Mass spectrometric mapping of the DNA adductome as a means to study genotoxin exposure, metabolism and effect

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ABSTRACT

Covalent binding of endo- or exogenous chemicals to DNA results in the formation of DNA adducts which are reflective of exposure of the human body to DNA-damaging molecules and their metabolic pathways. The study of DNA adduct types and levels in human tissue therefore offers an interesting tool in several fields of research, including toxicology and cancer epidemiology. Over the years, a range of techniques and methods have been developed to study the formation of endo- and exogenous DNA adducts. However, for the simultaneous detection, identification and quantification of both known and unknown DNA adducts, mass spectrometry (MS) is deemed to be the most promising technique. In this perspective, we focus on the analysis of multiple DNA adducts within a sample with the emphasis on untargeted analysis. The advantageous use of MS methodologies for DNA adductome mapping is discussed comprehensively with relevant field examples. In addition, several aspects of study design, sample pretreatment and analysis are addressed as these factors significantly affect the reliability of DNA adductomics studies.

INTRODUCTION

Although the underlying pathways are not fully understood, years of epidemiological research have demonstrated that approximately 90% of cancer deaths can be attributed to the presence of certain environmental factors, and not genetics.[1,2] Unfortunately, assessment of human exposure to the large spectrum of environmental factors and the direct or indirect role in disease onset and development has proven to be a challenge. In 2005, Wild introduced the 'exposome' as a complementary concept to the 'genome', a concept which originated at least 10 years earlier.[3] The exposome encompasses all of the encountered exposures of a certain individual over the course of his or her lifetime, from the very early stages of conception and embryonic development through to adulthood, old age and death. In contrast to the individual genome, which is set at conception, the individual exposome evolves continuously throughout one's life.[1-4]

In light of genotoxicity and carcinogenicity studies, the DNA adductome, which consists of all DNA adduct types and levels present in a certain DNA sample, and can be considered as a part of the exposome, is of particular interest. DNA adducts originate from the interaction and subsequent covalent bonding between an electrophilic molecule and nucleophilic sites in DNA (i.e. nucleobases).[5,6] The majority of DNA reactive molecules have the potential to lead to mutations and chromosomal alterations during DNA replication via formation of DNA adducts or DNA strand breaks, thus possibly resulting in carcinogenesis later on.[7] Therefore, DNA adduct formation is deemed to be the first step in chemically induced carcinogenesis.[5] However, whilst carcinogenesis due to genotoxin exposure and DNA adduct formation poses a convincing hypothesis, at the present time only a limited number of studies have successfully demonstrated a positive association between certain DNA adduct levels and cancer incidence i.e. 'biomarkers of effect'. In consequence, one may question the relevance of DNA adduct analysis in cancer risk assessment. Nonetheless, the association between, for example, aflatoxin B1 exposure and hepatocellular carcinoma[8], and the long undoubted association between tobacco smoking and cervical cancer[9] clearly demonstrate the merit of DNA adduct analysis in cancer risk assessment. Overall, evidence for the association between DNA adducts and cancer risk is accumulating.[10,11] Hence, the need for a fit-for-purpose analytical approach to study DNA adduct formation in different pathways has presented itself, along with the need to assemble all acquired knowledge on DNA adduct formation in a comprehensive database.

A multitude of analytical methods have been developed and optimized for the detection of DNA adducts in different biological matrices ranging from those based on antibodies and labeling such as immunoassays, immunohistochemistry and ^{32}P-postlabeling to advanced instrumental techniques. The latter invariably use chromatographic separation coupled with various detection methods e.g. gas chromatography-electron capture detection (GC-ECD), high performance liquid chromatography-fluorescence detection (HPLC-FD), GC- or (HP)LC-mass spectrometry ((HP)LC-MS), (LC-)nuclear magnetic resonance ((LC-)NMR), and accelerator MS (AMS).[12,13] The characteristic advantages of MS, currently recognized as the gold standard for DNA adduct detection, have been reviewed and argued in the past.[6,12,14] In this paper, we further explore the potential of DNA adductomics studies to map the DNA adductome. In light of this, the tools at hand and several important aspects of DNA adductomic studies, including the issue of DNA adduct and internal standard stability, sample preparation, analysis of target vs. surrogate tissue, method validation and study design are discussed comprehensively. Moreover, the promising use of MS as a tool for DNA adductomic studies focusing on the detection and identification of both known and unknown DNA adducts in the exposome is highlighted, explained and demonstrated with recent examples.

DNA ADDUCT ANALYSIS AS A MEANS TO STUDY GENOTOXIN EXPOSURE, METABOLISM AND EFFECT

DNA adducts originate from exposure of cellular DNA to both endo- and exogenous genotoxins. Tissues and cells are exposed to endogenously generated chemicals through several (patho)physiological processes on a daily basis, including attack of DNA by reactive oxygen and

carbonyl species, lipid peroxidation products, estrogens and S-adenosylmethionine (gene expression regulator and methyl donor).[15] However, the exogenous exposure to xenobiotics is deemed more important in toxicology and cancer risk assessment.[16] Examples of exogenous DNA adduct formation consists of DNA damage by dietary toxins such as mycotoxins, acrylamide and heterocyclic amines (HAAs).[17-19] Of course, several other environmental lifestyle factors can also significantly contribute to genotoxin exposure and exogenous DNA adduct formation; e.g. smoking, alcohol, certain industrial occupations and living conditions.[20-22]

The direct measurement of genotoxic chemicals in body tissues and fluids does not take into account important factors such as interindividual differences in exposure, absorption and distribution. Moreover, these chemicals may have a rapid turnover in the body, making direct measurement impossible. Hence, DNA adductome mapping offers a more thorough view of the different biological pathways involved in genotoxin exposure. This is especially pertinent since individual heterogeneity in genotoxin metabolism and DNA repair specifically complicates a straightforward assessment of the effect of certain genotoxins. Accordingly, holistic assessment of all DNA adduct types and levels ('mapping') provides a more appropriate tool to study the biological effect of a genotoxic chemical. The arguments raised above demonstrate the fact that DNA adducts show great potential as 'biomarkers of exposure' or 'biomarkers of internal dose'. Furthermore, since DNA adducts represent the amount of genotoxin that 'successfully' reached the DNA molecule in a certain individual, they can even act as a 'biomarker of the biological effective dose' of a certain genotoxic substance for that particular individual.[10,11]

Although interpretation of DNA adduct formation is complicated by several interfering factors, the field of DNA adductomics shows great potential in different areas of research. DNA adductome mapping does not only enable research into genotoxin exposure, but can also provide information on interindividual differences in genotoxin detoxification or activation. For example, Haugen and colleagues demonstrated a gender related difference in susceptibility to DNA adduct formation in tobacco smokers due to a significantly higher expression level of lung cytochrome P450 1A1 in women.[23] Genetic polymorphisms in DNA repair can also be a source of interindividual variation in DNA adduct levels[11]; e.g. Xia and co-workers recently published research on interindividual differences in aflatoxin B1 DNA adduct formation due to certain genetic polymorphisms in a DNA repair gene.[24]

In addition to information on exposure to genotoxins, interindividual differences in genotoxin metabolisation and individual susceptibility to DNA damage and repair, DNA adduct analysis also provides important evidence on the possible long-term adverse health effects of genotoxic chemicals. When DNA adducts are introduced to the DNA sequence, the resulting DNA damage may lead to mutagenesis and carcinogenesis.[7] There are at least three cases in which a causal link between the occurrence of DNA adducts and cancer incidence have been confirmed; firstly, the previously acknowledged aflatoxin B1 DNA adducts and their link to hepatocellular carcinoma[8]; secondly, the case of PAH-DNA adducts and cervical cancer[9], and thirdly, the link between aristolochic acid consumption (via consumption of *Aristolochia* plants), aristolactam DNA adduct formation and transitional cell (urothelial) carcinoma of the upper urinary tract.[25] For many other exo- or endogenous

DNA adducts, potential clues for the probable relationship between DNA adduct levels in tissue and cancer incidence are accumulating, emphasizing the significance of DNA adduct studies in the field of toxicology and cancer epidemiology.[10,11] Adductome mapping, like any technique, may not provide the full answer but can aid with the elucidation of cancer susceptibility and mechanisms, and potentially lead to improved cancer prevention and/or development of treatments for at-risk individuals. In Fig. 1, the position of DNA adduct formation in the pathway of genotoxin exposure, metabolism and effect is presented to illustrate the potential use of DNA adduct analysis for in-depth assessment of genotoxin exposure, metabolism and effect.

