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REVIEW ARTICLE

Assays for urinary biomarkers of oxidatively damaged nucleic acids

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Abstract
The analysis of oxidized nucleic acid metabolites can be performed by a variety of methodologies: liquid chromatography coupled with electrochemical or mass-spectrometry detection, gas chromatography coupled with mass spectrometry, capillary electrophoresis and ELISA (Enzyme-linked immunosorbent assay). The major analytical challenge is specificity. The best combination of selectivity and speed of analysis can be obtained by liquid chromatography coupled with tandem mass spectrometric detection. This, however, is also the most demanding technique with regard to price, complexity and skills requirement. The available ELISA methods present considerable specificity problems and cannot be recommended at present. The oxidized nucleic acid metabolites in urine are assumed to originate from the DNA and RNA. However, direct evidence is not available. A possible contribution from the nucleotide pools is most probably minimal, if existing. Recent investigation on RNA oxidation has shown conditions where RNA oxidation but not DNA oxidation is prominent, and while investigation on DNA is of huge interest, RNA oxidation may be overlooked. The methods for analyzing oxidized deoxynucleosides can easily be expanded to analyze the oxidized ribonucleosides. The urinary measurement of oxidized nucleic acid metabolites provides a non-invasive measurement of oxidative stress to DNA and RNA.

Keywords: *DNA oxidation, RNA oxidation, GC-MS, HPLC, tandem mass spectrometry, ELISA*

Abbreviations: *5HMdU, 5-hydroxymethyl-2′-deoxyuridine; 5HMUra, 5-(hydroxymethyl)uracil; 5OHUra, 5-hydroxyuracil; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoAde, 8-oxo-7,8-dihydroadenine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; BER, base excision repair; CE, capillary electrophoresis; dG, 2′-deoxyguanosine; EC, electrochemical detection; εCyt, 3,N*[4]*-ethenocytosine; ELISA, enzyme-linked immunosorbent assay; ESCODD, European Standards Committee on Oxidative DNA Damage; ESCULA, European standards committee of urinary (DNA) lesion analysis; FapyGua, 2,6-di-amino-4-hydroxy-5-formamidopyrimidine; GC-MS, gas chromatography coupled with mass spectrometry; Gua, guanine; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MIP, molecular imprinted polymer; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NER, nucleotide excision repair; SPE, solid phase extraction.*

Introduction

Already in 1960, it was reported that hydroxyl radicals react with pyrimidine and purine compounds[1,2], and Jean Cadet reported on the specific reactivity between nucleic acids and different reactive oxygen species [3]. Later it was found as a serendipity while examining the effects of sugars that guanine was oxidized in the 8-position [4–6]. Subsequent to this discovery the field of oxidative modifications to nucleic acids was fuelled by the simultaneous and independent publications showing that the 8-hydroxylation of guanine could be measured by HPLC, coupled with electrochemical detection [4,6,7]. The electrochemical detection provided the necessary sensitivity that conventional UV detection did not. Later analytical progress was due to the development of carbon-based

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electrochemical cells by ESA, cells that provided better stability, the advances in gas chromatography/mass spectrometry, and ion spray technology that provided sensitive and specific tools to detail several of the modifications induced by oxidation.

The oxidation of DNA attracted much attention and a dedicated effort in the laboratories of Miral Dizdaroglu at National Institute of Standards and Technology in the USA and Jean Cadet at 'Laboratoire Lesions des Acides Nucleiques', Grenoble, France, provided much knowledge about all the possible oxidation products from the reaction between DNA and oxygen. These discoveries were based on analysis with gas-chromatography and mass spectrometry [8,9].

