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The use of biomarkers for improved retrospective exposure assessment in epidemiological studies: summary of an ECETOC workshop*

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Abstract
During a scientific workshop the use of biological monitoring in characterization of retrospective exposure assessment was discussed. The workshop addressed currently available methodology and also novel approaches such as in different fields of 'omics'. For use in epidemiology requiring retrospective exposure assessment, biomarker levels should not vary too much over time. If variability in exposure over time is large and differences in exposure between individuals are relatively small, this may lead to underestimation of the exposure–response relationship. This means that, for a sound assessment of health risk, biomarkers that reflect cumulative exposure over a long period of time are preferred over biomarkers with short half-lives. Most of the existing biomarkers such as metabolites in body fluids usually have rather short half-lives, typically less than 1–2 days. Some adducts to DNA show somewhat longer half-lives. The current limit to persistence of biomarkers reflecting cumulative exposure over time is from adducts to haemoglobin with a half-life of 4 months. Some specific organic substances may be more persistent due to storage in adipose tissue or metals in kidneys, nails and hair. The metabonomics, proteomics and present gene expression profiling approaches do not provide a perspective to the availability of more persistent biomarkers and most approaches discussed to date show that it is difficult to interpret study outcomes in terms of exposure to a specific xenobiotic factor. Research efforts should focus on improvement and validation of currently available approaches in the field of addition products to DNA and proteins. Promising new developments may be phosphotriester DNA adducts and adducts to more long-lived proteins such as histones.

Keywords: *Metabonomics, proteomics, gene expression profiling, phosphotriester DNA adducts, histones*

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Introduction

How can biomarkers be used to improve retrospective exposure assessment? This question is justified by an increasing interest of health authorities to use population-based data in risk assessments. Epidemiologists are confident that these questions can be addressed but they are especially aware of limitations, specifically to the exposure

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characterization. In a workshop organized by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) this issue was discussed by epidemiologists with researchers active in the development and application of biomonitoring and some investigators who are involved in application of novel 'omics' approaches. This meeting report describes the current scientific knowledge base in this area and addresses some ideas and opinions that came up during presentations and discussions. The report will only cover the scientific assessment of where we stand now, and tries to identify research challenges for the future. Other more societal aspects will also be covered in an ECETOC publication that is currently being prepared (see http://www.ecetoc.org).

Better use of existing methods for exposure assessment using biomarkers

To set the stage for an indepth discussion about the role of biomarkers in reconstruction of exposures in the past, Dr Tim Gant (Medical Research Council and University of Leicester and Chair of the meeting) presented the overall question that the meeting would be addressing: Are there novel marker technologies or evolving modelling techniques which could be used or adapted to better define historical exposure to chemicals with the aim of using this information in the conduct of epidemiological studies? Dr Gant specified the type of information that would be needed to characterize the hazard: What chemical substance, mixture is involved in the exposure? When did the exposure take place? How much was the subject exposed to, quantitatively?

The role of a biomarker can be interpreted as a 'fingerprint' left behind in the body after exposure (with the analogy of the body as a 'crime scene'). The physical entity of this fingerprint can be an adduct, a change like DNA hypermethylation or an altered DNA sequence (e.g. microsatellite mutation), an altered gene expression, a protein structure modification, or any other change. For use in retrospective exposure assessment, the half-lives of these changes are important. How can this half-life be determined? Is the half-life of a modified macromolecule equivalent to the half-life of the parent unmodified macromolecule? For the assessment of factors which could attenuate individual response to chemical exposure, in addition individual susceptibility characteristics would have to be collected. In a population-based study these individual characteristics could be taken into account as splice variants or single nucleotide polymorphisms by full genome sequencing.

Dr Martin Wilks (Syngenta Crop Protection AG, Basel, Switzerland) stood in for Dr Alan Boobis of Imperial College, London, UK who could not attend the meeting. Because many diseases are multifactorial, more refined techniques are required to be able to identify causal relationships. In 1965 criteria were proposed to evaluate the strength of associations with respect to causality (Bradford Hill 1965). These criteria are known as the Bradford Hill criteria (in short: consistency, strength of association, temporal sequence, dose–response, specificity, coherence, biological plausibility, analogy and experimental evidence). Biomarkers may help to underpin a causal relationship; some biomarkers may help to mechanistically link the exposure to an (early health) effect. As biomarkers have often been validated in animal studies, biomarkers normally reflect toxicity mechanisms relevant to humans. Therefore, Dr Wilks also drew attention to the necessary validation of biomarkers in animal studies (preferably including a dose–response relationship). In addition coherent

results in human population-based cross-sectional and longitudinal studies would be needed to convince the epidemiologist of the validity of an approach.

Dr Chris Wild (University of Leeds, UK) addressed the value of biomarkers in establishing a relationship between exposure and health effects (Wild at al. 2008). In this context he defined 'environmental exposure' from a broad perspective (lifestyle, diet, environmental and occupational chemicals, etc.) and drew attention to the need to balance efforts to measure genetic effects with increased attention to accurate assessment of exposure. Only if this happens will the large prospective cohort studies, such as UK Biobank be able to elucidate gene–environment interactions adequately in chronic diseases. He proposes the term 'exposome' to indicate the scale and dynamic complexity of all environmental exposures throughout the life course of an individual (Wild 2005). Even a partial characterization of the exposome can give a significant reward in terms of identifying and quantifying a relevant causal factor in the onset of a disease, including helping to establish the biological plausibility of an exposure–disease relationship. Stratifying by genotype may reveal underlying associations between exposure and disease which are otherwise obscured by heterogeneity of response in the population. Past exposure assessment remains a challenge to the biomarker field, with only a limited number of examples where somatic mutation analyses have been used in population-based studies to link specific exposures to genetic alterations in tumours, e.g. *TP53* mutational spectra in tumours and plasma DNA (Kirk et al. 2005). Dr Wild also drew attention to the value of the use of biomarkers in evaluating the impact of intervention studies, such as in the study of blood aflatoxin-albumin adducts in Guinea (Turner et al. 2005).