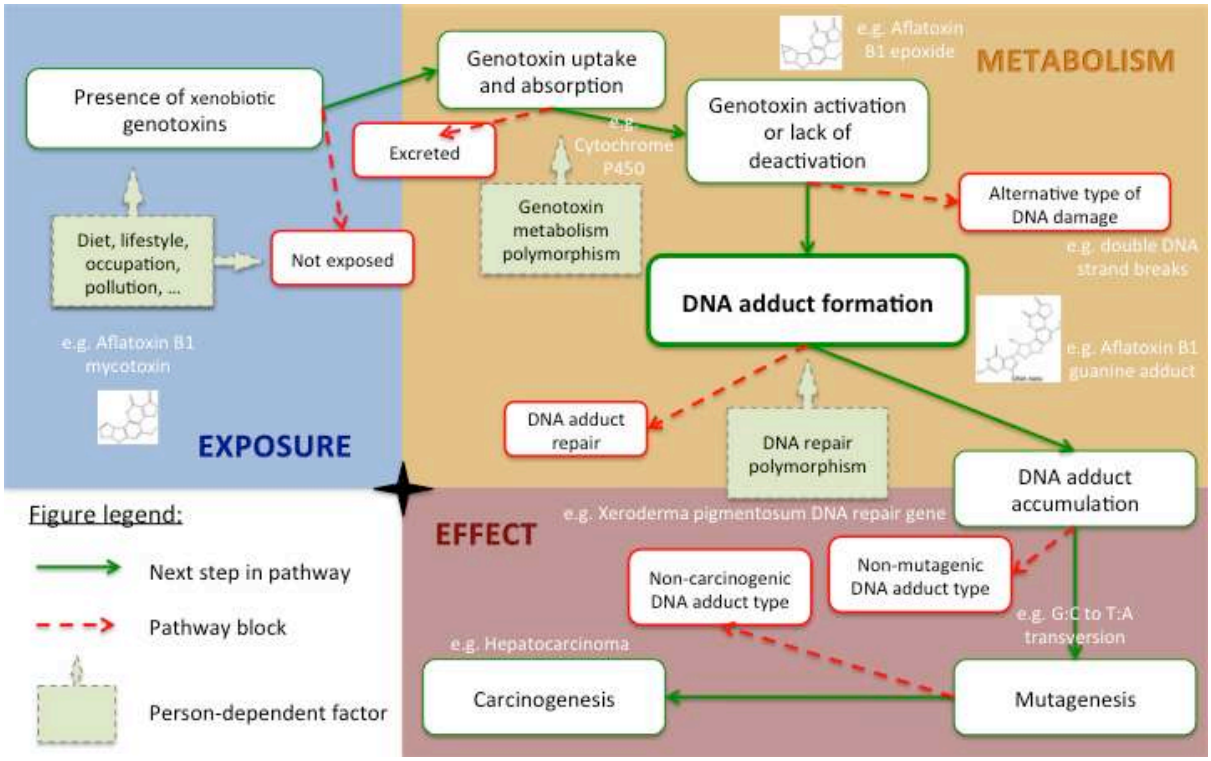

Figure 1. DNA adducts in relation to genotoxin exposure, metabolism and effect (aflatoxin B1 associated hepatocarcinogenesis as a case study).

THE UNIQUE POTENTIAL OF DNA ADDUCTOMICS

Exposomic studies comprise both external and internal exposure assessment in order to correctly link a certain exposure to a certain effect[2], and this also applies to DNA adductomic studies. The most common tool used to study environmental exposure to toxins are questionnaires. However, the major flaw of questionnaires in light of exposome mapping, is the fact that they can only focus on a limited amount of environmental factors and pollutants. Furthermore, questionnaires do not take into account exposure to yet unknown, but possibly also highly relevant environmental factors, and are often

subject to participant or response bias.[1,2]. Therefore, an in-depth exposure assessment requires a multi-disciplinary approach that can tackle these issues.

Focussing on the more detailed assessment of genotoxin exposure by means of analytical chemistry, two different types of studies or approaches can be distinguished; the bottom-up (targeted) and the top-down (untargeted) approach. The bottom-up approach envisions summing up all known exposure types or groups in order to characterize the exposome. The downside to this most commonly used approach is the fact that unknown exogenous and also endogenous environmental factors may be overlooked.[2,26] The alternative strategy on the other hand; the top-down approach, reflects both known and unknown exogenous and endogenous exposures.[2] Although the latter sounds very appealing, it requires specialized untargeted "omics" technologies and methodologies, and embodies extensive data processing by means of specialized software. Nevertheless, independent experts in the field have suggested that "omics" is the present and future of (cancer) epidemiology, despite its expense and complexity.[2,4] Therefore, application of these approaches to the field of adductomics, via the use of appropriately designed studies and analytical methodologies, is worthy of investigation.

MASS SPECTROMETRY AS THE METHOD OF CHOICE FOR DNA ADDUCTOMICS

DNA adduct analysis requires very sensitive and highly specific analytical techniques and methodologies. For years, ^{32}P-postlabeling was the most utilized technique and for some DNA adducts types, a sensitivity of 1 adduct per 10^{10} nucleotides could be achieved.[27] Unfortunately, false positives and artefacts are common when using this approach.[28] MS-based detection techniques on the other hand, enable accurate identification of DNA adducts and can also provide structural information, in which other analytical methods often fall short. Multiple analytical techniques have contributed to the current knowledge on DNA adducts and DNA adduct detection techniques have been reviewed extensively in the past.[12,13,29] However, it is clear that MS detection excels in specificity and structural identification.[12-14] More than a decade ago Koc *et al.* stated that the only disadvantage of MS in the field of DNA adduct analysis, was its sensitivity.[28] Over the years, sensitivity has continued to improve[6,14] as different research groups have focused on optimization of DNA adduct detection methods with MS[30-37], including research into non-manual data mining and sequencing to locate DNA adduction sites.[38,39] Due to ongoing technical advancements and the use of stable isotope labeled internal standards, MS currently offers a reliable tool to measure low DNA adduct levels with the highest specificity.[12,14,27,28] Coupling of MS with LC by means of the electrospray interface (ESI) has enabled analysis of DNA adducts in very complex biological matrices[40], whilst avoiding complex and labour-intensive sample preparation with derivatization for the initially envisioned use of GC-couplings.[41]

An important advantage of MS, in contrast to all other previously mentioned DNA adduct detection methods (besides NMR), is the possibility to detect both 'targeted' and 'untargeted' DNA adducts by means of full scan MS. Targeted DNA adduct detection (also known as 'profiling') refers to the detection of known types of DNA adducts, which implies that the MS system specifically scans for the

presence of certain compounds of interest to assess their presence and abundance, whilst all other molecules in the sample are disregarded completely. On the other hand, untargeted analysis (also known as 'fingerprinting'), refers to the detection of all compounds present, even if unknown or deemed irrelevant at the time.[42] The full scan data obtained of biological samples can be searched for the presence of other DNA adduct types (known or unknown) in parallel or retrospectively, providing potentially highly relevant additional information. The targeted detection of DNA adducts accords with the bottom-up approach, whereas the untargeted mapping or fingerprinting of DNA adducts facilitates a top-down approach.

MASS SPECTROMETRIC TOOLS IN USE FOR DNA ADDUCTOMICS

Triple Quadrupole Tandem Mass Spectrometry

LC-ESI-tandem MS (LC-MS/MS) by means of triple quadrupoles is currently the most applied technique/instrument for the targeted quantification of DNA adducts.[31] With LC-MS/MS, mapping of the DNA adductome is enabled through monitoring of the constant neutral loss (CNL) of 2'-deoxyribose (116 Da) from positively ionized 2'-deoxynucleoside adducts. This approach is demonstrated for four different adducts in Fig. 2, where the difference between the precursor ion and the base peak is always 116 Da.[13] One can focus on all $[M+H]^+$ to $[M+H - 116]^+$ transitions by applying a full scan approach in Q3, the third quadrupole, or alternatively use selected reaction monitoring (SRM) to view $[M+H]^+$ to $[M+H - 116]^+$ transitions in a more narrow, selected range. The narrow range of SRM can be compensated by multiple injections of the same sample, which are then analyzed in different mass ranges, although this requires more time for analysis.[27]