In those days, measurements were only done on a variety of cell cultures or, for example, liver tissue from experimental animals and on isolated mitochondria. The levels, however, showed a tremendous range [10,11] and were higher than the estimates from the Comet assay [12]. This clearly suggested methodological problems. It soon became evident that the most probable cause was spurious oxidation that occurred during the sample preparation process. Many of the DNA isolation procedures originated from the molecular biology field where DNA isolation protocols with slight oxidation, that is, 1% did not provide any problems for the PCR reaction. However, in a chemical measurement of guanine lesions present at very low frequency, that is, one out of a million, 0.1% spurious oxidation, or even 0.01% oxidation is deleterious as it will provide a result that is more than 10–100 times to high. The issues of different levels reported by different laboratories were addressed by a large European network effort funded by the EU framework program, called ESCODD, the European Standardization Committee on Oxidative DNA Damage, and was headed by Andrew Collins with Lennart Möller and one of the authors of the present paper (Henrik Enghusen Poulsen) in the Steering Committee [13–18]. This effort provided a standard protocol to reduce or eliminate spurious oxidation in the DNA extraction procedure, and also compared various methodologies. This brief historical summary details that in the scientific world, it is very difficult to reach such a standardization, which is in striking contrast to, for example, the huge standardization and accreditation effort done by hospital laboratories and the external quality assessment for laboratory data originating from hospital biochemistry and other laboratories (NIQAS, www.neqas.org.uk). It is interesting to notice that even though many lesions induced by oxidation have been described, the bulk of data in the literature is still mostly confined to 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG).

The laboratory of Bruce Ames in Berkeley, California, reported on the urinary excretion of the 8-hydroxylation of 2′-deoxyguanosine, 8-oxodG [19]. However, the first larger scale high pressure liquid chromatography (HPLC) coupled with electrochemical detection (HPLC-EC) analysis and the first systematic data in humans were reported from our laboratory[20] and independently by a group in Sweden [21]. In urine, the challenge was not spurious oxidation, as the 2′-deoxyguanosine (dG) is not excreted in much higher concentrations than the 8-oxodG. In analogy to the reasoning on tissue measurement, even a 1–2% oxidation of dG would not bias the results.

While sensitivity is an issue in both the analysis of urine and extracted DNA, specificity is a major problem in the analysis of oxidized bases and nucleosides in urine, because it contains a considerable number of compounds being very similar in mass and structure. This presents a major problem in the chromatographic analysis and shows up as large baseline noise, low signal to noise ratio and overlapping peaks.

In the present paper, we present and review methodologies to perform urinary analysis of oxidized nucleic acid metabolites, a compilation of the different lesions reported so far, a discussion of the most appropriate methodology, the origin of metabolites in the urine and how to interpret the results.

Analytical techniques

HPLC-MS/MS

The LC-MS/MS methods have traditionally had a reputation of being very specific and usually only require a simple sample preparation. It can, however, be questioned how specific an LC-MS/MS method is, if only one multiple reaction monitoring (MRM) pair is used for identification and quantification of each analyte. It is therefore appropriate that some of the recent methods uses two MRM pairs (quantifier and qualifier ions) [22,23]. This should be the minimum requirement for a reliable identification as it also adheres to international requirements for specific analysis [24]. In addition to using two MRM pairs for each analyte, it is also important to assure that the ratio between MRM pairs is the same in the real samples as for a pure standard of the analyte. Furthermore, the MRM pairs must be characteristic for the analyte in question. Relying on a single MRM transition and using a very common neutral loss as for instance loss of 28u [25] for the identification and quantification of 2,6-di-amino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 8-oxo-7,8-dihydroguanine (8-oxoGua) cannot be recommended because of the high risk of overlooking overlapping peaks and false identification.

Increased specificity can also be achieved by using high resolution MS/MS, and by using Ultra performance liquid chromatography (UPLC) (or Ultra high pressure liquid chromatography,UHPLC) instead of

HPLC in order to get sharper and higher chromatographic peaks. Unfortunately, using high resolution mass spectrometry and more MRM pairs usually comes at the expense of sensitivity. In contrast, higher resolution on the chromatography side has the added benefit of increased sensitivity since the peaks areas are constant. Thus, when the peaks get narrower, it follows that they get higher at the same time and thus leads to improved detection limits. Use of UPLC does, however, put demands on the mass spectrometer. It has to be able to scan fast enough to obtain a sufficient number of measurements across each chromatographic peak so that it is well defined.

