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Validation of biomarkers for the study of environmental carcinogens: a review

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Abstract

There is a need for validation of biomarkers. Our aim is to review published work on the validation of selected biomarkers: bulky DNA adducts, *N*-nitroso compounds, 1-hydroxypyrene, and oxidative damage to DNA. A systematic literature search in PubMed was performed. Information on the variability and reliability of the laboratory tests used for biomarkers measurements was collected. For the evaluation of the evidence on validation we referred to the ACCE criteria. Little is known about intraindividual variation of DNA adduct measurements, but measurements have a good repeatability irrespective of the technique used for their identification; reproducibility improved after the correction for a laboratory factor. A high-sensitivity method is available for the measurement of 1-hydroxypyrene in urine. There is consensus on validation of biomarkers of oxidative damage DNA based on the comet assay and chromatographic measurement in blood while urinary measurements by chromatographic assays are well validated, and ELISA-based assays appear to lack specificity. Immunoassays for the quantification of adducts of *N*-nitroso compounds are useful for large epidemiological studies, given their sensitivity, the small amount of DNA required and their potential for rapid and high-throughput analysis.

Keywords: *validation, DNA adducts, 1-hydroxypyrene, oxidative damage to DNA, N-nitroso compounds, environmental carcinogenesis*

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Introduction

The use of biomarkers in cancer epidemiology has a rather long history (the wording 'molecular epidemiology' was originally proposed by Perera and Weinstein in 1982

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(Perera et al. 1982)) and great successes have been achieved, such as the investigation of the predictive ability of chromosome aberrations (Delaunay et al. 2003), the relationship between aromatic amines in tobacco, the *NAT2* genotype and bladder cancer (Marcus et al. 2000), or the mechanisms by which benzene induces leukaemia (Hirabayashi et al. 2004). In spite of the fact that, in early stages of biomarker development, validation is incomplete and interpretation of results needs care, many biomarkers are introduced into research without proper validation, and this hampers successful research, introducing bias or simply blurring existing associations. In addition, new and complex issues are emerging through the introduction of high-throughput technologies, like the expected large number of false positives, or the complex interplay between environmental exposures, intermediate markers, confounders and disease. There is a strong need for validation of biomarkers through robust field studies. Validation should be interpreted as both technical validation in the laboratory and epidemiological validation in populations. Here we refer mainly to the latter.

In the present paper and in a more extensive report from the Environmental Cancer Risk, Nutrition and Individual Susceptibility (ECNIS) network (Vineis & Gallo 2007) we have considered in detail several examples of biomarker validation: bulky DNA adducts, *N*-nitroso compounds, 1-hydroxypyrene, oxidative damage to DNA and heterocyclic aromatic amines. These examples are not exhaustive both because we have not considered all potential biomarkers, but only those for which some kind of evidence on validation has been published, and because for each of the biomarkers only some aspects of validation have been covered. Nevertheless, this kind of methodological review is essential to assess the state-of-the-art of methods used for biomarker measurement and to identify gaps in knowledge.

Methods

A systematic literature search in PubMed was performed in order to identify all studies in which the relevant biomarkers (bulky DNA adducts, *N*-nitroso compounds, 1-hydroxypyrene, and oxidative damage to DNA) were measured. These are all biomarkers of genotoxicity for which extensive literature is available, particularly on sources of variation, and the review has been prepared in the context of ECNIS, a large European network of experts.

For each biomarker category, two independent readers extracted from each paper information on the study design, sample type and size, tissue and/or cells where measurements were carried out, type of exposure related to the biomarker level (if applicable), and laboratory technique used for the measurement of the biomarker. In addition, information on the variability and reliability of the laboratory tests used for biomarker measurements were collected: data on intraindividual variability of the test came from the results of several measurements of the same biomarker in the same subject over time (for example Kuljukka and colleagues report no significant difference between adduct levels measured in the winter and in the fall season) (Kuljukka et al. 1998). Data on reliability of the test came from test repeatability and reproducibility; the repeatability is the variation between different measurements in the same laboratory and with the same machinery on the same tissue/cell sample (also called intra-assay variation); for example in their nested case–control study on environmental air pollution and DNA adducts, Peluso et al. (2005) repeated the

measurement of the adducts in 27% of subjects ($n=311$) reporting a coefficient of correlation $r=0.93$ ($p<0.0001$). Test reproducibility, on the other hand, is the variation observed between measurements in different labs on the same sample (also called inter-lab variation) (see the work by Phillips and Castegnaro on validation of DNA adducts measurement described below) (Phillips & Castegnaro 1999). Usually these variations are reported in terms of coefficient of variation (CV) (%) or with a coefficient of correlation (r). Finally, additional information on confounders taken into consideration in the analysis, the presence of a dose–response relationship and the identification of other sources of variation were collected from each study.

For the evaluation of the evidence on validation we have referred to the ACCE criteria (Burke et al. 2002) (Table I). The acronym ACCE stands for the four key elements needed to evaluate any genetic test: analytical validity, clinical validity, clinical utility and ethical, legal and social implications.

Further details on the content of this review are given in the ECNIS report (Vineis & Gallo 2007); also extensive tables can be accessed on the ECNIS website (ECNIS 2007).

Results

Bulky DNA adducts

DNA adducts are among the most informative biomarkers of exposure to genotoxic agents. The measurement of DNA adducts gives a quantification of the biologically effective carcinogen dose reaching the DNA in cells, in the tissue under examination. That is, they represent the amount of carcinogen that has been absorbed by the body, undergone metabolic activation, become bound to cellular DNA and has not been repaired. Biologically they result from the exposure to genotoxic carcinogens such as polycyclic aromatic hydrocarbons (PAH) and aflatoxin B1, whose metabolized electrophilic intermediates bind covalently to DNA bases. The presence of these adducts prior to replication can lead to mutation in the DNA. DNA adducts are considered necessary, but not sufficient, in the pathway from genotoxic exposure to cancer development (Rundle et al. 2002).

Table I. The ACCE criteria (Burke et al. 2002).

Analytical validity
Analytical validity focuses on the ability of the test to measure accurately and reliably the marker/genotype of interest. The components of analytical validity are sensitivity, specificity, and test reliability. Sensitivity evaluates how well the test measures the marker/genotype when it is present. Specificity, on the other hand, evaluates the test to determine how well it measures the marker/genotype when it is not present. The reliability of a test measures how often the same results are obtained when a sample is retested.
Clinical validity
Clinical validity focuses on the ability of the genetic test to detect or predict the associated disorder (phenotype). Clinical validity is also the positive predictive value (PPV), that is, the proportion of individuals who develop the disease given that they have the marker/genotype.
Clinical utility
Clinical utility addresses the elements that need to be considered when evaluating risks and benefits associated with the introduction of the test into routine clinical practice. A test that has clinical utility, such as blood cholesterol, provides the individual with valuable information that can be used for prevention, treatment, or life planning, regardless of results.

Several methods have been developed to detect and identify the adducts from cellular DNA; sensitivity and specificity of each assay can vary greatly. On average, the detection range of 1 adduct per 10^8 nucleotides to 1 adduct per 10^9 nucleotides is sufficient to give information on dietary and environmental exposures in humans.

Humans are exposed to a wide range of genotoxic compounds; some assays measure mixtures of DNA adducts, others identify particular adducts. The choice of the assay is often based on multiple characteristics being evaluated: the amount of biological sample needed, costs and data available on each specific assay.

Each of these assays should be validated before interpreting the results: validation implies testing the repeatability of the test (in the same laboratory, with the same sample, and between samples from the same person), and its reproducibility (results on the same sample analysed in two different laboratories). In addition, few studies have been conducted to compare results of DNA adduct quantification carried out on the same samples using different methods of DNA adduct analysis.

^{32}P-postlabelling. In the ^{32}P-postlabelling assay, modified bases of DNA are tagged with a radioactive isotope and then detected by means of the radioactive decay. In order to select only the adducted nucleotides of the DNA, the DNA undergoes a digestion to mononucleotides which is followed by an adduct enrichment procedure involving further enzymatic digestion (nuclease P1 digestion) or solvent extraction (with butanol). Other techniques to select adducted nucleotides are available, such as high-performance liquid chromatography (HPLC), small cartridge columns or immunoaffinity columns with antibodies to the adducts of interest (Farmer & Emeny 2006).