Dr Dick Heederik of the University of Utrecht, The Netherlands gave an overview of recent developments in exposure assessment in occupational epidemiology. He showed that improved exposure assessment strategies lead to more optimal and flexible use of exposure information in the data analysis. With good-quality exposure information the shape of exposure–response relationships can be explored in epidemiological studies using spline analysis (Eisen at al. 2004). This perspective is of special interest in the low range of exposure and will stimulate discussions on the mechanistic assumptions, e.g. in genotoxic substances. These high-resolution exposure–response studies are also expected to improve the quality of quantitative risk assessments. A crucial issue in the optimal performance of exposure information in epidemiological studies is control over misclassification of exposure and measurement error. The analytical error of an exposure measurement is usually small (<10 to maximally 20% in some assays). However, when the aim is to estimate long-term exposure, variability over time in exposure determines how precise the average exposure can be estimated on the basis of a limited number of measurements. When variability over time (or intraindividual variability) is large, and differences in exposure between individuals are relatively small, this intraindividual variability leads to underestimation of the exposure–response relationship (Heederik & Attfield 2000). The following equation describes the relationship between the slope of an exposure–response relationship, the ratio of the intra- and interindividual variability in exposure (λ), and the number of repeated measurements (k). In this equation β_{true} is the slope of a straight line describing the relationship between the true mean exposure and the expected health outcome. β_{obs} is the observed slope in a particular dataset. The quality of the study is improved by increasing the number of observations k:

$$\beta obs/\beta true = \frac{1}{1 + \lambda/k}$$

However, if the intraindividual variability (noise) is large in comparison with the interindividual variability (signal), which increases the variance ratio λ to values >1, this will cause strong underestimation (or attenuation) of the slope of the exposure–response relationship to values closer to 1. Strong underestimation of an exposure–response relationship can be avoided by reducing the influence of σ^2_{intra} by taking more measurements per individual or by increasing σ^2_{inter} by involving more subjects in the study with a low or high exposure. These principles can also be applied to biomarker information. Biomarkers which vary strongly over time, for instance because of their short half-life in the human body, will lead to strong attenuation of exposure–response relationships (Lin et al. 2005). (In the context of this report it is worthwhile mentioning that Lin et al. (2005) concluded that biomarkers performed better than air sampling in terms of a lower variance ratio λ, as expressed in the policy implication that was published with this paper: 'Epidemiologists should consider using biomarkers instead of, or in addition to, air measurements for assessing levels of chemical exposures'.)

Dr Heederik is optimistic about the possibility of having better quality data from field studies because some of the crucial epidemiological principles are now being applied by occupational hygienists in exposure studies linked to epidemiological surveys. Some critical commentaries and reviews, and a new book cover the subject of the use of biomarkers in epidemiology (Lan et al. 2004, Pearce 1994, Wild et al. 2008).

Protein adducts

The author of this meeting report reviewed the current knowledge of adducts of chemicals to proteins. Proteins contain numerous nucleophilic groups (e.g. val, cys, his, try, ser, lys, asp, glu and tyr) that can be targets for electrophilic attack by reactive intermediates to form adducts (Törnqvist et al. 2002). These are mostly electrophilic, but are not only alkylating substances such as alkenes or alkylhalides, but are also arylating, nitrenium forming, carbonyl, acylating and phosphorylating organic species (including chemical species such as polycyclic aromatic hydrocarbons and their alkyl derivatives, aromatic amines and nitroarenes, aldehydes, isocyanates, anhydrides, halides, organophosphorus ester pesticides and some war agents). Metals and metal oxides are also known to be involved in protein adduct formation, in part through formation of free radicals, reactive oxygen species that may also be formed by organic peroxides and quinones. In toxicology most of these substances share mutagenic, carcinogenic and reprotoxic properties as part of their toxicity profile. Törnqvist et al. (2002) point out that not all of these adducts are exclusively of post-translational origin like adducts to N-terminal val that are much used as biomarkers for example for ethylene oxide, 1,3-butadiene, acrylamide, etc. Adducts formed to the side chain of nucleophilic amino acids could in theory be formed even prior to synthesis and faulty amino acids could thus be incorporated into *de novo*-formed proteins.

Much is known about the kinetics of formation and decay of protein adducts *in vivo*. Intracellular proteins such as haemoglobin (Hb) follow zero-order kinetics, leading to a steady-state level that is limited by the turnover of the cells. In the case of Hb the lifespan of the erythrocytes is limited to 126 days in humans, causing a gradual

decrease of the adduct after cessation of exposure with a half-life of 63 days in humans. This kinetic pattern was confirmed in experimental studies in animals for different adducts including sulfur mustard adducts in the marmoset (Noort et al. 2002). The formation and decay of adducts to proteins free in solution such as serum albumin (SA) follow first-order kinetics leading to an asymptotic maximum level and a concave-shaped curve describing the course of decay. For adducts to SA in humans this results in a half-life of 20 days. Adducts to SA and Hb follow these kinetics as long as they are chemically stable and as long as there is no biological cleavage mechanism. To date a mechanism of enzymatic repair of adducts to SA and Hb has not been reported. In the light of the purpose of the workshop the question was raised if other proteins could be identified that, with our current understanding of kinetic and dynamic behaviour, would be interesting as candidates for dosimeters of accumulated internal exposure. Among the candidate proteins that could possibly stretch the half-life of biomarkers, collagen and histones were mentioned.

