Measurement and meaning of oxidatively-modified DNA lesions in urine

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Running title: urinary oxidatively-modified DNA lesions

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Keywords: urine; DNA oxidation; DNA repair; immunoassay; HPLC.

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Amongst the authors of this review are partners of ECNIS (Environmental Cancer Risk, Nutrition and Individual Susceptibility), a network of excellence operating within the European Union 6th Framework Program, Priority 5: "Food Quality and Safety" (Contract No 513943).
Abstract

**Background:** Oxidatively generated damage to DNA has been implicated in the pathogenesis of a wide variety of diseases. The non-invasive assessment of such damage, i.e. in urine, and application to large-scale human studies, is vital to understanding this role, and devising intervention strategies.

**Methods:** We have reviewed the literature in order to establish the status-quo with regard the methods and meaning of measuring DNA oxidation products in urine.

**Results:** Most of the literature focuses upon 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxodG), and whilst a large number of these reports concern clinical conditions, there remains (i) lack of consensus between methods, (ii) possible contribution from diet and/or cell death, (iii) no definitive DNA repair source of urinary 2’-deoxyribonucleoside lesions, and (iv) no reference ranges for healthy or diseased individuals.

**Conclusions:** The origin of 8-oxodG is not identified, however recent cell culture studies suggest that the action of Nudix hydrolase(s) on oxidative modification of the nucleotide pool is a likely candidate for the 8-oxodG found in urine and, potentially, of other oxidised 2’-deoxyribonucleoside lesions. Literature reports suggest that diet and cell death have minimal, if any, influence upon urinary levels of 8-oxodG and 8-oxo-7,8-dihydroguanine (8-oxoGua), although this should be assessed on a lesion-by-lesion basis. Broadly speaking, there is consensus between chromatographic techniques, however, ELISA approaches continue to over-estimate 8-oxodG levels and is not sufficiently specific for accurate quantification. With increasing numbers of lesions being studied, it is vital that these fundamental issues are addressed. We report the formation of the European Standards Committee on Urinary (DNA) Lesion Analysis (ESCULA) whose primary goal is to achieve consensus between methods and establish reference ranges in health and disease.
1. Introduction

Reactive oxygen species (ROS) are produced continually, from normal cellular metabolism, but may be produced in excess, leading to oxidative stress, following exposure to xenobiotics, radiation etc. A consequence of ROS production is their interaction with, and subsequent modification of, cellular biomolecules, in particular, DNA, lipids and proteins. Damage to DNA is of particular importance, in part due to the possibility of inheritable, sequence alterations (mutations), although it should not be forgotten that ROS-induced damage to DNA may have non-mutational effects, such as the promotion of microsatellite instability, and acceleration of telomere shortening (1). Over 30 base modifications, such as 8-oxo-7,8-dihydroguanine (8-oxoGua, and its corresponding deoxyribonucleoside equivalent, 8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxodG; Figure 1), have been described (2), with the total number of DNA lesions approaching 70 (3). Furthermore, reactive intermediates, arising from the interaction of ROS with lipids and proteins, may also modify DNA, leading to adducts such as \(1,N^6\)-etheno-2'-deoxyadenosine (\(\epsilon\)dA), 3-(2-deoxy-\(\beta\)-D-erythro-pentofuranosyl)pyrimido[1,2-\(\alpha\)]purin-10(3\(\alpha\))-one (M\(_1\)dG) and DNA-protein cross-links (4)(Figure 1). A great deal of evidence exists which suggests that this plethora of lesions, arising from oxidative stress, may have an important role in the aetiology and/or pathogenesis of many diseases (reviewed in Cooke et al. (5)), such as cancer and aging.

To fully understand the extent to which such DNA lesions are involved in disease, methods for their analysis are essential. Numerous approaches have been applied to the study of oxidatively damaged DNA, including gas chromatography with mass spectrometry (GC/MS (6)), HPLC with electrochemical detection (HPLC-EC (7)), HPLC with single- (8) or tandem (9) mass spectrometry, \(^{32}\)P-post-labelling (10), immunoassay (11), alkaline elution (12) and Comet assay (13), plus other methods based upon the nicking of DNA at oxidised bases by means of repair enzymes (14, 15). However, following the publication of the
findings from the European Standards Committee on Oxidative DNA Damage (ESCODD (16)), a number of these techniques have fallen from favour (reviewed by Guetens et al. (17)). Assessment of damage to DNA by methods requiring invasive procedures, e.g. blood samples or tissue biopsy, imposes severe limitations in large-scale human studies, requiring staff with specialist training, and reducing the likelihood of consent. Furthermore, the possibility of adventitious oxidation during sample storage and DNA extraction has not been entirely ruled out, but methods have been developed to minimise the risk (15). Examining the products of oxidatively generated damage to DNA in extracellular matrices offers a means by which oxidative stress may be assessed non-, or minimally, invasively, and circumvents extraction and the resultant artefacts.

2. Methods of analysis

For the most part, methods that have been applied to the analysis of oxidatively damaged DNA lesions in urine are either chromatographic (principally, HPLC-MS/MS; liquid chromatography pre-purification prior to GC-MS, HPLC-GC/MS, HPLC-EC, GC-MS), or immunoassay. The majority of assays focus upon 8-oxodG as the analyte of choice, although the following have been reported to be present in urine, in many cases with their corresponding deoxyribonucleoside (Table 1).

Chromatographic techniques.

Urine is a very complex matrix, and therefore the common challenge for all chromatographic techniques has been to clean-up the urine sufficiently to simplify analysis, which very often also extends instrument life. At its simplest, column switching has meant that, following chromatographic separation of the urine’s constituents, only the fraction
containing the compound of interest (e.g. 8-oxodG) is applied to the final separation column and detector, either EC (18, 19) or MS (20); the remainder is diverted to waste.

