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Urinary Biomarkers of Oxidative Status

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Abstract

Oxidative damage produced by reactive oxygen species (ROS) has been implicated in the etiology and pathology of many health conditions, including a large number of chronic diseases. Urinary biomarkers of oxidative status present a great opportunity to study redox balance in human populations. With urinary biomarkers, specimen collection is non-invasive and the organic/metal content is low, which minimizes the artifactual formation of oxidative damage to molecules in specimens. Also, urinary levels of the biomarkers present intergraded indices of redox balance over a longer period of time compared to blood levels. This review summarizes the criteria for evaluation of biomarkers applicable to epidemiological studies and evaluation of several classes of biomarkers that are formed non-enzymatically: oxidative damage to lipids, proteins, DNA, and allantoin, an oxidative product of uric acid. The review considers formation, metabolism, and exertion of each biomarker, available data on validation in animal and clinical models of oxidative stress, analytical approaches, and their intra- and inter-individual variation. The recommended biomarkers for monitoring oxidative status over time are F_2-isoprostanes and 8-oxodG. For inter-individual comparisons, F_2-isoprostanes are recommended, whereas urinary 8-oxodG levels may be confounded by differences in the DNA repair capacity. Promising urinary biomarkers include allantoin, acrolein-lysine, and dityrosine.

Keywords

Reactive Oxygen Species; Oxidative stress; Urine; Biomarkers; F2-Isoprostanes; Malondialdehyde; 8-oxodG; Allantoin; Dityrosine; Acrolein

1. Introduction

The focus of this review is to evaluate the applicability of existing biomarkers of oxidative status to human studies or epidemiological research. This involves consideration of many factors (which are discussed later, Table 1); and therefore, such evaluation can only be conducted for already studied, as opposed to novel, biomarkers. For this reason, we focus on oxygen-derived damage to biological molecules, because biomarkers of damage produced by reactive nitrogen species are less studied.

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1.1 Conceptual framework: oxidative stress versus oxidative status

Reactive oxygen species (ROS) are constantly produced in aerobic organisms by normal metabolic processes, such as cellular respiration, antibacterial defense, and others [1]. In addition, external exposures (such ionizing radiation, smoking, and toxins) also induce production of ROS [1]. As a result, exposure to ROS is ubiquitous, and a certain level of oxidative damage is always present in any individual. To counteract their damaging effects, aerobic organisms have developed multiple defense systems [1]. These antioxidant agents include enzymes (such as superoxide dismutase, catalase, glutathione peroxidases), sequesters of metal ions, and endogenous antioxidants (e.g. glutathione, ubiquinol, bilirubin, uric acid, α-tocopherol, and ascorbic acid). The two opposing processes–ROS production and antioxidant defense–set constitutive levels of ROS within the tissues and at the systemic level. Differences in both the intensity of ROS generation and the effectiveness of the antioxidant defense produce variability in oxidative status between individuals [2]. Variability in oxidative status within an individual between tissues as well as between individuals results from a complex interaction of multiple factors, including genetic [3;4] and epigenetic differences, endogenous promoters of ROS (such as iron and copper) [1], chronic inflammation [5;6] or other chronic conditions. It should be noted that most chronic conditions occur at the tissue level, while most biomarkers consider oxidative stress at the systemic level, with the implicit assumption greater tissue-specific ROS production will be reflected by an increased systemic oxidative status. Although there is no evidence to support or refute this assumption, a distinction between tissue-specific and systemic oxidative status should be acknowledged.

The term "oxidative stress" is widely used, but, as noted by Halliwell, this term "is vaguely defined", referring to "a serious imbalance between production of reactive species and antioxidant defense"[7]. Because some levels of oxidative damage are present in every individual, the question arises as to which levels represent a "normal" (non-stress) range versus pathological elevation, which could be defined as oxidative stress. Because of this uncertainty, we believe that the term "oxidative status" is a term that can be more logically and consistently applied to both stress and non-stress states of oxidative load. For example, relatively large scale human studies (n 100) reveal a wide variation of any oxidative status biomarker in human populations. For example, in 2,828 subjects of the Framingham Heart Study, urinary levels of iPF 2α-III (a marker of lipid peroxidation) ranged from 10 to 1845 ng/mmol creatinine[8]. In 100 healthy children and adolescents, the range 8-OHdG (a marker of DNA oxidative damage) levels in urine was 4.6–27.2 ng/mg creatinine [9]. It is not clear which levels should be considered "normal" (non-stress) and which represent a serious imbalance between ROS generation and antioxidant defense (stress). The term "oxidative status" therefore seems more applicable.