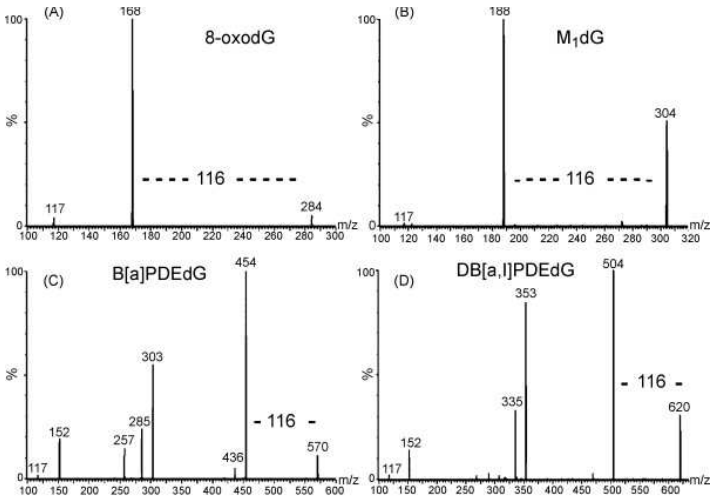

Figure 2. ESI MS/MS mass spectra documenting $[M+H]^+$ to $[M+H-116]^+$ transitions of four DNA different adducts; (A) 8-oxo-dG, the main oxidative stress related DNA adduct; (B) M1dG, the main malondialdehyde adduct; (C) B[a]PDEdG, the main benzo[a]pyrene diol epoxide DNA adduct; and (D)

DiB[a]PDEdG, a dibenzo[a,1]pyrene diol epoxide DNA adduct. Adapted from Farmer, P.B.; Singh, R. Mutat. Res.-Rev. Mutat. 2008, 659, 68-76 (ref 13) [13], copyright 2008 Elsevier.

The group of Kanaly *et al.* was one of the first to develop and apply an LC-MS/MS method for mapping of the DNA adductome. Analysis of human lung and esophagus DNA samples revealed the possible presence of more than 1000 putative DNA adducts in each tissue type. The use of analytical standards and isotope dilution allowed full identification of seven DNA adducts.[43,44] This demonstrates the vast amount of data that can be generated and the amount of time involved in positively identifying the adducts within a particular sample; the analysis itself takes 28 to 60 min. per sample and the authors describe that the time required for data processing and confident identification is 'manageable'. Examples of similar LC-MS/MS applications include work by several research groups, demonstrating the popularity of this type of instrumentation for DNA adductomics.[31,45-52]

An alternative approach to monitoring of the mutual loss of 2-deoxyribose, is the detection of altered DNA nucleobases instead of altered nucleosides. Inagaki *et al.* reported the presence of characteristic fragment ions for guanine at *m/z* 152 (≈ protonated guanine) and 135, which corresponds to fragmentation of the NH_2 group, and for adenine at *m/z* 136 (≈ protonated adenine) and 119[35], which is presumably the corresponding fragmentation although the authors did not show the data. Confirming part of these findings, Gregson *et al.* also documented deamination upon collision induced fragmentation of protonated guanine[53], whilst other independent research groups have also reported the occurrence of a product ion with *m/z* 152 for guanine[34,54] and *m/z* 136 for adenine[55,56] using different systems and focusing on different DNA adduct types. However, at the present time, it is not clear whether those exact same ions are formed upon fragmentation of all purine DNA adduct types.

The aforementioned research demonstrates that tandem MS can reveal hundreds of putative DNA adducts in DNA samples and thus holds great potential for biomarker discovery. However, one disadvantage of tandem MS/MS is the loss of sensitivity with CNL or the need for SRM transition optimization for each different DNA adduct in order to achieve sufficient detection sensitivity with pseudo-CNL.[27,31] Secondly, since triple quadrupoles only allow low resolution data acquisition, and DNA adduct databases providing MS/MS spectra are not available, DNA adduct identity confirmation is dependent on the availability of analyte standards or the use of additional analytical techniques. This renders triple quadrupole mass measurements to be less suited for untargeted compound analysis and confident compound identification compared to high resolution mass spectrometry (HRMS), e.g. time of flight (TOF) and orbitrap, which is discussed further on.[27]

Ion Trap Mass Spectrometry

Ion trap MS-analyzers allow multistage scan events (MS^n) that provide additional structural information. Just like most triple quadrupole methods, ion trap DNA adduct analysis depends on the detection of the neutral loss of the 2'-deoxyribose group. Bessette, Turesky and co-workers describe the use of a linear ion trap for data-dependent LC-MS^3 (DD-CNL-MS^3), where first, the detection of a

DNA adduct ion (listed in a targeted mass-list) in a limited *m/z* scan range leads to MS^2 acquisition. Subsequently, the detection of the $[M+H - 116]^+$ ion amongst the top ten of the most abundant MS^2 ions triggers MS^3 fragmentation. Bessette *et al.* used this acquisition type to study the formation of tobacco-associated DNA adducts of certain aromatic amines, HAAs, PAHs and aldehydes in rat livers, human hepatocytes and buccal cells.[33] MS^3 acquisition or multistage MS^n scanning in general, seems a major advancement compared to MS^2 CNL scanning techniques since MS^n provides a higher specificity and further DNA adduct characterization.[27,33] Unmistakable identification with the ion trap occurs through evaluation of the MS^n product ion spectrum and co-elution with an analytical standard. If necessary (e.g. no analytical standard available), the use of additional analytical techniques using accurate mass measurements may assist in the identification of unknowns. Co-workers of Bessette and Turesky applied the DD-CNL-MS^3 approach in research on 4-aminobiphenyl, HAA and aristolochic acid-related aristolactam DNA adducts with a clear focus on targeted DNA adduct detection.[57,58] Pietsch *et al.* adapted the method described by Bessette *et al.* to study DNA adduct formation by Illudin S, an antitumoral agent. They were able to study known DNA adducts in a colon cancer cell line, but were unable to detect or identify any untargeted DNA adducts.[59] This suggests that although the ion trap and DD-CNL-MS^3 method have proven their worth for structural characterization, identification and quantitation of (a limited number of) targeted DNA adducts, the low resolution methodology appears less suited for holistic, untargeted omics applications, including DNA adductomics.[60] Fig. 3 demonstrates compound identification by means of MS^3 fragmentation patterns for three different DNA adducts of the HAA N^2-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) (deoxyguanosine-N^2-MeIQx, deoxyguanosine-C^8-MeIQx, and deoxyadenosine-C^8-MeIQx)[33] whereby the MS^3 fragmentation patterns obtained allow confident compound identification.

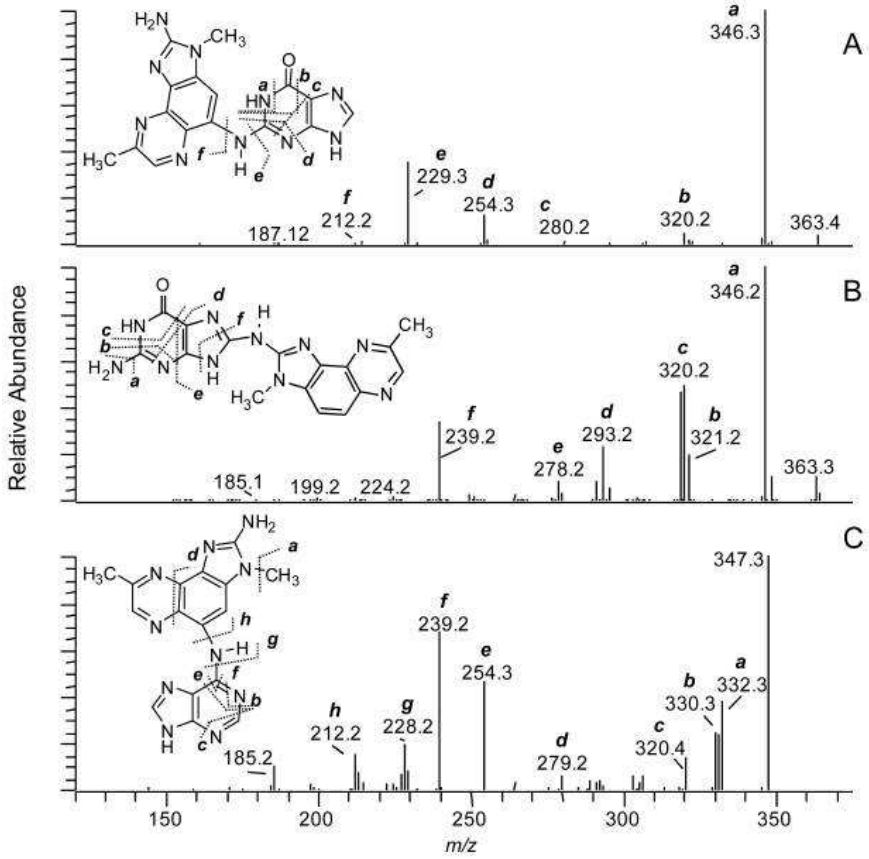

Figure 3. CNL-MS^3 product ion spectra of three MeIQx DNA adducts, from HAA exposure, clearly showing multiple fragmentations within the adducts; (A) dG-N^2-MeIQx, (B) dG-C^8-MeIQx, and (C) dA-N^6-MeIQx. Reproduced from Bessette, E. E.; Goodenough, A. K.; Langouet, S.; Yasa, I.; Kozekov, I. D.; Spivack, S. D.; Turesky, R. J. Anal. Chem. 2009, 81, 809-819 (ref 33) [33], copyright 2009 American Chemical Society.