Using HPLC, Tg that was termed a ‘significant marker’ of oxidative stress (21), was one of the first ROS-induced DNA lesions to be studied in urine. The methodology required the use of boronate, solid phase extraction columns and semi-preparative HPLC to isolate Tg and thymidine glycol (dTg) from urine; the lesions were then chemically reduced back to thymine and thymidine prior to analysis by HPLC with UV detection (22). The widespread preference for studies of 8-oxodG, however, derives from a one thousand-fold greater sensitivity of 8-oxodG detection using HPLC-EC, compared to the UV detection of Tg, along with the predominance of 8-oxodG over Tg, in human urine (23). This predominance may arise for reasons such as the reported instability of Tg, and greater levels of 8-oxodG in DNA, due to the relative ease of formation of the latter (23). Overall, this has led to urinary measurements of Tg being superseded by those of 8-oxodG, and the subsequent widespread use of HPLC-EC. A number of methods based upon column-switching HPLC with regular reverse phase (usually C18) separative columns and EC-detection for assay of 8-oxodG have been developed (24, 25, 26). The pre-, or extraction, columns have differed in their packing material, using generally C18 or ion-exchange separation approaches. The switching of the effluent from the first to the second column can be controlled automatically by adding to the sample a detectable marker which elutes close 8-oxodG from the first column and the peak is used as the trigger, and this has greatly enhanced the analytical capacity by electrochemical detection (27). An approach shown to be successful in a wide variety of biological matrices (including DNA, urine, plasma, red blood cell extracts, cerebrospinal fluid, saliva, sweat, kidney dialysate, brain and muscle microdialysate, food (rat, mouse, monkey and human chow), Caenorhabditis elegans, cell culture medium and rat faeces), utilises a column
comprising ‘treated’ carbon material, similar to that used for EC detector cells, together with Coularray, multichannel electrochemical detection (28).

Most recently, mass spectrometric techniques have been increasingly applied to study urinary lesions. Benefits of mass spectrometry include: the use of isotopically-labelled internal standards, simplifying quantification and accounting for loss during sample workup (and storage), differences in ionisation efficiencies due to matrix effects, and confirmation of analyte identity. The HPLC-MS/MS assay described by Weimann et al. (20) was the first report for the simultaneous analysis of the oxidised (8-oxoGua, 8-oxoGuo and 8-oxodG) and native (Gua, Guo and dG) moieties. Inclusion of the latter provided useful data for subsequent hypotheses (see the section concerning cell death). Similarly, the HPLC-GC/MS assay of Olinski’s laboratory entails HPLC pre-purification of the compounds of interest, which again includes native compounds namely 8-oxoGua, 8-oxodG, Gua, dG and 5-(hydroxymethyl)uracil (5-HMUra), (29, 30), prior to derivatisation and GC-MS. This approach was first applied to the simultaneous analysis of five urinary, oxidatively modified DNA base products including 8-oxoGua, 5-HMUra, 5-hydroxyuracil (5-OHUra), 8-oxodG, and 8-oxo-7,8-dihydoadenine (8-oxoAde); (31). Analysis time for HPLC-GC/MS is said to be comparable to that of HPLC-MS/MS and, at present, is capable of the simultaneous analysis of the greatest number of lesions.

Whilst solid phase extraction (SPE) had been used to isolate 8-oxodG, a major drawback of many SPE columns is the need to keep them wet during use. The Waters Oasis HLB SPE column, first used by Lin et al. (32), prior to GC-MS analysis of urinary 8-oxdG, benefits from allowing to become dry during use. These columns have also been used prior to HPLC-MS/MS which has minimised the incidence of interfering peaks reported by Lin et al. (32) in 10 – 20 % of their urine samples (33). The analysis of urinary 8-oxodA has been attempted previously (34), but levels were reported to be at, or below, the limit of detection.
for the HPLC-MS/MS assay (0.3 nM, 7.5 fmol injected, S/N = 3). A similar finding was noted with the SPE-based HPLC-MS/MS assay (Evans et al., submitted). We predict that SPE will receive increasing application in the future, particularly when on-line SPE is introduced.

**Immunoassay**

Competitive enzyme-linked immunosorbent assay (ELISA) is invariably the format of choice for the analysis of lesions in extracellular matrices, when antibody-based methods are employed. Predominantly, 8-oxodG has been the lesion of choice for commercially available (e.g. from the Japanese Institute for the Control of Aging, JICA; Stressgen; and Oxis, the latter simply being a distributor of the JICA kit), and custom made (35) ELISAs. It appears that there are very few commercially available kits presently on the market. Two are available from from JICA, named the “New” and “Highly Sensitive” formats (calibration range is 0.5 – 200ng/mL and 0.125 – 10 ng/mL, respectively). These kits are remarkably similar in description (including references cited) to those marketed by Northwest Life Science Specialties, and indeed both utilise the monoclonal antibody denoted N45.1 and have the same concentration range of standards. The other kit available is from Stressgen Bioreagents, now known as Nventa, (the DNA damage StressXpress ELISA kit) which is remarkably similar to that from Trevigen Inc. (HT 8-oxodG kit). Both have a range of standards in their calibration curve of 1.9 – 60 ng/mL, and essentially the same Microsoft Excel calculation spreadsheet, although the source of the primary antibody employed is less clear.

