasma)	Kinetex HILIC (100 mm x 2.1 mm; 1.7 µm)	10 min	To measure the effect of elite physical exercise by triathletes (5 amateur training women and 10 elite training men)	2015	[175]
3-NT	500 µL (plasma)	Restek ultra-PFPP (150 mm x 2.1 mm; 3 µm)	10 min	The development of a LC-MS/MS method to establish a reference interval and to investigate biological variability (n = 54)	2015	[208]
Isoprostanes/furans, neuroprostanes/furans	100 µL (serum)	Acquity BEH C18 (50 mm x 2.1 mm; 1.7 µm)	8 min	To determine a large number of lipid peroxidation markers in newborn serum samples (n = 20)	2015	[158]
Oxysterol, cholesterol, 25-hydroxy-vitamin D3	200 µL (plasma)	Supelcosil LC-18-S (250 mm x 4.6 mm; 5 µm)	45 min	The development of a robust and effective LC-MS/MS method (56 healthy controls, 148 multiple sclerosis patients and 16 patients with other neurological disorders)	2015	[187]
Cys34 adducts	Not specified (plasma)	PepSwift monolithic (250 mm x 0.1 mm)	Not specified	To investigate the association between Cys34 adducts and smoking status in healthy volunteers (n = 158)	2016	[229]
MDA	50 µL (plasma)	Zorbax Eclipse Plus C18 (50 mm x 4.6 mm; 1.8 µm)	2.5 min	To confirm the known associations between MDA concentrations and obesity in patients with major depressive disorder (n = 26)	2016	[174]
Ubiquinol-10 and ubiquinone-10	100 µL (plasma)	Zorbax SB-C18 (30 mm x 2.1 mm; 3.5 µm)	4.2 min	The development of a quantitative method combining extraction of plasma in isopropanol and direct injection of the extract (n = 10)	2016	[226]
Non-derivatized isoprostanes	1 mL (plasma)	Zorbax SB-C18 (100 mm x 2.1 mm; 1.8 µm)	28 min	The development of a complete quantitative profiling of isoprostanes (method was tested on mice)	2016	[161]
Derivatized isoprostanes			30 min			

8-OHdG	400 µL (plasma)	Acquity HSS T3 (100 mm x 2.1 mm; 1.8 µm)	NA	To gain insight into the determinants of both OS biomarkers, to understand the nature of these markers and to examine the association with metabolic syndrome and chronic disease	2016	[162]
8-isoPGF _{2α}		Acquity BEH C18 (100 mm x 2.1 mm; 1.7 µm)		(1117 patients without current psychiatric diagnosis)		

Table 2 continued: Newly developed methods quantifying oxidative stress biomarkers in human plasma, serum or whole blood using LC-MS/MS (2015-2020)

Oxidative stress biomarkers	Matrix volume	Analytical column	Analysis time	Application	Publication year	Ref.
Isoprostanes/furans, neuroprostanes/furans	100 µL (plasma)	Acquity BEH C18 (50 mm x 2.1 mm; 1.7 µm)	8 min	The development of a method to determine lipid peroxidation biomarkers in newborn plasma (method was tested on piglet plasma)	2016	[160]
7 eicosanoids	100 µL (plasma)	Acquity BEH Shield C18 (100 mm x 2.1 mm; 1.7 µm)	7.5 min	To evaluate the use of ferromagnetic particle enhanced deproteinization as suitable tool in the analysis of eicosanoids (no patients included)	2016	[191]
Methionine sulfoxides	5 µL (serum)	Capcell pack MGIII-H S3 (50 mm x 2.0 mm)	45 min	To accurately quantify methionine sulfoxides in serum proteins (23 patients with type 2 diabetes, 12 patients with diabetes-related chronic renal failure, 9 healthy smokers and 18 healthy non-smokers)	2016	[231]
POVPC	10 µL (serum)	Acclaim C18 (150 mm x 2.1 mm; 3 µm)	51 min	To investigate if POVPC is a modifiable marker of Alzheimer's disease that relates to cognitive performance (21 patients with Alzheimer's disease and 16 healthy controls)	2017	[199]
8-IsoPGF _{2α} , 8,15-PGF _{2α} , 11-PGF _{2α} , 15-PGF _{2α}	400 µL (plasma)	Kinetex C18 (100 mm x 2.1 mm; 2.6 µm)	10 min	To develop a sensitive, robust and fast method for the separation and determination of four isomers of F ₂ -isoprostanes (n = 8)	2017	[168]
28 isoprostanes	100 µL (plasma)	Acquity BEH C18 (100 mm x 2.1 mm; 1.7 µm)	7 min	To develop a comprehensive analytical approach for a broad range of lipid peroxidation products in newborn plasma samples (n = 150)	2017	[165]

