Use of Markers for Improved Retrospective Exposure Assessment in Epidemiology Studies
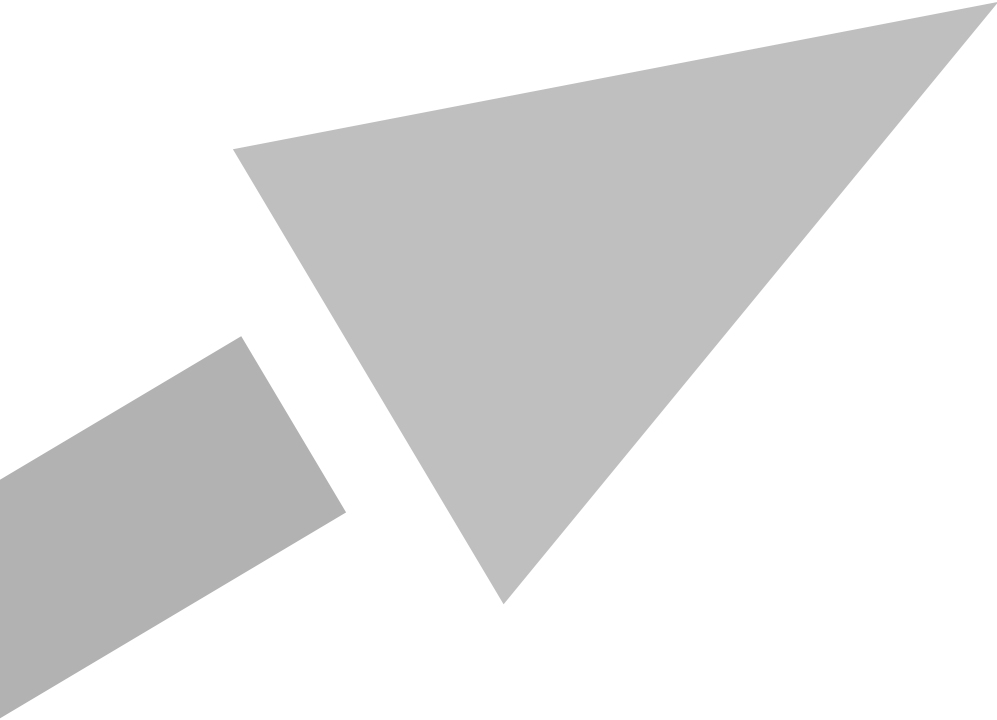## 24-25 June 2008, Brussels

Workshop Report No. 14

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ECETOC WORKSHOP REPORT No. 14

Use of Markers for Improved Retrospective Exposure Assessment

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1. EXECUTIVE SUMMARY

The workshop addressed the question whether new analytical and molecular techniques would allow the identification of biomarkers of historical exposure to chemicals. This would allow epidemiological researchers to use objective and quantitative data in place of surrogates of exposure such as data from questionnaires. The criteria for these biomarkers to be useful are: sensitivity to low levels of the chemical or the change induced; stability of the biomarker inside the exposed individual and specificity of the change that allows it to be unambiguously attributed to the exposure.

The techniques available from classical analytical methods to genomics and proteomics are all capable of extreme sensitivity. However, while the requirement for sensitivity is met by the methods discussed, the other two criteria are currently not attained at a feasible level.

In particular the biological matrices which can be readily obtained are turned over rapidly in the living organism. Red blood cells remain for a matter of months and other tissues are impractical to sample. At a sub-cellular level addition products (adducts) to DNA and proteins in cell populations with longer biological half lives may be useful. Certain adducts, notably adducts to histones, are potentially longer lasting.

The 'omics' (metabonomics, proteomics, genomics), have promise, but are subject to large interindividual variation in part due to environmental factors.

In conclusion, no single one of the techniques described can be used in the field to valid historical exposure. Exceptions to this require that the exposure window be relatively recent and the marker is present as an adduct to a macromolecule. The workshop identified several areas where research held the promise of improving on this situation, although it seems unlikely that a general approach would be rapidly forthcoming.

2. INTRODUCTION

The number of epidemiology studies looking at adverse health effects from long-term occupational and environmental exposure to chemicals and pesticides has significantly increased in recent years. Many of these studies report positive associations between assumed exposure and acute or chronic adverse health effects. There is often an inference that positive association equates to causation.

Estimation of historical exposure of the study subjects is a critical component of this type of epidemiology studies and is rarely subjected to critical evaluation. The lack of validated methods for quantifying historical exposure to chemicals including pesticides in epidemiology studies is a major limiting factor in the interpretation of findings. Currently, exposure is estimated primarily through individual questionnaire interviews and job exposure matrices. However, these have inherent problems of recall bias and variability. Rarely they are based on quantitative monitoring data or validated models for retrospective exposure assessment.

Rapid advances in molecular biology techniques and modelling have led to the emergence of molecular epidemiology as a new discipline concerned with the use of biomarkers in epidemiologic study design. However, the main area of research is focused on the use of biomarkers as early predictors of disease rather than as measures of exposure.

The workshop discussed the problems associated with retrospective exposure and assessment based on biomonitoring and looked for opportunities from other areas of novel biomarker discovery and modelling techniques that could be further developed and applied to the problem.

The Workshop
- Addressed the following question: "Are there novel marker technologies or evolving modelling techniques which could be used or adapted to better define historical exposure to chemicals (including pesticides) with the aim of using this information in the conduct of epidemiology studies?"
- Reviewed most recent advances in the field of marker technologies in epidemiology studies in terms of: the strengths these techniques have; and what could be improved.
- Looked for opportunities in other areas of novel marker technologies and modelling techniques that could be developed and applied to retrospective monitoring.
- Identified scope of further work (e.g. research proposals, task forces, expert groups) and in particular activities that might usefully be supported via LRI and ECPA.

The Workshop was an active event with approximately 30 invited participants, academic, regulatory and industry experts in marker technologies and including key epidemiologists. Keynote lectures described both the current approach of epidemiologists to assessing historical

chemical exposure and recent developments/improvements in current techniques (DNA adducts) – highlighting limitations. It was an interactive workshop that included discussion and critique of the available science with the aim of facilitating a collective review of the data presented and development of a consensus statement. The intended output is an ECETOC report, a review article and potential LRI RfPs.

3. PRESENTATIONS

3.1 Chairman's introduction

Tim Gant
University of Leicester

Epidemiology determines whether a relationship exists between chemical exposure and a biological consequence in its broadest context. When the exposure cannot be determined directly then traditionally methods such as questionnaires are used to gather the exposure data. The advent of new technology across a spectrum of molecular science has given rise to the intriguing possibility that chemical exposure may leave behind precise characteristic fingerprints at the scene of the crime. If so these could potentially be used to explore the three fundamental questions required in retrospective epidemiology, what, when and how much?

The best established of these fingerprints is that of a mutational spectrum arising from a strong genotoxin, for example Aflatoxin B1. More subtle techniques are available though, for example detection of adduct formation on DNA. This can be done very sensitively on a whole DNA scale. Of importance though is sequence specificity of adduct formation and methods for determining this are becoming more robust, though they lack the sensitivity of non sequence specific methods. DNA is not the only macromoelecule which may acquire adducts during chemical exposure, and this meeting spent some time considering the proteins, and in particular histones, as long lived proteins where the fingerprint of chemical exposure may remain long after the chemical has departed.

One of the potential consequences of alterations in DNA sequence, or adduct formation, may be altered DNA transcription which may establish a different pattern of gene expression in the cells and tissues exposed. Potentially, therefore, gene expression offers the possibility of fingerprint detection in retrospective epidemiological studies. Care needs to be taken though. Unlike adduct formation and mutation, alteration of gene expression in response to chemical exposure is not necessarily a bad event and may not be specific for any given chemical. Differential gene expression is necessary to deal with environmental changes and is a dynamic process. Identifying a permanent change of gene expression against this background could be difficult. Despite the large amounts of research already undertaken to establish reliability of gene expression patterns as fingerprints of chemical exposure, many of the responses detected are very generic. A lot more validation and research is required to establish this technique in retrospective epidemiology.