1.2 Requirements for oxidative status biomarkers applicable to human studies

Because ROS have short lifetimes and cannot be directly detected in humans[10], a reasonable alternative approach is the measurement of biomarkers that are the products of non-enzymatic reactions between biological molecules and ROS [1;7]. The involvement of enzymes in the formation of biomarkers would introduce an inaccessible level of variability, and so these products do not make good biomarker candidates. Assessment of non-enzymatically formed biomarkers circumvents this problem and provides a direct index of the extent of oxidative modifications produced by ROS. Although the levels of such oxidative modifications do not measure the ROS levels *per se*, they are assumed to be proportional to the ROS levels. Therefore, the core requirement for a biomarker of oxidative status is its validation *in vivo* against a known oxidative stressor, i.e. a compound that produces ROS in biological systems as measured by electron spin resonance spectroscopy directly. In response to this well-recognized need, the National Institute of Environmental

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Health Sciences (NIEHS) has established an initiative to conduct a comparative study of biomarkers of oxidative stress (BOSS). The BOSS project tests responsiveness and specificity of the commonly used oxidative indices in an established model of oxidative stress–carbon tetrachloride (CCl_4) poisoning in rodents [11–14]. Similar to this approach, we developed a clinical model of oxidative stress, based on doxorubicin (DOX)-based chemotherapy [15;16]. DOX has been demonstrated to generate superoxide and hydrogen peroxide *in vitro*; this ROS production has been observed in animals, at pharmacological levels, using electron spin resonance spectroscopy [17;18]. This and other important characteristics for evaluation of biomarkers are presented in Table 1. Currently, only a handful of oxidative status biomarkers have been validated in either animal or clinical models.

This review focuses on urinary biomarkers because they represent the least invasive way to assess individual oxidative status and can be used in large-scale human studies. Also, urine is a better matrix than blood/plasma for measurement of oxidative modifications of biological molecules, because it has a much lower organic as well as inorganic metal content, i.e. lower levels of the material that can be oxidized as well as lower levels of the ROS promoters. Therefore, urine is less liable to artificial increase of oxidative markers during sample collection and storage.

2. Oxidative Modifications of Lipids in Urine

2.1 F_2-isoprotanes

F_2-isoproatnes are formed during non-enzymatic oxidation of arachidonic acid by different types of free radicals, including reactive oxygen species [19;20]. Depending on the position where the oxygen molecule is added to arachidonic acid, four regioisomers are formed, giving each of the four F_2-isoprostane series. Furthermore, each series comprises 16 stereoisomers. Mainly two nomenclatures are used for isoprostanes (Taber *et al.*[21] and Rokach *et al.*[22]). However, other nomenclatures of isoprotanes may be found in the literature, potentially confusing readers [23].

F_2-isoprotanes can be measured in detectable quantities in human blood and urine in the general population as well as in pathological conditions [2;8;23;24]. F_2-isoprotanes and their metabolites, excreted in urine, are chemically stable compounds [25;26] and their urinary excretion levels are not sensitive to dietary intake of lipids [27–29]. The existing data indicate that levels of urinary F_2-isoprotanes are relatively stable within individuals (especially when assayed in first morning urine void) [30;31] but are widely variable in human populations[8;32], and are therefore, highly useful as biomarkers for human studies. Urinary F_2-isoprotanes levels have been validated as sensitive biomarkers of oxidative stress in animal [12] and clinical [16] models, making these biomarkers a valuable tool for assessing oxidative status.