Time of Flight High Resolution Mass Spectrometry

TOF instruments are most commonly used for qualitative analysis as a stand-alone instrument, although coupling to a second mass spectrometer offers several opportunities for DNA adductomics studies. Recently, Giese and co-workers developed a MALDI-TOF/TOF method (MALDI = matrix-assisted laser desorption/ionization) to enable untargeted DNA adduct detection (preceded by HPLC separation), facilitating investigation of unknown DNA adducts. To this purpose they employed a tedious but highly profitable sample preparation procedure based on benzoylhistamine labeling of altered nucleotides. This approach enabled the specific detection of altered deoxynucleotides with increased sensitivity and specificity (noise was reduced due to the use of negative ionization) in a semi-quantitative manner.[61,62]

By coupling of a TOF-MS to a quadrupole, the resulting hybrid instrument can also be easily employed for both identification and quantitation purposes since accurate mass measurements are important for confident compound identification and can also eliminate spectral noise due to matrix

interferences.[40] Esmans, Van den Driessche and colleagues published research[63,64] on the use of a QTOF instrument for DNA adduct screening and characterization. Unfortunately, and to the best of our knowledge, both the use of the QTOF (quadrupole coupled to TOF) and MALDI-TOF/TOF instruments for DNA adductome mapping has not been explored further which may be due to the lack of available instrumentation in appropriate research laboratories as tandem MS instrumentation has been favoured in recent years.

Orbitrap High Resolution Mass Spectrometry

Orbitrap technology enables very accurate mass detection due to a high resolving power and mass accuracy. The technology is particularly suited and implemented for small molecule analysis and untargeted omics applications. The orbitrap is often coupled to an ion trap instrument (early on) or a quadrupole (later on), but can also be used as a standalone instrument.[40]

Recently, Balbo *et al.* developed a high resolution DD-CNL-MS^3 method for DNA adductomics purposes, using a linear ion trap-orbitrap system.[32] Within the described application, the orbitrap ensures accurate mass measurements resulting in determination of possible elemental composition, selective identification of DNA adducts and avoidance of false positives. The CNL $[M+H]^+$ to $[M+H-116]^+$ transition triggers MS^3 acquisition, further contributing to molecular structure data and assisting with identification of untargeted DNA adducts (demonstrated in Fig. 4). Therefore, this methodology appears to be suitable for wider application to adductomics areas of research.

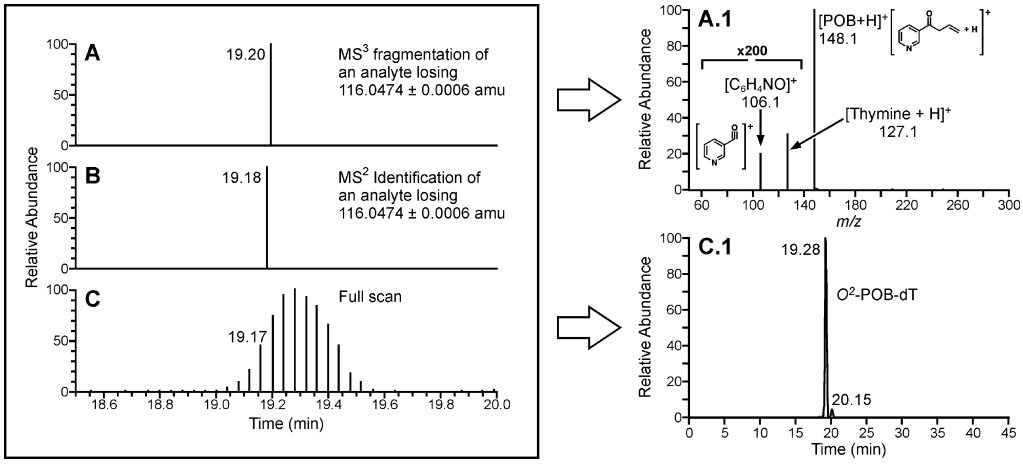

Figure 4. Demonstration of the CNL-MS^3 high-resolution/accurate mass adductomic approach with an O^2-POB-dT standard (a tobacco-specific nitrosamine related DNA adduct). (A) MS^3 scan event triggered by a mass difference of 116.0474 amu between an ion mass in the full scan (C) and an ion mass in the corresponding MS^2 spectrum (B). (A.1) MS^3 spectrum of O^2-POB-dT, (C.1) accurate mass full scan ion chromatogram of O^2-POB-dT (m/z = 390.1660 amu). Reproduced from Balbo, S.; Hecht, S. S.; Upadhyaya, P.; Villalta, P. W. Anal. Chem. 2014, 86, 1744-1752 (ref 32) [32], copyright 2014 American Chemical Society.

PERSPECTIVES FOR MASS SPECTROMETRY-BASED DNA ADDUCTOME MAPPING

Hybrid High Resolution Mass Spectrometry

For years, triple quads have dominated the field of trace quantitation.[40] Yet, when conducting DNA adductome mapping, tandem MS encounters some difficulties regarding DNA adduct identification; since accurate mass data are not available and, at the time, sufficient knowledge on MS/MS spectra of DNA adducts is somewhat lacking. This necessitates that comparison with analytical standards and/or the use of other analytical techniques are essential for confident compound identification. High resolution MS provides accurate mass measurements and thus more information regarding compound mass, elemental composition and identity. New hybrid systems like QTOF and ion trap-orbitrap may not always surpass triple quadrupole instruments for low level quantitation of DNA adducts in terms of peak areas, but full scan HRMS acquisition always results in lower signal to noise ratios compared to low resolution MS due to elimination of noise. Therefore, comparable limits of detection and limits of quantification for high resolution hybrids and triple quads are definitely within reach. In any case, hybrid HRMS systems offer indisputable advantages through accurate mass detection, which renders them to be an excellent tool for omics applications.[40]

Besides the QTOF[64] and linear ion trap-orbitrap instruments[32], other hybrid HRMS systems such as the quadrupole-orbitrap, quadrupole ion trap-TOF and linear ion trap-fourier transform cyclotron resonance MS could be highly accommodating for DNA adductome mapping.[65,66] For example, a quadrupole-orbitrap instrument like the Q-Exactive™ can be operated in different acquisition modes; full scan MS, selected ion monitoring (SIM) MS, MS^2, full scan data dependent MS^2 (DD-MS^2), SIM-DD-MS^2 and neutral loss DD-MS^2 (NL-DD-MS^2), enabling different approaches for targeted and untargeted analysis of complex biological matrices.[67] In particular, the use of NL-DD-MS^2 for untargeted DNA adduct detection should be explored further as this approach can provide $HRMS^2$ spectra of DNA adducts characterized by the loss of e.g. 2'-deoxyribose ($[M+H]^+$ to $[M+H - 116]^+$ transition) upon fragmentation; a distinctive feature of nucleoside DNA adducts that has been commonly exploited for DNA adductomic research by means of both triple quads and ion traps.[33,43] Employment of the neutral loss of 2'-deoxyribose by nucleosides, and the potentially characteristic loss of protonated bases (as reported by Inagaki and co-workers[35]) during DNA adduct fragmentation, could prove to be very rewarding as it allows analysts to focus their attention on the detection of potential DNA adduct biomarkers exclusively by ignoring all non-DNA adduct originating ions and molecules.