Recently another DNA modification has started to interest many in toxicological research altered epigenetic profile (epigenetics). Epigenetics is in essence a change in DNA methylation pattern and has application in retrospective epidemiology. DNA methylation is known to be a dynamic process and leads to altered gene expression. It is particularly important in embryogenesis and

gamete formation. Over the past few years, evidence has started to emerge that changes in cellular epigenetic status may be modified by chemical exposure and that this may be a permanent enough alteration to act not only as a retrospective biomarker, but also to be inherited across generations in the germline. This gives rise to the possibility that the progeny of an exposure individual may themselves carry an epigenetic fingerprint of chemical exposure that occurred to their ancestors. The evidence for this happening is at an early stage of derivation and a great deal or work is required. However the potential for such a marker is profound. Epigenetics controls the expression of genes so this mechanism gives credence for a change in gene expression pattern being a retrospective marker of chemical exposure, if this can be recognised against the background of the normal regulation to maintain homeostasis.

In a simplistic sense and ignoring some major regulatory steps, changes in gene expression lead ultimately to changes in proteins and activity. Ultimately changes in protein activity can give rise to altered biochemistry and profile thereof in bodily fluid. An important aspect of this conference was therefore to consider if changes in physiological metabolites in accessible bodily fluids could act as a retrospective marker of chemical exposure.

In forensics, time of death is an important parameter. No less so in epidemiology; when did exposure occur? The meeting explored the possibilities for modeling back to 'time of death' starting with a measure of exposure to the compound itself and using physiological parameter such as organ blood flow. Similar methods can be applied to determine amount of exposure if the time of exposure is known. The question to be addressed here though was; can the same methods be applied where there is not a measure of the chemical itself but only its fingerprint left at the scene of the crime?

Finally it must be considered 'what is the consequence'? Stratification of epidemiological data has often occurred using parameters such as gender, race, living standard, place of residence and others. High throughput sequencing will further differentiate what are now regarded as single populations into many smaller populations based on their genotype. This is no crystal ball science, but is already happening. A key question for epidemiology is then what is the consequence of a retrospectively determined chemical exposure for a subpopulation or individual according to their genotype.

The conference explored all of these complex issues. There are very few examples where full validation has been undertaken, and certainly with the more recently recognized phenomenon such as epigenetics potential is apparent, but quantitation of that potential is lacking. The meeting explored some exciting opportunities. As is so often the case all the explored methodology requires rigorous validation to different degrees and more work is required to distinguish those techniques with real retrospective epidemiological potential from those simply promising. For epidemiology exciting possibilities are apparent but realizing the possibility will be challenging.

The meeting brought together people from academia and industry as well government regulatory experts and included experts in the field of new biomarker technology, epidemiology and molecular toxicology.

3.2 Assessing exposure in epidemiology studies – What is it we want to know?

Alan Boobis
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Epidemiology provides a 'gold standard' in environmental risk assessment, in that it provides information on the consequences of real-world exposures in the species of concern. However, as relative risks are usually low, often $\leq$ 2-fold, the power of most studies to detect an effect is limited and hence whilst a positive result can be informative, a negative result does not mean that there is no risk. A key question is therefore, what confidence is required of a negative result. There is an important distinction between exploring a causal relationship and seeking to establish an upper bound for a relationship that is already suspected.

Much of the strength of an epidemiology study comes from the reliability of exposure assessment. However, it is widely acknowledged that current methods for assessing exposure retrospectively are not well suited for exploring associations with health outcomes that can take many years before they are manifest, for example remembering or even knowing exposures that occurred many years ago. Approaches used for exposure assessment, particularly in retrospective studies, rely on methods such as occupational categorization, which are subject to potential confounding.

In order to explore causal associations between exposure and effect, accurate estimates of the exposure of individuals to specific agents at defined periods of their lives are required. Where metabolites contribute to effect, an estimate of exposure to both parent and metabolite is required. Ideally, not only would external exposure be assessed, but also systemic and target site exposure as well. It would be necessary to obtain information on the inter-relationships between these, and factors affecting them, to enable appropriate interpretation of the data.

Retrospective exposure assessment can contribute to the weight of evidence arguments for a causal link with exposure, provide evidence of a biological gradient (dose-response relationship), provide information on levels of exposure without observable effects, provide mechanistic insight into toxicological effect, for example when combined with knowledge on specific genetic polymorphisms, help in identifying susceptible sub-populations, and in the identification of factors modifying risk, either increasing or decreasing it, for example smoking and asbestos in bronchogenic carcinoma.

Often, the results of epidemiological studies are used in risk assessment, i.e. in hazard identification and hazard characterisation. Where retrospective information on exposure is available, this can be used in dose-response assessment, which is part of hazard characterisation,

the basic premise being that risk increases with exposure and that for most risks, there is an exposure below which an effect is not observable. Hence, exposure assessment contributes to the application of the Bradford Hill (1965) criteria for assessing causation of environmental factors in human disease: i.e. consistency, strength of association, temporal sequence, dose-response, specificity, coherence, biological plausibility, analogy and experimental evidence.

In using retrospective exposure assessment in this way it is important to consider whether exposure has been determined unequivocally, or could it be confounded by some other factor, whether the data are consistent with a causal association, when the totality of the evidence, for example using the Bradford Hill criteria, is taken into account, and how the apparent potency compares with that in experimental animals, when such data are available. Where there is a marked difference in potency, is this consistent with available experimental evidence on species differences, for example in relevant cell systems, on biomarkers of effect, and on close structural analogues.

The value of retrospective exposure assessment can be enhanced by the use of suitable biomarkers of exposure. These can provide confirmation of exposure classification by confirming the level of exposure and reducing misclassification. They are of value in assessing combined exposures to similar compounds, i.e. cumulative exposure, in estimating internal or target dose, in bridging between studies in experimental animals and observations in humans, and in exploring interindividual variation in exposure. However, biomarkers of exposure have a number of potential limitations. They are often restricted to relatively recent exposures, intraindividual variation over time can complicate interpretation, and reconstructing exposure (external) is often difficult if not impossible, and here pharmacokinetic information can be invaluable. In addition, there is a need for individual sampling, which is often invasive to some extent and adequate analytical methodology, including sample handling and storage, is necessary. There is also potential for confounding, particularly when the biomarker is not specific for a given exposure, although issues such as extraneous factors that can influence the level of the biomarker also need to be considered.

Knowledge of external dose, reconstructed from a biomarker of exposure if necessary, is of value for a number of reasons. It provides quantitative linkage between the environmental source and internal exposure. It allows distinction between susceptibility due to differences in environmental exposure, which could be addressed by risk management strategies, and the determination of the impact of remediation strategies, rather than differences in toxicokinetics. It also allows identification of the important routes of exposure, again of value in determining risk management strategies.

In using a biomarker to assess exposure it is important to consider what it is that the biomarker measures, and whether the biomarker can be related to external dose. Is there an overall

advantage to its use when compared with more conventional means of assessing exposure. Has the biomarker been adequately qualified, i.e. is it suitable for its intended purpose and how should such qualification be undertaken. The amount of time it takes to develop and qualify a novel biomarker should not be underestimated.

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3.3 Current state of the art in retrospective exposure assessment methodologies and the needs in this area

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Exposure assessment has always been the Achilles heel in most occupational and environmental epidemiological studies. This was to some extent due to the fact that in early studies, adequate monitoring techniques were not available. However, concepts on how exposure should be measured and how measurements should be allocated to the workers and over time were not yet developed either. Gradual technological development resulted in a range of techniques for personal exposure assessment and this development is still ongoing. A major breakthrough formed insights in how variability in exposure over time and space needed to be dealt with to avoid the effect of exposure misclassification on measures of association like the Risk Ratio and the Odds Ratio. We now know that exposure misclassification can bias exposure response relationships. The bias depends on the type of error and the specific scale on which the exposure has been measured. Depending on the specific situation, these methodological insights can be used to design optimal exposure assessment strategies for a given situation and guide the analysis of exposure response relationships in the data analysis phase. Guiding rules are now available which help to reduce exposure misclassification. Together, these developments resulted in a new generation of epidemiological studies, which make use of large exposure data bases, and elaborate statistical analysis. The same guiding rules should also be applied to biomarkers which are more and more available. Application of these rules indicated that only few biomarkers of exposure exist which are applicable in large scale epidemiological studies.

3.4 How is Exposure currently assessed in epidemiology studies – With examples of successes and limitations

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Background

The majority of cancers have a complex etiology where environmental risk factors interact with genetic background, age, sex, socio-demographic status and other factors. However, the contribution of individual environmental exposures and their interaction with genotype is difficult to elucidate. This is partially due to the challenge of accurately measuring exposure. Whilst exposure misclassification introduces uncertainty and limits the power of epidemiological studies, biomarkers promise to resolve some of these challenges by improved assessment at the individual level.