Quantification of F_2-isoprotanes in urine presents a challenge as to detecting specific isomers in the mix of highly structurally similar 64 stereoisomers and their metabolites. For a comprehensive review of analytical techniques used to quantify F_2-isoprotanes in urine we refer the reader to several reviews published earlier [23;33]. Briefly, the three main techniques used to assay F_2-isoprotanes in urine are gas-chromatography with mass spectrometry detection (GC-MS), liquid chromatography with tandem mass spectrometry detection (LC-MS/MS), and enzyme-linked immunosorbent assay (ELISA). The ELISA-based assay is inherently inferior as compared to the chromatography-based techniques due to cross-reactivity [34]. Some assays report not specific isomers but "urinary F_2-isoprostanes"; whether or not this represents a value for all F_2-isoportanes in urine is not defined. These assays are based on quantification of composite peaks, using a specific

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isomer that has similar elution time [35;36]. These peaks comprise many isomers and possibly some interfering compounds [37;38]. Correlation between such composite measurements and a single-isomer measurement sometimes is low (approximately 0.3), suggesting that these two assays do not measure the same physiological parameter [39]. At the same time, correlation between four individual isomers in our studies was approximately 0.5 and higher. Therefore, interpretation of the composite peak measurements is not straightforward, as opposed to the measurements of specific isomers. It is especially important to quantify specific isomers as opposed to composite peaks, because specific isomers may produce different epidemiological results, some showing an association with a chosen outcome and some not. In our studies, all four F_2-isoprostane isomers have been validated in a clinical model of oxidative stress [16]; however, their associations with different outcomes may or may not vary [24;32;40]. For example, the associations between four urinary F2-isoprostane isomers and type 2 diabetes risk were similar [24], whereas the association with the risk of weight gain varied between the same isomers [32]. Thus, specific F_2-isoprostane isomers may be more or less sensitive to various outcomes, which could be explained by an assumption that different sources of free radicals favor differential production of F_2-isoprotane isomers and their metabolites.

Halliwell and Lee [41] raise a question on whether differences in the rate of hydrolysis of F_2-isoprotanes from esterified phospholipids and their beta-oxidation, (a pathways that is not related to oxidative stress) can influence the results of human studies. The authors considered a scenario when hydrolysis of F_2-isoprotanes from phospholipids may be reduced due to some chronic processes, resulting in lower levels of urinary F_2-isoprotanes [41]. There are some *in vitro* data, suggesting that intra-individual differences exist in the rate at which F_2-isoprotanes are hydrolyzed from plasma lipids [42]. The same *in vitro* study suggests that hydrolysis from cellular membranes may vary between individuals [42]. Whether or not the suggested intra-individual differences in hydrolysis of F_2-isoprotanes influence its steady-state urinary excretion and confounds the estimate of oxidative status remains unknown.

Finally, we would like to comment on a fundamental epidemiological fallacy so commonly found in studies on the relationships between urinary F_2-isoprotanes and chronic conditions. A large majority of such studies are cross-sectional and frequently show a positive association between F_2-isoprostanes and some chronic disease [23]. Such elevations are commonly interpreted as causal despite the fact that cross-sectional study design cannot distinguish whether the detected association is a cause or a consequence of the outcome of interest. In contrast, a handful of prospective studies show inconsistent results for the association with cancer risk [43–45] and inverse associations with the risk of weight gain [32;46] and type 2 diabetes [24]. This discrepancy between the cross-sectional and prospective results strongly suggest that elevation of urinary F_2-isoprotanes most likely present a consequence rather than a cause of chronic diseases and calls for rigorous epidemiological methodology in testing whether the detected cross-sectional associations are causal or not.

2.2 Aldehydes Formed in Lipid Peroxidation

Similar to F_2-isoprostanes, several highly reactive and therefore toxic aldehydes are formed as a result of peroxidation of polyunsaturated fatty acids [1;47]. Some of these aldehydes have been used as biomarkers of oxidative damage, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), 4-oxo-2-nonenal (4-ONE), and acrolein. One of the major problems in quantifying these compounds in biological matrices is their metabolism *in vivo* [48]. This will be discussed in more detail below.