Mass spectrometry. Mass spectrometry was formerly used only for compound characterization, today it is also viable for quantitation of DNA adducts levels in humans after genotoxic exposure. Different techniques are available: liquid chromatography-electrospray ionization–mass spectrometry (ESI-LC-MS); gas chromatography–electron capture–negative chemical ionization–mass spectrometry (GC-EC-NCI-MS). Its use mainly depends on the characteristics of the DNA adduct of interest (Farmer & Emeny 2006).

Immunoassay. In immunoassays poly- or monoclonal antibodies against structural modifications of DNA are used. The antibodies employed are adduct-structure specific or structural-class specific, in the latter case existing cross-reactivity with closely related adduct structures. Many techniques are now available, with different sensitivities (Farmer & Emeny 2006).

Reproducibility of the test. A total of 36 studies conducted from 1998 to date have been included in this review (Rundle et al. 2002, Binkova et al. 1998, van Delft et al. 1998, Kuljukka et al. 1998, Pan et al. 1998, Whyatt et al. 1998, Arnould et al. 1999, Autrup et al. 1999, Pavanello et al. 1999, Phillips & Castegnaro 1999, Schoket et al. 1999, Viezzer et al. 1999, Palli et al. 2000, Rojas et al. 2000, Rundle et al. 2000, Georgiadis et al. 2001, van Delft et al. 2001, Ruchirawa et al. 2002, Teixeira et al. 2002, Farmer et al. 2003, Sorensen et al. 2003b, Baranczewski & Moller 2004, Ibanez et al. 2005, Peluso et al. 2005) including 12 studies carried out on cigarette smoking as a specific exposure (Godschalk et al. 1998a, Schoket et al. 1998, Mollerup et al. 1999, Ozawa

et al. 1999, Wiencke et al. 1999, Cheng et al. 2000, Piipari et al. 2000, Butkiewicz et al. 2000, Schoket et al. 2001, Godschalk et al. 2002, Gyorffy et al. 2004, Bak et al. 2006) (see also ECNIS 2007). The work coordinated by Phillips and Castegnaro (1999) was specifically aimed at standardizing and validating the ^{32}P-postlabelling technique for DNA adduct detection and quantitation. The entire work was composed of two inter-lab trials: in a first trial, 15 labs were required to analyse four samples (B[*a*]P-modified DNA, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-modified DNA, aminobiphenyl (ABP)-modified DNA and the unmodified calf thymus DNA that had been used to prepare the modified DNA samples) with both a batch of polynucleotide kinase (PNK) from the trial and with the one currently used in the participants' laboratories. With the B[*a*]P–DNA samples, for example, and using the PNK provided, the average values obtained with the nuclease P1 digestion and butanol extraction methods were 93 and 77%, respectively, of the value of 111 adducts per 10^8 nucleotides obtained from measurement of the ^{3}H label in the DNA samples. Some laboratories were closer with the trial PNK than with their own method, others obtained similar values with both methods and others were closer with their own method. After this first trial, lab procedures were standardized further. A second trial was then carried out on the same samples and again participants were required to analyse them both with a batch of PNK from the trial and with one currently used in their lab, twice. The second measurement was normalized using values for their level of modification provided. When compared with the first interlaboratory trial, no apparent improvement in the uncorrected results obtained with both the low and high modified samples was noticeable from the coefficients of variation. However, there was a distinct improvement in the shape of the distribution curves, with mean and median values much closer than in the first trial. For the corrected values, the improvement in both the shape of the curves and in the statistics amounted to a reduction of 20% in the coefficient of variation for both the low and high B[*a*]P-modified DNA samples compared with the first trial.

Repeatability of the test. Among the reviewed articles, 32 used ^{32}P-postlabelling to detect DNA adducts. Of these studies, 14 reported testing the repeatability of the assay. In some cases, repeatability was tested on a subset of specimens and an estimated CV was reported (Binkova et al. 1998, Godschalk et al. 1998a, Schoket et al. 1998, Ozawa et al. 1999, Viezzer et al. 1999, Wiencke et al. 1999, Georgiadis et al. 2001, van Delft et al. 2001, Godschalk et al. 2002, Sorensen et al. 2003b, Bak et al. 2006). When reported, the CV was around (or below) 20%. In other studies, all the measurements were conducted in duplicates or triplicates and results reported were the mean of these repeated measurements (Pan et al. 1998, Arnould et al. 1999, Phillips & Castegnaro 1999, Cheng et al. 2000, van Delft et al. 2001, Ruchirawa et al. 2002, Teixeira et al. 2002, Gyorffy et al. 2004). In the latter cases, usually the CV is not shown, nor are the original measurements. Finally, in a few reports an intraclass correlation coefficient was calculated, and usually highly significant values were found (Palli et al. 2000, Rundle et al. 2000, Ibanez et al. 2005, Peluso et al. 2005). The reproducibility of the ^{32}P-postlabelling assay across labs has not been tested except in the work of Phillips and Castegnaro (1999) and also of Godschalk et al. (1998a), who carried out an analysis of differences in aromatic DNA-adduct levels between alveolar macrophages and subpopulations of white blood cells from smokers, pooling data from different populations. The authors (Godschalk et al. 1998a) acknowledged that

the differences in DNA isolation methods may partly be responsible for interlaboratory differences.

The majority of the studies on DNA adducts measured by ^{32}P-postlabelling procedure took into account few potential confounders such as smoking habits, age, body mass index, diet, alcohol intake, physical activity and genetic polymorphisms. Other specific confounders, such as occupational exposure levels, were considered where appropriate.

In 10 studies, immunochemical methods were used to analyse the presence of DNA adducts, alone or in comparison with other methods (Whyatt et al. 1998, Arnould et al. 1999, Pavanello et al. 1999, Schoket et al. 1999, Rojas et al. 2000, Rundle et al. 2000, Ruchirawa et al. 2002, Rundle et al. 2002, Farmer et al. 2003, Gyorffy et al. 2004). The antibodies most often used for immunoassays were raised against DNA that had been reacted with anti-BPDE (benzo[*a*]pyrene-trans-7, 8-dihydrodiol 9, 10-epoxide), thus containing the DNA adduct formed *in vivo* by B[*a*]P. The majority of the studies reported that negative control samples were used in each determination and that the DNA adduct detection was carried out twice in a subset of the samples. Raw data are reported only seldomly.

In particular, Rundle and colleagues (2002) analysed the amount of DNA adducts contained in tumour and normal breast tissue of breast cancer patients and controls using immunochemical techniques, using monoclonal antibody 5D11. After a first scoring of all samples performed by one technician, a second one re-scored a randomly selected subset of them: the resulting intraclass correlation coefficient was found to be 0.80 ($p = 0.0001$) for tumour tissue, 0.72 ($p = 0.0006$) for benign tissue and 0.93 ($p < 0.0001$) for non-tumour tissue.

In a study (Pavanello et al. 1999) involving occupationally exposed workers (coke oven, chimney sweeps and aluminium anode plant workers), using HPLC/fluorescence to detect anti-BPDE tetrols in urine or released by hydrolysis from blood DNA, Pavanello and colleagues carried out a calibration with calf thymus DNA alone, and spiked with different amounts of anti-BPDE tetrol. The correlation coefficient so obtained was 0.98, and the mean coefficient of variation for analyses repeated on different days was 16%.

Dose–response relationships. Several experimental studies looked at dose–response relationships between carcinogen exposure and DNA adduct levels. In the work of Teixeira and colleagues (2002), a correlation between levels of DNA adducts in blood and the number of cigarettes smoked was found among unexposed workers of a coke oven plant. Conversely, Schoket and colleagues (1998) found no correlation between daily or cumulative cigarette dose and DNA-adduct levels in bronchial tissue, but they found a highly significant linear inverse relationship between DNA-adduct levels and the logarithm of the time from abstinence from smoking, suggesting an exponential elimination of DNA adducts from the bronchial tissue of former smokers (Schoket et al. 1998). In addition, a modest but significant linear relationship was observed between aromatic DNA-adduct levels in mononucleate cells and tar exposure by Godschalk and colleagues (1998a). The dose–response relationship between exposure level and DNA adducts has also been studied by Pavanello and colleagues (1999) who reported that the risk of having high levels of anti-BPDE-DNA adducts increases according to the occupational exposure to PAH.