Histone adducts as possible new dosimeters

Histones initially have been looked upon as structure proteins that are intimately intertwined with DNA in the nucleus of the cells. Four types of histones (H2A, H2B, H3 and H4) form a double octamer or histone core. The DNA is wrapped around these cores in nucleosomes of 11 nm in diameter, like beads on a string. The distance between two nucleosomes is covered by 54 base pairs of spacer DNA that is kept in place by a fifth type of histone, H1 (Ebralidse et al. 1988). It became clear that histones have a much more important role than controlling condensation and decondensation of the chromosome during the replication of cells. It was discovered that the flexible free-floating N-terminal lysine tails are often acetylated, each time taking away a positive charge. In the nucleosome there are 26 of these N-terminal lys positions. It was found that acetylation stimulates transcription by RNA-polymerase and that this 'acetylation switch' has an important impact on most chromatin-templated processes of the cells (Jenuwein & Allis 2001). The finding that deacetylation activity by histone deacetylase (HDAC) could influence the growth of tumours, triggered research in the discovery of possible new drugs for cancer treatments that attenuate HDAC activity (Esteller 2008). Several other so-called post-translational modifications (PTMs) have been identified in addition to acetylation. Studies have shown that electrophilic substances can form covalent bonds to N-terminal lys and other nucleophilic targets in different types of histones. Adducts of benzo[a]pyrene diol-epoxide (BaPDE), aflatoxin B1 (AFB1), phosgene and several different aldehydes have been characterized in *in vitro* experiments, and also, but to a very limited extent, in experimental animals. Interestingly, Özbal and co-workers (1994) reported that histone adducts of BaPDE and AFB1 were passed through to daughter cells together with unadducted histones in a culture of human B-lymphoblasts. It is tempting to speculate that histones as well as adducts to histones could persist far beyond the limit of 126 days and perhaps even across generations similar to methylated DNA. They suggested the use of histone adducts for molecular dosimetry purposes. This is a very promising finding but there are still many questions such as: What is the lifespan of histones in humans and to what extent are histone adducts diluted due to *de novo* synthesis and due to the turnover of cells in different tissues? So far only one study in mice has reported on the lifespan of histones. In this

study an average lifespan of 117 days was reported in liver cells and 223 days in the brain. So the lifespan of histones could be longer than that of, for example Hb in humans but it is still uncertain how the dilution of histone adducts would affect the possibility for detection, e.g. in peripheral blood lymphocytes. Despite several uncertainties it is intriguing to suggest that reactive xenobiotic substances might disrupt the histone code that is said to give the cell a memory and identity (Turner 2002).

Novel biomarkers originating from conventional approaches

Metabolites

Anthony Tsarbopoulos of the University of Patras and GAIA Research Center (Greece) introduced the audience to the current state of the art in the targeted ultrasensitive mass spectrometry (MS)-based detection of metabolites. [In addition to what Prof. Tsarbopoulos presented it should be noted that most parent chemical substances have an excretion half-life of hours to 1 or 2 days. There are exceptions, notably chemicals that accumulate in adipose tissue because of their lipophilic properties (e.g. polyhalogenated hydrocarbons, polycyclic aromatic hydrocarbons, dioxins and dibenzofurans). Some of these chemicals also have a high affinity to serum proteins. In addition, metals in particular may have slow kinetics and accumulate in certain tissues due to their binding to specific proteins such as metallothioneins (e.g. accumulation of cadmium in kidneys), protoporphyrins (e.g. lead in zinc protoporphyrin), or metals in hair and nails. Slow kinetics can also be caused by limited bioavailability leading to a slow uptake by those substances that are insoluble to scarcely soluble and that are deposited in the non-ciliated parts of the lungs, sometimes also adsorbed on the surface of fine particles (e.g. chromates in deposited welding fume particles, nitroarenes adsorbed to diesel exhaust soot particles, dyes encapsulated in toner particles). Once chemicals have reached the liver or other metabolizing tissues, biotransformation usually results in formation of metabolites and conjugates with increased water solubility that can be effectively excreted via urine and/or bile.]

Introduction of electrospray ionization (ESI; Fenn 2002) is an important improvement for sensitive analysis of small molecules like metabolites but also for peptides. The ideal configuration for these analyses is a liquid chromatography (LC)-ESI MS/MS triple quad instrument. Triple quad in this case refers to the mass spectrometers comprising two separate quadrupole analysers and a collision quadrupole in between, a set-up that enables MS/MS and pseudo-MS^3 analyses in addition to the so-called single- or full-scan mode analyses. Multiple MS enables more reliable positive identification due to more detailed structure information from the specific MS/MS spectra obtained by further fragmentation of the parent ion into smaller daughter ions. Trace analyses are usually performed in the multiple reaction monitoring (MRM) mode. In this mode a single precursor ion and a selected product ion thereof are selected for quantification (after reappraisal of the mass spectrum to confirm the identity of the substance). As an example the analysis of exposure to carbofuran and carbaryl in the potato seed production on the Island of Naxos was presented. The parent pesticide and their metabolites could be detected in blood samples. A particular problem in the interpretation of the results of the urinary excretion is that carbaryl is rapidly metabolized to its metabolite 1-naphthol, a product that is also

formed in the metabolism of anthracene usually observed in smokers. One way to solve this is also to analyse a second metabolite from anthracene metabolism: 2-naphthol. In this way the results of urinary analysis could be corrected for the contribution of cigarette smoke by looking at the 1-naphthol/2-naphthol ratio.