Results

Progress has been made by the application of biomarkers of internal dose (e.g. chemicals and their metabolites) or biologically effective dose (DNA and protein adducts) into population-based studies. These categories of biomarkers are generally best suited to prospective cohort studies where samples are collected prior to onset of the disease, both because the biomarkers generally reflect only recent past exposure and because of the risk that the disease state alters the biomarker level. An example of a successful application is the association between urinary aflatoxin-DNA adducts, hepatitis B virus and liver cancer risk in China (1). Similar biomarkers have been extremely valuable in contributing to the biological plausibility of exposure-disease associations and as endpoints in "proof-of-principle" intervention studies (2). In order to try and detect past exposure the mutational "fingerprint" associated with specific chemical exposures has been explored, but the number of examples to date is limited (3). Some biomarkers are longer-lived, such as pesticides in adipose tissue or heavy metals in nails or hair samples for example, but these are the exceptions and are not applicable to many exposures of interest.

A notable feature of the above first generation of exposure biomarkers in cancer epidemiology was an emphasis on genotoxic compounds. What is needed now is an analogous set of biomarkers for exposures acting through other mechanisms of carcinogenesis, namely via epigenetic effects including DNA methylation, histone modification and small non-coding RNAs. The ability to detect DNA methylation in gene promoter regions in circulating plasma DNA offers promise in translating this new mechanistic understanding into appropriate biomarkers.

The new technologies of transcriptomics, proteomics and metabonomics may provide a step change in exposure assessment if the level of expression of RNA, proteins and metabolites is shown to reflect specific environmental exposures over an extended time period. This work is at an early stage but merits further investigation (4).

One general drawback with many candidate exposure biomarkers, both the first generation and those promised by the newer 'omics' technologies is that they have not been fully validated in terms of their relationship with external exposure prior to application in epidemiological studies. This may partially reflect the difficulty in obtaining funding for both technical assay developments and pilot studies aimed at comparing exposure with biomarker in a limited number of study subjects.

Conclusions

The accurate assessment of many environmental exposures remains an outstanding challenge with a need to improve assessment of the 'exposome' to complement the genome (5). This is of importance because a significant investment is being made in large prospective cohort studies incorporating banks of biological samples and these are predicated on the ability to study gene-environment interactions. Exposure biomarkers can contribute to such studies but strategic funding is required now for their development and validation. At the same time as these initiatives it is important to note that refinement of exposure assessment is also being addressed by complementary approaches, including geographic information systems, personal and environmental monitoring and increasingly sophisticated questionnaires; it is eventually a combination of tools which is most likely to provide the answers required.

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3.5 Metabonomics and xenometabolome metabolite signatures in epidemiology studies

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Metabonomics/Metabolomics is a rapidly emerging field of research combining sophisticated analytical tools such as NMR spectroscopy and mass spectrometry with multivariate statistical analysis to generate complex metabolic profiles of biofluids and tissues. Metabonomics provides a systems approach to measuring dynamic biochemical responses of organisms to pathological stimuli or genetic modification and operates by profiling the metabolic responses of key intermediary biochemical pathways. Such analysis has been shown to be of considerable value in providing detailed information regarding the metabolic status of an organism, in characterizing the metabolic phenotype of genetically modified organisms and in discerning and predicting a wide range of pathological conditions. Moreover, this approach has proven value in assessing the efficacy of therapeutic interventions in animals and man.

More recently metabolite profiling strategies have been applied in epidemiological studies. Large scale screening of human populations is now possible and models of metabolic phenotype can be constructed for populations. However, the complexity and interactive nature of biological systems introduce confounding variation into the metabonomic data. Various chemometric and bioinformatic strategies for optimizing the characterization and prediction of pathological conditions can be adopted in order to increase the sensitivity of metabonomic analysis by reducing the influence of confounding random and systematic noise, accommodating the presence of large dynamic range in the measurement variables and/or incorporating the temporal dependence of pathologies. These strategies can also be applied to exploring the xenometabolome and report on the presence of drugs, dietary supplements and other exogenous chemicals present in the biofluids and tissues of humans.

Using a range of multivariate analytical strategies, metabonomic data can be integrated with gene expression and proteomic data to provide a more holistic vision of biological processes at a whole systems level. Gene-metabolite interactions can be probed using a range of chemometric tools and the metabolic signature used to direct appropriate sampling points for genomic/proteomic analysis. Examples of this integrative approach will be drawn from a number of fields including dysmetabolic syndrome and insulin resistance, cardiovascular disease, neurodegenerative disorders, cancer and infectious diseases. Metabolic responses to therapeutic and nutritional intervention and strategies for the recovery of xenobiotic signatures are also considered.

3.6 Personalised exposure assessment, integrated transcriptomics, proteomics and metabolite analysis

Jos Kleinjans
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Background

Two decades of experience with biomarker research in cases of environmental and occupational health risks have demonstrated the utility of molecular markers in understanding underlying mechanisms. However, analysis of current biomarkers of effect tend to be laborious while simultaneously, there are doubts concerning their specificity and sensitivity. It has therefore been postulated that genomics-based biomarkers may represent an improvement. Also, it has also been suggested that through taking the toxicogenomics approach towards unraveling gene-environment interaction, personalized exposure assessment becomes within reach.

In this respect, we decided to conduct a case study (1,2):

- To generate whole genome gene expression data from peripheral lymphocytes sampled from environmentally exposed subjects and controls: children and parents from different areas in Czech Republic

- To compare obtained transcriptomic profiles with a classical and well-validated marker of environmental cancer risk, e.g. lymphocytic micronuclei frequency.

- To evaluate whether personalized exposure assessment though genomics analysis may become feasible.

Results

Differences in biological responses to exposure to hazardous airborne substances between children and adults have been reported, suggesting children to be more susceptible. The aim of this study was to improve our understanding of differences in susceptibility in cancer risk associated with air pollution by comparing genome-wide gene expression profiles in peripheral blood of children and their parents. Gene expression analysis was performed in blood from children and parents living in two different regions in the Czech Republic with different levels of air pollution. In addition, gene expressions in both children and adults were investigated for associations with micronuclei frequencies. Gene expression analysis returned considerably more

genes or gene groups and pathways that significantly differed between children from both regions than between parents. Very little overlap was observed between children and adults. The two most important biological processes or molecular functions significantly modulated in children, but not in adults, are nucleosome and immune response related. Our studies therefore suggest differences between children and adults in relation to air pollution exposure at the transcriptome level.

We thereupon asked whether an exposed individual can be discriminated from a group of non-exposed subjects through gene expression profiling of peripheral lymphocytes from that person. We therefore performed hierarchical clustering of all single gene expressions significantly modified ($p < 0,0001$) in parents and their children from Teplice versus Prachatice, in order to investigate whether a specific exposure-related gene expression profile could be created. However, discrimination between exposed versus non-exposed parents or children appeared to be less than 100%.

Conclusions

- It appears feasible to generate discriminative modulations of gene expression and of genetic pathways at rather low differences in environmental exposure levels.

- Concomitantly, a wealth of mechanistic information has been gathered, the full biological relevance of which has to be thoroughly explored.

- Transcriptomic analysis thus represents a very promising tool for human biomonitoring.

- However, results on gene expression analysis seem too a-specific to be applied at the individual level for the purpose of personalized exposure assessment.

- This may be improved by integrating gene expression analysis with results from other genomics technologies: proteomics, metabonomics, whole genome epigenetics, microRNA analysis.

- This indicated future directions in developing genomics-based biomarkers for analysing gene-environment interactions.

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3.7 Proteomic analysis – 'Protein Imprinting' – Possible use in the clinical situation and what might possibly be learnt from retrospective analysis in epidemiological studies

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Background

Proteins can undergo posttranslational modifications leading to covalently bound adducts on amino acid residues. For example, phosphonic acids (hydrolysis products of organophosphates) can adduct to serine residues. In a different area, reducing sugars and lipid oxidation products are able to form adducts on certain amino acid residues within protein, including lysine and arginine.

Mass spectrometry is a very powerful tool for determining protein adducts. Theoretically, the intact protein can be analysed, but when the extent of adduct formation is small and the protein is large, the mass difference between the native and adducted protein and the low abundance of the adducted form may make it difficult to detect. An alternative strategy is to digest the protein, typically with an enzyme, and then to analyse the resulting peptides by mass spectrometry. This permits the determination of the site of modification of the protein and protein identification. When the protein identity or the site of modification is of little interest, another analytical approach is to subject the protein to digestion with acid or enzymes to give the constituent amino acids and adducts. These components may then be quantified by mass spectrometry.