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MDA is a frequently used biomarker that is measured in plasma, urine, or tissue as thiobarbituric acid-reactive (TBAR) material. Although the simple TBAR assay has been extensively criticized as being non-specific for MDA [7;49], it remains widely used. The assays with improved specificity for MDA include HPLC/GC-based isolation of (MDA)-TBAR product [12;50;51] and spectrophotometric detection of a colored pigment–produced by MDA and N-methyl-2-phenylindole [2]. Previous attempts to measure MDA in urine had been unsuccessful, and their use in such assays as markers of oxidative status was discouraged [52]. Several assays involving different MDA derivatizations have been proposed, which may revive the use of these assays in humans [53–55]. However, information on intra-individual variation for these assays, including urinary levels, could not be found.

Animals model showed that plasma and urine MDA measured by a GC-based assay increase in response to administration of carbon tetrachloride, a known oxidative stressor, suggesting that plasma and urinary MDA are potentially sensitive biomarkers of oxidative status [12]. However, even improved assays involving the HPLC-based isolation of (MDA)-TBA product failed to increase in response to DOX administration in humans, suggesting differential validity of oxidative status measurements for using MDA in animals and humans [15]. This discrepancy may have been caused by several non-mutually exclusive explanations. For example, DOX is given at a physiological dose in the clinic, where causing oxidative stress is not the primary aim and the oxidative stress levels caused by the drug itself may be lower than in animal models. Conversely, the doses of CCl_4 that are used in animal studies are given at pharmacological doses, specifically to cause oxidative stress, which may explain the differences in MDA measurements between the studies. Also, DOX and CCl4 may vary significantly based on their location of ROS generation and in the type of damage they tend to produce. However, the extent of oxidative damage produced by DOX-based therapy is likely to be one of the strongest experienced by humans, as all urine samples collected 24 hours after DOX injection showed heavy precipitate of dead cells. Thus, if such a strong systemic oxidative exposure as DOX injection does not produce changes in plasma MDA measured by the improved assay, it is doubtful that MDA can serve as a biomarker of oxidative status in humans populations. Also, MDA formation is not specific to free radical-induced oxidation of lipids [49;56;57]; and therefore, its contribution to the entire pool may depend on the avenue of oxidative assault. Also, besides non-specificity, it appears that urinary MDA levels are confounded by dietary MDA content [52;58], which also may explain the discrepancy between the animal and human models of oxidative stress. Taking into account non-specificity of MDA formation in relation to ROS-induced oxidation, lack of association in a clinical model of oxidative stress, and the possibility for dietary confounding, MDA cannot be recommended as a systemic biomarker of oxidative status.

4-HNE and 4ONE are highly reactive aldehydes and easily form covalent bonds with protein thiol and amino groups and with other biological molecules[59]. Therefore, their free levels are unstable. 4-HNE is rapidly metabolized by inducible enzymes[60], with glutathione S-transferases playing a key role in this pathway[61;62]. Also, the likelihood of high intra-individual variation due to inducibility of 4-HNE-metabolizing enzymes, an important source of inter-individual variation (not related to the production of ROS) is introduced by known polymorphisms in glutathione S-transferases [63]. There have been attempts to quantify urinary levels of 1,4-dihydroxynonane-mercapturic acid (DHN-MA), the end-product of 4-HNE metabolism, as a biomarker of oxidative status in animals and humans [64–67]. It appears that this metabolite is sensitive to oxidative stress produced in rats by administration of bromotrichloromethane, a known inducer of lipid peroxidation [68]. However, as noted above, formation of this metabolite may be confounded by genetic polymorphisms in human populations. The data related to intra- and inter-individual

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variation of 4-HNE and its metabolites has not been found. Also, formation of 4-HNE has been detected in meat [69], although it is likely that such a reactive compound would be exerted in urine as amino acid or protein adducts.

4-ONE is less studied as a biomarker of oxidative status as compared to 4-HNE. However, similar principles in evaluating this compound as a biomarker can be applied. 4-ONE is also a reactive aldehyde and its free levels are most likely to be unstable. Metabolism of 4-ONE is similar to 4-HNE and involves inducible and polymorphic enzymes. Thus, quantification of 4-HNE and 4-ONE metabolites in urine [70] is liable for uncontrolled variation due to reasons other than ROS production within an individual.