DNA adducts

Dr Peter Farmer of the University of Leicester gave an overview of the developments in the analysis of DNA adducts. The new developments in this field progress from a longstanding tradition on characterization of adducts relying mostly on nuclear magnetic resonance (NMR) and MS for structure characterization. The method of ^{32}P-postlabelling used to be the preferred method for detection of DNA adducts due to its great sensitivity of at least one adduct per 10^8 nucleotides ($1/10^8$). This technique is limited as to its capabilities of identifying an adduct of interest, due to the use of co-elution with reference standard by chromatography as the sole basis for identification, which is quite far from the state of the art so-called tentative or (very close to) positive identifications achieved using MS-based techniques (Singh & Farmer 2006). There have been attempts to correlate results of the analysis of the DNA adduct of BaPDE (BaPDE-N^2-dG) by ^{32}P-postlabelling to results obtained by LC-MS/MS analysis. The correlation found by Singh et al. (2006) is very high ($r = 0.962$, $p < 0.001$) but it is rather worrying that there appears to be an unexplained 3.7-fold discrepancy between these two methods.

The important news in this presentation is that the achievements in sensitivity of novel MS-based instruments are presently in the range of $1/10^7$ to $1/10^9$ for some adducts (Marsden et al. 2007), including the renowned BaPDE-N^2-dG. Sensitivity may be further improved by using an LC system with column switching (a refinement of conventional single-column LC applications, e.g. Doerge et al. 2000) and analysis on an LC/ESI-MS/MS instrument.

MS also has a promise for the future, as analysis of different adducts to 2′-deoxyguanosine has shown that there is a pattern of loss of a mass of 116 amu. These so-called $[M+H\text{-}116]^+$ ions are formed after neutral loss of 2′-deoxyribose. From this a multireaction-monitoring map (also called 'adductome' map) is produced that adds a global 'adductomics' strategy to the existing targeted analysis of DNA adducts (Kanaly 2006).

Apart from the detection of DNA adducts in peripheral lymphocytes there is yet another possibility involving products of enzymatic DNA repair which leads to rapid urinary excretion of DNA repair products like adducts at N^7-guanine and N^3-adenine. The excretion of these repair products cannot be linked to a tissue. Also it is important to note that some repair systems may be induced by chemical exposures. Despite these efficient mechanisms of enzymatic repair the aforementioned BaPDE-N^2-dG adducts can still be detected until 4 weeks after exposure (Singh et al. 2006). It must be concluded that these bulky adducts are repaired after a much longer period of time than many smaller adducts.

As a challenge or promise for the future Dr Farmer suggests the use of phosphotriester adducts as more persistent species of DNA adducts. There appears to be no enzymatic repair of these adducts in eukaryotic systems. These adducts have been found for N-nitroso compounds (Beranek et al. 1980, Singer 1985), cyanoethylene oxide (Yates et al. 1994), cyclophosphamide (Maccubbin et al. 1991) and phenyl

glycidyl ether (Deforce et al. 1998). Long lifetimes have already been demonstrated *in vivo* in human fibroblasts (Bodell et al. 1979).

Novel biomarkers using developments in the field of 'omics'

Metabonomics

Dr Elaine Holmes of Imperial College, London, UK addressed the role of metabonomics and xenometabolome signatures in epidemiological studies. These complex metabolic profiles are obtained by NMR spectroscopy and MS analysis from body fluids and tissues. Characteristic in this approach is the computational analysis of large datasets using principal-component analysis (PCA), uni- and bidirectional signal correlation, Bayesian probabilistic models and statistical correlation spectroscopy (STOCSY) to analyse patterns. With these techniques it is possible to detect systematic changes that can be used to assess efficacy of therapeutic interventions but also dietary interventions. A nice example of the latter is a study on Camomila tea (extracted from the flower of the *Matricaria chamomilla*). In a well-controlled volunteer study the metabolic patterns were followed in a group of individuals consuming Camomila tea. Interesting changes were noted indicating a change in the profile of metabolites over a period of several weeks. It was surprising to observe that the metabolic pattern deviated from the pattern that was observed before the consumption of the tea and did not return to this initial pattern, suggesting that the consumption of Camomila tea caused a changed pattern that appeared to persist. Further studies are needed to find out how long it takes for the metabolite profile to return to the initial pattern.

This study illustrates the difficulty in interpreting changes in metabolic profiles in terms of biological significance: should the changes be interpreted as beneficial or hazardous? This type of analysis can be hypothesis driven but at the same time using this approach, it can happen that changes are observed that you are not looking for. To reduce the influence of confounding and systemic noise, various chemometric and bioinformatic techniques are required. As limitations encountered in the metabonomics approach Dr Holmes mentioned the lack of sufficient annotation, everything is visible, and the metabolome of body fluids provides a representation of multiple organs, tissues and even of an entire organism. Then there are bottlenecks in the data analysis: there appears to be a lack of protocols for computations that are needed in the interpretation of the data, problems are also expected in integration of the metabonomics approach in conventional epidemiological study designs and, last but not least the phenomenon that 'omics' approaches are moving away from the common way of 'univariate thinking' in analytical chemistry.