The shape of the dose–response relationship has been addressed in a comprehensive meta-analysis, including 13 occupational cohorts exposed to air pollution, by Peluso and co-workers who investigated the dose–response relationship between DNA adducts in blood cells and occupational exposure to air pollutants (classified into three categories: industrial workers, urban workers and referents) (Peluso et al. 2001). A frequency ratio (FR) index was calculated as the mean DNA-adduct levels of PAH-exposed workers over the level of the referent group. The dose–response relationship between frequency ratios and external benzo(a)pyrene (B[a]P) concentrations in work environments has been estimated. In the inset, the same dose–response curve at very low exposure doses is also shown, assuming a linear dose–response relationship for B[a]P levels below the detection threshold.

The role of DNA adducts as a valid biomarker of low environmental PAH exposure has been reviewed subsequently by Castaño-Vinyals and co-workers (2004). They included a total of 17 studies on DNA adducts which also estimated the PAH exposure by measuring B[a]P or a sum of PAHs in the air, or by using a proxy variable for estimating the exposure level. They showed the comparison between levels of DNA adducts among subjects exposed and unexposed to environmental PAH using ^{32}P-postlabelling and immunoassay. With the first assay, in 15 out of 25 pairs, exposed subjects had higher DNA adducts compared with unexposed; with the second, this was true in three out of four pairs. A correlation between the log-transformed stationary measured amount of B[a]P levels in air and DNA adducts, by assay was also calculated. The correlation of the overall scatter plot showed a Pearson coefficient was $r = 0.60$; when considering only the adduct levels measured by ELISA, the Pearson coefficient was $r = 0.99$; when including only those measured by ^{32}P-postlabelling, the Pearson coefficient was $r = 0.21$. These results are encouraging and support the use of PAH-DNA adducts for assessing environmental exposure to PAHs at group level.

Assay comparability. With regard to the comparisons between different assays, Arnould et al. (1999) conducted a study on occupationally exposed subjects comparing the ^{32}P-postlabelling method and immunoassay in detecting DNA adducts. DNA-adduct detection with the ELISA (BPDE-DNA) method was carried out twice in five different series and negative control samples were used in each series of determination, while for the ^{32}P-postlabelling, detection was performed in triplicates (data not shown). They also reported a regression line between the two assays ($r^2 = 0.81$; $p < 0.001$); the values obtained by the immunoassay method were significantly higher than those obtained by the ^{32}P-postlabelling method ($t_{14} = 5.40$; $p < 0.001$). The lowest levels of adducts (by the two methods) were recorded in the less exposed non-smoking workers (Mann–Whitney U test, $p = 0.010$). No relationship was found between the levels of adducts and the concentration of B[a]P in the workplace.

Schoket et al. (1999) reported data from three biomonitoring studies analysing DNA adducts with different measurement techniques. In particular, two methods (^{32}P-postlabeling and BPDE-DNA ELISA) provided similar qualitative evidence of genotoxic exposure in workers. However, there was a weak negative correlation between the two DNA adduct biomarkers in individual pairs of data ($r = -0.232$; $p = 0.028$).

Finally Gyorffy and colleagues (2004) documented bulky DNA-adduct formation in lung tumour, distal lung tissue, bronchial tissue and peripheral blood lymphocytes taken from smoking and non-smoking patients with lung malignancy. The

DNA-adduct levels were determined by ^{32}P-postlabelling with nuclease P_1 digestion adduct enrichment. In addition, PAH-DNA adduct levels were determined in lung tumours and peripheral lung tissue by BPDE-DNA CIA. The results demonstrate smoking-related primary DNA-damaging processes in lung, and the use of peripheral blood lymphocytes as surrogate tissue in molecular epidemiological studies of human genotoxic exposures to complex environmental mixtures. No significant difference was found in the DNA-adduct levels in any tissue, regardless of the method, between males and females after stratification for histological type or smoking category. An *in vitro*-modified BPDE-DNA standard was used in duplicate as an external standard to reduce interassay variability. In lung tumour DNA, a weak correlation between values obtained by ^{32}P-postlabelling and BPDE-DNA immunoassay was observed; while in normal lung DNA samples there was no correlation.

Intraindividual variability. Data on intraindividual variability are sparser, and generally quite contradictory. Apart from toxicant exposure levels (often used as main exposure), the main cause of intraindividual variation being studied as a potential source of variation (blurring the association between the exposure and the biomarker under investigation) is seasonality, but general trends and possible mechanisms of action are far from clear. In a study involving 50 students living in central Copenhagen (Sorensen et al. 2003a), blood and urine DNA-adducts level were measured over a 11-year period and significant differences between seasons were found. The highest concentration of PAH adducts was observed during spring and summer. Removing season from the logistic model, external temperature was a significant predictor of adduct level causing a 3% increase in PAH adducts per 1°C increase in average outdoor temperature ($p = 0.01$). In contrast, in another study (Butkiewicz et al. 2000) on gene–environment interaction in carcinogen susceptibility conducted in Poland on 170 healthy men (smokers and non-smokers), *GSTP1* and *GSTM1* polymorphisms were analysed in relation to PAH exposure. Mean levels of adducts by genotype were found to be higher in winter compared with summer for all polymorphism except for the *GSTM1* (*null*)/*GSTP1*-AG or –GG.

On the other hand, in a study conducted in Greece among non-smoking students living in the city of Athens and in the region of Halkida (Georgiadis et al. 2001) to assess the relationship between DNA adducts and airborne PAHs, measurements in lymphocytes were carried out twice, in winter and the following summer. Winter versus summer correlations of subject ranking according to adduct levels and exposure parameters were not significant, except for those subjects recruited in the campus area of Halkida.

Some causes of intraindividual variation that are not related to seasonality have been investigated: in a study conducted at Bangkok (Thailand) (Ruchirawa et al. 2002) on high (traffic policemen) and low (office policemen) road pollution-exposed policemen, those who spent their day outside air-conditioned buildings were found to be exposed to a higher level of genotoxic substances, and facemasks only gave minor protection against this exposure.

Finally, Godschalk and colleagues (2003) reported on a sample of 24 healthy smoker volunteers; quitting smoking significantly decreased their blood DNA-adduct levels.

Conclusions

The ideal biomarker of exposure is one that shows a low measurement error, i.e. high repeatability (same assay carried out on the same sample in the same laboratory) and reproducibility (same assay carried out on the same sample in different laboratories). The range of intra-individual variability mainly depends on the amount of exposure and the individual co-factors influencing the biomarker level in the body.

Little is known yet about the intra-individual variation of DNA adducts measurements, making comparison between individuals particularly difficult. DNA adduct levels in the body seem to be influenced by season, although it is not clear in which direction (Sorensen et al. 2003b; Butkiewicz et al. 2000) and with which mechanism: temperature-mediated or influenced by other factors, such as diet. Moreover, it is not clear how seasonality interacts with exposure levels, biomarker level or both, thus being a factor difficult to handle.

The importance of accurately estimating intra-person variability relies on the fact that this parameter, together with the reliability of the test, is a key factor in interpreting overall variability. In fact, correct interpretation of the amount of DNA adducts as a marker of exposure is possible only if results (i.e., differences between subjects) are not overshadowed by intra-person variations or uncertainty in laboratory measurements (Godschalk et al. 2003).

Overall, DNA adduct measurements have been found to have good repeatability, irrespective of the technique used for their identification. Reproducibility has also been shown to be acceptable and improved after the correction for a laboratory factor which makes results more comparable (Phillips and Castegnaro 1999). The main concern in interpreting research results, therefore, comes from the relation between inter- and intra-individual variability, i.e. how much of the observed difference between individuals might be not explained by a different level of exposure but by other determinants of DNA adduct levels. It is important, therefore that researchers presenting findings on DNA adduct measurements, and with only one sample per subject collected, report carefully the timing and seasonality of collection of the biological samples and take this into consideration in their analysis of results. In addition, comparison between studies and pooled analyses need to be approached very carefully and to take into consideration the adjustment for the laboratory factor in comparing results.

1-Hydroxypyrene

PAHs are well-known ubiquitous environmental pollutants, and numerous representatives of this chemical class of compounds are potential carcinogens. PAHs are formed during incomplete combustion and represent complex human exposure. Pyrene, which is a non-genotoxic component of the PAH mixture, is transformed into 1-hydroxypyrene (1-OHPY) in a cytochrome P450-catalysed reaction. 1-OHPY is conjugated by UDP-glucuronyltransferase and sulfotransferase to form water-soluble conjugates which are excreted by urine. Urinary 1-OHPY is a widely used marker for biomonitoring human exposure to PAHs.