Acrolein is the most reactive of the aldehydes produced from lipid peroxidation. Excretion of its lysine adducts has been used, although rarely, as a biomarker of oxidative status in a pediatric population using ELISA [9]. The obvious pitfall of this assay is cross-reactivity, which may be a barrier in experimental application of this biomarker, presenting a problem in application to human populations due to intra- and inter-individual variability of cross-reacting molecule levels. For example, we found such correlations between urinary F_2-isopronates detected by ELISA and GS-MC depended on urine diluteness, which was measured by the concentration of urinary creatinine (unpublished data). Another possible pitfall of this assay is the evidence that acrolein-lysine is not an end product and undergoes further thiolation [71]. Whether or not the latter is recognized by the same antibodies remains unknown.

In summary, aldehydes produced in lipid peroxidation have been used as biomarkers of oxidative status. However, high reactivity of these aldehydes questions the detection of their free levels as valid measurements of oxidative status. Moreover, these aldehydes are quickly metabolized. Furthermore, the levels of these urinary metabolites are likely to depend more on individual exposures, which induce xenobiotic metabolizing enzymes, and on genetic polymorphisms. Therefore, use of these biomarkers in human populations is not recommended [48].

3. Oxidative Modification of Proteins in Urine

3.1 Protein adducts produced by lipid peroxidation products

These biomarkers have been partially discussed in the section 2.2. In this section, we summarize the mechanism of their formation and their qualities as biomarkers. As indicated in the previous section, the more commonly studied products of lipid peroxidation include MDA, 4-HNE, 4-ONE, and acrolein. Each of these products can form adducts to the amino acid residues of protein, predominately targeting cysteines, histidines, and lysines [72–74]. Many of these reactions occur via Michael addition reactions; in some cases these products can further form a more stable Schiff base. Formation of 4-HNE adducts was detected *in situ* and *in vivo* in response to oxidative damage. Specifically, 4-HNE-amino acid adducts were formed in hepatocytes oxidized with tert-butylhydroperoxide or metal ions [75] and in rat plasma in response to iron overload, a known promoter of ROS [76]. Acrolein-lysine adducts were formed *in vitro* in LDL, as a result of copper-catalyzed oxidation [77]. There are some indications that acrolein-lysine is the main product caused by the reaction of acrolein to protein [78].

Because these adducts are in general more stable than the aldehydes from which they were derived, they have been considered as better biomarkers of oxidative status [79]. Another potential advantage is that these adducts may reflect the extent of oxidative damage to proteins that cause protein dysfunction. However, this potential has not been fully realized for different reasons. MDA-amino acid adducts, as MDA alone, cannot be considered

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specific to ROS-related oxidation; their urinary levels may be confounded by diet [80]. Information on urinary levels of amino acid adducts produced by 4-HNE and 4-ONE could not been found. However, acrolein-lysine adducts have been measured by ELISA in human populations, including healthy individuals as well as patients with type 2 diabetes, Alzheimer's disease, and atopic dermatitis [9;81–83]; positive cross-sectional associations have been found with all three conditions [81–83]. However, cross-reactivity of ELISA continues to be a major barrier to application of this biomarker. Overall, aldehyde-amino acid adducts represent potentially understudied biomarkers of oxidative status and oxidative protein damage. Further research in this field will be needed before the exact role and utility of these compounds can be clearly understood.

3.2 Dityrosine

Dityrosine is formed by free-radical attack on a wide range of proteins and has been proposed as a good indicator of protein oxidation [7;84–88]. Dityrosine arises from the tyrosine radical, which can be generated by many reactive species, including hydroxyl radicals and peroxynitrite [89]. Its production is proportional to the overall extent of oxidative insult as well as the rate of radical formation [7;85]. However, since many reactive species may participate in dityrosine formation, the biomarker may not even be able to discriminate between oxidative versus nitrosative stress.

Formed in proteins, dityrosine marks damaged proteins for degradation and is excreted in urine [90;91]. Importantly, radiolabeled dityrosine injected in animal models is quantitatively excreted in urine [90], and is not reincorporated into newly synthesized proteins or metabolized to other compounds [87]. Urinary dityrosine can be measured *via* chromatographic separation with mass spectrometry detection; these methods are reviewed by DiMarco and Giulivi [92]. Currently, absence of a commercially available standard and internal standard present the major challenges for wide application of these methods.