Gene expression profiling

The approach of gene expression profiling was introduced in a contribution by Dr Jos Kleinjans (Maastricht University, The Netherlands). In this application the challenge was to show that these profiles are more than just a genome-wide fingerprint, in such a sense that they do also represent biomarkers of health and/or disease status. Dr Kleinjans illustrated this concept by presenting the results of a transcriptomic analysis with data from a small-scale study in the Czech Republic. This study involved children of around 6 and 9 years of age and their parents living in two regions: Teplice

(part of the black triangle industrialized northern region) and Prachatice (a rural region in the south of the Czech Republic). Characterization of the air quality showed a fourfold difference in air concentrations of polycyclic aromatic hydrocarbons (PAH), also for a selection of PAH that are classified as carcinogens, a fivefold difference in NO and a twofold difference in NO_2. The air quality was not different with respect to carbon monoxide, PM-2.5 and PM-10. Small differences were observed in the number of micronuclei (MN) but only in children of around 9 years of age. An important finding was that the gene expression patterns observed in children were substantially different from those observed in adults. In the analysis T Profiler was used. This is a computational method that determines whether an *a priori* defined set of genes shows statistically significant regulation of concordant difference between two biological states. Surprisingly no statistically significant differences were observed between the adults in both regions, whereas a series of gene ontology terms were significantly up- or downregulated in children from Teplice in comparison with children from Prachatice. The genetic pathways that discriminated between the children of the two regions included gene sets related to immune repression and response to viruses and gene sets related to the nucleosome. The latter may be reflected in the results obtained for MN. Dr Kleinjans will attempt to use the gene expression profiles observed for benzo[a]pyrene exposure in *in vitro* studies with human cells and use these patterns for interpretation of the gene expression profiles obtained from the human population.

Proteomics

In a third contribution, the analysis of the proteome was discussed by Dr Jennifer M. Ames of Queen's University Belfast. Lysine and arginine are the amino acids most reactive towards reducing sugars and their oxidation products such as methylglyoxal (MGO). The covalent binding of MGO is used as an example. As a first analytical approach to locate adducts, a protein can be digested by restriction enzymes that hydrolyse covalent bonds between different amino acids. For example, trypsin cleaves proteins predominantly at the C-terminal side of arg and lys. Sometimes it is useful to combine trypsin with other restriction enzymes such as endoproteinase glu-C to achieve peptides of appropriate size for analysis. The mixture of peptide fragments can be analysed by LC-MS in the full-scan mode. A so-called single mass spectrum can be reconstructed from the full-scan data for the native protein and compared with that for the modified protein. This allows ions that are unique to the modified protein to be located. These ions are probably due to protein adducts. Protein fragments can be further characterized in a daughter ion scan in which the molecular ion is fragmented. In cases where members of a chemical class of adducts give a characteristic daughter ion, a parent ion scan may be used to locate all analytes in that class. A third option for analysis of a protein digest is the neutral loss experiment for specific detection of loss of a neutral fragment like phosphoric acid. When the masses of the molecular ion and a prominent daughter ion are known, multiple reaction monitoring (MRM) experiments may be used for accurate and sensitive quantification. All these experiments may be applied to mixtures of peptides or amino acids. So for example, if a protein is completely hydrolysed to its constituent amino acids and amino acid adducts, MRM experiments may be used to quantify the global amount of each individual adduct in a mixed protein hydrolysate.

Modelling of exposure data

Dr G. Loizou of the Health and Safety Laboratory (Buxton, UK) used this opportunity to illustrate the use of physiologically based pharmacokinetic (PBPK) models to assist biomonitoring applications, e.g. to predict the internal dose in target tissues, based on external exposure and/or levels of biomarkers such as end-exhaled breath, blood concentrations or urinary excretion levels. A PBPK model is a realistic but simplified model to describe the kinetic pattern of absorption, distribution, metabolism and elimination (ADME) processes in the body. Input data to describe realistic anatomical and physiological properties of individuals can be taken from the peer-reviewed literature (Brown et al. 1997). If model predictions do not correspond to the measured data, the model can be adapted but again not by fitting the data but rather by changing assumptions with respect to the role of certain components in the model, each representing true physiological compartments such as lung and skin as organs important for uptake of industrial chemicals, but also liver for metabolism and kidney for urinary excretion.

Population-based PBPK modelling is undertaken by incorporating interindividual differences in all model parameters by ascribing appropriate distributions for variability in organ and tissue masses and blood perfusion rates. Distributions for organ and tissue masses and blood perfusion rates for various ethnic groups, ages and gender can be obtained from a freely available resource such as the P^3M software (http://www.thelifelinegroup.org/p3m/index.htm). Through Monte Carlo sampling of the distributions, simulation of interindividual variability is taken into account. In this way the influence of for example obesity can be modelled by incorporating a compartment representing adipose tissue and uploading population-based distributions of body mass index data. Bayesian techniques are applied to distinguish between model parameter uncertainty due to experimental measurement error and normal biological variability (Jonsson & Johanson 2002). PBPK models can be applied in forward dosimetry, e.g. in the prediction of an arterial blood concentration after exposure of a subject to an air concentration. It is also possible (but sometimes more difficult) to reconstruct an external exposure that happened in the past from biological monitoring data (Clewell et al. 2008, Hays et al. 2007). Dr Loizou gave an example of pregnant women in Iraq who had been exposed to methyl mercury. From the levels of methyl mercury in hair it was possible to reconstruct the average daily dose and the internal dose for the unborn child (Clewell et al. 1999, 2000, Shipp et al. 2000). The effects and implications of different workloads on ADME can also be estimated using PBPK modelling (Jonsson et al. 2001).

Discussion

Usefulness of 'omics' approaches

During a discussion about the usefulness of the various biomonitoring approaches and different types of biomarkers it appeared that most of the available new omics-oriented techniques do not offer a clear perspective for new applications that could be useful in the field of retrospective exposure assessment. These techniques often have most valuable properties as markers of effects induced as a response to the presence of chemical substances rather than identification and quantification of chemical substances. A direct relationship with the type and amount of a specific chemical

substance is not obvious and sometimes even very difficult to identify (metabonomics, proteome characterization, gene expression profiling). This does not make these approaches less valuable in the context of public health-related questions at this moment but in the field of retrospective exposure assessment little work is carried out and there is a need for further study to evaluate the potential in this field.