Our present summary reviews nearly 40 research papers of the past two decades. We have focused attention on the following main aspects of the relevant publications: study design and sample size; laboratory method; type of exposure; intraindividual and interindividual variation; dose–response; other sources of variation.

Laboratory methods. Measurement of 1-OHPY most often occurs by the detection of 1-OHPY by HPLC-fluorometry following enzymatic decomposition of its conjugates by glucuronidase and sulfatase followed by solid-phase extraction of the free metabolite (Jongeneelen et al. 1987). An alternative approach is the measurement of 1-hydroxypyrene glucuronide (1-OHPYG) by immunoaffinity chromatography – synchronous fluorescence spectroscopy (IAC-SFS) (Strickland et al. 1994).

Recovery of the analyte was 85% or higher (Jongeneelen et al. 1987, Buckley & Lioy 1992). The intraday variation of the assay was between 3.7% and 6% (Jongeneelen 1996, Godschalk et al. 1998b). The typical mean coefficients of variation were between 11.6% and 16.2% for 1-OHPY (Jongeneelen et al. 1987, Buckley & Lioy 1992, Jongeneelen 1996, van Schooten et al. 1995), and 8–10% for 1-OHPYG (Kang et al. 1995, Lee et al. 2003), which values may be used as an estimation of the between-day variation. The detection limit was between 23 and 80 ng of 1-OHPY per litre of urine (Godschalk et al. 1998b, Buckley & Lioy 1992). 1-OHPY concentrations are usually expressed in μmol 1-OHPY mol^{-1} creatinine, although some authors report the results in nmol l^{-1} urine. It is important to note that the unit of measure may affect the result of the statistical analysis. For example, Siwinska et al. (1998) found statistically significant difference in urinary 1-OHPY between boys and girls when 1-OHPY concentration was calculated per volume of urine but when 1-OHPY concentration was corrected for the creatinine concentration in the urine, the difference was not statistically significant. 1-OHPY is considered a cost-effective indicator of PAH exposure, and it can be measured by a well-established high-sensitivity method.

Validation studies. PAHs can be absorbed in the respiratory tract, in the gastrointestinal tract and can penetrate through the skin. The main types of exposure represented in the literature are occupational, medicinal, environmental and dietary exposures.

Occupationally exposed populations. There are specific industries in which workers are at significantly increased risk of cancer of the lung and skin and other organ sites. In a number of these industries the increased cancer risk has been attributed to technologies that cause high PAH exposure to the workers (IARC 1994). The most typical worksites include coke ovens (Jongeneelen et al. 1990, Pan et al. 1998), aluminium production plants (van Schooten et al. 1995, Schoket et al. 1999), electrode paste plants (Bentsen et al. 1998), creosote impregnating plants (Jongeneelen et al. 1985) and vulcanizing rubber plants (Schoket et al. 1999). Other jobs with PAH exposure include asphalt pavers (Jongeneelen et al. 1988), garage mechanics (Schoket et al. 1999), shipyard painters (Lee et al. 2003), and traffic policemen (Merlo et al. 1998, Perico et al. 2001).

The half-life for urinary excretion of 1-OHPY is in the relatively large range of 4–35 h, with a mean of about 13–18 h, which is a substantial source of intra- and interindividual variation of 1-OHPY concentration. The timing of urine sample collection, therefore, is a critical point in the biomonitoring of occupational exposure. Typical post-shift versus pre-shift differences were observed in many studies that reported increased urinary 1-OHPY concentrations at the end of the work day. In coke-oven workers, the pre-shift 1-OHPY was positively associated with the post-shift concentrations but the association was not statistically significant. Whilst there is a

fluctuation of the biomarker level within 24 h, a trend of increase of 1-OHPY concentration was often observed during consecutive working days (Wu et al. 1998).

Among coke-oven workers a 10-fold increase of daily exposure expressed in benzene-soluble fraction of air particulates resulted in up to 2-fold increase of 1-OHPY level (Wu et al. 1998). Due to PAH exposure many-fold increases may occur in the individual 1-OHPY values. For example, in aluminium plant workers, the highest values were about 60 μmol mol^{-1} creatinine compared with the control level of about 0.3 μmol mol^{-1} creatinine (Schoket et al. 1999). 1-OHPY levels were in good association with job categories in many studies. There was a statistically significant good correlation between personal pyrene levels and 1-OHPY in coke-oven workers (Pan et al. 1998). However, correlation between personal or ambient PAH exposure and individual 1-OHPY level was not straightforward in the various publications (Marczynski et al. 2005). A significant effect of smoking on the excretion of 1-OHPY was observed in many studies (Jongeneelen et al. 1990, van Schooten et al. 1995, Merlo et al. 1998). Due to the individual variability of the minimum–maximum of 1-OHPY range within a job category and among job categories as well (Jongeneelen et al. 1985, 1990, van Schooten et al. 1995, Wu et al. 1998), it was recommended to sample urine at the end of shift at the end of a routine workweek for obtaining representative information (Jongeneelen et al. 1988). The most relevant data on occupational exposure studies are presented in the tables available in the ECNIS report (Vineis & Gallo 2007).

Thus, highly elevated levels of PAH exposure in specific industrial workplaces are detectable by urinary 1-OHPY measurement. However, the relative proportion of pyrene in PAH mixtures is not constant. The variation of relative pyrene levels in various PAH sources, particularly in occupational settings means that additional analysis of the PAH profile is important when different PAH sources are compared. 1-OHPY as a biomarker of occupational PAH exposure is especially appropriate for the follow-up of exposure in longitudinal studies rather than between different work-sites. Jongeneelen gives the interpretation of 1-OHPY levels in details in the Guidelines for Biological Monitoring of Workers in Aluminium Production (Jongeneelen 2004).

Medicinal exposure. Medicinal exposure to PAHs occurs from treatment of psoriasis and eczema patients with coal tar ointment (Jongeneelen et al. 1985, Santella et al. 1994, Godschalk et al. 1998b). Due to daily topical treatment of a skin patient with 40 g ointment containing 10% coal tar (approx. 67 mg pyrene) for 4 days, the urinary 1-OHPY concentration increased approximately 200 times (Jongeneelen et al. 1985). The difference in the mean 1-OHPY values between patients and controls was 3900-fold. The range of 1-OHPY concentration was 10–5160 μmol mol^{-1} creatinine in the patients treated with estimated 20–100 g tar per day, and 0.02–0.98 μmol mol^{-1} creatinine in the controls (Santella et al. 1994).

The 1-OHPY level increased sharply from the baseline level of 0.39–96.5 μmol mol^{-1} creatinine after topical application of a 3% coal-tar ointment for 1 day. Continuation of treatment for 1 week was reflected in a further but not significant increase of the 1-OHPY concentration (Godschalk et al. 1998b). There was also no significant association between the number of days of treatment and the level of 1-OHPY in a study by Santella et al. (1994). Cessation of treatment resulted in a quick decrease, after 1 week, to the baseline level. Smoking had no significant effect on

1-OHPY level at high-level dermal exposure to PAHs (Santella et al. 1994). Major information from the relevant papers is given in the tables available in the ECNIS report (Vineis & Gallo 2007).

Environmentally exposed general population. Environmental exposure to ambient air pollutants has been investigated in adults and children in industrialized regions, big cities/urban settlements and rural settlements. Background values may vary from country to country, probably due to variations in environmental and/or dietary PAH exposure. Mean urinary 1-OHPY levels of non-smoking adult study populations were in the range of 0.03–0.68 μmol mol^{-1} creatinine (Levin 1995). In children, the 1-OHPY levels were somewhat elevated compared with the non-smoking adult populations, between 0.08–0.74 μmol mol^{-1} creatinine.

Similarly to the time-related pattern in the occupational settings, 1-OHPY was significantly higher in the evening than in the morning in adults (van Rooij et al. 1994). Morning, afternoon and following morning comparison of urinary 1-OHPY in school children in Bangkok and rural Thailand also indicated characteristic time-dependent changes of the biomarker (Ruchirawat et al. 2005).