Chromatographic Innovations

As MS is usually coupled to chromatography, further advances could still be achieved by means of modern LC techniques. Within this framework, the use of capillary or nano capillary LC coupled to micro- or nano-ESI-MS could provide a rise in sensitivity.[14,31] Due to the lower sample flow rates of capillary LC, the ionization and ion sampling efficiency in the electrospray source increase

significantly, resulting in a higher amount of ions in the MS system, an improved sensitivity and low mass detection limits.[14] With micro- or nano-ESI, a higher electrospray efficiency and improved MS sensitivity are achieved in the same manner.[68] Both (nano) capillary LC and micro- or nano-ESI-MS have been implemented for targeted DNA adduct analysis[69-71] and allow sensitive DNA adduct analysis with a limited amount of sample.

To eliminate non-altered nucleosides from the sample, two-dimensional (2D-)LC can be implemented.[71] 2D-LC is another on-line chromatography application that could definitely assist with detailed DNA adduct mapping because this technique allows one sample (or its most interesting 'section') to be chromatographically separated twice (with 2 different columns), significantly adding to the separation power required for the analysis of complex biological samples like DNA. 2D-LC has already demonstrated its potential in metabolomics and proteomics[72], and has been used for DNA adductomics at least once by Singh *et al*., who used a trap column to isolate PAH-dihydrodiolepoxide DNA adducts in order to facilitate subsequent separation by means of an analytical column thereafter.[31] This approach enabled an increased sample throughput and a significant reduction of ionisation suppression and other matrix effects. Besides the elimination of unmodified DNA building blocks by means of a trap column, which significantly enhances the sensitivity of the analysis and also reduces the risk of artifacts[14], 2D-LC also has the potential to assist with the combined and more adequate separation of different types of DNA adducts with different chemical attributes during one single chromatographic run when using two analytical columns. Unfortunately, according to the available literature, this has not been investigated yet.

An additional technique that could be used to achieve an increase in sensitivity, consists of miniaturized separation techniques like LC-chip.[31] This state-of-the-art development improves sensitivity by a gain in ionization efficiency and also significantly reduces the required sample size.[73,74] However, up to date, there are very few promising papers on DNA adduct analysis by means of LC-chip MS, although the technique was introduced over a decade ago. It appears that the specialized nature of LC-chip technology and the need for specific LC and MS equipment may pose important restrictions for its widespread application. Although the technique definitely seems very promising for DNA adduct biomarker research, its optimisation and subsequent application seems to be rather complex and difficult; e.g. Bani-Yaseen *et al*. documented persistent problems with the separation of similar molecular structures.[75] In contrast, Vouros and co-workers were able to use (commercialized) HPLC-chip MS methodology quite easily for the detection of dG-C8-4-ABP (a 4-aminobiphenyl DNA adduct), although they do not discuss its optimisation and practical use in detail.[69]

Unfortunately, none of the above-mentioned techniques have been applied for untargeted DNA adductome mapping, merely leaving us with the promise of a giant leap forward in this field of research. It has, at least in part, been demonstrated for targeted DNA adduct analysis, but still needs to be established and confirmed for DNA adductomics purposes.

PRACTICAL CONSIDERATIONS FOR (MASS SPECTROMETRY-BASED) DNA ADDUCTOME MAPPING

Internal Standards

In recent years, the sensitivity of MS for DNA adduct quantitation purposes has increased significantly. The use of stable isotope labeled internal standards has provided a means to quantitate with high accuracy.[13,14,28] An internal standard can be manufactured by replacing one or several atoms in the DNA adduct structure with their ^{2}H, ^{13}C, ^{15}N or ^{18}O isotopes. Due to the nearly identical chemical characteristics, these types of internal standards are well-suited for correction of variation due to losses during sample handling and preparation, local matrix effects, and possible fluctuations in sensitivity during analysis.[14,28] In addition, the use of labeled (and unlabeled) (internal) standards facilitates compound identification by enabling comparison of the retention times that were obtained for different compounds.

Unfortunately, appropriate internal standards for DNA adducts are not always easily obtained commercially.[12] Furthermore, there may be some concerns regarding the stability of the labeled internal standards.[14] Some deuterated DNA adduct analogues (e.g. d_2-O^6CMG) lack chemical stability and are prone to decomposition due to exchange of deuterium for hydrogen.[14,54] The stability of ^{13}C, ^{15}N and ^{18}O labeled DNA adducts seems to be less cause for concern, as their degradation likely parallels the breakdown of their unlabeled counterparts.[28] Furthermore, the internal standard should ideally differ in mass by 3 units from that of the compound under investigation. Hence, the use of ^{13}C, ^{15}N and ^{18}O labeled DNA bases is the obvious choice of internal standards.

If there is no appropriate and stable internal standard available at the time, analysis and quantitation by means of an external calibration line offers a possible alternative, although less accurate approach for DNA adduct quantitation.[14] However, this offers no scope for correction due to sample preparation issues.

DNA Adduct Stability

Sufficient knowledge on chemical stability of DNA adducts in biomarker studies is extremely important for correct interpretation of results.[10,13] However, it appears that only a limited number of studies have studied the stability of a limited number of adduct types, which was discussed in detail by Himmelstein *et al.*[12] DNA adduct stability depends on several factors including pH (e.g. M_1dG is not stable under alkaline conditions[76]) and composition of storage buffers (e.g. Tris buffer induces M_1dG instability[77]). Sample matrix (e.g. embedded in liver vs. kidney or other sample types[78]), sample processing (e.g. contamination by RNA can add to N^7-methylguanine levels[79]), storage temperature (M_1dG and benzo(a)pyrene DNA adducts, among others[77,80]) and, last but not least, DNA adduct type or chemical composition (e.g. O^6-methylguanine appears to be more stable than N^7-methylguanine and N^3-methylguanine[81]) also influence DNA adduct stability. In contrast, it appears that the number of freeze-thaw cycles and long term storage might not significantly reduce DNA adduct stability.[82-84]

In order to avoid incorrect interpretation and loss of results, sample handling and storage should be considered in a case-by-case manner and executed carefully and consistently. To improve knowledge on DNA adduct stability, researchers should opt to conduct more DNA adduct stability studies focusing on different DNA adduct types, sample handling and storage conditions; e.g. taking the use of certain DNA buffers and the optimum storage temperature into account. In the meantime,

DNA should preferably be stored at -80°C, whilst evaporation to dryness may prevent early decomposition of DNA adducts.[12]

Sample Preparation

Sample preparation is considered to be one of the most critical steps in analytical chemistry in general, but in particular for DNA adduct quantitation since DNA adducts are embedded in a complex matrix of abundantly present unmodified DNA building blocks.[28] To enable the simultaneous detection of a multitude of DNA adducts, sample preparation should be kept to a minimum, as extensive sample preparation procedures may induce artefacts (e.g. formation of oxo-dG during sample preparation), loss of sample and relevant information (due to e.g. instability issues). In addition, sample preparation (clean-up and enrichment) can be quite time consuming and labour intensive. However, sufficient release of DNA adducts from the DNA sequence (DNA hydrolysis efficiency), enrichment and removal of unwanted matrix constituents are indispensable.[12,14,28]

DNA adducts can be detected individually as adducted nucleotides, nucleosides or nucleobases upon DNA hydrolysis, DNA adduct extraction and enrichment. The choice of measuring either adducted oligonucleotides, nucleotides, nucleosides or nucleobases greatly influences sample preparation needs. Analysis of (oligo-)nucleotides is least common and requires enzymatic digestion of DNA. Likewise, analysis of nucleosides requires enzymatic digestion and is the most common method of sample preparation for mass spectrometry-based analyses. Thermally labile modified nucleobases can be released by means of thermal hydrolysis. More stable adducted nucleobases can be retrieved with thermal hydrolysis at high temperature or strong acid hydrolysis. By combining acid and thermal hydrolysis, both altered and unaltered nucleobases are cleaved from the DNA sequence.[14] Nonetheless, Kato *et al*. found that a single approach may not release all adducts and had to employ two different enzymatic hydrolysis methods to their samples, resulting in a doubled workload.[46] These procedures are lengthy and have multiple steps that may cause changes to the DNA and the adduct profile, which must be thoroughly investigated during method development.

To improve the sensitivity, sample clean-up and enrichment upon DNA hydrolysis or digestion are highly recommended.[12,27] The envisioned removal of unmodified DNA building blocks and interfering contaminants (e.g. highly polar compounds that interfere with ionization) is required to minimize signal and ionization suppression.[14,28] Frequently utilized on- or off-line techniques for DNA adduct enrichment include immunoaffinity column purification, HPLC column switching and solid phase extraction.[14,27,28] However, care must be taken with selection of the appropriate stationary phases and elution buffers to avoid degradation/loss of the adducts. In addition, immunoaffinity column purification can only be implemented prior to targeted analysis due to the specificity of the antibodies in use.[85] Therefore, this particular technique is only suited for targeted DNA adduct analyses, but not DNA adductomics.