Adducts to macromolecules

The more conventional approaches such as analysis of addition products from body fluids, such as adducts of DNA and proteins offer a better perspective to determine exposures to specific chemicals in retrospect. Sensitivity is good due to the use of increasingly sensitive MS-based techniques. Specificity is also good in the sense that an adduct contains the chemical structure information that is required to find out which ultimate chemical reaction product was involved in its formation. The scope of application may be somewhat limited, i.e. to chemicals that are sufficiently reactive to form covalent bonds to nucleophilic sites in macromolecules. However, this is a property of most if not all genotoxic substances and most chemicals toxic to reproduction except for hormone-disrupting species and other receptor-binding substances that interfere for example with the Ah receptor complex. Especially for protein adducts, relationships with dose are well established, primarily in experimental work using animal models. These markers of exposure take into account the bioavailability and bioactivation of chemicals. Existing analytical methods are well validated but there is a lack of validation of many candidate biomarkers in terms of the quantitative relationship with the level of environmental exposure.

Persistent protein adducts

Persistence of a modification has been identified as a key property of a candidate biomarker. The main interest is to find biomarkers that are more persistent than the chemical they are derived from, but the trace should still contain a pattern specific for the chemical substance that caused the modification (previously referred to as a 'fingerprint'). Compared with the required persistence for applications in endpoints with long lag-times such as cancer, the persistence of all known adduct applications is rather limited compared with the lag-time of 10–30 years. For protein adducts the lifespan is well established in certain specific animals. There are no indications for a decrease of this lifespan of protein adducts due to enzyme-based repair mechanisms. It should be noted however that the lifespan of certain intracellular proteins is determined by the lifespan of the cell. This brings the discussion to the preferred body fluid and cell type. Despite its invasive nature, adducts appear to require collection of peripheral venous blood samples. Populations of erythrocytes have a well-predictable lifespan of 126 days in humans (Törnqvist et al. 2002). For lymphocytes this is more difficult. The turnover appears to be more dynamic and the lifespan appears to be 30 ± 7 days in sheep (Yong et al. 1995). In this respect it would be interesting to focus efforts on the isolation of (subpopulations of specific) long-lived cells such as peripheral lymphocytes. Another interesting suggestion for further research is the use of adducts to histones. *In vitro* studies have indicated that histones, and also their adducts, are passed on to their daughter cells in human lymphoblasts. In the literature suggestions have been made that histone modifications might be hereditary. It should be noted that it is still uncertain if and how the structural

properties of histones are involved in epigenetic imprinting. For the moment it is sufficient to conclude that histone adducts may be persist (far) beyond the limit of 4 months that is the maximum of presently validated protein adduct approaches.

It should be noted that there is another limitation to detecting even very persistent adducts and that is the constant dilution of histones (like all proteins) due to *de novo* synthesis, especially in proliferating tissues. Therefore, it might be interesting to analyse histone adducts from non-proliferation cells like differentiated lymphocytes, e.g. (sub) populations of highly specialized lymphocytes such as B-memory cells. There are still many practical and technical questions such as how efficiently can histone adducts be isolated from a standard volume of human blood for sufficiently sensitive detection. Kinetics of formation and dilution decay and relationships between (accumulated) exposure and adduct formation should be studied in animal studies and later also in volunteer studies to validate this approach. It is also suggested that novel biomarkers be validated in prospective studies. Ideally these and other novel biomarkers should reflect in retrospect the person-months of accumulated exposure estimated by different conventional techniques for exposure assessment, including perhaps the accumulated value calculated from repeated collection of more conventional less-persistent biomarkers (prospective or forward validation).

Persistent DNA adducts and DNA hypermethylation

For DNA adducts the lifespan is limited to a few days and for some specific adducts appears to have a limit of around 3–4 weeks such as the BaPDE-^{2}N-dG. A limitation is the disappearance of these adducts due to enzymatic repair. There are indications that the repair activity is influenced by chemical exposures. Some adducts, e.g. bulky adducts, appear to be less prone to repair, and adducts such as phosphotriesters have been shown not to be subject to repair in eukaryotic cells. It would be interesting to look further into this aspect. In addition it would be interesting to find out about the suitability of DNA hypermethylation patterns in relation to chemical exposure. It is suggested that hypermethylation of clusters of cancer gene promoter islands (so-called CpG islands) may result in heritable transcriptional silencing of for example DNA repair genes (Jones & Baylin 2002).

Mechanistic based or not

An important question raised by Dr Gant was: Should biomarkers be mechanistically anchored? In view of the message brought by Dr Wild the answer should be yes. If biomarkers are used in intervention studies as surrogates for disease then it is important to be confident that the biomarker truly reflects the mechanism in the cause-effect chain. Dr Wild showed that this link to health endpoints would have to be explored in a population-based design. In fact he identified only two studies that have examined the role of protein adducts and DNA damage in the prediction of cancer at the individual level in rats: AFB1 albumin adducts and hepatocellular carcinoma (Kensler et al. 1997), and *N*-ethyl nitrosourea, sister chromatid exchange and brain tumours (Aitio et al. 1988). Both studies concluded that biomarker approaches may be useful in populations but not to predict cancer on an individual basis. Dr Wild therefore advocated the use of biomarkers in population-based intervention studies to establish a proof of principle that interventions can reduce either exposure or modulate steps on the causal pathway, prior to larger-scale interventions with disease

as an outcome. In some cases using biomarkers simply to monitor the effectiveness of interventions to reduce exposure, for example in an occupational hygiene setting, would be of value and here a mechanistic link to a disease endpoint is not required.