Simply, the assay format of these ELISAs comprises: 8-oxodG-protein conjugate bound to the bottom of a 96-well plate, a calibration curve of buffered aqueous 8-oxodG standards, containing 0.9 % NaCl, and a monoclonal antibody. Both 8-oxodG in the samples,
and on the plate, compete for antibody binding. Following a wash step, antibody that remains bound to the plate can be detected using an enzyme-labelled secondary antibody. Colour intensity (absorbance) of the enzyme-catalysed product is proportional to the amount of primary antibody bound which, in turn, is inversely proportional to the concentration of antigen in the test sample.

The benefits of ELISA are (i) ease of use; (ii) no specialist (or indeed expensive) equipment is required; (iii) potential application to numerous extracellular matrices (serum (36), plasma (37), saliva (38, 39), urine (40), CSF (41, 42), cell culture medium (43-45) and sputum (Kulkarni et al., in press); (iv) other than centrifugation of cloudy specimens, no pre-treatment of urine is required (v) high throughput. The kit from JICA has, by far, received the most published use, and represents the basis of the bulk of observations to follow. It should be noted that only the JICA kit is recommended for use in plasma, such an application has only recently been reported in the literature, unlike serum. In the case of plasma, the manufacturer recommends ultra-centrifugation of the specimen through a 10 kDa filter (JICA kit insert), to remove ‘interfering substances’ (JICA, personal communication), the identity of which is unknown to us. This has been discussed recently by Cooke et al. (33), who speculated that the competitive format of the ELISA is prone to interference from high molecular weight compounds (e.g. proteins and carbohydrates), present in some body fluids, that resulted in high 8-oxodG readings (e.g. serum), or readings in the absence of significant amounts of 8-oxodG (e.g. saliva).

**Analysis of secondary DNA products of oxidation reactions in urine**

Although not as prevalent as methods for analysing primary DNA products of oxidation in urine, this is a rapidly growing area of interest. Several methods have been developed for the measurement of lipid peroxidation-induced etheno-DNA adducts in human urine. An HPLC-
fluorescence detection method has been developed for εdA (46) and applied in several studies. For example, in Japanese women urinary εdA levels were positively associated with increased ω-6 polyunsaturated fatty acid intake and NaCl excretion, providing evidence of salt-induced inflammation and lipid peroxidation (47). It has also been reported that short-term fasting does not affect urinary εdA adduct, or indeed 8-oxodG, levels in healthy Korean women, despite a reduction in primary lipid peroxidation products (e.g. urinary MDA and 8-isoprostaglandin F$_{2α}$) (48). Isotope dilution-gas chromatography-negative ion chemical ionization/mass spectrometry (GC-NICI/MS) methodology have been developed for 3,N$^4$-ethenocytosine (εCyt), 3,N$^4$-ethenodeoxycytidine (εdC), with higher levels detected in the urine of smokers compared to non-smokers (49, 50). Furthermore, there was good agreement between urinary levels of 1,N$^6$-ethenoadenine (εAde), in healthy individuals, measured by HPLC-electrospray ionisation (ESI)-MS/MS method and isotope dilution GC-NICI/MS methods (51). An HPLC MS/MS method has been developed to analyse urinary εdA (52) and εdC (53), and have been applied to demonstrate a significant association between etheno-DNA adduct excretion rate and the dietary intake of linoleic acid in healthy men (54). A $^{32}$P-postlabeling method has also been reported that measures εdC in human urine (Sun et al, 2006)

Background levels of 1,N$^2$-ethenoguanine and N$^2$,3-ethenoguanine have been detected in the urine from healthy subjects, who have not been exposed to the recognised sources these adducts e.g. vinyl chloride and ethyl carbamate (55). An HPLC-ESI-MS/MS method was developed and used for the detection of low levels of 1,N$^2$-ethenoguanine, which were found to be higher in cigarette smokers (56).
Method comparison

A principal source of criticism of ELISA derives from the discrepancy between chromatography and immunoassay in the determination of ‘background’ or baseline levels of urinary 8-oxodG, in healthy individuals. This difference can be anywhere between four and ten-fold, although recent improvements in the JICA kit (narrowing calibration curve range; recommendation for strict temperature control), have reduced this margin (Figure 2). For the most part, however, 8-oxodG measurements by the two approaches have shown significant correlation: $r = 0.46$, $p < 0.001$ using HPLC-ECD (57) and $r = 0.73$, $p = 0.002$ using HPLC-GC/MS (58), although this is not always the case (59). Nevertheless, this does suggest that both techniques share a common analyte, with the ELISA perhaps recognising additional compounds. In accounting for the discrepancy, some reports in the literature fail to distinguish between antibodies, and their respective specificities, applying a blanket statement to the unsuitability of all ELISAs. Whilst some of this criticism is well-founded, clearly some antibodies are highly specific, and their application to urine, seems entirely appropriate. For example, according to characterisation data reported by Toyokuni et al. (60), N45.1 is highly specific for 8-oxodG. In addition to being specific for the hydroxylated C8 position of Gua, it would appear to discriminate the C6 carbonyl group of 8-oxoGua (and C2 NH$_2$), from the C6 amino group of 8-oxoAde. Furthermore, unlike many antibodies, N45.1 displays preference for the 2-deoxyribose moiety of 8-oxodG, until recently, a prerequisite to the assessment of urinary DNA oxidation products. The closest competitor for the antibody is 8-oxo-7,8-dihydroguanosine (8-oxoG), which needs to be present in concentrations two orders of magnitude higher than 8-oxodG, in order to compete to the same extent. Weimann et al. (20) later showed that 8-oxoG was present at a concentration not dissimilar to 8-oxodG itself (8-oxoG: 48 nmol/24 h vs. 8-oxodG: 28 nmol/24 h), and hence, it was concluded, that 8-oxoG