Study Design

The choice of an appropriate study design is considered to be one of the most important factors in DNA adductomics studies. The exposome is very complex, dynamic, and continuously changing.[4] Therefore, measurement of the exposome or DNA adductome at one isolated moment in time will not

answer all related research questions. Thus, assessment of individual exposure requires longitudinal studies.[2,10] According to Wild, full characterization of the individual exposome requires an extensive number of sequential measures throughout a lifetime, or at the least, a smaller number of measures to assess exposures over a series of extended periods.[4] In addition, exposomics studies should also consider the interfering influence of other very important factors like sample handling, fixation, storage and tracking, lack of tissue homogeneity, differences in individual susceptibility and genetic polymorphisms.[10,86] Consequently, only well thought-out long-term and large-scale (e.g. many individuals, appropriate controls and different tissue types) studies will enable correct and thorough assessment of the DNA adductome.

Surrogate *vs.* Target Tissue

Ideally, DNA adduct formation should be monitored in the considered target tissue. A technical and ethical difficulty is that target tissue is not always easily obtained and/or available. A possible solution to that problem is the use of appropriate surrogate tissues like blood, urine and exfoliated (e.g. buccal or gastrointestinal epithelial) cells, provided that DNA adduct levels in target and surrogate tissue are distinctly related and a sufficient amount of DNA can be collected. Typically, procedures use an initial amount of 100 µg DNA although some require a lot less; e.g. the most recent method by Kanaly *et al.* used 15 µg DNA per injection.[45] If no data on correlation of particular DNA adducts in surrogate vs. target tissue are available, correlation studies should be performed during or prior to bio-monitoring studies. Researchers must also consider the possibility that a certain type of surrogate tissue may be more appropriate than others, or that a well-suited surrogate tissue simply does not exist.[10,87] For example, although Wiencke *et al.*[88] were able to demonstrate the use of mononuclear blood cells as an appropriate surrogate tissue for lung tissue to study tobacco-associated DNA adduct formation, Kriek and co-workers were unable to correlate PAH-DNA adduct levels in white blood cells and lung tissue in lung cancer patients.[89]

Method Validation

Over the past thirty years, several papers have reported the development of new methods for the detection of single or plural DNA adducts in different matrices. The use of analytical methods for DNA adduct detection and quantification in biomonitoring studies necessitates thorough evaluation of reliability and fit-for-purpose. This requires assured specificity, accuracy, precision and sensitivity, acceptable recovery and reproducibility, information about the assay and compound stability, and the assessment of detection and quantification limits.[90] Moreover, intra- and interlaboratory variability need to be assessed and properly addressed.[12] However, at present, detailed and specific guidelines concerning the validation and interpretation of validation parameters of an analytical method for the detection of DNA adducts or possible biomarkers do not exist.

Since biomarkers could provide interesting opportunities for the pharmaceutical industry, both the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) are aware of the urgency to establish proper validation guidelines for biomarker assays. In consequence, suitable guidelines are to be published as soon as possible.[90,91] In 2013, the FDA published draft guidance for

industry on bioanalytical method validation. In this draft guidance, the agency stated the following: "The accuracy, precision, selectivity, range, reproducibility, and stability of a biomarker assay are important characteristics that define the method. The approach used for pharmacokinetic assays should be the starting point for validation of biomarker assays, although FDA realizes that some characteristics may not apply or that different considerations may need to be addressed".[90] In agreement, the EMA published a concept paper on good genomics biomarker practices in 2014[91], which acknowledged and documented the need for guidance concerning choice and proper use of technology and methodology for genomic biomarker analysis in a clinical setting.

Since DNA adducts are regarded as biomarkers of exposure and (possible) biomarkers of effect, the future FDA and EMA guidelines could provide a basis for validation of DNA adduct detection methods as well.

CONCLUSIONS

The field of DNA adduct research is a highly promising area due to the proposed causal link between the prevalence of certain environmental genotoxins, the formation of DNA adducts and the onset of certain non-hereditary cancer types.[8,9,25] Furthermore, DNA adduct research does not only enable investigation of genotoxin exposure, uptake and metabolism, but can also provide us with information on the individual rate of DNA repair and individual susceptibility to permanent DNA damage, mutagenesis and carcinogenesis.[11,24] Luckily, a multidisciplinary approach and the continuously evolving field of analytical apparatus available provide us with the appropriate tools for extensive DNA adductomic research in several specialized areas of research.

In recent years, DNA adductomics is slowly emerging as a new omics tool that aims to study the formation and prevalence of a multitude of DNA adducts *in vitro* or *in vivo.*[45] Omics technology and methodology allows top-down exposome and DNA adductome mapping, enabling potential discovery of yet unknown DNA adduct biomarkers in different biological samples. Contrary to most other methods, MS analyses can reliably generate both qualitative and quantitative DNA adduct data.[32] MS-based DNA adductomics therefore is particularly suited for research on the exposure of the human body to both known and unknown endo- and exogenous hazardous chemicals and any subsequently formed DNA adducts.[27] Nevertheless, the search for answers does not end with DNA adduct mapping or biomarker establishment, as the described top-down approach does not evidently link genotoxin exposure to a certain environmental factor as a causal risk factor on the one hand, or disease outcome on the other.[4] Any information obtained from top-down omics studies will only prove its value if combined with bottom-up targeted analyses in both long-term studies and purposeful short-term intervention studies.[2,4] In addition, epidemiological information on human exposure to genotoxins or certain environmental factors by means of validated questionnaires or modern tracking technologies does still prove its worth.[2] Hence, there is a clear need for large-scale and highly collaborative high resolution hybrid MS-based DNA adductomic studies combining the knowledge and effort of different researchers (e.g. epidemiologists, clinicians, pathologists, analysts and statisticians) to further unravel the non-genetic basis of chronic disease initiation and development due to genotoxin exposure. The

earlier discussion on study design, DNA adduct stability issues and method validation has clarified that DNA adductomic studies should not be embarked upon without a prudent hands-on approach, distinctly underlining the importance of multidisciplinary research.

Insufficient knowledge of DNA adduct fragmentation patterns and limited availability of DNA adduct standards currently act as a bottleneck for the full characterization and correct identification of untargeted and unknown DNA adducts with MS.[27] In this context, the need for accurate mass measurements is indisputable, whereas the establishment of a database to assemble all information on chemical structure and characteristics of DNA adducts, fragmentation patterns, stability, prevalence and origin (= initiating genotoxin + route of exposure) would provide a major advance by facilitating investigation of DNA adduct formation and its potential role in different pathophysiological pathways.

Different research groups have explored triple quadrupole MS/MS, ion trap MS, TOF HRMS and orbitrap HRMS for DNA adductomics purposes. Up until now, triple quad and ion trap technology have been applied most frequently as MS^n accommodates the need for low level DNA adduct measurements. However, for untargeted omics applications HRMS is the more rational choice since accurate mass measurements simplify compound identification. A relatively recent trend in MS technology is the more widely spread and commercial use of hybrid MS instruments that combine the accuracy of HRMS with the specificity and sensitivity of MS^n. Accordingly, these hybrid MS instruments currently bring the best to the world of MS DNA adductomics, although additional work is required to further optimize the use of MS for DNA adductome mapping.

Hybrid HRMS/MS systems are particularly well suited for the detection and tentative identification of unknowns because the implementation of accurate mass measurements with HRMS offers a major advantage over low resolution MS to accurately study the obtained fragmentation pattern of an unknown compound. After all, accurate mass measurements provide essential information on the exact mass of the precursor and fragments, their elemental composition and thus also the unknown identity of the precursor. In the field of DNA adduct research, the development and use of neutral loss HRMS/MS methodologies can push the investigation of unknown DNA adducts. By means of neutral loss, DNA adducts can be recognised due to the loss of a typical fragment; the loss of a nucleobase (DNA adduct research at the nucleobase level) or the deoxyribose moiety (DNA adduct research at nucleoside level) upon fragmentation of the precursor. Further in-depth investigation of the remaining fragments can then provide more information on the presence and exact chemical composition of both known and unknown DNA adducts in different sample types.[33] At the time, the number of published DNA adductomics methods that make use of hybrid HRMS/MS technology are very limited (n = 2).[32,66] However, due to the still ongoing establishment of these hybrid MS systems in the field, the number of DNA adductomics applications could increase significantly in the near future.

CONFLICT OF INTEREST DISCLOSURE

There is no conflict of interest to declare.