Biobanks

Related to the approach of biomonitoring is the concept of biobanking of biological materials specifically for occupationally exposed populations. Such an initiative would be valuable in addition to existing biobanks that have a focus on exposures in the general population (Wild 2005). Many hypotheses concerning exposure to specific industrial chemicals can be examined, based on analysis of biological materials as part of epidemiological study designs. Because of the experiences with limited chemical stability of some biomarkers, it is recommended that tissues such as blood samples be fractionated in different fractions and also to consider extracting certain biomarkers to be stored on solid-phase materials prior to long-term storage (Ross et al. 1992).

Concluding remarks and future challenges

For use as biomarkers in the reconstruction of past exposure the persistence of a dosimeter and the presence of structural information pointing to the causative chemical substance were both identified as key properties. Such biomarkers would have to be validated in animal studies in order to verify dose–response relationships and kinetic patterns, including information on the lifespan of such dosimeters. Successful new candidate biomarkers would then require further validation in epidemiological studies to verify sufficient sensitivity in human populations and identify possible confounders that could mask a direct relationship with exposure. Such biomarkers would preferably be mechanism based, but this is not an absolute requirement as biomarkers that (only) reflect exposure could also very well be used in primary prevention strategies.

Retrospective exposure assessment may be undertaken in some instances by estimating biologically effective dose or tissue dosimetry with PBPK modelling. Tissue concentrations of chemicals can then be correlated with validated biomarkers using reverse dosimetry modelling.

Most parent substances and their metabolites do not offer much of a perspective for use in retrospective exposure assessment because of limited persistence in body tissues. Of all established methods addition products (adducts) are the most promising dosimeters for retrospective exposure assessment as indicated by a substantial number of successful applications in epidemiological studies. Chemically stable adducts to globin are detected up to 4 months and this appears to be the current limit of persistence among known general approaches for multiple chemical exposures. Beyond a persistence of 4 months adducts to histones have been identified as promising but they are not yet available for application in humans. Where adducts to proteins follow predicted kinetics during formation and decay, the kinetics of DNA adducts appear less predictable due to enzymatic repair that appears to be inducible by chemical exposure. However, adducts formed by aromatic hydrocarbons (bulky adducts) may be less prone to repair and can be detected up to 4 weeks. Also biomonitoring applications may be developed for phosphotriester DNA adducts, because there does not appear to be a repair system for these DNA adducts in eukaryotic systems.

Another interesting avenue for future research efforts is the hypermethylation of cytosine bases in CpG islands that could lead to persistent heritable changes in DNA structure. A drawback is that these modifications are not specific to the chemical substance(s) involved in their formation. At present 'omics' approaches do not offer a clear perspective of application in this field except for the introduction of new laboratory-based technologies such as the characterization of protein adducts by analysing adducted peptide fragments from enzymatic digests using sensitive ESI-MS. New more persistent biomarkers will have to be validated in animal and volunteer studies but also in epidemiological studies before they can be applied in routine.

References

Aitio A, Cabral JM, Camus AM, Galendo D, Bartsch H, Aitio ML, et al. 1988. Evaluation of sister chromatid exchange as an indicator of sensitivity to N-ethyl-N-nitrosourea-induced carcinogenesis in rats. Teratogenesis, Carcinogenesis, and Mutagenesis 8:273–286.

Beranek DT, Weis CC, Swenson DH. 1980. A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. Carcinogenesis 1:595–606.

Bodell WJ, Singer B, Thomas GH, Cleaver JE. 1979. Evidence for removal at different rates of O-ethyl pyrimidines and ethylphosphotriesters in two human fibroblast cell lines. Nucleic Acids Research 6: 2819–2829.

Bradford Hill A. 1965. The environment and disease: association or causation? Proceedings of the Royal Society of Medicine 58:295–300.

Brown RP, Delp MD, Lindstedt SL, Rhomberg LR, Beliles RP. 1997. Physiological parameter values for physiologically based pharmacokinetic models. Toxicology and Industrial Health 13:407–484.

Clewell HJ, Gearhart JM, Gentry PR, Covington TR, VanLandingham CB, Crump KS, et al. 1999. Evaluation of the uncertainty in an oral reference dose for methylmercury due to interindividual variability in pharmacokinetics. Risk Analysis 19:547–558.

Clewell HJ, Crump KS, Gentry PR, Shipp AM. 2000. Site-specific reference dose for methylmercury for fish-eating populations. Fuel Processing Technology 65–66:43–54.

Clewell HJ, Tan YM, Campbell JL, Andersen ME. 2008. Quantitative interpretation of human biomonitoring data. Toxicology and Applied Pharmacology 231:122–33.

Deforce DL, Lemiere F, Hoes I, Millecamps RE, Esmans EL, De Leenheer A, et al. 1998. Analysis of the DNA adducts of phenyl glycidyl ether in a calf thymus DNA hydrolysate by capillary zone electrophoresis-electrospray mass spectrometry: evidence for phosphate alkylation. Carcinogenesis 19:1077–1086.

Doerge DR, Churchwell MI, Fang JL, Beland FA. 2000. Quantification of etheno-DNA adducts using liquid chromatography, on-line sample processing and electrospray tandem mass spectrometry. Chemical Research in Toxicology 13:1259–1264.

Ebralidse KK, Grachev SA, Mirzabekov AD. 1988. A highly basic histone H4 domain bound to the sharply bent region of nucleosomal DNA. Nature 331:365–367.

Eisen EA, Agaliu I, Thurston SW, Coull BA, Checkoway H. 2004. Smoothing in occupational cohort studies: an illustration based on penalized spines. Occupational & Environmental Medicine 61:854–860.

Esteller M. 2008. Epigenetics in cancer. New England Journal of Medicine 13:1148–1159.

Fenn JB, Tanaka K, Wütrich K. 2002. The Nobel Prize in Chemistry, 2002. Available at: http://nobelprize.org/nobel_prizes/chemistry/laureates/2002/index.html [last accessed 10 October 2008].