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$^7$ Cooke et al. unpublished data.
was unlikely to be a competitor (61). Similarly, whilst 8-oxoGua is present in urine at significant concentrations (136 +/- 12 nmol/24 h), characterisation data suggests reactivity of N45.1 towards the modified base to be, at worst, negligible. Some of the comments in the literature which state that N45.1 recognises 8-oxoGua, are likely to derive from misinterpretation of a statement in the abstract of the paper by Toyokuni et al. (60) “N45.1 recognized both the modified base and 2-deoxyribose structure of 8-oxodG”. Clearly, some authors have taken this to refer to the modified base and 2'-deoxyribonucleoside, as separate structures, not in combination, as intended by Toyokuni et al. (60). It is important to note that whilst the JICA assay is reported to be specific for 8-oxodG, the specificity of the Trevigen/Stressgen kit(s) includes recognition of 8-oxoGua and 8-oxoG, likely to account for the higher levels of urinary 8-oxodG reported by these kits. The appropriateness of a calibration curve consisting solely of 8-oxodG, as used by these latter two suppliers’ kits, is also questionable.

Identifying the basis of the discrepancy has proven to be problematic. We have speculated that, as chromatographic assays can only detect ‘free’ 8-oxodG, other compounds structurally related to 8-oxodG, recognisable by N45.1 specifically, may exist and be present in urine (62). For example, 8-oxodGMP, a product of the activity of enzymes such as the human Mut T homologue (hMTH1 (63)), which may have the potential to leave the cell (64); along with 8-oxodG-containing oligomers, putative products of nucleotide excision repair (62). The issue 8-oxodG containing oligomers in urine does not appear to have been resolved, as there have been two reports both indicating (65), and disputing their presence (66), although both reports agree that oligomers are present. Patel et al. (65) ascribe the failure of Weimann et al. (66) to detect 8-oxodG-containing oligomers to a sensitivity issue, arising from the absence of sample concentration steps, despite LC-MS/MS having greater than three times the sensitivity of the ELISA. At best, this would indicate that, if present, 8-
oxodG-containing oligomers are not at a concentration detectable in urine by the ELISA, and hence not the source of the discrepancy between this and chromatographic techniques. Furthermore, use of an independent technique, i.e. not one relying upon antibody specificity, would provide a more definitive answer.

Reardon et al. (67) demonstrated that 8-oxodG is a substrate for NER, which excises lesions as a single-stranded, lesion-containing oligomer (see ‘Repair’, below). Galloway et al. (68) showed that, post-excision, cyclobutadithymine (T<>T) containing oligomers at least, are subsequently degraded to lesion-containing 7-mers. What then happens to the oligomers is unknown, although, from the findings of Le Curieux and Hemminki (69), who detected the presence of urinary T<>T as a monophosphate nucleotide dimer, it may be implied that further processing does occur, the nature of which remains to be described. Despite all this circumstantial evidence, the precise reason for the higher ELISA values remains unknown.

The HPLC-EC method is not without analytical difficulties, Bogdanov et al. (28) reported the presence of peaks which co-elute with 8-oxodG, identified by multi-channel coulometric electrochemical detection. Furthermore, if DNA oxidation products are indeed present in urine in forms other than bases or 2'-deoxyribonucleosides, such as oligomers, mono- or diphosphates, then the vast majority of chromatographic methods will not recognise these products. Despite these issues, within technique agreement of levels in control subjects between laboratories is strong, and supports the continued use of both chromatographic and ELISA approaches. Furthermore, in longitudinal or comparative studies, the significance of absolute levels is of less importance, compared to the ability to detect differences in 8-oxodG levels.
Urinary lesions: issues of artefact and stability

The measurement of oxidatively generated damage to DNA has been plagued by the issue of artefact generated, for example, during DNA extraction, or sample workup. This issue has been critically examined, in detail, by the European Standards Committee on Oxidative DNA Damage (ESCODD), as part of its attempts to achieve consensus between different techniques and different laboratories. Similarly, urinary lesions may arise from the artefactual oxidation of DNA, or its constituent nucleobases, or 2’-deoxyribonucleosides, following exposure to metabolic enzymes, or other oxidising species, after release into the systemic circulation, or in the urine. However, Shigenaga et al. (70) showed that dG is not artefactually oxidised in the systemic circulation, or following incubation with microsomal enzymes. Equally, 8-oxodG, Tg and dTg are not subject to degradation upon release into the systemic circulation (22, 70), the stability of 8-oxoGua not having been examined. Experimental studies with 8-oxodG injected intravenously have shown that recovery is almost complete in urine in pigs, indicating that further metabolism is an unlikely event (71).

Furthermore, as significant concentrations of hydrogen peroxide have been reported to be present in urine (72), Lin et al. (32) examined the potential for 8-oxodG to be generated from dG, present in the urine. Combinations of dG (1 nmol) and hydrogen peroxide (50 or 100 µM), were incubated in a number of urine samples, for 24 hours, at room temperature (32). The authors concluded that hydrogen peroxide has a negligible effect upon 8-oxodG production, under these circumstances, agreeing with previous results from Ames’ laboratory.