This report summarises analytical outcomes from the determination of advanced glycation endproducts (AGEs) on a specific arginine residue of ribonuclease A (RNase) (1) and the determination of Nε-(carboxymethyl)lysine in acid hydrolysates of protein isolated from foods (2).

Results

Differences between tryptic digests of native RNase and AGE-modified RNase were located within the mass spectral data by generating a single mass spectrum for each sample from the MS data collected in full scan mode, even when using a triple quadrupole MS which is a low resolution instrument. This method is simple but may be limited by sensitivity. Ions detected only in the AGE-protein were targeted for sequencing using the higher resolution quadrupole-time of flight (Q-TOF) instrument.

Trypsin is commonly used to digest protein prior to MS sequencing. However, it is not the best enzyme for all applications and alternatives exist, such as chymotrypsin and endoproteinase glu-C. Each enzyme cleaves at specific sites. The use of multiple enzymes may also be helpful, for example endoproteinase glu-C followed by trypsin. In the pesticide field, pepsin rather than trypsin or pronase or thermolysin has been used to determine methylphosphonic acid adducts on serine residues in butyrylcholinesterase.

Certain adducts on protein undergo 'neutral losses' in the MS, e.g., water and phosphoric acid. This can happen in both the ionization source (where the ions are formed) and in the collision cell (where fragment or daughter ions are formed). Especially when quantifying adducts, it is important to know if any type of degradation occurs in the mass spectrometer because it can lead to underestimation of the amounts in the sample. Depending on the adduct, it may be possible to adjust the MS settings to avoid degradation.

If adducts are stable in hot acid, protein may be hydrolysed to its constituent amino acids by heating in 6M HCl at 100 °C for 24 h followed by the determination of global amounts of individual adducts, e.g., N^{ε}-(carboxymethyl)lysine. This is best done by sensitive and specific multiple reaction monitoring involving monitoring the molecular ion and a prominent daughter ion. Adducts may be accurately quantified by adding deuterated adduct (standard) to the sample.

Conclusions

MS is a powerful technique for analysis of protein adducts. In clinical analysis, the main limitation is likely to be the lifetime of readily available physiological proteins, e.g. from blood. Inter- and intra-subject variations would also need to be established.

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3.8 Detection of xenobiotics and metabolites thereof using mass spectrometry-based methodology

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Background

Pesticides are one of the major types of worldwide pollutants. The use of pesticides has increased over the last two decades not only in agricultural fields, but also in households. This broad use has raised concerns among hygienists, epidemiologists, physicians and other health officials for causing adverse health effects in humans. Preventive medicine considers the determination of pesticide residues in food, water and air as extremely important in order to estimate the degree of human exposure. Persistent pesticides such as organophosphates have been replaced by the so-called non-persistent ones, like carbamates. In the last five years, serious health disorders have been observed by the scientific community due to the extensive use of carbamates. These health problems may indicate that even though pesticides like carbaryl (CRB) or carbofuran (CF) were not considered highly toxic and dangerous to humans, their polar degradation products may have higher toxicity than the parent compound. In this study, analytical methodology based on gas chromatography-mass spectrometry (GC-MS/MS) has been developed for the simultaneous determination of the CRB, CF pesticides and their metabolites in human urine and blood plasma samples.

Results

In the analysis of urine samples both N-methylcarbamates and their metabolites have been converted into stable TFAA derivatives, thus allowing us to estimate the exposure by detecting both the main compound and the metabolites excreted from the metabolic routes of humans, without resorting to chemical hydrolysis of urine samples. In correlation to applicators' specimens, all five applicators were found positive to 1-naphthol (1N) and 2-naphthol (2N). In four of them though, an exposure to CRB could be addressed compared to the results obtained from smokers. For the fifth subject passive exposure to naphthalene could not be excluded. Higher concentrations of 1N in applicators can be easily explained, because they take no precautions and application duration lasted over 2 h for each individual in high summer temperatures. All five volunteers were also found positive to 7-phenol, CFphenol-3-keto, major urine metabolites of CF, but only two of them were found positive in 3-HO-CF in levels above the LOQ levels. Neither CRB nor CF was found in measurable levels in any of these samples.

In the analysis of blood samples, the method employed a simple pre-treatment step of protein removal and the isolation of the analytes by solid-phase extraction. This method had the advantage to include the quantitation of four metabolites of CF, and 1N for CRB, in the presence of 2N from the naphthalene metabolic pathway; thus exhibiting higher specificity than methods previously reported. The method has been applied to a suitable number of samples from pesticide users and urban area citizens in order to confirm the applicability and usefulness of our method, especially in the area of biomarker research and bio-monitoring studies such as low-, medium-, high-level ones, and incidental, residential or occupational research. CRB was detected at quantitative levels in all five farmers. 1N and 2N were detected in three samples, but it was not correlated to smoking habits of the subjects and no research has been reported yet on detectable levels of 2N in plasma samples from passive smoking, therefore these data cannot be further evaluated.

Conclusions

The GC-MS/MS developed methodologies were successfully developed and validated to provide reliable, sensitive and specific data for the biological monitoring of the most widely used methyl carbamates; CRB, CF and their metabolites in human urine and blood plasma samples. This methodology as well as LC-MS/MS methodology employing electrospray ionization (ESI) could be valuable in the area of biomarker research and biomonitoring studies. In addition, LC-ESI MS methodology can be applied for the detection and monitoring of noncovalent interactions between small xenobiotics and macromolecules which are responsible for the appearance of certain diseases.

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3.9 Advantages and limitations of DNA adduct measurement in identifying retrospective exposure

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Background

A key stage in the genotoxic carcinogenic process is the formation of interaction products ('adducts') of the carcinogen with DNA. Measurement of such adducts indicates the dose of active compound that has reached the cells under study (biologically active dose), and is being increasingly used in molecular epidemiology as an exposure biomarker. Considerable progress has recently been made in improving the sensitivity and selectivity of DNA adduct measuring techniques and in the development of screening systems. The advantages and limitations of these approaches were considered, concentrating particularly on three of the essential parameters described in the ECETOC Document 44 on 'Guidance for the interpretation of biomonitoring data' (2006): the analytical integrity of the data, the relationship between the biomarker and effects, and particularly the data's ability to describe exposure.

Results

Adducts are formed at all the nucleophilic sites within the DNA bases and also at the phosphate oxygens. Some repair products (e.g. N7-alkylguanine and N3-alkyladenine) are excreted in urine within a few days after exposure. The analytical methods for DNA adducts include ^{32}P-postlabelling, immunoassay, LC-fluorescence, LC-electrochemical detection, and mass spectrometry [GC-MS/MS and LC-MS(/MS)]. An example of the use of mass spectrometry to make a highly sensitive determination of a purine base adduct from an environmental carcinogen is that of N7-(2-hydroxyethyl)guanine, which is the major adduct formed by ethylene oxide (Marsden et al, 2007). This may be released by thermal depurination of DNA and analysed with a limit of detection of 6 adducts/10^9 nucleotides. This method was sensitive enough to detect the presence of 'background levels' of this adduct in unexposed animals, caused by endogenous production of ethylene oxide. It is clearly important to determine if a biomarker is being produced endogenously as this may have an important impact on the risk assessment process.

New methods using LC-MS/MS have been developed for determining 2'-deoxynucleoside adducts obtained after enzymatic degradation of DNA. A common fragmentation [MH$^+$-(MH-116)$^+$] is found in the positive electrospray MS/MS spectra of many such adducts, and this has so far been used to determine individual adducts with much improved selectivity. To date there are

no mass spectral screening systems fully developed for monitoring the total adduct content of a DNA sample but attempts are currently being made to use this common fragmentation to develop such a system.

The lifetime of adducts is very variable depending on where they are formed (e.g. glutathione adducts ca 1 day, globin ca 4 months, DNA hours to weeks). For DNA adducts the lifetime is governed by site of the adduct on the deoxynucleotide, the nature and level of repair enzymes, and the location of the adduct within the DNA strand. Long lifetime adduct biomarkers would be of considerable value in epidemiological studies and for risk assessment. Although most DNA base adducts are of relatively short lifetime (a few weeks or less) there is the possibility that phosphotriester adducts may be very long-lived, as there is no known eukaryotic repair system for them. Several analytical systems for these have been developed (e.g. LC-MS/MS, ^{32}P-postlabelling, transalkylation and HPLC) and the potential of phosphotriesters as biomarkers is currently being investigated. Long-lived protein adducts such as those on collagen and histones may be an alternative solution. Several epidemiological studies have shown that bulky DNA adducts are associated with an increased carcinogenic risk on a population basis, but other studies have not. The timing of sample collection and the stability of biomarkers (if the biological samples are stored) may be critical factors in such studies.