Traditionally, true high resolution MS/MS instruments are not the best for quantification. Their sensitivity and linearity has not been as good as the triple quadrupole instruments that now are the gold standard for measuring 8-oxodG and other oxidized nucleosides by LC-MS/MS. However, the traditional limitations of high resolution MS/MS instruments now seem to be addressed by the instrument manufacturers. So, may be this is about to change and in the near future oxidized nucleosides can also be measured by true high resolution mass spectrometers.

When quantifying by LC-MS or LC-MS/MS, it is important to use stable isotope labelled internal standards for each analyte, since the sensitivity of the mass spectrometer can change dramatically during a chromatographic run. This signal suppression (or enhancement) is mainly caused by co-eluting compounds and can change considerable from sample to sample.

Quite a few analytes (Table I) have been detected in urine by LC-MS/MS, among them 8-oxoGua, 8-oxodG, 8-oxoGuo [26], (5′R)- and (5′S)-8,5′-cyclo-2′-deoxyadenosine[27], Fapyguanine[25], 1,N^6-ethenoadenine, 1,N^6-etheno-2′-deoxyadenosine, 3,N^4-etheno-2′-deoxycytidine [28], Malondialdehyde-2′-deoxyguanosine [29], 1,N^2-ethenoguanine [30], N^2,3-ethenoguanine [31] and 5-hydroxymethyl-2′-deoxyuridine [32].

The LC-MS/MS methods are usually fast and can also be very specific. The major drawback is that the equipment is quite expensive.

As an example of the performance of an LC-MS/MS method with qualifier ions the method of Henriksen et al. [22] shows a lower limit of quantification (LLOQ) for 8oxoGuo and 8oxodG of 1nM for both analytes. The within-day precision was 4.4 and 3.7% and the between-day precision 4.0 and 2.3% respectively. The average recovery for 8oxoGuo and 8oxodG were 106.2% and 106.9% respectively. The values for precision and accuracy are comparable to what is previously reported for similar methods. The sensitivity, however, is very instrument setup and compound dependent. Often urinary LC-MS/MS methods require very little sample preparation, as in this example only a heating, mixing, dilution, addition of internal standards and centrifugation. The total LC-MS/MS runtime was 23 minutes.

A method like this can be close to fully automated and with newer more sensitive mass spectrometers it is expected that the precision, accuracy and the LLOQ will improve. With more sensitive mass spectrometers it is also expected that more nucleic acid oxidation products will be found and quantified in urine.

GC-MS

The reliability of the results obtained by use of GC-MS for the quantification of DNA oxidation products in tissue has caused an intense debate, due to the high risk of introducing artifact oxidation of the overwhelming amount of unoxidized nucleobases (A ratio of approximately 10^6:1 for guanine (Gua):8-oxoGua is typical in tissue) during sample preparation (DNA extraction, hydrolysis and derivatization). The situation in urine is much better, as no DNA extraction and hydrolysis is needed. Also, the ratio between Gua and 8-oxoGua is only approximately 14:1, and for dG and 8-oxodG, it is approximately 1:2 [26], so the risk of artifact oxidation is much reduced. Because of this the urine collection and handling at atmospheric oxygen pressure is in general not considered as causing any measurable artifact oxidation, which is supported by the works of Shigenaga et al. [33] who stored urine spiked with [^{3}H]dG for 19 days at 4°C without being able to detect any formed [^{3}H]8-oxodG and the work by Lin et al. [34] who spiked urine with dG and H_2O_2 without being able to detect any formation of 8-oxodG.

Derivatization of the polar DNA oxidation products from urine is needed before analysis by GC-MS. The salt content in the urine does, however, interfere with the derivatization [35]. A pre-purification of the urine samples before derivatization is thus required. This pre-purification has been performed either by Solid phase extraction (SPE) [34] or by HPLC [35,36].