REFERENCES

(1) Rappaport, S. M. *J. Epidemiol. Community Health* **2012**, *66*, 99-102.
(2) Coughlin, S. S. *Am. J. Epidemiol.* **2014**, *180*, 1188-1195.
(3) Wild, C. P. *Cancer Epidemiol., Biomarkers Prev.* **2005**, *14*, 1847-1850.
(4) Wild, C. P. *Int. J. Epidemiol.* **2012**, *41*, 24-32.
(5) Poirier, M. C. *Nat. Rev. Cancer* **2004**, *4*, 630-637.
(6) Farmer, P. B.; Brown, K.; Tompkins, E.; Emms, V. L.; Jones, D. J.; Singh, R.; Phillips, D. H. *Toxicol. Appl. Pharmacol.* **2005**, *207*, 293-301.
(7) Colin Garner, R. *Mutat. Res., Fundam. Mol. Mech. Mutagen.***1998**, *402*, 67-75.
(8) Qian, G. S.; Ross, R. K.; Yu, M. C.; Yuan, J. M.; Gao, Y. T.; Henderson, B. E.; Wogan, G. N.; Groopman, J. D. *Cancer Epidemiol., Biomarkers Prev.* **1994**, *3*, 3-10.
(9) Angerer, J.; Ewers, U.; Wilhelm, M. *Int.J. Hyg. Environ. Health* **2007**, *210*, 201-228.
(10) Rundle, A. *Mutat. Res.* **2006**, *600*, 23-36.
(11) Perera, F. P.; Weinstein, I. B. *Carcinogenesis* **2000**, *21*, 517-524.
(12) Himmelstein, M. W.; Boogaard, P. J.; Cadet, J.; Farmer, P. B.; Kim, J. H.; Martin, E. A.; Persaud, R.; Shuker, D. E. *Crit. Rev. Toxicol.* **2009**, *39*, 679-694.
(13) Farmer, P. B.; Singh, R. *Mutat. Res., Rev. Mutat. Res.* **2008**, *659*, 68-76.
(14) Singh, R.; Farmer, P. B. *Carcinogenesis* **2006**, *27*, 178-196.
(15) De Bont, R.; van Larebeke, N. *Mutagenesis* **2004**, *19*, 169-185.
(16) Wogan, G. N.; Hecht, S. S.; Felton, J. S.; Conney, A. H.; Loeb, L. A. *Semin. Cancer Biol.* **2004**, *14*, 473-486.
(17) Wang, J. S.; Groopman, J. D. *Mutat. Res.* **1999**, *424*, 167-181.
(18) Besaratinia, A.; Pfeifer, G. P. *Mutat. Res., Genet. Toxicol. Environ. Mutagen.* **2005**, *580*, 31-40.
(19) Turesky, R. J.; Vouros, P. *J. Chromatogr. B* **2004**, *802*, 155-166.
(20) Wiencke, J. K. *Oncogene* **2002**, *21*, 7376-7391.
(21) Hemminki, K.; Grzybowska, E.; Chorazy, M.; Twardowska-Saucha, K.; Sroczynski, J. W.; Putman, K. L.; Randerath, K.; Phillips, D. H.; Hewer, A.; Santella, R. M.; et al. *Carcinogenesis* **1990**, *11*, 1229-1231.
(22) Yu, H. S.; Oyama, T.; Isse, T.; Kitagawa, K.; Pham, T. T.; Tanaka, M.; Kawamoto, T. *Chem. Biol. Interact* **2010**, *188*, 367-375.
(23) Mollerup, S.; Ryberg, D.; Hewer, A.; Phillips, D. H.; Haugen, A. *Cancer Res.* **1999**, *59*, 3317-3320.
(24) Long, X. D.; Huang, H. D.; Huang, X. Y.; Yao, J. G.; Xia, Q. *Int. J. Clin. Exp. Med.* **2015**, *8*, 1197-1204.
(25) Jelakovic, B.; Karanovic, S.; Vukovic-Lela, I.; Miller, F.; Edwards, K. L.; Nikolic, J.; Tomic, K.; Slade, N.; Brdar, B.; Turesky, R. J.; Stipancic, Z.; Dittrich, D.; Grollman, A. P.; Dickman, K. G. *Kidney Int.* **2012**, *81*, 559-567.
(26) Lioy, P. J.; Rappaport, S. M. *Environ. Health Perspect.* **2011**, *119*, A466-467.
(27) Balbo, S.; Turesky, R. J.; Villalta, P. W. *Chem. Res. Toxicol.* **2014**, *27*, 356-366.
(28) Koc, H.; Swenberg, J. A. *J. Chromatogr. B* **2002**, *778*, 323-343.
(29) Phillips, D. H. *Mutat. Res.* **2005**, *577*, 284-292.
(30) Gamboa da Costa, G.; Churchwell, M. I.; Hamilton, L. P.; Von Tungeln, L. S.; Beland, F. A.; Marques, M. M.; Doerge, D. R. *Chem. Res. Toxicol.* **2003**, *16*, 1328-1337.
(31) Singh, R.; Teichert, F.; Seidel, A.; Roach, J.; Cordell, R.; Cheng, M. K.; Frank, H.; Steward, W. P.; Manson, M. M.; Farmer, P. B. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2329-2340.
(32) Balbo, S.; Hecht, S. S.; Upadhyaya, P.; Villalta, P. W. *Anal. Chem.* **2014**, *86*, 1744-1752.
(33) Bessette, E. E.; Goodenough, A. K.; Langouet, S.; Yasa, I.; Kozekov, I. D.; Spivack, S. D.; Turesky, R. J. *Anal. Chem.* **2009**, *81*, 809-819.
(34) Goggin, M.; Loeber, R.; Park, S.; Walker, V.; Wickliffe, J.; Tretyakova, N. *Chem. Res. Toxicol.* **2007**, *20*, 839-847.
(35) Inagaki, S.; Hirashima, H.; Esaka, Y.; Higashi, T.; Min, J.; Toyo'oka, T. *Chromatographia* **2010**, *72*, 1043-1048.
(36) Jaruga, P.; Kirkali, G.; Dizdaroglu, M. *Free Radical Biol. Med.* **2008**, *45*, 1601-1609.
(37) Wang, J.; Yuan, B.; Guerrero, C.; Bahde, R.; Gupta, S.; Wang, Y. *Anal. Chem.* **2011**, *83*, 2201-2209.
(38) Klaene, J. J.; Sharma, V. K.; Glick, J.; Vouros, P. *Cancer Lett.* **2013**, *334*, 10-19.
(39) Sharma, V. K.; Glick, J.; Liao, Q.; Shen, C.; Vouros, P. *J. Mass Spectrom.* **2012**, *47*, 490-501.
(40) Tretyakova, N.; Villalta, P. W.; Kotapati, S. *Chem. Rev.* **2013**, *113*, 2395-2436.