GSSG, GSH	60 µL (blood)	HySphere C18 HD (10 mm x 2.0 mm; 7 µm)	1.5 min	To develop a high-throughput assay for GSH after derivatization and GSSG in blood samples (no patients included)	2018	[210]
27 isoprostanes and 8 isofurans	500 µL (plasma)	Zorbax Eclipse Plus C18 (150 mm x 2.1 mm; 1.8 µm)	30.5 min	The development of a LC- MS/MS method covering a comprehensive set of isoprostanes and isofurans from six biologically relevant PUFAs (method was tested on HCT116 cells)	2018	[166]
HETEs, EETs, DiHETEs	500 µL (plasma)	Acquity BEH C18 (100 mm x 2.1 mm; 1.7 µm)	6.4 min	The development of a UPLC- MS/MS method for assessment of different eicosanoids and the quantification of metabolite levels in plasma samples of healthy controls to evaluate the association between niacin administration and eicosanoid response (n = 8)	2018	[195]
Prostaglandins		Acquity BEH C18 (150 mm x 2.1 mm; 1.7 µm)	12 min			
Ethanolamine and choline plasmalogen	300 µL (serum)	Acquity BEH C18 (100 mm x 2.1 mm; 1.7 µm)	21.5 min	To evaluate the distribution of plasmalogens of serum lipoproteins (11 healthy volunteers and 5 patients with NASH)	2019	[201]

Table 2 continued: Newly developed methods quantifying oxidative stress biomarkers in human plasma, serum or whole blood using LC-MS/MS (2015-2020)

Oxidative stress biomarkers	Matrix volume	Analytical column	Analysis time	Application	Publication year	Ref.
GSH and GSSG	100 µL (plasma)	Acquity HSS T3 (50 mm x 2.1 mm; 1.8 µm)	8 min	To investigate how sample preparation procedures affect the level of GSH/GSSG in human plasma (n = 8)	2019	[217]
8-IsoPGF _{2α} , 8-IsoPGE ₂ , PGE ₂	50 µL (blood)	Polaris 3 C18-A (50 mm x 4.6 mm; 3 µm)	18 min	The development of a reliable analytical method combining micro-extraction by packed sorbent and UHPLC in dried blood spots (no patients included)	2020	[169]
Oxidized Met(147) of human serum albumin	5 µL (serum)	Capcell pack MGIII-H S3 (50 mm x 2.0 mm)	45 min	The quantification of the levels of oxidized and non-oxidized Met(147) to determine whether the oxidation status reflects OS induced during	2020	[232]

disease (124 patients with type 2 diabetes and 40 healthy volunteers)						
12 oxidized phosphocholines	10 μ L (plasma)	Luna Omega C18 (50 mm x 2.1 mm; 3 μ m)	24 min	To investigate the distribution of plasma oxidized phosphatidylcholines in patients with CKD and periodontitis as co-morbidity (20 healthy controls, 13 CKD patients, 17 periodontitis patients and 20 patients with CKD and periodontitis)	2020	[197]
9 exocyclic DNA adducts induced by 8 aldehydes	Not specified (plasma)	Acquity HSS C18 SB (150 mm x 1.0 mm; 1.8 μ m)	7 min	The development of a UHPLC-MS/MS method for the quantification of 9 exocyclic DNA adducts derived from 8 main exogenous and endogenous aldehydes (1 smoker and 1 non-smoker)	2020	[178]
3-NT, 3-chlorotyrosine, 3-bromotyrosine	100 μ L (plasma)	Kinetex PFP (100 mm x 2.1 mm; 1.7 μ m)	8.5 min	To determine the association between the products of tyrosine modifications and colorectal cancer presence and progression and their dynamic in the early postoperative period (43 healthy controls and 75 colorectal cancer patients)	2020	