It is possible to quantify 8-oxodG directly (after purification and derivatization) by GC-MS [34]), but often the 8-oxodG is hydrolyzed to 8-oxoGua before derivatization [35,36] as this gives better sensitivity [35]. In order to be able to distinguish between the urinary content of 8-oxoGua and the 8-oxoGua formed after hydrolysis of 8-oxodG, the 8-oxoGua and the 8-oxodG from urine are initially fractionated by HPLC.

Several other analytes have been detected in urine by GC-MS, among them 8-oxo-7,8-dihydroadenine (8-oxoAde), 5-hydroxyuracil (5-OHUra), 5-(hydroxymethyl)uracil (5-HMUra) [35], 5-hydroxymethyl-2′-deoxyuridine (5-HMdU) [37] and 3,N^4-ethenocytosine (εCyt) [38].

As with all quantitative mass spectrometric methods, it is highly recommended to use stable isotope labelled internal standards.

The GC-MS method is very specific provided that a sufficient number of characteristic fragment ions are

used during GC-MS analysis, and it is assured that the ratio between them is the same as for a pure standard of the analyte. A drawback with this method is the time-consuming sample preparation and pre-purification.

HPLC-EC

An electrochemical detector is a less specific detector than a mass spectrometer. This means that there is a higher demand for extensive chromatography before detection of the analytes if the same specificity is to be achieved by HPLC-EC as compared with chromatography coupled with mass spectrometry. In order to obtain sufficient specificity for electrochemical detection in a matrix as complex as urine, two-dimensional HPLC is often used to obtain the desired chromatographic resolution [20].

In addition to the limited specificity of an electrochemical detector, it is also less versatile than a mass spectrometer. In order to be able to measure an analyte by electrochemical detection, the analyte has to be electroactive, and the analyte has to possess sufficient electrochemical activity to be measured at the usually low concentrations of oxidized DNA nucleobases and nucleosides present in urine. In practice, among the relevant analytes only 8-oxoGuo, 8-oxodG and 8-oxoGua are suitable for quantification by HPLC-EC due to their sufficiently high electrochemical activity in combination with the usually relatively high concentrations of these analytes in urine.

The LC-EC equipment is sensitive, but here the main problems are limited specificity and that only few nucleic acid oxidation products have sufficient electrochemical activity to be detected in urine.

Chromatographic techniques

A common trait for the techniques based on chromatography coupled with mass spectrometry is that they can be applied to the measurement of several different nucleic acid oxidation products. Another advantage is the possibility of using stable isotope labelled internal standards. These standards will thus behave chemically as the unlabelled analytes. It is, however, usually very expensive to buy the stable isotopic labelled analytes if they can be bought at all.

Several attempts for obtaining more selectivity on the liquid chromatography side have been tried. A paper that used a carbon column for trapping the DNA oxidation products in a column switching system seemed promising [39], but unfortunately the production of more of these columns failed. Others have used immunoaffinity pre-purification before HPLC separation [29,40], but the immunoaffinity columns are not commercially available, and thus this has gained limited use. Currently, C-18 reversed phase HPLC columns seem to be most frequently used. Recently, a paper have described the use of a molecular imprinted polymer (MIP) for use as a Solid phase micro extraction (SPME) column in combination with reversed phase HPLC with UV-detection for the quantification of 8-oxodG in urine [41]. This technique seems very promising, but unfortunately these SPME columns are at present not commercially available.

Table I. DNA oxidation products found in urine.