Conclusions

Many highly sensitive methods for analyzing DNA adducts now exist, although further validation of many of these is still needed. Development of longer term biomarkers and of screening systems, and studies of adduct stability under storage are important current research needs.

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3.10 Do histonic data contain historic information?

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Background

Proteins contain numerous nucleophilic groups that can be targets for electrophilic attack by reactive intermediates to form adducts (1). These are mostly electrophilic but not only alkylating substances like alkenes or alkylhalides, but also arylating, nitrenium forming, carbonyl, acylating, and phosphorylating organic species (including chemical species like polycyclic aromatic hydrocarbons and their alkyl derivatives, aromatic amines and nitroarenes, aldehydes, isocyanates, anhydrides, halides, organo phosphorous 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 and co-workers (1) point out that not all of these adducts are exclusively of post-translational origin like N-terminal val that are much used as biomarkers for e.g. ethylene oxide, 1,3-buadiene, acrylamide, etc. Much is known about the kinetics of formation and decay of protein adducts in vivo. Intracellular proteins such as hemoglobin follow zero-order kinetics leading to a steady state level that is limited by the turnover of the cells. In the case of hemoglobin the life span 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. The formation and decay of adducts to proteins free in solution such as serum albumin (SA) follows 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. So far a mechanism of enzymatic repair of adducts to SA and Hb was not 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 behavior, would be interesting as candidates for dosimeters of accumulated internal exposure. Among the candidate proteins that could possibly stretch the half life of biomarkers histones were mentioned as possible candidates.

Results

Histones initially have been looked upon as structure proteins that are intimately interwined 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 like beads on a string. The distance between two nucleosomes is covered by 54 base pairs of spacer DNA that is anchored in place by a fifth type of histones: H1. 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. Several other so-called post translational modifications (PTMs) have been identified in addition to acetylation. Some 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 BaPDE, 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 (2) reported that histone adducts of BPDE 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. This is a very promising finding but there are still many questions like: 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 reported on the life span of histones. In this study an average life span of 117 days was reported in liver cells and 223 days in the brain. So the life span of histones could be higher than that of e.g. hemoglobin 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.

Conclusions

Beyond a persistence of 4 months adducts to histones have been identified as promising but they are not available for application in humans as yet. Histone adducts have been shown to be passed on to daughter cells in human lymphoblasts implying that persistence could be infinite and possibly extend beyond one generation. Detection capability will be determined by recovery of sufficient amounts of histone adducts from peripheral lymphocytes and sensitivity of the analysis.

This will be a trade off with on-going zero-order dilution due to de novo synthesis of histones in blood cell populations.

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3.11 Modelling techniques available and being developed

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Background

Physiologically-based pharmacokinetic (PBPK) modelling is increasingly being used in quantitative, biologically-based chemical risk assessment. It is a powerful means of simulating the factors that determine tissue dose within any biological organism and consequently, correlation with health effects. The value of PBPK models is that they are tools for integrating *in vitro* and *in vivo* mechanistic, pharmacokinetic and toxicological information through their explicit mathematical description of important anatomical, physiological and biochemical determinants of chemical uptake, disposition and elimination.

PBPK models are appropriate tools for the quantitative interpretation of human biomarkers (also referred to as biological monitoring data) and associated health implications. The estimation of internal exposure in studies characterising the toxicity of a chemical is known as 'forward dosimetry'. The estimation of environmental exposure consistent with measured biomarker data is known as 'reverse dosimetry'. The measurement of chemicals or their metabolites in human tissues or specimens, such as blood, exhaled breath, urine, saliva and hair are known as biomarkers of exposure. However, the estimation of subcellular tissue concentrations could be correlated with biomarkers of early biological effects such as, chromosome aberrations and somatic mutations. Therefore, the identification of biomarkers of exposure and effect may provide a feasible means of reconstructing historical exposures by reverse dosimetry.

The presentation will give an overview of PBPK model construction, evaluation and application with a focus on the interpretation of biomarkers of exposure and effect and the potential for reconstruction of past exposures.

Results

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, not by fitting to the data, but rather by changing assumptions with

respect to the role of certain components in the model, each representing true physiological compartments like lung and skin as important organs for uptake of industrial chemicals, but also liver for metabolism and kidney for urinary excretion.

Population-based PBPK modeling is undertaken by incorporating interindividual differences in all model parameters by ascribing appropriate distributions for variability to 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[1]. Through Monte Carlo sampling of the distributions simulation of interindividual variability is taken into account. In this way the influence of e.g. obesity can be modeled by incorporating a compartment representing adipose tissue and uploading population-based distributions of BMI-data. Bayesian techniques are applied to distinguish between model parameter uncertainty due to experimental measurement error and normal biological variability (Jonsson and Johanson, 2002). PBPK models can be applied in forward dosimetry e.g. in the prediction of a arterial blood concentration after exposure of a subject to an air concentration. It is also possible (but sometimes more difficult) to reconstruct external exposure that happened in the past from biological monitoring data (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 methylmercury if was possible to reconstruct the average daily dose and the internal dose of the unborn child (Clewell *et al.*, 2000; Clewell *et al.*, 1999; Shipp *et al.*, 2000).

The effects and implications of different workloads on ADME can also be estimated using PBPK modeling (Jonsson *et al.*, 2001).

Conclusions

Retrospective exposure assessment may be undertaken in some instances by estimating biologically effective dose or tissue dosimetry with PBPK modeling. Tissue concentrations of chemical can then be correlated with validated biomarkers using reverse dosimetry modeling (Clewell *et al.*, 2008; Hays *et al.*, 2008; Hays *et al.*, 2007).

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[1] www.thelifelinegroup.org/p3m/index.htm

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4. REPORTS FROM BREAKOUT GROUPS

4.1 Breakout Group I

Chair: Stefano Bonassi
Rapporteur: Gerard Swaen

Based on the information presented, what are the most promising leads in the field of novel biomarkers which could be developed/adapted in the short term (1-3 years) for the use in retrospective exposure assessment in epidemiology studies with chemicals/pesticides, and what are their possible limitations?

First the group discussed the context in which the charge question has to be placed. It was decided to evaluate the new biomarkers in a timeframe of 10+ years, meaning that the biomarker should be capable of assisting in assessing exposures that occurred 10 years or more ago. Next, a list of biomarker groups presented at the workshop was compiled. The biomarker groups were: Metabonomics, Transcriptomics, Proteomics and DNA or protein adducts, including histones. Not considered groups were DNA adducts in lipids (lipidomics?) and the chemicals themselves or metabolites stored in tissue such as fat and bone.

Next, the process for evaluating the biomarker groups was discussed. The group decided to make a SWOT analysis by applying the following aspects:

- Persistence (minimum persistence of 10 years)

- Sensitivity (correlation with past exposure at low, realistic exposure ranges)

- Specificity for the chemical (source specificity not required)

- Relationship with dose (will the biomarker provide information on intensity of exposure?)

- High throughput

- Costs and time required

- Have the analytical methods been validated?

Persistence was seen as the most discriminating aspect. If the biomarker is thought not to be persistent for at least 10 year it would not be eligible. Group 1 evaluated each biomarker group against the aspects mentioned above. However it realized that for some groups insufficient information is available at this moment and may become available in the near future.

Metabonomics

These biomarkers were regarded not to be persistent since cells have a relatively short lifetime in the context of the persistence looked at in the workshop. Sensitivity at trace levels was thought to be low and not to be specific for a chemical to the extent that chemical specific exposures could be identified with genetic profiling. Since the metabonomic biomarkers are thought not to be specific it is unlikely that they will provide reliable dose-response information. The biomarkers are high throughput and have become reasonably priced with limited time expenditure. The analytical methods are not yet validated.

Transcriptomics

These are probably not persistent since data indicate that intra-individual gene expression levels vary significantly over time. However a change to the genome, such as an epigenetic change could give rise to a persistent change in gene expression. The epigenetic change per se could act as the biomarker and is likely to be more sensitive and specific than the hypothesised consequential change in gene expression. There are no data available to indicate if this is a realistic persistent biomarker. There are not enough data to evaluate sensitivity and the transcriptomic biomarkers are likely not to be specific for the chemical in question. It will therefore be difficult to get dose/exposure intensity information by means of these biomarkers. These biomarkers are high throughput and reasonably priced and some of the techniques have been validated in terms of repeatability and inter laboratory variation, but not with respect to their interpretation or biological significance.