8-hydroxy-2'-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), 3-nitro-L-tyrosine (3-NT), 8-nitroguanine (8-NO₂Gua), 8-iso-prostaglandin F_{2 α} (8-IsoPGF_{2 α}), 11 β -prostaglandin F_{2 α} (11-PGF_{2 α}), 2-deoxyguanosine (2dG), Prostaglandin E₂ metabolite (PGE-M), malondialdehyde (MDA), Hydroxyeicosatetraenoic acid (HETE), epoxyeicosatrienoic acid (EET), dihydroxyeicosapentaenoic acid (diHETE), 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphatidylcholine (POVPC), cyclic guaninemonophosphate (cGMP), 8-nitroguanosine 3',5'-cyclic monophosphate (8-NO₂-cGMP), N ϵ -carboxymethyl-lysine (CML), N ϵ -(1-carboxyethyl)-lysine (CEL), glyoxal hydroimidazolone-1 (G-H1), 3-deoxyglucosone hydroimidazolone (3DG-H), methylglyoxal hydroimidazolone-1 (MG-H1), advanced glycoxidation end products (AGEs), polyunsaturated fatty acid (PUFA), non-alcoholic steatohepatitis (NASH), chronic kidney disease (CKD)

Table 3: Summary of the review, including references to papers related to the significance of an OS biomarker in a clinical context

ROS / RNS	Attack site	Terminal stress metabolites	Disease	Matrix	OS biomarker	Behavior	Fold change	p-value	Ref.
LIPID OXIDATION AND LIPID PEROXIDATION									
Free radicals	PUFAs (mainly arachidonic acid)	Isoprostanes, isofurans, neuroprostanes, neurofurans, eicosanoids, neurofurans, MDA, HNE and ONE	Bronchopulmonary dysplasia	Urine	Isofurans	Increase d: day 1 Increase d: day 3 Increase d: day 4	2-fold 1.5-fold 1.5-fold	p < 0.01 p < 0.01 p < 0.05	[126]
			Incident stroke	Urine	8-IsoPGF _{2α} HNE-MA	Increase d No difference	1.6-fold /	p = 0.004 p = 0.84	[131]
			Epilepsy	Urine	Isoprostanes Neuroprostanes	Increase d No difference	1.5-fold /	p < 0.01 Not significant	[128]
			Prediabetes Diabetes Mellitus	Urine	8-IsoPGF _{2α}	Increase d	2-fold 1.2-fold	p = 0.006 p = 0.28	[112]
			COPD Mechanical ventilation	Urine	MDA	No difference Decreased	/ 1.5-fold	Not significant p < 0.005	[132]
			Brain aneurysm Subarachnoid hemorrhage	Plasma	F2-isoprostanes F4-isoprostanes F2-isoprostanes F4-isoprostanes	Increase d	2.4-fold 8-fold 2.6-fold 9.5-fold	p-value not mentioned (All significant)	[157]
			Colorectal cancer Enteritis	Serum	5-HETE 15-HETE 12-HETE 5-HETE 15-HETE 12-HETE	Decreased Decreased Decreased Increased Increased No	2.6-fold 2-fold 8-fold 3.6-fold 3.3-fold /	p < 0.01 p < 0.01 p < 0.01 p < 0.01 Not significant	[190]

						difference			
			Alzheimer's disease	Serum	8-IsoPGF _{2α}	No difference	/	Not significant	[199]

Table 3 (continued): Summary of the review, including references to papers related to the significance of an OS biomarker in a clinical context