	Nucleobase	2′-deoxynucleoside	Ribonucleoside
Adenine	8-oxo-7,8-dihydroadenine[35]		
		(5′R)- and (5′S)- 8,5′-cyclo-2′-deoxyadenosine[27]	
	1,N^6-ethenoadenine[28,65]*	1,N^6-etheno-2′-deoxyadenosine[65–67]*	1,N^6-ethenoadenosine[65]*
	N^6-furfuryladenine (kinetin)[68,69]		
Cytosine	3,N^4-ethenocytosine[70]	3,N^4-etheno-2′-deoxycytidine[28,46,66,71]	
Guanine	8-oxo-7,8-dihydroguanine[25,26,35,72]	8-oxo-7,8-dihydro-2′-deoxyguanosine[26,35,39]	8-oxo-7,8-dihydroguanosine[22,26]
	Fapyguanine[25]		
	1,N^2-ethenoguanine[30,31]#		
	N^2,3-ethenoguanine[31]#		
	Malondialdehyde-Guanine[73]	Malondialdehyde-2′-deoxyguanosine[29]	Malondialdehyde-Guanosine[74]
Thymine	Thymine glycol[45,75]	Thymidine glycol[45,75]	
		Cyclobutane Thymidine Dimers[76]	
	5-(hydroxymethyl)uracil[37,52,77]¤	5-hydroxymethyl-2′-deoxyuridine[37,77]	
	5-hydroxyuracil[35]		

*In rat urine

¤In mouse urine

#Apparently the sum of the nucleobase, deoxynucleoside and ribonucleoside

Table 1 shows which DNA oxidation products so far have been identified and quantified in urine. In addition to the oxidative DNA lesions found in urine it has been attempted to quantify other oxidative DNA lesions in urine e.g. 8-oxo-7,8-dihydro-2′-deoxyadenosine[78,79] and 5-hydroxy-2′-deoxyuridine[80]. That these analytes were not found does not mean, that they are not present in human urine, but rather that with the sensitivity of the used methods they were not detected – it is still very possible that they are present below that level or present in higher concentrations under special conditions.

Capillary electrophoresis

Capillary Electrophoresis (CE) has also been used for the quantification of 8-oxodG in urine. Although the method could appear promising because of the narrow peaks, and thus good selectivity, it usually suffers from poor sensitivity. LOD of 42 nM [42] and 550 nM [43] have been reported. However, a single report of a detection limit of 0.22 nM following SPE purification and 20x concentration has been reported [44]. Capillary electrophoresis has also been used for the quantification of 5-hydroxymethyl-2′-deoxyuridine with a reported detection limit of 980 nM [43].

Because the sensitivity usually is poor capillary electrophoresis has found limited use for the quantification of nucleic acid oxidation products in urine.

Immunoassays

ELISA

ELISA is widely used for the measurement of DNA oxidation products in urine and especially the kit from the Japanese Institute for the Control of Aging (JaICA) is very popular. The advantages of using ELISA are that no expensive equipment is required, it is easy to use, no pre-treatment of urine is required (except for a centrifugation of cloudy samples), a possibility for high throughput and no specialist skills are required to perform the analyses. The main drawback by using ELISA is that the specificity is often limited and thus too high values are reported (see next paragraph). Another disadvantage is that a separate kit for each specific nucleic acid modification is required.

Other techniques

The current review focuses on the main techniques for quantifying urinary nucleic acid oxidation products (Table II). Other techniques have also been used including HPLC with UV-detection [45], ^{32}P-postlabelling [46] and immunoaffinity purification followed by HPLC with fluorescence detection [40], but none of these techniques have, for different reasons, gained widespread use.

Comparisons of methodologies

A few comparisons of different methodologies for quantification of urinary 8-oxodG have been published in recent years [47–49].

Harri et al. compared the levels of 8-oxodG in 246 urine samples measured by HPLC-EC with measurement on the same samples by HPLC-MS/MS and found good agreement with an R^2 value of 0.8707.