Dityrosine can be measured in the urine of healthy individuals [87;93;94] as well as in pathological conditions thought to be associated with oxidative stress [90;93;95;96]. Although this biomarker has not been validated in a model of oxidative stress, it has been shown to be sensitive to the increased oxidative load induced by exercise [94]. The range of inter-individual variation of urinary dityrosine in healthy subjects is at least fourfold [94]. Intra-individual variation has not been rigorously studied, although it was shown that urinary dityrosine levels do not vary significantly during the day [94]. Thus, dityrosine appears to be a promising biomarker of oxidative status. Availability of a chemical standard could help to further studies of this biomarker.

3.3 Advanced glycation products

Advanced glycation end-products (AGE) have been considered as urinary biomarkers of oxidative stress. Nε-(caroboxymethyl)lysine (CML) and pentosidine are among some of the more commonly studied biomarkers [97]. The formation of AGEs does not directly involve oxidative stress, although Halliwell and Gutteridge suggested that oxidative stress contributes to AGE formation [1]. AGEs form as a result of non-enzymatic glycation reactions to proteins and DNA. These slow reactions with the DNA or protein, which reach equilibrium over days or weeks, begin by formation a Schiff base which can then be converted to a more stable Amadori product [98–100]. These non-ROS products are stable enough to be excreted and easily detected in urine [101;102].

AGEs themselves are thought to be associated with increased ROS production; however, these associations are extremely complex. Elevated levels of carbohydrates, which would favor the formation of AGEs, have been shown to increase the amount of ROS produced

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from mitochondria [103;103]. CML may act as a chelator of redox active copper and thereby increase oxidation of ascorbate, reducing antioxidant potential [104]. AGEs have also been shown to cause the activation of NF-κB, a transcription factor with known roles in cellular response to oxidative stress [105–109]; this in turn may induce upregulation of antioxidant enzymes (e.g. manganese superoxide dismutase and thioredoxin-1). On the other hand, this activation of NF-κB is also capable of increasing ROS production *via* upregulation of ROS producing genes (e.g. xanthine oxidase and NADPH oxidase-2) and by generally enhancing a pro-inflammatory state [109]. Thus, AGEs can have paradoxical relationships to both increasing and decreasing cellular oxidative stress, depending on conditions.

AGEs have gained interest because of their observed associations with several disease states, including cardiovascular disease, Alzheimer's disease, cancer, and in particular diabetes [110–116]. As such, AGEs may be useful biomarkers of metabolic disorders and disease, but because they are not derived directly from ROS-mediated processes and their connections to oxidative stress are complex, their use and interpretation as urinary biomarkers of oxidative status should be avoided.

4. Oxidative Modifications of DNA in Urine

ROS attack on DNA produces a large number of purine and pyrimidine-derived lesions [117]. The most studied biomarker is 8-hydroxy-2′-deoxyguanosine (8-oxodG), a stable end product of non-enzymatic DNA oxidation. Urinary 8-oxodG levels have been validated as a sensitive biomarker of oxidative stress in an animal model using the administration of CCl_4 [12]. Similar validation against a known oxidative stressor in humans has not been conducted, although an increase in urinary 8-oxodG was documented in cancer patients undergoing chemotherapy with cisplatin [118].

Most of the issues regarding applicability of 8-oxodG to human studies have been extensively discussed [119–121] so we will briefly summarize this discussion. Urinary levels of 8-oxodG represent the product of DNA excision repair with involvement of multiple enzymes that have a complex regulation [120;122]. Neither cell death [118;120] nor diet [123;124] contribute considerably to urinary 8-oxodG; and its levels are not influenced by long-term storage of urine specimens at −20°C [125]. Measurements of urinary 8-oxodG include chromatography-based methods and ELISA, with chromatography-based techniques showing low inter-assay variability [120]. Although several improvements of ELISA have been discussed [119], cross-reactivity continues to be a significant problem [126].

Diurnal variation of urinary 8-oxodG measured by ELISA was found to be substantial; however, the same study found good correlation (r=0.75) between 24-hour urine collection and morning samples, although this study is based on a small sample size (n=5) [127]. Two other intra-individual variation studies also included small number of subjects (n=2 and 3), concluding that intra-individual variation of 8-oxodG levels in spot urine is high [128;129]. A study with a sufficiently high number of individuals (n=68) came to the same conclusion [130], although the results of the statistical analysis are presented as coefficient of variation for each individual, and partitioning total variance observed into intra- and inter-individual variation would have been more informative. As a result, the question of whether or not intra-individual variation of this biomarker presents the main source of total variation remains open.