There were significant differences in 1-OHPY or 1-OHPYG levels between children with urban and rural residences (Hansen et al. 2005, Kang et al. 2005, Ruchirawat et al. 2005), and between children exposed to indoor coal-burning stoves and other (gas, electric) heating and cooking facilities (Siwinska et al. 1999, Mielzynska et al. 2006). A positive correlation was found between hours spent outdoors and 1-OHPY concentration in children in urban residences (Hansen et al. 2005).

Seasonal variation has also been observed in some studies (Ovrebo et al. 1995). 1-OHPY did not differ in the age range of 1–6 years (van Wijnen et al. 1996). Significant age-dependent difference was observed recently by Huang et al. (2006), in which 6–12-year-old children had higher 1-OHPY levels than adolescents and adults. Females tended to have higher 1-OHPY levels than males; however, the difference was not significant (Kyrtopoulos et al. 2001). Smoking increased baseline 1-OHPY level in adults. The range of mean 1-OHPY values in smokers' study populations was between 0.25 and 1.01 μmol mol^{-1} creatinine. The number of cigarettes smoked daily correlated with 1-OHPY rather well (van Rooij et al. 1994, Pastorelli et al. 1999) or weakly but significantly (Joseph et al. 2005). Environmental tobacco smoke exposure did not appear to have a strong effect on 1-OHPY level in children (Siwinska et al. 1998, Mucha et al. 2006). Most characteristic data from the relevant papers are given in the tables available in the ECNIS report (Vineis & Gallo 2007).

Dietary exposure. Dietary exposure to PAH has been assessed to be as substantial as some occupational exposures. Dietary exposure was investigated under controlled conditions in studies with hamburger and char-broiled beef (Buckley & Lioy 1992, van Maanen et al. 1994, Kang et al. 1995). A 100- to 250-fold increase of dietary benzo[a]pyrene dose paralleled a 4- to 12-fold increase in urinary 1-OHPY level (Buckley & Lioy 1992). The study by van Maanen et al. (1994) gives an interesting and complex example of intra- and interindividual variations in a time- and dose-dependent manner. Daily consumption of 170 g charcoal-broiled hamburger, which contained 1.5 μg benzo[a]pyrene and 4.5 μg pyrene, for 5 days caused significant maximal 1-OHPY excretion on day 3 in the study subjects. A lower daily intake of 0.038 μg benzo[a]pyrene and 0.9 μg pyrene did not increase 1-OHPY excretion

significantly. Segregation of the subjects into separate response groups was observed in the levels of 1-OHPYG in a similar study by Kang et al. (1995), suggesting the existence of special determinants that may regulate absorption, metabolism and/or excretion of ingested pyrene. The information from these two feeding studies is included in the tables available in the ECNIS report (Vineis & Gallo 2007).

Predictive value of 1-OHPY. Urinary 1-OHPY concentration reflects very recent PAH exposure. The reliability of a single 1-OHPY measurement for the assessment of long-term average PAH exposure is therefore low. No epidemiological data are available yet which are based on the effect of a long-term average of urinary 1-OHPY level to cancer outcome. However, an attempt was made to establish an indirect relationship between lung cancer mortality risk and 1-OHPY as the biological exposure indicator for coke-oven workers. Exposure at the level of a suggested tentative biological exposure limit of 2.3 μmol mol^{-1} creatinine was estimated to be equal to a relative risk of lung cancer of approximately 1.3 (Jongeneelen 1992). Depending on the type of occupational exposure the suggested tentative biological exposure limit varies due to the PAH profile at a given workplace (Jongeneelen 2001).

Conclusions. 1-Hydroxypyrene is a metabolite of pyrene, and pyrene is always present in PAH mixtures. Urinary 1-OHPY is an indicator of recent PAH exposure via multiple exposure routes at a wide range of exposure levels. A well-established high-sensitivity cost-effective method is available for the measurement of 1-OHPY in urine. Trace amounts of 1-OHPY are detected in urine of environmentally exposed subjects of the general population. Background values may vary from country to country, probably due to variations in environmental and/or dietary PAH exposure. Smoking increases the 1-OHPY concentration. 1-OHPY is especially appropriate for the follow-up of PAH exposure in longitudinal occupational studies rather than between industrial worksites with different PAH profiles. No epidemiological data are available that are based on the effect of long-term average of urinary 1-OHPY level to cancer outcome; therefore urinary 1-OHPY is currently not suitable for direct cancer risk estimation.

Oxidative damage to DNA

Oxidized DNA lesions can be measured in both cellular DNA and urine. The most studied biomarkers in this respect are the comet assay-based measurement of strand breaks (SB), oxidized pyrimidines as endonuclease III (ENDOIII)-sensitive sites and oxidized purines as fapyguanine-DNA-glycosylase (FPG)-sensitive sites and 8-oxodeoxyguanosine (8-oxodG) in cellular DNA, especially from mononuclear blood cells (MNBC), as well as urinary excretion of 8-oxodG, which may be a product from oxidation of guanine in the nucleotide pool and/or so far unknown repair pathways of DNA. Data are available on variation, confounding factors and predictive value of cancer risk for these biomarkers.

The true level of oxidized base lesions in cells has been subject to heated debate over the past decade. Extraction of DNA for chromatographic or immunochemical analysis of 8-oxodG has inherent problems of spurious oxidation. In keeping with the conclusions from the ESCODD project, we should be sceptical about measurements of over five lesions per 10^6 dG in cells (European Standards Committee on Oxidative

DNA Damage – ESCODD 2002, Collins et al. 2004), given the dynamic range of the comet assay and the currently used calibration procedures (Moller & Loft 2006).

The two most used ways of detecting 8-oxodG in urine are based on chromatographic and immunochemical methodology. The latter consistently yields higher estimates of the 8-oxodG excretion than the former and there is considerable variation between the two methods, which is believed to be due to insufficient specificity of the antibodies. For validation purposes, this report only includes data obtained by the chromatographic assays because they may be closer to the true excretion levels of 8-oxodG in urine. Too little data are available on measurement of 8-oxoguanine in urine to discuss validation at the present time.

Variation studies. The sources of variation in assays for detection of oxidized DNA in cells or urine originate as a consequence of variability of subjects (intra- and interindividual variation), laboratories and assay variation. The intraindividual variation of SB measured by the comet assay endpoints of unexposed subjects studied over a period of 6 months revealed coefficient of variation (CV) as follows: 42% (95% confidence interval (CI) 18–72), 26% and 21% for intraindividual, interindividual, and assay variation, respectively (Holz et al. 1995). In contrast, a similar study with samplings taken over 14 months and examining seasonal variation in the level of SB reported that the intraindividual and assay variation were of similar magnitude (Moller et al. 2002). The intraindividual variation of urinary 8-oxodG excretion in two subjects investigated over a 10-day period showed high variation (37% and 57%, respectively) (Pilger et al. 2002). A study with six experimental series over a period of eight months yielded interindividual, intraindividual, and assay variation as follows (mean CV and range): 57% (53–68 in various serious), 48% (18–106), and 14%, respectively (Pilger et al. 2001). The DNA damage biomarkers appear to vary over time in the same subject; this variation is dependent on external exposures that could be diet (Dusinska et al. 2002, Giovannelli et al. 2002), air pollution (Sorensen et al. 2003a, Vinzents et al. 2005, Brauner et al. 2007) or sunlight (Moller et al. 2002, Sorensen et al. 2003a).