The two other comparisons are interlaboratory comparisons. In one of the papers [47], the 8-oxodG levels measured with HPLC-EC, HPLC – GC/MS and ELISA were compared. HPLC-EC and HPLC – GC/MS showed good agreement with $r = 0.89$. On the contrary comparing HPLC-EC with ELISA and HPLC – GC/MS with ELISA showed poor agreement with $r = 0.56$ and 0.43, respectively. In addition, the 8-oxodG concentrations measured by ELISA were 5–7 times higher than the levels measured by the other two methods. In contrast, the concentrations measured by HPLC-EC were on average only ~10% higher than the concentrations measured by HPLC – GC/MS.

The most recent interlaboratory comparison paper [49] is a result of the newly started ESCULA (European Standards Committee of Urinary (DNA) Lesion Analysis) program. The program is similar to the previous ESCODD program for measuring 8-oxodG in tissue [16], but instead ESCULA focuses on the measurement of 8-oxodG in urine. In the first ESCULA trial, results were returned from 20 different methods broadly classified as mass spectrometric (MS), electrochemical detection (EC) or ELISA. The results showed that there was good intra-technique agreement, with the majority of results lying within one SD of their consensus mean. But ELISA showed more intra-technique variation than the other techniques and reported higher urine values for 8-oxodG. In addition, the trials showed good agreement between MS and EC based techniques but concentration-dependent deviation for ELISA.

To sum up the results, there is, in general, good agreement between the chromatography-based techniques, but ELISA still causes concern. It seems like specificity is the main issue, especially, between the chromatographic methods and ELISA. Specificity

Table II. Advantages and disadvantages with different techniques. It shows the pros and cons of the different methodologies in a tabulated form.

	Sensitivity	Specificity	Skills required	Identical int. std.	Sample preparation	Number of Ox. prod.	Price
LC-EC	+++	++	++	-	++	+	++
GC-MS	++++	++++	+++	+	+++	++++	+++
LC-MS/MS	++++	++++	++++	+	+	++++	++++
CE	+	+++	++	(+) Dependent on detector type	++	+	++
ELISA	++++	+	+	-	+	+	+

High, ++++; Low, + (or + for present and – for absent). int. std., internal standard; Ox. prod., oxidized products.

may also be the reason why the EC-based methods tend to give higher values than the MS based methods. It has been suggested that the difference is because the MS-based methods can use stable isotopic labelled internal standards [47]. However, if the possibility of using stable isotopic labelled standards was the cause, it would be expected that the precision would be better in the MS-based methods than in the EC-based methods, but that the mean of the methods would be the same. If the specificity of the EC detector, however, is the problem, then the EC-based methods will tend to give too high values, which is the case.

Interpretation of the urinary excretion of oxidized nucleobases and oxidized nucleosides

In principle, one could expect urine to contain the excised DNA repair products from Base excision repair (BER) and Nucleotide excision eepair (NER): Nucleobases and small oligonucleotides. There have been conflicting reports about the presence of 8-oxodG containing oligonucleotides in urine. One report which was based on LC-MS/MS detected no presence of 8-oxodG containing oligonucleotides in urine [50], whereas presence of 8-oxodG containing oligonucleotides has been reported based on ELISA detection [51]. The concentration was, however, very low. No matter whether 8-oxodG containing oligonucleotides are present in urine or not, it can be concluded that if they are present then they are only present in low concentrations and since the oligonucleotides are of varying length and sequence, the signal will be divided over several peaks and thus give low sensitivity if chromatographic measuring techniques are used. Instead mononucleotides or nucleosides can be quantified, and here the best results have been obtained on the measurement of nucleosides. So far, however, the link between NER and the excretion of modified deoxynucleosides in urine is not well documented.