As part of a study, representing a great deal of foresight, Loft et al. (73) reported that storage of urine samples at –20 °C, and repeat measurements, did not lead to a decrease in the concentration of 8-oxodG over a 15 year period, confirming the stability of this lesion in urine. This also applies to HMUra that has been stable upon storage at – 80°C for periods up to 4 months (74).
Whilst the above provide evidence of stability in extracellular matrices, it is worth noting that 8-oxodG is more prone to oxidation than dG, due to its lower redox potential (75, 76) and is preferentially oxidised, even in the presence of an excess of Gua (77, 78). By the same token, oxidation of Thy to Tg is less likely to occur, than Gua to 8-oxoGua, due to the lower oxidation potential of Gua, compared to Thy. However, it would appear that most of these studies have examined the oxidation of 8-oxoGua in situ in DNA (79), rather than as a post-excision product of DNA repair, which may alter the likelihood of oxidation and its ‘oxidisability’. In contrast, M\textsubscript{1}G and M\textsubscript{1}dG both appear to undergo further oxidative metabolism in rat liver cytosol, with the base adduct being a better substrate for such enzymic oxidation than the deoxyribonucleoside adduct (80, 81). There is also some evidence to suggest that M\textsubscript{1}G is further oxidised when administered intraveously, although M\textsubscript{1}dG was not examined (80).

Overall, it would appear that 8-oxodG, Tg and dTg, and probably 8-oxoGua, are not formed artefactually in vivo, in biological matrices of mammals, including humans, and there is no published evidence for their degradation upon release. In contrast, there is strong evidence that M\textsubscript{1}G and M\textsubscript{1}dG may undergo oxidation, either enzymically, or in the systemic circulation.

3. Sources of extracellular, oxidatively-modified DNA lesions.

Broadly speaking, the possible sources of extracellular, oxidatively-modified, DNA lesions are (i) diet, (ii) cell death/turnover, mitochondrial turnover and (iii) DNA repair.

(i) Diet

Urine is the extracellular matrix upon which dietary influence has been most studied. The majority of reports measuring oxidatively modified DNA lesions in urine, have focussed upon 2'-deoxyribonucleoside lesions, and 8-oxodG specifically. This is largely a consequence
of early work from Bruce Ames’ laboratory, in rats, demonstrated that diet could affect levels of urinary 8-oxoGua, but not 8-oxodG and, whilst they concluded Tg to be unaffected by diet (discussed in detail by Cooke et al. (62)). There is little human data on the subject of dietary contribution, and some disagreement with the animal findings. Gackowski et al. (82) have presented evidence to suggest that, in humans, neither 8-oxoGua, nor 8-oxodG, are affected by diet. Pivotal to this study was the assumption that, like rats (83), humans reach a minimum level of 8-oxoGua excretion 2-3 days after switching to a nucleic acid-free diet. Similarly, it was assumed that a maximum level of 8-oxoGua excretion was reached 3-5 days after reverting to a normal diet. Additional sampling points throughout this study would make these conclusions more definitive. Studies utilising radio-labelled lesions are preferred over those involving nucleic acid-free diets as the use of radiolabels provides a degree of stringency not afforded by nucleic acid-free diets. However, for ethical reasons, radio-labelled lesions are to be avoided in human studies. To circumvent this issue, Cooke et al. (30) recently completed a feeding study using heavily oxidised, stable isotopically-labelled $^{15}$N-DNA (98 % incorporation). Healthy, male volunteers were fed 5, 15 or 25 mg of oxidised $^{15}$N-DNA, and first void, mid-stream urine samples were collected for up to 14 days later. The presence of $^{15}$N$_5$-8-oxodG, and $^{15}$N$_5$-8-oxoGua in urine was examined using HPLC-GC/MS (82). Neither lesion was detected in any of the urine samples. To confirm that the absence of lesion was not a sensitivity issue, urinary $^{15}$N$_5$-dG and $^{15}$N$_5$-Gua, which should be present at significant levels, was also examined. Again, neither of these labelled compounds were detected in any of the urine samples. The authors also investigated whether any of the ingested $^{15}$N$_5$-Gua/dG had been degraded to $^{15}$N$_5$-uric acid, the final product of purine metabolism, prior to excretion. Again, no labelled material was detected. As with previous studies (70), the authors could only presume that the isotopically labelled DNA components/adducts pass through the gastro-intestinal tract to appear in faeces.
Nevertheless, these results, coupled with the findings of Gackowski et al. (82), provide the most compelling argument that diet is not a significant contributor to both urinary 8-oxoGua and 8-oxodG levels in human urine. This is consistent with the animal data relating to 2'-deoxyribonucleoside lesions (70), which includes dTg (22), but disagrees with early data for 8-oxoGua and Tg (22, 84). Species differences may be proposed to account for this discrepancy, although the focus of our attention must be upon the more relevant, human data. Earlier animal studies should be repeated using the highly specific and sensitive mass spectrometric techniques.

(ii) Cell death/turnover.

In 1993, it was asserted that urinary 8-oxodG did not reflect DNA repair, as it was not a product of base excision repair (85), rather it was a product of non-specific nucleases, acting upon DNA released from during cell death, liberating dG which is subsequently oxidised. This discounted the potential existence of other, as yet undiscovered, repair pathways, which may yield 8-oxodG. Whilst repair processes, whose reaction product may be 8-oxodG, have been described (see below), the possible contribution from cell turnover remains to be addressed, although some data are beginning to emerge. For example, it has been reported recently that rat liver homogenates can release 8-oxodG from oxidatively modified DNA (86). The authors suggest that, during DNA degradation, most of the 8-oxodG, present in DNA, is released by enzymes, and subsequently appears in the urine, implying this to be a major source. However, they do acknowledge their system does not accurately represent the in vivo situation, not least as it is unrealistic – multiple, uncompartmentalised nucleases acting upon circulating DNA, particularly as the majority of cell death in healthy humans will be via apoptosis.
The evidence against a contribution from cell turnover has, for the most part, been anecdotal. There exists a number of reports in which urinary 8-oxodG has been measured in patients undergoing chemotherapy, in the absence of concomitant increase in urinary 8-oxodG (87, 88), despite evidence of extensive cell death i.e. significant increases (p<0.01) in urinary uric acid (a biochemical index of cell turnover); and a reduction in tumour mass. Indeed, any reported increases in urinary 8-oxodG following chemo- or radiotherapy have been attributed entirely to DNA repair (89, 90). As yet, the most decisive argument against the contribution of cell death to urinary levels of 8-oxodG and 8-oxoGua comes from a recently published report from the Olinski group (91). This study revealed that a statistically significant (p = 0.0003) increase (60%) in 8-oxoGua was observed 24 h after infusion of the cisplatin, a period when no loss of blood cells was reported. In the “nadir days”, when the most distinct cell death can be observed, urinary excretion of 8-oxoGua dropped significantly in comparison with the aforementioned time point. These results indicate that cell death does not appear to contribute to urinary 8-oxoGua and 8-oxodG in humans. This is supported by the results of Weimann et al., in a study examining oligonucleotides in urine, concluding that the limited excretion of oligonucleotides into urine argues against oligonucleotides, or indeed nucleosides, originating from cell death (66). On balance, these results suggest that the contribution of cell death to urinary 8-oxodG levels is minimal, but clearly further work needs to be performed.