One rarely discussed, although important, aspect of urinary 8-oxodG levels is variation in DNA repair capacity, which presents a source of inter-individual variability that is not related to ROS-induced damage [131–134]. For example, healthy volunteers and lung cancer patients showed similar urinary excretion of 8-oxodG, but the activity of DNA repair was

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greater in healthy volunteers, suggesting that lung cancer patients accumulate oxidative damage in DNA at higher rates [135]. Moreover, intra-individual variations in DNA repair responsible for excision of 8-oxodG has been shown to vary in human populations [136].

Although many conditions and exposures have been associated with urinary 8-oxodG levels [120], the only epidemiological study examining a prospective association with lung cancer showed a definitively null association: the relative risk estimate for the association of lung cancer per doubling of 8-oxodG excretion was 0.96 (95% confidence interval 0.78–1.19) [125]. Stratified analysis showed null associations for former and current smokers and a positive association among never-smokers with the relative risk estimate of 11.8 (95% confidence interval: 1.21–115). Based on a small sample size (eight cases and eight comparison subjects) and the corresponding wide confidence interval, this estimate can be considered only as a preliminary indication that the association between urinary 8-oxodG levels and lung cancer differs by smoking status. Thus, this prospective study does not provide evidence that increased levels of urinary 8-oxodG are associated with increased risk of lung cancer.

To summarize, urinary 8-oxodG levels are often presented as a valid biomarker of oxidative status in humans. Although this biomarker was shown to have many characteristics necessary for applicability to human studies, several questions remain unanswered. The most important unknown is how much inter-individual variation of DNA repair capacity contributes to urinary 8-oxodG variation in human populations. Even though the null association with lung cancer was detected by Loft and others [125], the question of whether oxidative DNA damage contributes to the risk of other chronic conditions remains unknown.

5. Allantoin

Allantoin is the predominant product of non-enzymatic oxidation of uric acid by many types of free radicals [7;137;138]. Whereas in most mammals allantoin is formed by enzymatic oxidation of uric acid (that is catalyzed by urate oxidase), in humans allantoin is formed only by non-enzymatic oxidation of urate(because urate oxidase is silenced in humans by several mutations) [139]. Thus, uric acid presents as the terminal product of purine metabolism in humans, with basal urate levels much higher than in other species. As a potent scavenger of ROS, uric acid can serve as an abundant, systemic antioxidant and as a natural trap for ROS [140]. Importantly, variation of uric acid levels do not correlate with variation in allantoin, indicating that formation in allantoin is independent of uric acid levels [141]; therefore, allantoin can serve as a human-specific biomarker of systemic oxidative status.

Allantoin is measurable in the urine of healthy individuals [24;141;142]. Historically, allantoin has been measured by the colorimetric assay based on the Rimini–Schryver reaction [143]. Currently, several improved methods using LC-MS/MS have been published [142;144;145] with some involving minimal sample preparation [142]. Urinary allantoin varies widely between individuals (more than seven-fold in our sample of 320 individuals) [24]. Although rigorous analysis of intra-individual variation of urinary allantoin is not available, there are indications that mean values of the systemic levels of allantoin remain stable over different time periods [146–148]. Allantoin concentration in urine is not affected by freeze/thaw cycles, sample preparation, or by storage at room temperature for up to 6 days, 12 days at 4°C, or, at minimum, 15 weeks at −70°C [142;149].

Allantoin increased in response to DOX injection in a clinical model of oxidative stress with slightly different dynamics compared to urinary F_2-isoprostanes [16;150]. Additional data, such as responses to exercise (increase in urine as well as in plasma and muscle) [151] and

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ozone challenge (increase in nasal-lining fluids) [152], provide additional evidence that allantoin is a valid biomarker of oxidative status in humans.