If one wants to be able to measure urinary nucleic acid oxidation products, then, at some point, the question arises if the method should focus on measurement of nucleobases, nucleosides or both. Traditionally the focus has been on measuring the nucleosides, as it has been assumed that the urinary nucleobases could originate from the diet. The assumption is based on the paper by Park et al. [19]. More recent investigation does, however, indicate that the diet contributes to neither 8-oxoGua nor 8-oxodG in urine [36,52]. In contrast to the conflicting results for the nucleobases, all studies of oxidatively modified deoxynucleosides have agreed that the contributions from diet are minimal [53]. To decide which oxidation damage(s) to look for is often a combination of what is possible (which analyte is present in sufficiently high concentrations to allow quantification with the available technology) and what one wants to investigate. It is possible to measure either direct nucleic acid oxidation or monitoring of specific lipid peroxidation products (such as α,β-unsaturated aldehydes and ketoaldehydes) reaction with nucleobases [28]. The latter may provide information about endogenous exposure to alkylating carcinogens and oxidative stress.

It is recommended to analyze for more than one oxidation damage, since if only one damage product is measured, then there is a risk that that specific damage may not be a good marker of the oxidative stress level, and thus not give a true picture of the amount of oxidative stress the subject in question is exposed to.

The origin of oxidized urinary nucleic acid metabolites is not finally established. One possibility is that they originate from the DNA repair processes, where specific repair enzymes recognize the modifications induced by oxidation; excise them and adjacent nucleotides, followed by hydrolysis of the sugar phosphate bindings and desphosphorylation. The nucleobases can be assumed to originate from BER and the nucleosides from NER. The oxidized nucleic acid metabolites, that is, bases and nucleosides, diffuse from the cell into plasma and are excreted into urine. This theory is based on the biochemical knowledge of the individual processes, but direct and quantitative data have not been presented.

Another possibility is that the oxidized nucleosides and nucleobases originate from oxidation in the nucleotide pool [54]. Presently, sensitivity of the methodologies to measure the free exchangeable nucleotides is not sufficient to detect the oxidized part of the pools, so this has to be improved before direct measurement can be done.

Assuming that the first possibility is valid, the interpretation of urinary excretion of oxidized nucleic acid metabolites is as follows.

In most experimental *in vivo* situations, the level of oxidized modifications are constant, for example, a normal control person, or a person with an intervention , for example, a treatment. In such situations, one can assume a steady state between the number of insults formed and the number of damage products excreted into urine as illustrated in Figure 1. Therefore, the number of oxidized lesions that are formed in 24 h are measured, that is, a 24-hour-urine collection. This is a rate measurement and can thus be interpreted as oxidative stress to nucleic acids. It is worth to note that a change in repair will lead to a change in the tissue levels; however, since the repair enzymes function by the first-order kinetics a new steady state will occur where formation equals excretion. This means that oxidative stress is independent of changes in DNA repair, even though it relies on functioning DNA repair enzymes at substrate concentrations following first-order kinetics. The time

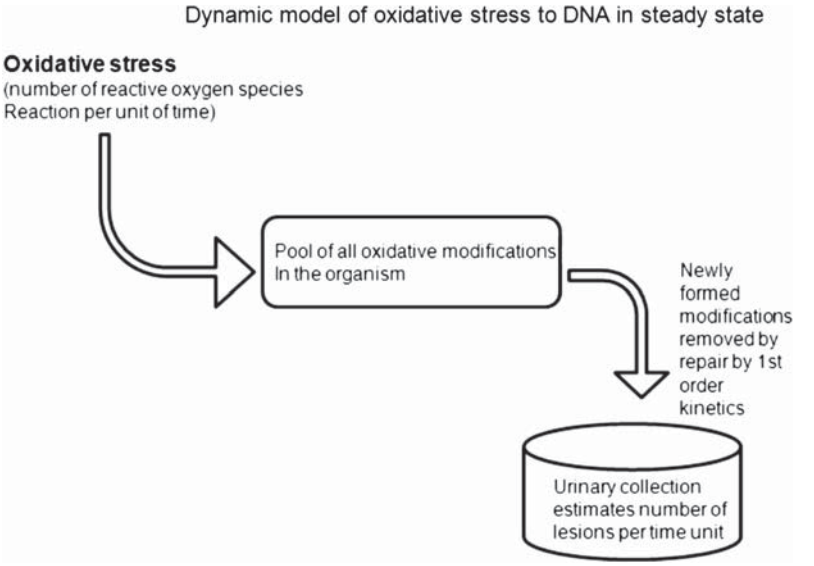

Figure 1. The formation of oxidative insults in DNA is induced by oxidative stress. Because of DNA repair mechanisms, these lesions are removed from DNA, and subsequently, removed from the organism by urinary excretion. According to mass conservation, the number of modified purines/pyrimidines formed per time unit must equal the excreted number, it's because the urinary excretion is a measure of global or average oxidative stress to DNA.