(iii) DNA repair.

With respect to DNA repair, the source of oxidatively modified DNA bases in extracellular matrices seems clear. The DNA N-glycosylases responsible for removing modified bases are increasingly well defined (92), and have been discussed thoroughly elsewhere (2). 8-Oxoguanine DNA glycosylase 1 (OGG1) is the major enzyme involved in
the removal of 8-oxoGua from cellular DNA. Recently homozygous OGG1 -/- null mice were generated (93). To assess the involvement of this enzyme in generation of urinary 8-oxoGua, and to clarify further the issue concerning the origin of this DNA modification, 8-oxoGua levels was determined in OGG1 deficient mice and compared to a wild type strain. If OGG1 was the only enzyme to remove 8-oxoGua, one would expect no excretion of 8-oxoGua. On the other hand if there were efficient back-up glycosylase(s) no change in urinary excretion of the modified base should be observed between OGG1 defective and wild type strains (assuming that the repair process is the main source of the compound). The results of Rozalski et al. indicated that there was about a 26 % reduction in levels of urinary 8-oxoGua in the deficient strain, compared to wild type, and no significant changes in 8-oxodG were observed (94). This indicates that mouse OGG1 glycosylase is a significant, but by no means unique, source of urinary 8-oxoGua.

The results clearly suggest the existence of back up DNA glycosylase(s), that cannot entirely compensate for OGG1 deficiency. The reversion to a less efficient back up system should result not only in reduction of 8-oxoGua in urine, but also is expected to lead to the simultaneous, gradual increase in the background level of 8-oxoGua in cellular DNA. Indeed, it has been demonstrated that nuclear DNA 8-oxoGua levels in the liver of OGG1 deficient mice increase with age (93, 95, 96). At 14 weeks of age a 7-fold increase in nuclear 8-oxoGua levels, from liver tissue, was observed in OGG1 deficient male mice, compared to wild type, rising to 40-fold at 50 weeks of age (95, 97). In female ogg1-/- mice of 8-10 weeks of age, no significant increase in 8-oxoGua, assessed as formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites, was found in the liver, compared to wild type mice; whereas the level in lung tissue was 3-fold greater than wild type (98). The faster accumulation in lung could be due to high level of oxygen exposure, compared to liver. The ogg1-/- mice also accumulate much more 8-oxodG in target organs following exposure to oxidative stress-inducing agents, such
as diesel emission particles or potassium bromate. In the mitochondrial genome, from liver tissue, a 20-fold increase in the 8-oxoGua level was observed in the OGG1 defective mice, compared to wild type (99). The recent finding of Seeberg’s group supports the existence of back-up glycosylases for OGG1. This group described a back up glycosylase (Nei-like glycosylase that, depending upon sequence context, is approximately 10-fold less effective than human OGG1 for 8-oxoGua removal (100).

However, this reasoning alone cannot fully explain the above findings, since the buildup of 8-oxoGua appears to be limited to certain organs, such as liver, lung and possibly skeletal muscles. Plus, urinary excretion of modified bases has been thought to represent the average rate of DNA damage in the whole body (50). This suggests the existence of another repair pathway, the product of which is not 8-oxoGua, or indeed 8-oxodG, that maintains lowered 8-oxoGua levels, in most tissues, even in the absence of OGG1. It is worth considering that the rate of 8-oxoGua accumulation in DNA may be determined by the proliferation rate of the tissue, so that rapid proliferation may dilute the effect of OGG1 deficiency, whereas 8-oxodG accumulates in liver, lung and muscles with a low proliferation rate.

The presence of 2’-deoxyribonucleoside lesions in extracellular matrices is very much less well defined, as there are no reports of a single DNA repair enzyme whose activity yields 8-oxodG. Based upon existing evidence, we have proposed the following three DNA repair routes (Figure 3) as the most likely contributors to the presence of oxidatively-modified 2’-deoxyribonucleosides in urine:

(A) Nudix hydrolases. There is an imperative for preventing modified DNA precursors from being incorporated into the genome. The best characterised enzyme which performs such a role is the 8-hydroxy-2’-deoxyguanosine triphosphatase (8-oxodGTPase(63)) activity of NUDT1 (MutT homologue, MTH1), hydrolysing 8-oxodGTP to 8-oxodGMP.
It has been suggested that further processing, perhaps by 5’(3’)-nucleotidases, may give rise to 8-oxodG, which is not charged and can be removed from the cell, ultimately appearing in the urine (63). Recent data from the group of Harms-Ringdahl strongly imply that NUDT1 activity, and thus the nucleotide pool, represents a major source of extracellular 8-oxodG (101). These studies in cell culture used siRNA gene expression knockdown methodology directed at human NUDT1. However, the roles of other Nudix hydrolases such as NUDT15 (MTH2) and NUDT5, which include 8-oxodGTP and 8-oxodGDP amongst their substrate repertoire respectively, remain to be defined (102, 103). These new observations on the contribution of NUDT1 to extracellular 8-oxodG, coupled with any potential negation of the role of diet and cell turnover, could mean that we are very close to determining the biological significance of urinary 8-oxodG levels as markers of oxidation in, and sanitisation of, the nucleotide pool.