6. Conclusions

Urinary biomarkers present a great opportunity to conduct large-scale studies because specimen collection is non-invasive and the organic and metal content is low, minimizing sample oxidation during collection and storage. Also, urinary levels of the biomarkers present intergraded indices of redox balance over a longer period of time compared to blood levels, which may make them more sensitive to predicting chronic conditions while also decreasing intra-individual variability of the measurements.

Studies of oxidative status in humans rely on biomarkers of oxidative damage. Only valid biomarkers may produce reliable results. Therefore, validation of a proposed biomarker *in vivo* against a known oxidative stressor is the key requirement for applicability of the biomarker to human studies. Only four biomarkers presented in this review have been validated in animal or clinical models; this includes F_2-isoprostanes, MDA detected by HPLC, 8-oxodG, and allantoin. The unanswered questions about these biomarkers are summarized below.

1. Urinary F_2-isoprostanes: This is a well-studied and validated biomarker of oxidative status, with known inter- and intra-individual variations. However, there is some concern that inter-individual variation in F_2-isoprostane hydrolysis form esterified lipids that could contribute to the differences in their steady-state urinary levels.

2. Urinary MDA: MDA has been heavily criticized on many accounts but mostly as an unstable and non-specific biomarker of oxidative status, although this biomarker has been validated in an animal model of oxidative stress. A clinical model of oxidative stress showed that plasma MDA, using HPLC separation, is not a sensitive index of oxidative status in humans, questioning the validity of urinary MDA as well.

3. Urinary 8-oxodG: 8-oxodG has been validated as a biomarker for oxidative stress in animal models, but so far this has not been shown in human studies. The impact of inter-individual variability in DNA repair capacity on the levels of 8-oxodG in urine remains unknown. Intra-individual variability is also uncertain. However, this biomarker can be useful as a monitoring tool.

4. Urinary allantoin: Allantoin has been validated in a clinical model of oxidative stress, and it has shown good stability in a variety of storage conditions. Also, handling methods prior to sample analysis seem to be relatively minor and might introduce a small number of artifacts. However, inter- and intra-individual variation of this biomarker needs to be investigated further.

There are also promising understudied biomarkers, such as acrolein-lysine and dityrosine, both of which reflect oxidative protein damage. Addition of these biomarkers to the panel of validated indices of oxidative status will diversify the existing tools in studying the role of oxidative status in human health and disease.

Application of urinary biomarkers of oxidative status has been for the most part limited to cross-sectional studies. As cross-sectional study design does not allow for distinguishing between cause and consequence of a health condition, more prospective studies are needed. Therefore, all prospective studies including clinical trials are encouraged to collect and store urine specimens for future analyses.

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Highlights

- Urine presents a valuable resource to study redox balance in human populations.
- A complex set of characteristics are required for urinary biomarkers to be valid biomarkers of oxidative status.
- F_2-isoprostanes and 8-oxodG are the best characterized urinary biomarkers and are recommended for monitoring the oxidative status of individuals over time.
- F_2-isoprostanes are recommended for comparisons of oxidative status between individuals; such comparison using 8-oxodG may be confounded by individual capacity of DNA repair.
- Allantoin, acrolein-lysine, and dityrosine are recommended for future studies as promising urinary biomarkers of oxidative status.
- Most comparisons of oxidative status biomarkers between patients with chronic conditions and controls are cross-sectional; no conclusion on causality can be made based on cross-sectional association.
- For ROS-related damage, prospective studies are needed to prove or disprove their causality to chronic conditions.

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Table 1

Required characteristics of biomarkers for epidemiological research

Important considerations	Required Characteristics	
Relevance to biology of free radicals	**1**	The biomarker should be a specific product of ROS-induced oxidation
	2	The biomarker should increase in response to a known oxidative stressor (validation criterion)
Analytical issues	**3**	An assay for detection of the biomarker should be specific and not interfere with other substances
	4	The biomarker should be a chemically stable compound
Specimen collection and storage	**5**	Specimen collection should be non-invasive
	6	Storage of specimens should not produce artefactual increase of the biomarker
Application to human studies	**7**	The biomarker should be detectable in biological fluids of healthy individuals before the onset of a disease
	8	Measurements should not be confounded by diet or by the concentration of non-oxidized parent molecule
	9	Levels of the biomarker should have low within-person variability