Probably the most reliable overall assessment of the interindividual variation comes from the ESCODD study, which used standardized assay protocols and analysed the level of DNA damage in MNBC DNA from young healthy male subjects recruited from different countries. The median (range) interindividual variation (CV) in 8-oxodG or FPG sites of MNBC DNA were 43% (33–188) and 49% (30–100), respectively (Gedik & Collins 2005). In comparison, the CVs for standard samples were 68% and 66%, respectively. Another report of 99 subjects with measurements of FPG sites and 8-oxodG in lymphocyte samples from the same subject indicated a somewhat lower CV for the FPG sites compared to 8-oxodG measurements, i.e. the means (SDs) were 0.24 (0.08) and 0.92 (0.42) lesions per 10^6 dG, respectively (Hofer et al. 2006). The CV of SB in MNBC of subjects in the control groups of biomonitoring studies has been reported to be 36% (95% CI 27–46) (Moller et al. 2000). An analysis of the reported data on the comet assay endpoints indicates that the level of DNA damage measured by the comet assay varied as follows (mean (SD)): SB 10 (7.2), ENDOIII 13.9 (12.6) and FPG 10.1 (9.5) arbitrary units in a 0–100 comet assay score (Moller 2006a). In this analysis some labs contributed with several reports; a subanalysis where each lab only contributed with one data point yielded essentially similar results indicating that the variation between separate investigations

contribute the most to the overall investigation (Moller 2006a). In the comet assay, an important contributor to the variation is slide scoring, which has been investigated in two studies. In one study, 19 investigators from seven laboratories scored the same slides of cells exposed to H_2O_2; all but one of the investigators detected a dose–response relationship, but the variation in slide scoring (CVs) ranged from 10% (highest dose) to 100% (control) (Garcia et al. 2004, Moller 2006b). Using a similar approach, another group of researchers found that both experienced and non-experienced investigators were able to distinguish between three slides of X-ray irradiated cells with clear difference in the average level of damage (Moller et al. 2004). The corresponding CVs of slide scoring were 20% (high dose), 38% (low dose) and 93% (non-irradiated) (Moller et al. 2004). Although the variance in slide scoring was large in this study, it appeared to depend on experience and was lowest among the most experienced investigators.

Reports of interlaboratory assessments of variation in urinary excretion of 8-oxodG are sparse, but very good linear relationship ($r = 0.95$ and $r = 0.99$) were obtained in two exercises where labs analysed the same samples (Poulsen et al. 2003, Loft et al. 2006).

Dose–response relationship. Most studies are designed for the purpose of detecting qualitative difference between exposed and referent groups of subjects. Quantitative relationships necessitate the measurement of continuous variables of oxidized DNA and exposure markers (e.g. concentration of chemicals in ambient media or bodily fluids), and correlations should be biologically plausible and preferably an integrated part of the study design. By far the most compelling examples of such dose-response relationships come from intervention studies where individual exposure–effect relationships are obtained. Positive linear dose–response relationships have been shown for FPG sites and 8-oxodG in MNBC related to particulate air pollution exposure, although with 42–49% unexplained variation (Sorensen et al. 2003a, Vinzents et al. 2005). Negative linear dose–response relationships have been reported for urinary excretion following ingestion of polyphenol-rich olive oils or green tea polyphenols in dose-dependent manners (Weinbrenner et al. 2004, Luo et al. 2006). However, there were no dose–response relationships in FPG or ENDOIII sites in MNBC after daily ingestion of 1–3 supplementary kiwi fruits, in spite of an overall beneficial effect (Collins et al. 2003). A study investigating the effect of different doses of vitamin C in MNBC showed no beneficial effect in terms of 8-oxodG (Herbert et al. 2006).

Predictive value of DNA damage biomarkers relation to cancer. Assessments of the predictive value of biomarkers of DNA damage in relation to the risk of cancer relies on the use of prospective cohort studies with biobank samples. Unfortunately very few biobanks have urine samples and these are often only spot urine samples, which are less reliable than samples collected over 24 h. Biobank material of blood cells appears to be limited to leukocytes that have not been cryopreserved optimally and therefore are not suitable for detection of oxidized DNA because of massive oxidation. Consequently the predictive value of oxidized DNA in blood cells has only been investigated in case–control studies. These have shown elevated odds ratios for various cancers in subjects having high levels of SB in MNBC (Moller 2006b), although not prostate cancer patients (Lockett et al. 2006). However, it should be noted that the

level of oxidized DNA in blood cells and urine of cancer patients may be elevated compared with referents, which increases the likelihood of reverse causality and is the main reason for the need of biobank-based prospective cohort studies (Loft & Moller 2006). Only one study to date has investigated the predictive value of 8-oxodG excretion in a prospective setting; the incidence rate ratio of lung cancer was 11.8 (95% CI 1.21–115) per doubling of 8-oxodG excretion in never smokers, whereas no association between 8-oxodG excretion and risk of lung cancer was observed among current and former smokers (Loft et al. 2006).

Conclusions. Biomarkers of oxidative damage to DNA in MNBC based on the comet assay and chromatographic measurement of 8-oxodG in extracted and hydrolysed DNA have been subject to some interlaboratory assay validation, problems have been characterized and there is some consensus as to the true levels. Similarly, urinary excretion of 8-oxodG measured by chromatographic assays has been validated in a few interlaboratory studies, whereas ELISA-based assays at present appear to lack specificity. Many studies of these biomarkers have demonstrated consistent associations with exposures, interventions, host factors and risk factors relevant for cancer. Case–control studies support a role of DNA base oxidation and repair in cancer risk, although they may be compromised by reverse causality. Biobank-based prospective studies on oxidative damage in DNA are difficult because of spurious oxidation during storage. So far, only one study has addressed urinary excretion of 8-oxodG as a risk factor for cancer in a prospective setting.

Adducts of N*-nitroso compounds*

Sources of exposure and formation of NOCs. Humans are exposed to a wide range of *N*-nitroso compounds (NOCs) from diet, tobacco smoking, work place (mainly rubber, leather and metal industries) and drinking water (Tricker & Preussmann 1991, Bartsch & Spiegelhalder 1996), diet being the major source of exposure for the general population (Tricker 1997). Preformed exogenous nitrosamines are found mainly in cured meat and cured cheese products, smoked preserved foods, beer and whisky, and pickled and salty preserved foods (Tricker & Preussmann 1991). The major preformed nitrosamines in the diet are *N*-nitrosodimethylamine (NDMA), *N*-nitropyrrolidine and *N*-nitrosopiperidine. The observed range of dietary intake of NDMA varied between 0.08 and 0.55 μg daily (Jakszyn et al. 2006). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosonornicotine (NNN) are specific nitrosamines from tobacco; heavy smokers have an intake of up to 48 μg per day (Bartsch & Spiegelhalder 1996).

On the other hand, nitrosamines are formed endogenously from nitrate and nitrite. Although levels have been reduced during the last 20 years, sodium nitrite is still widely used as food preservative in cured meat products. Nitrite is also formed in the human body, from oral reduction of salivary nitrate. Vegetables and water are the main sources of nitrate intake. Nitrites are transformed into nitric oxide by gastric acid catalysed formation, which acts as a nitrosating agent of amines and amides, forming NOCs (Tricker & Preussmann 1991). Under chronic inflammatory conditions, such as in precancerous lesions of the digestive tract, nitrosating agents are overproduced (Bartsch & Spiegelhalder 1996). Studies in volunteers have shown that red meat intake has a consistent dose response in the endogenous formation of NOC that are measured in faecal samples as apparent total NOCs (ATNC) (Bingham et al. 1996).

This effect seems to be associated with the content of haem, a red organic pigment containing ferrous iron (Cross et al. 2003). Under certain conditions, haems are known to be nitrosated, and act as nitrosating agents (Bonnett et al. 1975). Nitric oxide (NO) has been shown to react directly with haemoglobin and myoglobin to produce NOCs (Wade & Castro 1990). The formation of *N*-nitrosoarginine by haem enzymes under anaerobic conditions has also been demonstrated (Hirst & Goodin 2000). Endogenous production of nitrosamines is substantiated by the markedly elevated levels of faecal output (in the order of 500 μg per day) compared with dietary intakes of only 13 μg daily (Bingham et al. 1996).

Relatively few epidemiological studies have evaluated the direct effect of nitrosamine intake. The estimation of dietary exposure to NOC and their precursors have been done indirectly through foods identified as sources of them. This could be due, until recently, to the absence of a complete food composition table on NOC content in foods (Jakszyn et al. 2004). On the other hand food frequency questionnaires do not usually collect detailed and complete information about preservation and processing methods of all potential food sources of nitrosamines. Therefore, it is difficult to achieve an accurate assessment of the dietary intake of these compounds. Furthermore, none of them had information about endogenous NOC. This means that they are actually measuring a small part of the total dietary human exposure to NOC, and therefore underestimating their effect.

There are also other factors than could modify the effect of NOC, including presence of inflammatory chronic conditions, intake of vitamin C or *Helicobacter pylori* infection. Finally, it could also be important to take into account interactions with genes, particularly with polymorphisms of metabolic genes involved in the metabolism of NOC or DNA repair genes, which so far have been poorly studied.