(B) Nucleotide excision repair. Despite appearing to be directed principally towards bulky lesions, such as cyclobutane thymine dimers (T<>T), there is some evidence that NER (Figure 3) may act upon non-bulky lesions such as 8-oxoGua (67). Indeed, the rate of 8-oxoGua removal appears comparable to that for T<>T (67), seemingly generating a lesion-containing oligomer, approximately 24-32 nucleotides long (104). A recent report failed to demonstrate 8-oxoGua-containing oligomers in urine (66) implying that further processing occurs, perhaps ultimately yielding 8-oxodG, or that they do not exist. However, under normal circumstances, the role of NER in the removal of 8-oxoGua, and perhaps other small oxidatively generated DNA lesions, would appear to be negligible (105-109), although results from XP cell lines have not entirely excluded the possibility (67, 106, 110, 111).

(C) Endonuclease(s). A poorly characterised endonuclease has been reported by Bessho et al. (112) which, lacking a glycosylase activity, is predicted to give rise to 3’·5’-8-oxodGDP as the putative product. We have previously proposed that this may be
subsequently hydrolysed to 8-oxodG by nucleotidase(s). The recently described nucleotide incision repair (NIR) process generates lesion-containing X'-dNMP products, again these could be subsequently metabolised to a 2’-deoxyribonucleoside. However the activity of NIR towards 8-oxodG-containing substrates is reported to be negligible. While this may be the case, it is entirely feasible that this process could be a potential source of other extracellular lesion-containing 2’-deoxyribonucleosides. Although the types of lesion that have been examined as substrates have yet to be detected in urine.

5. European Standards Committee on Urinary (DNA) Lesion Analysis (ESCULA)

There is growing evidence that, rather than simply being a non-invasive marker of whole body oxidative stress, measurement of urinary lesions, such as 8-oxodG, may also reflect DNA repair activity. As noted above, there is a discrepancy in basal urinary 8-oxodG levels when comparing chromatographic techniques with ELISA, although all techniques have been shown to discriminate between diseased and healthy subjects. ELISA has received widespread use, and is clearly amenable to the greatest number of laboratories, however, this discrepancy continues to raise questions regarding its utility. Understanding the basis of this discrepancy will aid our understanding of the significance of urinary lesions. Furthermore, performing inter-lab validation of assays for urinary 8-oxodG measurement would provide robust methods for widespread dissemination and application. Hitherto, this has been performed in a limited fashion, and the discrepancies remain unaddressed. Serious consideration also needs to be given to how results for urinary lesion measurements are expressed, and the applicability of correcting for creatinine. Finally, there is growing clinical interest in the measurement of urinary 8-oxodG, as a means to determine the role of oxidative stress in disease, and evaluate intervention strategies. As with other clinical parameters, a reference range must first be determined.
Primary objectives of ESCULA:

1. To compare a multiple methods for the analysis of urinary 8-oxodG.

2. To identify consensus and investigate the basis for any discrepancy between techniques.

3. Provide validated assays for further application.

4. Establish a reference range for urinary 8-oxodG (accounting for factors such as: age, sex, smoking status, ethnicity), determined by the above methods.

5. Measurement of other oxidised bases and nucleosides as possible biomarkers
References


20. Weimann, A., Belling, D., and Poulsen, H. E. Quantification of 8-oxo-guanine and guanine as the nucleobase, nucleoside and deoxynucleoside forms in human urine by


110. Lipinski, L. J., Hoehr, N., Mazur, S. J., Dianov, G. L., Senturker, S., Dizdaroglu, M., and Bohr, V. A. Repair of oxidative DNA base lesions induced by fluorescent light is


115. Rozalski, R., Gackowski, D., Roszkowski, K., Foksinski, M., and Olinski, R. The level of 8-hydroxyguanine, a possible repair product of oxidative DNA damage, is


Figure Legends

**Figure 1.** Structures of DNA base adducts analysed in urine. I, $1,N^2$-εGua; II, $N^2,3$-εGua; III, $M_1$Gua; IV, 8-oxoGua; V, 8-oxoAde; VI, $1,N^6$-εAde; VII, 5-OHUra; VIII, 5-HMeUra; IX, Tg; X, $3,N^4$-εCyt; XI, 2’-deoxyribose which forms a N-glycosidic bond with the N$^9$ in purines and N$^1$ in pyrimidines in the 2’-deoxyribonucleoside analogues of these base modifications.

**Figure 2.** Comparison of urinary 8-oxodG levels by chromatographic techniques (HPLC-ECD, LC-MS/MS) and the Japanese Institute for the Control of Aging ELISA (‘original’ 8-OHdG Check and ‘New’ 8-OHdG Check).

**Figure 3.** DNA repair sources of extracellular 8-oxodG and 8-oxoGua. NER, nucleotide excision repair; hOGG1, human 8-oxoguanine glycosylase
Table 1. DNA markers of oxidative stress reported in human urine.