required to reach a new steady state level after an intervention that changes oxidative stress or DNA repair is probably short. This is evidenced by the short half-life of 8-oxodG in plasma after injection [55] and the return of 8-oxodG values after administration of 2-nitropropane [56].

By measuring the urinary excretion of 8-oxodG one gets quantitative estimates of global oxidative stress to DNA. This method is, particularly, useful in situations where all cells in the body are influenced. Prime examples of this is our report on the effect of smoking [20], smoking cessation [57], iron overload in hemochromatosis [58] and diabetes [59].

It is noteworthy that, for example, in hemochromatosis the oxidative stress measured as the 8-oxoGuo excretion in urine is much more prominent on RNA than on 8-oxodG from DNA. Also the treatment of colon cancer with 5-FU induces more RNA oxidation than DNA oxidation [60] and the latest news is that RNA oxidation, but not DNA oxidation has shown to have prognostic value in the development of Diabetes II complications and death [61]. The origin of urinary oxidized RNA residues is, at present, unresolved whether it is from RNA or the nucleotide pool, but no matter where it comes from, it can give valuable information since it can give prognostic information. A few reports have also reported increased RNA oxidation in the early stages of Alzheimer's Disease (AD) measured as the concentration of 8-oxoGuo in CSF [62], and Shan et al. found that 30–70% of the mRNAs isolated from AD frontal cortices were oxidized [63]. In general, however, very little has been published on RNA oxidation, and RNA oxidation could be an overlooked target for oxidative stress.

Evidently, high oxidative stress in a small organ only, for example, the prostate, will not be detectable from measuring urinary excretion as the contribution will be minor compared to the remaining part of the organism.

In many cases, it is very impractical to collect 24-hour urine. Instead, one can use spot urine samples with correction for urinary creatinine, especially, since there does not seem to be circadian variations [64]. This is particularly well suited for paired experiments or randomized trials where equal urinary creatinine excretion can be assumed in the groups compared. On the contrary, in experiments where there are huge differences in creatinine excretion, this may cause a bias and wrong interpretation. Examples of this are comparison of newborn babies with bodybuilders, comparison of centenarians with young subjects and cachectic cancer patients with controls.

Conclusion

The conclusions fo the study are as follows:

- Measurement of 8-oxodG and 8-oxoGuo in urine provides estimates of global DNA and RNA oxidation *in vivo* and is applicable to human investigations as a non-invasive method.
- It is preferable to measure on 24-hour urine, but in some cases, for example, paired experiments and randomized trials, spot urine samples with creatinine correction can be used.
- For the highest specificity, the preferred method is liquid chromatography with tandem mass spectrometry, but liquid chromatography with

electrochemical detection is an acceptable alternative also.

- The analysis by immunological methods consistently provides high and biased results and cannot be recommended at present.
- Indirect evidence suggests that the origin of the oxidized nucleic acid metabolites found in urine is from DNA and RNA, and that the contributions from the nucleotide pools are negligible.
- There are some conditions with extensive RNA oxidation, for example, hemochromatosis, and the role of RNA oxidation warrants much more attention.
- Global nucleic acid oxidation is particularly suited for conditions where a global oxidative stress can be expected, for example, hemochromatosis, diabetes, septic shock, cardiac failure and possibly neurodegenerative diseases.
- There is a dire need for continued interlaboratory comparisons and quality control measures.

Declaration of interest

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All authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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