Biomarkers of NOC exposure. Measurement and quantification of DNA adducts of nitrosamines in humans may be the most direct way to assess both sources (exogenous and endogenous) and could provide a good biomarker of biologically relevant exposure that will facilitate the epidemiological investigation of their causal relationship with cancer (Shuker & Bartsch 1994). Metabolic activation (α-hydroxylation) of nitrosamines is required to convert them to a methylating agent. In most cases, this pathway is due to oxygenation of the α-carbon, mediated by CYP450 enzymes (Yang & Smith 1996) and progress to the formation of α-hydroxyalkylnitrosamines. These compounds decompose to give alkylating agents. Alkyldiazonium ions are considered the last compounds formed from oxidative metabolism of nitrosamines that react with DNA bases and give rise to alkyl adducts.

The most important methylated products generated in DNA from NDMA, NNK and other nitrosamines are: N^7-methylguanine (N^7-MeG) which is the most common, because alkylating agents react preferentially at the N7-position of guanine (about 70% of total methylated adducts formed), O^6-methylguanine (O^6-MeG) (about 7%), N^3-methyladenine (about 3%), O^4-methylthymine (<1%), methylphosphotriester (about 12%) as well as other minor base modifications (Kyrtopoulos 1998). It has been observed that nitrosated glycine derivatives react with DNA to give rise to several other adducts including O^6-carboxymethylguanine (Cupid et al. 2004). O^6-MeG found in DNA from human gastrointestinal tissues may also be derived from intragastric nitrosation of glycine or related compounds (Shuker & Margison 1997).

The N^7-MeG which is the most abundant products of DNA alkylation by nitrosamines, is not directly promutagenic, although it can undergo metabolic changes to lesions that are. It is repaired relatively slowly (the half-life in rat liver or lymphocytes is about 55–60 h (Kyrtopoulos 1998)). The O^6-MeG adduct is well established as promutagenic. There are several studies that show that tumours induced in rats by a high dose of NDMA are associated with the formation of O^6-MeG in the DNA of the target organs (Souliotis et al. 1995). It has been shown that the formation of O^6-MeG in DNA, especially from gastrointestinal and urogenital organs is increased (more than ten-fold) by ethanol co-exposure (Anderson et al. 1996). Formation of O^6-MeG in the DNA of the target organs seems to be a necessary step for tumour development (Shuker & Bartsch 1994). O^6-MeG on the other hand is a substrate for the DNA repair enzyme O^6-alkylguanine-DNA-alkyltransferase (Atase) (Margison et al. 2003) which specifically repairs O^6-MeG adducts. In contrast O^6-carboxymethylguanine is not repaired by this enzyme (Shuker & Margison 1997).

Validation studies. Several studies have been carried out to measure nitrosamine-derived DNA adducts in humans and animals in different specimens (normal tissue, tumour tissue, from surgical or endoscopic biopsies, blood cells). Some studies were carried out in samples exposed to a very high level of nitrosamines (NDMA poisoning; exposed to cytostatic alkylating agents) in subjects living in high-risk areas for oesophageal and gastric cancer (Shuker & Bartsch 1994) or with normal levels of exposure. Several techniques have been used to detect and quantify N^7-methyldeoxyguanosine (N^7-MedG) and O^6-MeG in DNA but very few studies have been conducted to assess the validity, repeatability and reproducibility of the techniques for measuring nitrosamine adducts.

Harrison et al. (2001) carried out a study to validate an immunoslot blot (ISB) assay for quantification of N^7-MedG in human DNA, using polyclonal antibodies against the imidazole ring-opened form of N^7-MedG. The limit of detection was 0.10 ± 0.009 μmol of adduct per mol of dG (or 2.5 ± 0.22 adducts per 10^8 normal nucleotides) using only 1 μg of DNA/slot, which is in the same order of sensitivity as that achieved by ^{32}P-postlabelling assay using 10 μg of DNA. Then, they assessed the dose-dependent formation of N^7-MedG in liver and brain DNA from mice receiving a dose of the methylating agent temozolamide. They observed a strong linear relationship with correlation coefficients of 0.97 and 0.94 in liver DNA and brain DNA, respectively, indicating that the adduct was formed in proportion to the dose over the entire dose range that was studied. The reproducibility of the assay was determined on different assays ($n = 3$) in 22 samples, observing a mean coefficient of variation of $11.6 \pm 8.3\%$ (SD). The same samples run in triplicate on the same ISB assay ($n = 94$) gave an average coefficient of variation of $8.7 \pm 8.3\%$ (SD). Then the ISB was validated against the HPLC/^{32}P-postlabelling method, using 24 pyloric DNA samples of rats treated with methylating agents. The results showed a high degree of correlation ($r = 0.98$) between the two methods. Five human bladder DNA samples were also analysed by both methods and showed a high correlation, although one had a higher level by the IBS assay than by the HPLC/PPL method. Finally, the ISB assay was applied to DNA from normal colorectal mucosa and colon carcinoma from individuals not known to be exposed to methylating agents, observing levels between 0.11 and 1.34 μmol of N^7-MedG per mol of dG, while peripheral blood mononuclear cell DNA samples from patients treated with methylating agents had levels between

0.22 and 320 μmol of N^7-MedG per mol of dG. This technique seems to be useful for large epidemiological studies, as it has a degree of sensitivity similar to other laborious methods, needs small amounts of DNA, and offers the potential for rapid and high-throughput analysis of DNA from different specimens (tumour specimen, normal tissue and white blood cells).

Zhang et al. (2006) conducted a study to validate measurement of N^7-MeG and O^6-MeG adducts by liquid chromatography-positive electrospray tandem mass spectrometry (LC/ESI-MS/MS) using standard solution, sample blanks and quality controls samples. The calibration curves obtained for both adducts showed correlation coefficients over 0.999. The limit of detection was 75.8 fmol for N^7-MeG and 22.7 fmol for O^6-MeG. The intra-day precision values (analysis of samples at different times during the same day) were $<9.3\%$ for O^6-MeG and $<11.1\%$ for N^7-MeG, and accuracy values (mean of determined concentration/actual concentration) ranging from 90.8 to 118% for O^6-MeG and from 92.9 to 119% for N^7-MeG were observed. The interday precision values (repeated analysis of quality samples over four consecutive days ($n=16$)) were $<8.0\%$ for O^6-MeG and $<7.2\%$ for N^7-MeG, and accuracy values ranging from 94.5 to 116% for O^6-MeG and from 95.2 to 110.2% for N^7-MeG. Then the method was applied to quantification of adducts on samples of salmon testis DNA treated with different concentration of *N*-methyl-*N*-nitrosurea (MNU). Values ranged from 69.4 to 1164.5 O^6-MeG adducts per 10^6 bases and from 493.0 to 7513.0 N^7-MeG adducts per 10^6 bases. The ratio N7-MeG to O^6-MeG was 6.5.

Yang et al. (2002) validated measurement of N^7-MeG and O^6-MeG adducts by liquid chromatography-UV tandem mass spectrometry (LC-UV-MS/MS) also using standard solution. The limits of detection were 64 and 43 fmol, respectively. The coefficients of variation (CV) were 6.7 and 7.9%, respectively. The intra-assay reproducibility (SD of five injections of the same standard solution) was 2.3% for N^7-MeG and 1.7% for O^6-MeG. The interassay CV ($n=5$) were 6.5 and 3.4%, respectively. Then the method was applied to liver DNA from rats treated with different doses of MNA, with levels of 95.2 adducts of N7-MeG per 10^5 dG and 14.8 O^6-MeG per 10^5 dG.

^{32}P-postlabelling has greatest sensitivity and is considered to be the most appropriate approach (Haque et al. 1994), particularly when the amount of DNA is limited, but is extremely time-consuming and laborious (Harrison et al. 2001). Povey and Cooper (1995) developed a combined immunoaffinity purification ^{32}P-postlabelling procedure and conducted a validation study on it using internal standard: less than 1 fmol of O^6-methyldeoxyguanosine (O^6-MedG) could be detected with high degree of precision (coefficient of variation $<12\%$). Then the assay was applied to human DNA samples exposed to chemotherapeutic methylating agents. O^6-MedG was detected in all samples analysed, ranging from 0.026 to 23.2 mmol O^6-MedG per mol dG. Comparing this result with those obtained in the same samples with HPLC/radioimmunoassay the investigators found a correlation of 0.99 between the two methods (Povey & Cooper 1995). N^7-MeG adducts were determined in normal bronchial specimens and peripheral blood lymphocytes in 13 smokers and 11 non-smokers undergoing pulmonary surgery (Mustonen et al. 1993) also using the ^{32}P-postlabelling assay with anion exchange chromatography as an adduct enrichment method. The adduct levels in smokers were significantly higher than in non-smokers. The mean adduct N^7-MeG levels in bronchial tissue were 17.3 and 4.7 adducts per

10^7 nucleotides for smokers and non-smokers and 11.5 and 2.3, respectively, in lymphocyte DNA. Among smokers for whom DNA from both tissues was available, N^7-MeG levels correlated ($r = 0.77$) in the two tissues.