<table>
<thead>
<tr>
<th>Nucleobase</th>
<th>Modification</th>
<th>Abbreviation</th>
<th>Representative method(s) of analysis</th>
<th>Reported levels</th>
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<tbody>
<tr>
<td>Gua</td>
<td>8-oxo-7,8-dihydroguanine</td>
<td>8-oxoGua</td>
<td>HPLC-GC/MS(113)</td>
<td>9.4 nmol/mmol Cr</td>
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<td></td>
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<td>LC-MS/MS(20)</td>
<td>136 nmol/24 hr</td>
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<td></td>
<td>HPLC-GC/MS(82)</td>
<td>130 nmol/24 hr</td>
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<td>8-oxo-7,8-dihydro-2’-deoxyguanosine</td>
<td>8-oxodG</td>
<td>HPLC-EC(70)</td>
<td>~308 pmol/kg/24 hr</td>
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<td></td>
<td>HPLC-GC/MS(113)</td>
<td>1.5 nmol/mmol Cr</td>
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<td></td>
<td></td>
<td></td>
<td>LC-MS/MS(33)</td>
<td>0.4 nmol/mmol Cr</td>
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<td></td>
<td>ELISA(88)</td>
<td>0.4 nmol/mmol Cr</td>
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<td></td>
<td></td>
<td>GC/MS (32)</td>
<td>19.4 nmol/mmol Cr</td>
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<td></td>
<td>CE-EC(114)</td>
<td>1 - 3 nmol/mmol Cr</td>
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<td></td>
<td></td>
<td>HPLC-GC/MS(115)</td>
<td>13.5 nM</td>
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<td></td>
<td>HPLC-MS/MS(9)</td>
<td>35 nmol/24hr</td>
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<td>8-oxo-7,8-dihydroguanosine</td>
<td>8-oxoG</td>
<td>HPLC-EC(116)</td>
<td>405 pmol/kg/24 hr</td>
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<td>LC-MS/MS(20)</td>
<td>48 nmol/24 hr</td>
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<td></td>
<td>Pyrimido[1,2-a]purin-10(3H)-one</td>
<td>M₁Gua</td>
<td>LC/MS(117)</td>
<td>&lt; limit of detection</td>
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<tr>
<td>3-(2-deoxy-β-D-erythro-</td>
<td>M$_{1}$dG</td>
<td>LC-MS/MS(118)</td>
<td>(LOD; 500 pmol/L)</td>
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<tr>
<td>pentofuranosyl)pyrimido[1,2-α]purin-10(3H)-one</td>
<td></td>
<td>LC/APCI-MS/MS(119)</td>
<td>12 fmol/kg/24 hrs</td>
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<tr>
<td>1, N$^{2}$-ethenoguanine</td>
<td>1, N$^{2}$-εGua</td>
<td>LC/ESI/MS/MS(55)</td>
<td>&lt;0.3 (LOD) – 10 nmol/L</td>
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<tr>
<td>N$^{2}$-3-ethenoguanine</td>
<td>N$^{2}$-3-εGua</td>
<td>LC/ESI/MS/MS(55)</td>
<td>&lt;0.3 (LOD) – 10 nmol/L</td>
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<tr>
<td><strong>Ade</strong></td>
<td>8-oxo-7,8-dihydroadenine</td>
<td>8-oxoAde</td>
<td>7 nmol/L</td>
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<td></td>
<td>8-oxo-7,8-dihydro-2'-deoxyadenosine</td>
<td>8-oxodA</td>
<td>(LC-MS/MS(34))</td>
<td>&lt; LOD</td>
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<td>1,N$^{6}$-etheno-2'-deoxyadenosine</td>
<td>εdA</td>
<td>LC/APCI-MS/MS(52)</td>
<td>38.6 pmol/24 h</td>
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<td>Immuno-HPLC-Fluoresence detection(47)</td>
<td>6 - 113 pmol/24 hr</td>
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<td>Immuno-HPLC-Fluoresence detection (48)</td>
<td>(1.2-17 pmol/mmol Cr)</td>
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<td>6.4 pmol/mmol Cr</td>
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<tr>
<td><strong>Thy</strong></td>
<td>Thymine glycol</td>
<td>Tg</td>
<td>HPLC-UV(22)</td>
<td>1.9 nmol/mmol Cr</td>
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<tr>
<td></td>
<td>Thymidine glycol</td>
<td>dTg</td>
<td>HPLC-UV(22)</td>
<td>1.2 nmol/mmol Cr</td>
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<tr>
<td>5-(hydroxymethyl)uracil</td>
<td>5-HMUra</td>
<td>HPLC-GC/MS(74, 120)</td>
<td>8.5 nmol/mmol Cr</td>
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<tr>
<td>5-hydroxymethyl-2’-deoxyuridine</td>
<td>5-HMdUrd</td>
<td>HPLC-GC/MS(120)</td>
<td>At limit of detection (5 nmol/L)</td>
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<tr>
<td>5-hydroxyuracil</td>
<td>5-OHUra</td>
<td>HPLC-GC/MS(31)</td>
<td>58 nM</td>
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<tr>
<td>Cyt</td>
<td>Cyt</td>
<td>GC/NICI/MS(121)</td>
<td>84.6 nmol/L/mmol Cr</td>
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<tr>
<td>3,N⁴-ethenocytosine</td>
<td>εCyt</td>
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<tr>
<td>3,N⁴-etheno-2’-deoxycytidine</td>
<td>εdC</td>
<td>³²P-postlabelling(122)</td>
<td>2.5 pmol/mmol Cr</td>
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</tbody>
</table>
Urinary 8-oxodG:creatinine (ratio)

- Chromatography
- ELISA
- ELISA (improved)

Method of analysis