Proteomics

Proteomic biomarkers are also thought not to be persistent since they have a substantial intra-individual variation over time. They seem to have a good sensitivity and can be highly specific for the chemical. There are also technical problems associated with detection of the full complement of proteins in a cell, specifically the difficulty to detect low abundant proteins and peptides. Proteomic profiling is a high throughput application, reasonably quick but at the moment still expensive. Some validation work has been done.

DNA or protein adducts including histones

These biomarkers are also not persistent over longer periods of time. Histones may be the exception since they may be transferred to next generation cells at cell division. The sensitivity appears to be good as well as specificity and relationship with dose/exposure intensity. They are not high throughput and expensive and time consuming at the moment. Few adducts formations have been validated but more work is needed.

In conclusion most if not all potential biomarkers for retrospective exposure assessment do not appear to meet the requirement to be persistent over time, the histones and to a lesser extent the other DNA adducts being the exception. However the disadvantage of DNA-adducts is that they may only be relevant for genotoxic carcinogens.

Table 1. Summary of SWOT analysis of the biomarker groups and their application for retrospective exposure assessment described at the workshop.

Biomarker group	Metabonomics	transcriptomics	proteomics	DNA or protein adducts
Persistence	No	No	No	Histones perhaps
Sensitivity	Not high	Unknown	Probably yes	High
Specificity	No	Not likely	Yes	Good
Dose/exposure intensity info	No	Not likely	Yes potentially	Good
High throughput	Yes	Yes	Yes	No, but may change
Costs, time	Reasonable	Reasonable	Expensive but quick	Expensive and time consuming
Analytically validated	Not yet	Yes, some	Depends on biomarker	Few have been validated

4.2 Breakout Group II

Chair: Dirk Pallapies
Rapporteur: Neil Carmichael

What further work should industry sponsor to build on leads which may not be readily useable, but could be promising in the medium term (3-5 years)?

The group agreed that the presentations had not revealed any techniques which were instantly useable for analysis of retrospective biomarkers in the sense the workshop was discussing. This meant methods capable of finding quantifiable evidence of exposure several months or years after a conventional analysis of residual chemical are not available. The group therefore concentrated on a discussion of the methods most likely to have this capability if developed with this in mind. The parameter chosen was a method capable of being developed for use within 3-5 years on the basis of current technology.

The methods considered were:

- DNA adduct quantification (chromosomal DNA)

- Histone adducts

- Metabonomics

- Proteomics

- Epigenetic DNA modifications e.g methylation

- PBPK modeling

It was not clear that any of these techniques were on the brink of becoming operational methods for retrospective biomarkers in the immediate future. There was some discussion whether it was preferable to measure a biomarker that was a surrogate for the hazard under consideration (such as DNA changes and cancer risk) or whether it was sufficient to measure any trace of exposure independently of its biological significance.

There was no clear conclusion about the method most likely to achieve the objective, but the most promising technologies appeared to be (at least on theoretical grounds):

- Metabonomics using exquisitely sensitive mass spectrometry techniques. It was noted that ICPMS (inductively coupled mass spectrometry) which was originally mostly used for metal analysis was now showing great promise for measuring biological macromolecules.

- Proteomics or protein adduct formation in very long lived cell populations such as the B memory cells.

- DNA methylation analysis.

Questions arising during the discussion were of two types: technical and ethical. The technical questions concerned principally the persistence of any biomarkers and the turnover of the biological material. There was some doubt as to the feasibility of purifying long lived cell types with a sufficient yield to be analysed

Ethical questions mostly concerned the validation required and the acceptability of research which would potentially require animal studies at the early stages of proof of concept and later human volunteer studies for confirmation. In this respect it was also recommended that techniques concentrate on biological samples that could be obtained in the least invasive manner which was practicable for the method.

4.3 Breakout Group III

Chair: Claudio Colosio
Rapporteur: Martin Wilks

How can novel biomarkers be linked with the currently used methods for exposure characterisation in epidemiology studies to allow an integrated retrospective assessment of exposure to chemicals/pesticides?

Introduction

The group made a number of assumptions based on the presentations and discussions during the workshop. 'Retrospective' was taken to mean the reconstruction of exposure patterns which had occurred some time in the past. The definition of 'novel biomarker' was understood to exclude the 'classical' forms of biological monitoring (i.e. measurement of chemicals or metabolites), but focused on detection of some kind of biochemical or cellular alteration as marker of an early biological or sub clinical effect where the relationship with exposure is defined. The presentations had demonstrated that the use of such biomarkers in retrospective studies is restricted mainly by their limited time span (< 120 days). Other limitations which were pointed out are a low or even absent specificity to individual chemicals (although the response patterns may give clues to the chemicals involved) and lack of knowledge on dose response and dose effect relationships. Dosimetry was seen as an important aspect: does the marker reflect long-term exposure, or a more recent, higher dose? This can only be addressed by relating the presence of the biomarker (which may need to be measured more than once) to the pharmacokinetics of the chemical or chemicals under investigation.

Currently used methods for exposure characterisation

Questionnaires are the primary source of information on exposure in epidemiology. Depending on the nature of the exposure and the time interval between exposure and interview they are all subject to varying degrees of information and recall bias.

Job exposure matrices (JEMs) are established by integrating information on the chemical exposure of specific work processes with the occupational history of workers. Depending on the accuracy and extent of the information available they may result in estimates with very different levels of quality.

Retrospective exposure can also be estimated, with different levels of quality, from expert opinion including the use of models. In this context, questionnaire interviews may provide the basis for the estimate.

A large number of markers of exposure or effect are already in existence with the most frequently used methods relating to measurement of chemicals and their metabolites. With few exceptions (e.g. persistent organic pollutants (POPs), heavy metals) they are limited to short-term exposure assessment. A further problem is often presented by the choice of the most adequate biological material to collect: for example, for POPs it might be body fat, but for obvious reasons this is not acceptable for large epidemiological studies. For some metals or pesticides information on exposure levels might be obtained from more easily collectable matrices, for example hair, but here the limitations are the reference values and the quantity of sample needed.

In theory, prospective studies are the most appropriate way forward since they allow the simultaneous collection of data on exposure and effect. The actions and strategies defined today may also become the basis for retrospective epidemiological studies in the future. Collecting and storing relevant information and even biological specimens for future use might be a step in the right direction.

How could new markers be used?

Two scenarios were developed in which novel biomarkers could be used to improve on existing exposure information:

1. Guide or refine existing methods

   The primary aim would be to develop better questionnaires and JEMs. For example, a biomarker could be used to identify exposure patterns in specific occupational or environmental exposure situations. This may in turn help to design more targeted questionnaires, or develop more precise JEMs. In this context the question was raised whether this concept would also be feasible for markers of effects since they relate more to the consequences of exposure rather than the exposure itself.

2. Screening approach

   Many of the proposed new markers allow the assessment of multiple and complex interactions of chemicals with biologic systems. It may therefore be possible to differentiate

between a person's exposure to a specific chemical and other exposures which may have contributed to the disease (e.g. cigarette smoking).

Conclusions/Recommendations

The group felt that in current retrospective studies of chronic disease novel markers do not appear to add substantive new insights to existing exposure information, principally due to their limited ability to extend into the past. However, novel markers may help in the future to do retrospective assessment in a better way. This will require the use of conventional approaches (questionnaires, JEMs, biological monitoring) in prospective studies to validate novel markers in populations with defined exposures. In this context it was mentioned that many large companies in the EU have collected exposure information on their workforce since the early 1980s; this could be a starting point either for validation studies or for conducting good quality retrospective studies. However, a common problem when taking biological samples is making decisions on processing and storing, e.g. should cells and plasma be separated, should samples be kept frozen, and at what temperature, should they be processed and trapped on columns, etc.? This requires careful consideration of possible future uses. A final thought was given to establishing a 'Biobank' for occupational health purposes. Biobanks are now being established to facilitate long-term studies, often linked to birth cohorts. The concept may find an application in the setting of occupational chemical exposures. In the subsequent plenary discussion the point was made that this does not only apply to occupational but also other environmental exposures.