ROS / RNS	Attack site	Terminal stress metabolites	Disease	Matrix	OS biomarker	Behavior	Fold change	p-value	Ref.
DNA AND RNA OXIDATION									
Hydroxyl radical, ONO ⁻ , MDA	DNA / RNA	8-OHdG, 8-OHG, 2-dG, 8-oxoGua, 8-NO ₂ Gua	Diabetes	Urine	8-OHdG 8-OHG	Increased Increased	1.1-fold 1.2-fold	p = 0.02 p < 0.001	[87]
			Prediabetes Diabetes Mellitus		8-OHdG	Increased Increased	3-fold 2-fold	p < 0.0001 p = 0.031	[112]
			Obesity w/o hypertension	Urine	8-OHdG 8-OHG	Increased	1.3-fold 1.2-fold	p = 0.03 p = 0.002	[92]
			Obesity + hypertension		8-OHdG 8-OHG		1.2-fold 1.3-fold	p = 0.03 p = 0.001	
			Alzheimer's disease	Urine	8-OHdG 2-dG 8-OHdG/2-dG	Increased	1.5-fold 1.2-fold 1.8-fold	p < 0.001 p = 0.479 p = 0.019	[94]
			Incident stroke	Urine	8-OHdG 8-NO ₂ Gua	No difference	/	p = 0.78 p = 0.97	[131]
			Several cancers	Urine	8-OHdG 8-OHG	Increased No difference	2-fold /	p-value not mentioned	[117]

			Colorectal cancer	Urine	8-OHdG 8-OHG	Increase d	2-fold 1.5- fold	p < 0.0001	[103 – 105]
			COPD		8-oxoGua 8-OHdG 8-OHG		1.1- fold 1.2- fold 2-fold	p < 0.05 p < 0.05 p < 0.001	
			Mechanical ventilation	Urine	8-oxoGua 8-OHdG 8-OHG	Increase d	3-fold 4-fold 6.5- fold	p < 0.05 p < 0.01 p < 0.01	[132]

Table 3 (continued): Summary of the review, including references to papers related to the significance of an OS biomarker in a clinical context

ROS / RNS	Attack site	Terminal stress metabolites	Disease	Matrix	OS biomarker	Behavior	Fold change	p-value	Ref.
PROTEIN OXIDATION, PROTEIN CARBOXYLATION, OXIDATIVE MODIFICATION OF AMINO ACIDS									
Free radicals, ONE, MDA, HNE, MPO	Proteins and lipoproteins	Protein carbonyl compounds, 3-NT, chlorotyrosine, AGE, oxidized low-density lipoproteins	Alzheimer's disease	Urine	3-NT	No difference	/	p = 0.835	[94]
				Serum	POVPC	Increased	2-fold	p = 0.017	[199]
			Interstitial Lung Disease	Plasma	3-chlorotyrosine 3-NT o,o'-Dityrosine	Increased	4.2-fold 3.1-fold 20.7-fold	p < 0.0001 p < 0.0001 p < 0.0001	[205]
			Niemann-Pick C1 disease	Plasma	3β,5α,6β-triol 7-KC	Increased	5.5-fold 7.9-fold	p < 0.001 p < 0.001	[181 – 183]
			Multiple sclerosis	Plasma	7-KC 7-α-OHCh 24-OHCh 27-OHCh	No difference Decreased No difference Decreased	/ Not given / Not given	Not significant Significant Not significant Significant	[187]

			Diabetes Mellitus Type 1		3 β ,5 α ,6 β -triol 7-KC		3.4-fold 2.3-fold	p < 0.001 p < 0.001	[184]
			Diabetes Mellitus Type 2	Plasma	3 β ,5 α ,6 β -triol 7-KC	Increased	4.3-fold 6.2-fold	p < 0.001 p < 0.001	
			Abdominal aortic aneurysm	Plasma	7-KC 7- β -OHCh 7- α -OHCh 24-OHCh 27-OHCh	Increased No difference Decreased Decreased Decreased	1.8-fold / 1.6-fold 1.5-fold 1.3-fold	p < 0.001 Not significant p < 0.001 p < 0.001 p < 0.001	[185]
			Colorectal cancer	Plasma	3-NT 3-chlorotyrosine 3-bromotyrosine	Increased Increased Increased	7.7-fold 2.3-fold 5-fold	p = 0.003 p = 0.044 p = 0.005	[209]

8-hydroxy-2'-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), 2'-deoxyguanosine (2-dG), 8-oxo-7,8-dihydroguanine (8-oxoGua), 8-nitroguanine (8-NO₂Gua), malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 4-oxo-2-nonenal (ONE), chronic kidney disease (CKD), 3-nitro-L-tyrosine (3-NT), advanced glycation end products (AGE); chronic obstructive pulmonary disease (COPD), 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC), cholestan-3 β ,5 α ,6 β -triol (3 β ,5 α ,6 β -triol), 7-ketocholesterol (7-KC), hydroxycholesterol (OHCh)