HPLC with immunopurification through specific antibodies and electrochemical detection or ELISA detection showed detection limits of 0.5 and 2 pmol N^7-MeG per DNA sample, respectively (Bianchini et al. 1993). Also using polyclonal antibodies, through a combined HPLC and immunoassay method, Degan et al. (1988) quantified N^7-MedG at levels as low as 0.05 pmol in lymphocyte DNA from rats treated with NDMA. Using 1 mg of DNA a level below 1 adducts per 10^7 nucleotides can be measured. At time points up to 48 h post-treatment the levels seen in DNA from lymphocytes were very similar to those seen in liver DNA. In another validation study, van Delft et al. (1993) compared immunochemical analysis of specific monoclonal antibodies (by ELISA) with electrochemical analysis (HPLC with electrochemical detection) of N^7-MeG adducts in liver DNA from rats treated with hydrazine, a methylating agent. They observed similar results with both methods, with a dose-dependent increase in the level of DNA methylation after a dose of 1 mg kg^{-1} or higher. The detection limit was 1 adduct per 10^6 nucleotide N^7-MeG for the ELISA and 0.4 for the HPLC-EC method. In a complementary study, antibodies of N^7-MeG were measured by ELISA in peripheral blood cells from patients treated with dacarbazine, an indirectly methylating cytostatic drug. During the first hour after the end of the treatment, a rapid increase in the level of methylation was observed, that then reached maximum levels after 4 h (23 N^7-MeG adducts per 10^6 nucleotides) followed by a slow decrease (the level was approximately 9 N^7-MeG per 10^6 nucleotides 72 h after treatment).

A combination of HPLC and ^{32}P-postlabelling was validated using radiolabelled DNA and liquid scintillation counting, which accounts for adduct loss from enzymatic digestion to detection (Kato et al. 1993). The detection limit using 100 μg of DNA was 1 adduct of N^7-MedG in 10^8 unmodified dGp. (Using the same combination of methods, a previous study (Shields et al. 1990) also using 100 μg of DNA obtained a limit of detection for N^7-MeG of 1 adduct in 10^7 normal dGp, suggesting that between 1990 and 1993 the sensitivity of the method was improved.)

Studies on dose-response. During chemotherapy of seven patients exposed to three daily doses for 10 days with procarbazine (a methylating agent), the accumulation of O^6-MeG was linearly correlated ($p < 0.01$) with the cumulative dose of procarbazine, with a slope of 0.011 fmol of O^6-MeG per μg DNA per mg kg^{-1} of body weight or 2.68×10^4 fmol of O^6-MeG DNA per mg m^{-2} (Souliotis et al. 1990). In another similar study (Souliotis et al. 1991) in patients treated with dacarbazine, it was observed that the levels of O^6-MeG adducts decreased by approximately 30% over the 24 h following exposure and were not detectable 49 h after exposure. No correlation between the extent of adduct formation and lymphocyte levels of the repair enzyme O6-alkylguanine-DNA alkyltransferase was observed. The levels of accumulation of O^6-MeG adducts in human blood leukocytes in humans exposed to therapeutic procarbazine are about fivefold less than those in treated rats (Souliotis et al. 1990). In another study in rats (Souliotis et al. 1995) with a daily intake of NDMA between 28 and 372 μg kg^{-1} for up to 28 days, O^6-MeG adducts accumulated rapidly in liver and blood leukocytes, reaching a steady state within 2–7 days in the range 0.08–0.45 μmol per mol G, similar in young and adult animals. Accumulation of adducts in blood was

30% lower that in liver. Following cessation of NDMA treatment, adducts were lost rapidly from DNA of both tissues, with an apparent $t_{1/2}$ of 19–23 h for liver and 30–35 h for leukocytes.

Samples from tumour tissue vs. normal tissue. Some studies have found that the N^7-MeG levels in bladder cancer patients are higher in the DNA from tumour tissue than in DNA from adjacent normal epithelium (Saad et al. 2006). In 42 Egyptians with bladder cancer, using the ^{32}P-postlabelling technique, N^7-MedG was detected in 93% of the DNA from the tumour and 74% of the normal bladder tissue sample. The adduct ratio (tumour DNA/normal DNA) varied between 0.2 and 136 (median 4.6) and the levels were not associated with gender, age or the presence of schistosomiasis. Another study in 46 patients with larynx tumours (Szyfter et al. 1996), also using ^{32}P-postlabelling, found higher average levels of N^7-alkylguanine adducts in tumour DNA (26.2 per 10^7 nucleotides) than in non-tumour DNA (22.7 per 10^7 nucleotides) or in blood leukocyte DNA (13.1 per 10^7 nucleotides). There were higher levels in males than in females and in smokers than in non-smokers.

In contrast, in a study on stomach cancer the levels of total adducts of O^6-alkylguanine and the proportion of samples with detectable levels were higher in non-involved mucosa than in the tumour tissue (DNA from stomach tissue samples of 24 gastric cancer patients) (Palli et al. 2001). In 62 subjects undergoing surgery for colorectal tumours in UK (Seiler et al. 1993) the O^6-MedG was detected in 43% of normal DNA samples and 52% of tumour DNA samples, and the levels of adduct were highest in areas of the colon and rectum where the highest incidence of large bowel tumours occurs.

Levels of DNA adducts from dietary or environmental exposure to nitrosamines. It has been estimated that current daily human exposure to DMN (between 0.1 and 10 μg) would be expected to give rise to O^6-MeG levels below 1×10^{-9} mol per mol guanine (Kyrtopoulos et al. 1993).

Only a few studies have evaluated the correlation between adduct levels and dietary or environmental exposures. In a study in healthy volunteers, measuring O^6-carboxymethylguanine DNA adducts in exfoliated colonic cells (Lewin et al. 2006), the percentage of positive cells was higher with a red meat diet than with a vegetarian diet, and a high correlation was observed between the level of adducts and ATNC in faeces. In another study (Palli et al. 2001) a high correlation was observed between the total levels of O^6-alkylguanine from the non-involved gastric mucosa and the estimated intake of DMA and NDMA. In contrast, in another study (Georgiadis et al. 2000) the level of O^6-MeG in maternal blood DNA and cord blood DNA was not associated with smoking, consumption of meat, fish or nitrate-treated foods, while fruit and vegetable intake showed non-statistically significant trends. The explanation was that given that as O^6-MeG in human WBC has a short half-life (approximately 22–25 h) the adduct levels are determined by recent exposure. This could be an important limitation for the use of these biomarkers in epidemiological studies on diet and cancer, in which we are interested in the habitual exposure.

O^6-MeG adducts have been measured in blood leucocyte DNA from a cross-sectional study in 407 samples from 17 populations (EUROGAST Study Group 1994). The presence of adducts was detected only in 5% of the samples, most of them from populations (Japan and Portugal) with extremely high gastric cancer incidence.

Furthermore, prevalence of adducts was observed mainly in those with detectable levels of serum pepsinogen A (a marker of severe chronic atrophic gastritis), consistent with the hypothesis that adducts may have arisen from the action of endogenously formed methylating agents, whose formation is increased in the presence of a chronic inflammation condition. The authors estimate that the half-life of N^7-MeG adducts in human leukocytes is about 40 h, reflecting a short period of exposure to NOCs (EUROGAST Study Group 1994).

Conclusions. Quantification of N^7-MeG and O^6-MeG in DNA from lymphocytes may be the most direct way to assess sources of both exogenous and endogenous nitrosamines in epidemiological studies and could provide a good biomarker to investigate their causal relationship with cancer. Immunoassays seem to be especially useful for large epidemiological studies, because of their degree of sensitivity, use of lesser amounts of DNA and potential for relatively easy, rapid and high-throughput analysis. However given the short half-life observed for these adducts, they are not very useful as a long-term marker of exposure.

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