4.4 Breakout Group IV

Chair: Michael Dunn
Rapporteur: Werner Mellert

What limitations are there likely to be regarding interpretation of information derived from novel biomarkers for retrospective exposure assessment?
How would we address these?

General Observations

The group concluded that there is no magic bullet regarding how information derived from novel biomarkers can be meaningfully interpreted to facilitate retrospective exposure assessment. There is still a lack of basic information on the underlying science (it tends to focus around a few groups of substances) and particularly the role that genes play. While biomarker technologies do appear to be able to reasonably reflect recent exposures, there are few reliable marker that are able to reflect past exposures, as there is a need to distinguish between the nature of chronic/cumulative and acute mechanisms for disease causation. The fact that most exposures are complex and involve co-exposures to several agents adds a further complication, as the concept of a reliable 'exposure sentinels' is probably often restricted to a limited number of settings.

In respect of the above, there would appear to be a need to make better use of a range of biomarkers and to apply suitable sample stratification/statistical techniques to help reduce uncertainties. Similar approaches could also be applied to help better address any consequences of inter-individual variability e.g. re-sampling or "population calibration". There are no obvious biomarkers for quantifying exposure during any critical 'window' of exposure for disease, especially for early life cohorts.

Specific Observations

The discussion focused on 2 areas:

1. the extent to which the existing technologies were sufficient and/or being suitably deployed, and
2. the research needs resulting from any deficiencies in our understandings

Biomarker Technologies

NMR was felt to present many advantages when applied to biomarkers because of its non-destructive nature. As such, it appears to be an under-utilised technology, although it does not possess the required specificity and sensitivity for some applications e.g. low level environmental exposures. Following on from this, it was noted that a lot of biomarker techniques were developed from 'high end' exposures and it was again noted that more work is required to ensure that the techniques are sufficiently validated at low end (environmental) exposures.

In terms of more recent advance in biomarker methods, it was felt that transcriptomics may be too 'robust' (i.e. not offer suitable specificity and discriminating power) to be capable of being routinely applied for 'subtle' exposures. The challenge of how analytical methods needed improvement if they were not to serve as a constraint to the desire to obtain certain analytes e.g. PCBs measurements require a large plasma volume, which represents both a hurdle for studies as well as reduced the likelihood that subsequent analyses can be undertaken on any sample/aliquot.

Research Needs

The major portion of the discussion focused on what key areas of research could usefully be implemented to both improve the understanding of the basic science and to increase the uptake within studies. In this respect it was noted that histome proteins are long lived and there appears to be opportunities for novel biomarkers to be developed based around histomes. At the same time, it was also noted that our understanding of histomes is not extensive e.g. how long lived they are; how they replicate; how errors are passed on, etc.. This lack of understanding also applies to other potentially useful biomarkers such as phosphotriesters and collagen. In this respect, there also appears to be a need not only to develop non-invasive biopsies techniques if, for example, collagen adducts are capable of representing the past *x* years exposure, but also to ensure that the supporting analytical techniques are sufficiently sensitive to make them more useable?

The importance of the adequate validation of BM techniques (repeatability, sensitivity, background rates, etc) was emphasised by many participants. Any technique needed to be able to answer the fundamental question "Is it measuring what we believe it to be measuring?". In this respect, it was noted that the relationship between exposures and adducts remains unclear in many key areas and as a consequence the relationship is often assumed by many researchers c.f. ochratoxin, aflatoxin P1.

Several participants pointed to the extent to whether enough is known about population variability and how this uncertainty affects the consequent interpretability of biomarker findings. The potential role of such variability during different life events was also raised e.g. is there a need for different BMs to be applied in combination in order to meaningfully characterise the exposures at different life stages? The example of how changes in DNA methylation patterns may affect the programming of the foetus was noted (diet of the mother influences offspring methylation capability) together with the potential role that the EU NewGeneris project may contribute to offering a methods to characterise the impact of exposures.

5. SUMMARY AND RECOMMENDATIONS

To study chemical exposures which have occurred historically, epidemiologists usually use combinations of registrations, questionnaires and job exposure matrices. These methods have some limitations that make it difficult to evaluate possible underlying causative relationships between chemical exposures and health effects. The workshop addressed this issue by considering the possibility of analysing body fluids and tissues for biomarkers which would allow a quantitative linkage between a chemical exposure and a biological, or health, effect. Established and emerging techniques were both evaluated.

During the workshop, desirable biomarker properties were identified in order to satisfy the requirements for applications in retrospective exposure assessment. Ideally, the biomarker should be persistent over a long period of time (years rather than months). The biomarker should contain a chemical signature that makes it possible to identify the cause of the observed change in terms of a specific chemical exposure.

Analytical techniques should have sufficient sensitivities, i.e. be able to quantify biomarkers at concentrations corresponding to realistic (low) levels of exposure. The quantity, or intensity, of the biomarker should also provide unambiguous information on the level of exposure.

In this light, the merits and limitations of different methods as dosimeters for retrospective exposure assessment were presented. The following options were discussed: parent substances and metabolites, metabonomics, DNA adducts, protein adducts, proteomics, transcriptomics, and computer models for reverse dosimetry. The conclusions for each of these options are given below:

1. New, ultrasensitive analytical techniques have made it possible to detect parent substances and their metabolites in urine and blood samples at very low levels. An important limitation is the lack of persistence of most substances and their metabolites. They are excreted in a time frame of hours to days. Only few substances tend to accumulate in adipose tissues and in other organs and tissues such as nails and hair, extending the half life of excretion to weeks, months, and sometimes years.

2. Extending from this targeted analysis, metabonomics is a rapidly emerging field where complex metabolite patterns are analysed in tissues and biological fluids using different mass spectrometry-based and multivariate statistical techniques. Currently, most applications are related to therapeutic interventions in animals and humans. This holistic approach could also be used to explore the influence of chemical exposures on health, but for the moment, and in addition to the limited persistence, it is not clear how to interpret metabonomics data with respect to historic exposures to specific chemicals.

3. The analysis of addition products (adducts) to macromolecules is an established approach. Adduct formation shows the bioavailability of the parent substance and the extent to which the parent substance is metabolised before it can covalently bind to macromolecules. Inter-individual differences in metabolic activation, e.g. due to genetic polymorphisms of involved enzyme activities, are taken into account. DNA-adduct analysis is relevant only to exposures to genotoxic substances. The determination of these adducts relies increasingly on mass spectrometry-based detection and quantification, which improves the capability of characterizing, and tentatively identifying, reactive intermediate products involved in the formation of these adducts. An important limitation is that DNA-adducts persist for only hours to weeks, due to enzymatic repair systems that appear to be inducible by chemical exposure, thus complicating direct links with exposure. Recently, however, phosphotriester adducts were suggested as novel markers that appear to be more persistent because there is no known repair system in eukaryotic cells. Adduct formation is also well established in proteins and in this application is not limited to genotoxic substances (some pesticides and nerve agents also form protein adducts in humans). These adducts are more persistent than DNA-adducts because they are not repaired. The persistence depends on the life span of the cell, or of the protein itself, and extends up to 4 months for adducts of hemoglobin. The kinetic pattern of formation and decay of protein adducts is well established both in animal models and humans. Adducts to histones are much more long-lived, but at present are established only in animals.

4. Proteins adducts can be analysed using the same mass spectrometry-based technique that is used for the characterization of endogenous, post translational modifications to the primary structure of proteins. This is part of the much wider field of proteomics which sets out to characterise native peptides and proteins as part of the proteome of tissues and body fluids. Efficient procedures have been developed to analyse the primary structure of proteins by mass spectrometry analysis of enzymatic digests, followed by a computer-aided reconstitution and identification of proteins. Similarly to metabonomics, it is difficult to determine what the inter- and intra-subject variations will indicate as to historic exposures.

5. Transcriptomic profiles have been proposed as new biomarkers of effect, using techniques developed in the field of genomics. Gene expression profiles suggest a profound influence on homeostasis due to environmental factors, including specific modulations of biological processes and molecular functions. Currently, however, gene expression patterns do not appear to be sufficiently specific to be applied at the individual level. Also, persistent epigenetic changes, such as DNA methylation, could have an application in retrospective epidemiology in the future. Further research efforts are needed to be able to link these data to specific chemical exposures.

6. Physiologically-based pharmacokinetic (PBPK) models can be used to calculate an exposure level at a certain moment in the past, preferably if the exact moment in time and duration of this exposure is known. In principle, these models could be applied to any quantified biomarker level for reverse dosimetry so long as sufficient data on physiological processes

in humans are available. For this analysis, repeated determination of the biomarker in the course of time, in the same subject, is recommended.