Hays SM, Becker RA, Leung HW, Aylward LL, Pyatt DW. 2007. Biomonitoring equivalents: a screening approach for interpreting biomonitoring results from a public health risk perspective. Regulatory Toxicology and Pharmacology 47:96–109.

Heederik H, Attfield M. 2000. Characterization of dust exposure for the study of chronic occupational lung disease: a comparison of different exposure assessment strategies. American Journal of Epidemiology 15:982–990.

Jenuwein T, Allis CD. 2001. Translating the histone code. Science 293:1074–1080.

Jones PA, Baylin SB. 2002. The fundamental role of epigenetic events in cancer. Nature Review Genetics 3:415–428.

Jonsson F, Bois F, Johanson G. 2001. Physiologically based pharmacokinetic modeling of inhalation exposure of humans to dichloromethane during moderate to heavy exercise. Toxicological Sciences 59:209–218.

Jonsson F, Johanson G. 2002. Physiologically based modeling of the inhalation kinetics of styrene in humans using a Bayesian population approach. Toxicology and Applied Pharmacology 179:35–49.

Kanaly RA, Hanaoka T, Sugimura H, Toa H, Matsui S, Matsuda T. 2006. Development of the adductome approach to detect DNA damage in humans. Antioxidants & Redox Signaling 8:993–1001.

Kensler TW, Gange SJ, Egner PA, Dolan PM, Munoz A, Groopman JD, et al. 1997. Predictive value of molecular dosimetry: individual versus group effects of oltipraz on aflatoxin-albumin adducts and risk of liver cancer. Cancer Epidemiology, Biomarkers & Prevention 6:603–610.

Kirk GD, Lesi OA, Mendy M, Szymanska K, Whittle H, Goedert JJ et al. 2005. 249(ser) TP53 mutation in plasma DNA, hepatitis B viral infection, and risk of hepatocellular carcinoma. Oncogene 24:5858–5867.

Lan Q, Zhang L, Li G, Vermeulen R, Weinberg RS, Dosemeci M, et al. 2004. Hematotoxicity in workers exposed to low levels of benzene. Science 306:1774–1776.

Lin YS, Kupper LL, Rappaport SM. 2005. Air sampling versus biomarkers for epidemiology. Occupational & Environmental Medicine 62:750–760.

Maccubbin AE, Caballes L, Riordan JM, Huang DH, Gurtoo HL. 1991. A cyclophosphamide-DNA phosphoester adduct formed in vitro and in vivo. Cancer Research 51:886–892.

Marsden DA, Jones DJL, Lamb JH, Tomins EM, Farmer PD, Brown K. 2007. Determination of endogenous and exogenously derived N7-(2-hydroxyethyl)guanine adducts in ethylene oxide-treated rats. Chemical Research in Toxicology 20:290–299.

Noort D, Benschop HP, Black RM. 2002. Biomonitoring of exposure to chemical warfare agents: a review. Toxicology and Applied Pharmacology 184:116–126.

Özbal CC, Velic I, SooHOo CK, Skipper PL, Tannenbaum SR. 1994. Conservation of histone carcinogen adducts during replication: implications for long-term molecular dosimetry. Cancer Research 54: 5599–5601.

Pearce N. 1994. Occupational exposures to carcinogens in developing countries. Annals of the Academy of Medicine, Singapore 23:684–689.

Ross RK, Yuan J-M, Yu MC, Wogan GN, Qian G-S, Tu J-T, Groopman JD, Gao Y-T, Henderson BE. 1992. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. Lancet 339:943–946.

Shipp AM, Gentry PR, Lawrence G, Van Landingham C, Covington T, Clewell H J, et al. 2000. Determination of a site-specific reference dose for methylmercury for fish-eating populations. Toxicology and Industrial Health 16: 335–438.

Singer B. 1985. In vivo formation and persistence of modified nucleosides resulting from alkylating agents. Environmental Health Perspectives 62: 41–48.

Singh R, Famer PB. 2006. Liquid chromatograph-electrospray ionization-mass spectrometry: the future of DNA-adduct detection. Carcinogenesis 27:178–196.

Singh R, Gaskell M, Le Pla RC, Kaur B, Azim-Araghi A, Roach J, et al. 2006. Detection and quantitation of benzo[a]pyrene-derived DNA adducts in mouse liver by liquid chromatography-tandem mass spectrometry: comparison with ^{32}P-postlabeling. Carcinogenesis 19:868–878.

Törnqvist M, Fred C, Haglund J, Helleberg, H, Paulsson B, Rydberg P. 2002. Protein adducts: quantitative and qualitative aspects of their formation, analysis and applications. Journal of Chromatography B 778:279–308.

Turner BM. 2002. Cellular memory and the histone code. Cell 111:285–291.

Turner TC, Sylla A, Gong YY, Diallo MS, Sutcliff AE, Hall AJ. 2005. Reduction in exposure to carcinogenic aflatoxins by postharvest intervention measures in West Africa: a community-based intervention study. Lancet 365:1950–1956.

Wild C. 2005. Complementing the genome with an 'exposome': the outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiology, Biomarkers & Prevention 14:1847–1850.

Wild C, Vineis P, Garte S, eds. 2008. Molecular Epidemiology of Chronic Diseases. Chichester, UK: John Wiley and Sons.

Yates JM, Fennell TR, Turner MJ, Recio L, Sumner SC. 1994. Characterization of phosphodiester adducts produced by the reaction of cyanoethylene oxide with nucleotides. Carcinogenesis 15:277–283.

Yong AJ, Hay JB. 2005. Rapid turnover of the recirculating lymphocyte pool in vivo. International Immunology 7:1607–1615.