Following presentations of available knowledge and discussion on possible avenues of future development some research needs were formulated:

1. Persistence appears to be the most important limitation of biomarkers currently available. Adducts not prone to repair, such as hemoglobin adducts, are established methods in humans for exposure assessments relating to the preceding four months. Now, research should be initiated to develop even more persistent biomarkers. There is a prospect for the development of biomarkers, such as phosphotriester DNA adducts and adducts to histones or other proteins, which have life spans longer than four months.

2. Since the persistence of a biomarker is related not only to the chemical stability of the biomarker itself, but also to the life span of the cell that contains the biomarker, it is useful to try and identify cell types which could be isolated in substantial quantities. The identification of the least invasive methods to obtain cell populations for this purpose and the characterization of new intra-cellular biomarkers are worthwhile studies. It would be useful to also explore the technical possibilities for pre-treatment and storage of such samples in tissue banks.

3. Techniques available in proteomics for the characterization of native post translational modifications could be used to interrogate the proteome for persistent modifications to the primary structure of proteins caused by xenobiotic chemicals. This approach may be applied for the screening of relevant multiple exposures in different populations.

4. Techniques used in genomics could be used to explore the possible linkage of chemical exposures to persistent changes such as DNA hypermethylation, e.g. in clusters of cancer gene promoter islands that result in transcriptional silencing of DNA repair genes. This approach will probably not yield information to identify specific chemicals as the cause of these changes in the structure of the DNA.

5. Candidate biomarkers should be thoroughly validated, first in animal studies, and then in studies involving human subjects. Prospective population based-studies allow forward validation of currently established, and new, biomarkers as dosimeters of exposure and allow comparison of these data with outcomes of conventional methods of exposure characterisation such as the use of registrations, questionnaires and job-exposure matrices.

ABBREVIATIONS

ADME	Absorption, distribution, metabolism and elimination
AFB1	Aflatoxin B1
AGE	Advanced glycation endproducts
BaPDE	Benzo[a]pyrenediolepoxide
BM	Biological monitoring
BMI	Body Mass Index
CF	Carbofuran
CRB	Carbaryl
DNA	Deoxyribonucleic acid
ECPA	European Crop Protection Association
ESI	Electrospray ionization
GC-MS/MS	Gas chromatography-mass spectrometry
Hb	Hemoglobin
HCl	Hydrogen chloride
HPLC	High performance liquid chomatography
JEM	Job exposure matrices
LC-MS/MS	Liquid chromatography mass spectrometry
LOQ	Limit of quantification
LRI	Cefic Long-range Research Initiative

MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PBPK	Physiologically-based pharmacokinetic
POPs	Persistent organic pollutants
PTM	Post translational modification
Q-TOF	Quadrupole-time of flight
RfPs	Requests for Proposal
RNA	Ribonucleic acid
SA	Serum albumin
SWOT	Strengths, weaknesses, opportunities, threats
TFAA	Trifluoroacetic anhydride

APPENDIX 1: WORKSHOP PROGRAMME

Tuesday 24 June 2008

12.00-13.30	Lunch and Registration	
13.30-13.40	**Welcome, Format for the Workshop**	Neil Carmichael ECETOC
13.40-13.50	**Chairman's Introduction**	Tim Gant University of Leicester
13.50-14.15	**Assessing Exposure in Epidemiology Studies – What is it we Want to Know?**	Alan Boobis Imperial College London
14.15-15.00	**Current State of the Art in Retrospective Exposure Assessment Methodologies and the Needs in this Area**	Dick Heederik Institute for Risk Assessment, Utrecht
15.00-15.15	Coffee Break	
15.15-1600	**How is Exposure Currently Assessed in Epidemiology Studies – Examples of Successes and Limitations**	Chris Wild Leeds University
16:00-16:45	**Metabonomics and Xenometabolome Metabolite Signatures in Epidemiology Studies**	Elaine Holmes Imperial College London
16.45-17.30	**Personalised Exposure Assessment, Integrated Transcriptomics, Proteomics and Metabolite Analysis**	Jos Kleinjans Maastricht University
18:30-19:30	Cocktail	
19:30-22.00	Dinner	

Wednesday 25 June 2008

08:30-09.15	**Proteomic Analysis - "Protein Imprinting" – Possible Use in the Clinical Situation and what Might Possibly be Learnt from Retrospective Analysis in Epidemiological Studies**	Jennifer Ames Belfast University
09.15-10.00	**Detection of Xenobiotics and metabolites Thereof Using Mass Spectometry-Based Methodology**	Anthony Tsarbopoulos University of Patras
10:00-10:30	Coffee Break	

Wednesday 25 June 2008 (cont'd)

10:30-11:15   **Advantages and Limitations of DNA Adduct Measurement in Identifying**   Peter Farmer
               Retrospective Exposure   University of Leicester

11:15-12:00   **Do Historic Data Contain Historic Information?**   Paul Scheepers
                                                                                                Radboud University Nijmegen

12.00-12:30   **Modelling Techniques Available and Being Developed**   George Loizou
                                                                                                UK HSL, Health Sciences Group

12.30-12:45   **Questions to be addressed in Breakout Groups -**
               6 People/Group, Groups begin Discussions over Lunch
               And 'Engage' with other Experts

12:45-14:00   Lunch

14:00-15:30   **Breakout Groups:**

               Introduction   Martin Wilks

               I   Based on the information presented, what are the most promising leads in the field of novel
                     biomarkers which could be developed/adapted in the short term (1-3 years) for the use in
                     retrospective exposure assessment in epidemiology studies with chemicals/pesticides, and what
                     are their possible limitations?

                     Chair: Stefano Bonassi
                     Rapporteur: Gerard Swaen

               II   What further work should industry sponsor to build on leads which may not be readily useable,
                     but could be promising in the medium term (3-5 years)?

                     Chair: Dirk Palapies
                     Rapporteur: Neil Carmichael

               III   How can novel biomarkers be linked with the currently used methods for exposure
                     characterisation in epidemiology studies to allow an integrated retrospective assessment of
                     exposure to chemicals/pesticides?

                     Chair: Claudio Colosio
                     Rapporteur: Martin Wilks

               IV   What limitations are there likely to be regarding interpretation of information derived from novel
                     biomarkers for retrospective exposure assessment? How would we address these?

                     Chair: Michael Dunn
                     Rapporteur: Werner Mellert

15.30-16.30   **Report from Breakout Groups and Summary**

17.00   Close of Workshop

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ECETOC WORKSHOP REPORTS

No.   Title

No. 1   Workshop on Availability, Interpretation and Use of Environmental Monitoring Data.
20-21 March 2003, Brussels

No. 2   Strategy Report on Challenges, Opportunities and Research Needs Arising from the Definition, Assessment and Management of Ecological Quality Status as Required by the EU Water Framework Directive Based on the Workshop EQS and WFD versus PNEC and REACh - Are They Doing the Job? 27-28 November 2003, Budapest

No. 3   Workshop on Use of Human Data in Risk Assessment. 23-24 February 2004, Cardiff

No. 4   Influence of Maternal Toxicity in Studies on Developmental Toxicity. 2 March 2004, Berlin

No. 5   Workshop on Alternative Testing Approaches in Environmental Risk Assessment. 7-9 July 2004, Crécy-la-Chapelle

No. 6   Workshop on Chemical Pollution, Respiratory Allergy and Asthma. 16-17 June 2005, Leuven

No. 7   Workshop on Testing Strategies to Establish the Safety of Nanomaterials. 7-8 November 2005, Barcelona

No. 8   Workshop on Societal Aspects of Nanotechnology, 9 November 2005, Barcelona

No. 9   Workshop on the Refinement of Mutagenicity / Genotoxicity Testing. 23-24 April 2007, Malta

No. 10   Workshop on Biodegradation and Persistence. 26-27 June 2007, Holmes Chapel

No. 11   Workshop on the Application of 'Omics in Toxicology and Ecotoxicology: Case Studies and Risk Assessment. 6-7 December 2007, Malaga

No. 12   Workshop on Triggering and Waiving Criteria for the Extended One-Generation Reproduction Toxicity Study.
14-15 April 2008, Barza d'Ispra

No. 13   Counting the Costs and Benefits of Chemical Controls: Role of Environmental Risk Assessment in Socio-Economic Analysis, 4 June 2008, Brussels

All ECETOC reports can be downloaded from www.ecetoc.org/workshop-reports