(41) Wu, K.-Y.; Chiang, S.-Y.; Shih, W.-C.; Huang, C.-C. J.; Chen, M.-F.; Swenberg, J. A. *Mass Spectrom. Rev.* **2011**, *30*, 733-756.
(42) Dettmer, K.; Aronov, P. A.; Hammock, B. D. *Mass Spectrom. Rev.* **2007**, *26*, 51-78.
(43) Kanaly, R. A.; Hanaoka, T.; Sugimura, H.; Toda, H.; Matsui, S.; Matsuda, T. *Antiox. Redox Signaling* **2006**, *8*, 993-1001.
(44) Kanaly, R. A.; Matsui, S.; Hanaoka, T.; Matsuda, T. *Mutat. Res.* **2007**, *625*, 83-93.
(45) Kanaly, R. A.; Micheletto, R.; Matsuda, T.; Utsuno, Y.; Ozeki, Y.; Hamamura, N. *MicrobiologyOpen* **2015**, *4*, 841-856.
(46) Kato, K.; Yamamura, E.; Kawanishi, M.; Yagi, T.; Matsuda, T.; Sugiyama, A.; Uno, Y. *Mutat. Res.* **2011**, *721*, 21-26.
(47) Spilsberg, B.; Rundberget, T.; Johannessen, L. E.; Kristoffersen, A. B.; Holst-Jensen, A.; Berdal, K. G. *J. Agric. Food Chem.* **2010**, *58*, 6370-6375.
(48) Compagnone, D.; Curini, R.; D'Ascenzo, G.; Del Carlo, M.; Montesano, C.; Napoletano, S.; Sergi, M. *Anal. Bioanal. Chem.* **2011**, *401*, 1983-1991.
(49) Matsuda, T.; Tao, H.; Goto, M.; Yamada, H.; Suzuki, M.; Wu, Y.; Xiao, N.; He, Q.; Guo, W.; Cai, Z.; Kurabe, N.; Ishino, K.; Matsushima, Y.; Shinmura, K.; Konno, H.; Maekawa, M.; Wang, Y.; Sugimura, H. *Carcinogenesis* **2013**, *34*, 121-127.
(50) Chou, P.-H.; Kageyama, S.; Matsuda, S.; Kanemoto, K.; Sasada, Y.; Oka, M.; Shinmura, K.; Mori, H.; Kawai, K.; Kasai, H.; Sugimura, H.; Matsuda, T. *Chem. Res. Toxicol.* **2010**, *23*, 1442-1448.
(51) Ishii, Y.; Takasu, S.; Kuroda, K.; Matsushita, K.; Kijima, A.; Nohmi, T.; Ogawa, K.; Umemura, T. *Anal. Bioanal. Chem.* **2014**, *406*, 2467-2475.
(52) Ishino, K.; Kato, T.; Kato, M.; Shibata, T.; Watanabe, M.; Wakabayashi, K.; Nakagama, H.; Totsuka, Y. *Int. J. Mol. Sci.* **2015**, *16*, 3474-3492.
(53) Gregson, J. M.; McCloskey, J. A. *Int. J. of Mass Spectrom. Ion Processes* **1997**, *165–166*, 475-485.
(54) Vanden Bussche, J.; Moore, S. A.; Pasmans, F.; Kuhnle, G. G.; Vanhaecke, L. *J. Chromatogr. A* **2012**, *1257*, 25-33.
(55) Iannitti-Tito, P.; Weimann, A.; Wickham, G.; Sheil, M. M. *Analyst* **2000**, *125*, 627-634.
(56) Chaudhary, A. K.; Reddy, G. R.; Blair, I. A.; Marnett, L. J. *Carcinogenesis* **1996**, *17*, 1167-1170.
(57) Yun, B. H.; Rosenquist, T. A.; Sidorenko, V.; Iden, C. R.; Chen, C. H.; Pu, Y. S.; Bonala, R.; Johnson, F.; Dickman, K. G.; Grollman, A. P.; Turesky, R. J. *Chem. Res. Toxicol.* **2012**, *25*, 1119-1131.
(58) Nauwelaers, G.; Bessette, E. E.; Gu, D.; Tang, Y.; Rageul, J.; Fessard, V.; Yuan, J. M.; Yu, M. C.; Langouet, S.; Turesky, R. J. *Chem. Res. Toxicol.* **2011**, *24*, 913-925.
(59) Pietsch, K. E.; van Midwoud, P. M.; Villalta, P. W.; Sturla, S. J. *Chem. Res. Toxicol.* **2013**, *26*, 146-155.
(60) Bessette, E. E.; Spivack, S. D.; Goodenough, A. K.; Wang, T.; Pinto, S.; Kadlubar, F. F.; Turesky, R. J. *Chem. Res. Toxicol.* **2010**, *23*, 1234-1244.
(61) Wang, P.; Fisher, D.; Rao, A.; Giese, R. W. *Anal. Chem.* **2012**, *84*, 3811-3819.
(62) Wang, P.; Gao, J.; Li, G.; Shimelis, O.; Giese, R. W. *Chem. Res. Toxicol.* **2012**, *25*, 2737-2743.
(63) Van den Driessche, B.; Lemiere, F.; Van Dongen, W.; Esmans, E. L. *J. Chromatogr. B* **2003**, *785*, 21-37.
(64) Van den Driessche, B.; Van Dongen, W.; Lemière, F.; Esmans, E. L. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 2001-2007.
(65) Glish, G. L.; Burinsky, D. J. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 161-172.
(66) Hemeryck, L. Y.; Decloedt, A. I.; Bussche, J. V.; Geboes, K. P.; Vanhaecke, L. *Anal. Chim. Acta* **2015,** *892,* 123-131.
(67) Zubarev, R. A.; Makarov, A. *Anal. Chem.* **2013**, *85*, 5288-5296.
(68) Cristoni, S.; Bernardi, L. R. *Mass Spectrom. Rev.* **2003**, *22*, 369-406.
(69) Randall, K. L.; Argoti, D.; Paonessa, J. D.; Ding, Y.; Oaks, Z.; Zhang, Y.; Vouros, P. *J. Chromatogr. A* **2010**, *1217*, 4135-4143.
(70) Soglia, J. R.; Turesky, R. J.; Paehler, A.; Vouros, P. *Anal. Chem.* **2001**, *73*, 2819-2827.
(71) Embrechts, J.; Lemiere, F.; Van Dongen, W.; Esmans, E. L.; Buytaert, P.; Van Marck, E.; Kockx, M.; Makar, A. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 482-491.
(72) Stoll, D. R.; Li, X.; Wang, X.; Carr, P. W.; Porter, S. E. G.; Rutan, S. C. *J. Chromatogr. A* **2007**, *1168*, 3-43.
(73) Zhao, C.; Wu, Z.; Xue, G.; Wang, J.; Zhao, Y.; Xu, Z.; Lin, D.; Herbert, G.; Chang, Y.; Cai, K.; Xu, G. *J. Chromatogr. A* **2011**, *1218*, 3669-3674.
(74) Gauthier, G. L.; Grimm, R. *Drug discovery today: Technol.* **2006**, *3*, 59-66.

(75) Bani-Yaseen, A. D.; Kawaguchi, T.; Price, A. K.; Culbertson, C. T.; Jankowiak, R. *Anal. Bioanal. Chem.* **2011**, *399*, 519-524.
(76) Mao, H.; Schnetz-Boutaud, N. C.; Weisenseel, J. P.; Marnett, L. J.; Stone, M. P. *Proc. Natl. Acad. Sci.* **1999**, *96*, 6615-6620.
(77) Niedernhofer, L. J.; Riley, M.; Schnetz-Boutaud, N.; Sanduwaran, G.; Chaudhary, A. K.; Reddy, G. R.; Marnett, L. J. *Chem. Res. Toxicol.* **1997**, *10*, 556-561.
(78) Inskeep, P. B.; Koga, N.; Cmarik, J. L.; Guengerich, F. P. *Cancer Res.* **1986**, *46*, 2839-2844.
(79) Bianchini, F.; Montesano, R.; Shuker, D. E.; Cuzick, J.; Wild, C. P. *Carcinogenesis* **1993**, *14*, 1677-1682.
(80) Izzotti, A.; Bagnasco, M.; Scatolini, L.; Rovida, A.; De Flora, S. *Carcinogenesis* **1993**, *14*, 2185-2187.
(81) Osborne, M. R.; Phillips, D. H. *Chem. Res. Toxicol.* **2000**, *13*, 257-261.
(82) Hillestrøm, P. R.; Weimann, A.; Poulsen, H. E. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 605-610.
(83) Binkova, B.; Hubalek, F.; Sram, R. *Mutat. Res.* **1996**, *371*, 229-235.
(84) Zhang, F.; Bartels, M. J.; Pottenger, L. H.; Gollapudi, B. B.; Schisler, M. R. *J. Chromatogr. B* **2006**, *833*, 141-148.
(85) Singh, R.; McEwan, M.; Lamb, J. H.; Santella, R. M.; Farmer, P. B. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 126-134.
(86) Liotta, L. A.; Petricoin, E. F., 3rd. *Cancer Epidemiol., Biomarkers Prev.* **2012**, *21*, 1229-1235.
(87) Rockett, J. C.; Burczynski, M. E.; Fornace, A. J.; Herrmann, P. C.; Krawetz, S. A.; Dix, D. J. *Toxicol. Appl. Pharmacol.* **2004**, *194*, 189-199.
(88) Wiencke, J. K.; Kelsey, K. T.; Varkonyi, A.; Semey, K.; Wain, J. C.; Mark, E.; Christiani, D. C. *Cancer Res.* **1995**, *55*, 4910-4914.
(89) van Schooten, F. J.; Hillebrand, M. J.; van Leeuwen, F. E.; van Zandwijk, N.; Jansen, H. M.; den Engelse, L.; Kriek, E. *Carcinogenesis* **1992**, *13*, 987-993.
(90) US Food and Drug Administration, Draft Guidance for Industry for Bioanalytical Method Validation, **2013,** *Available at www.fda.gov*.
(91) Committee for Medicinal Products for Human Use, E. M. A., **2014,** Available at *www.ema.europe.eu*.

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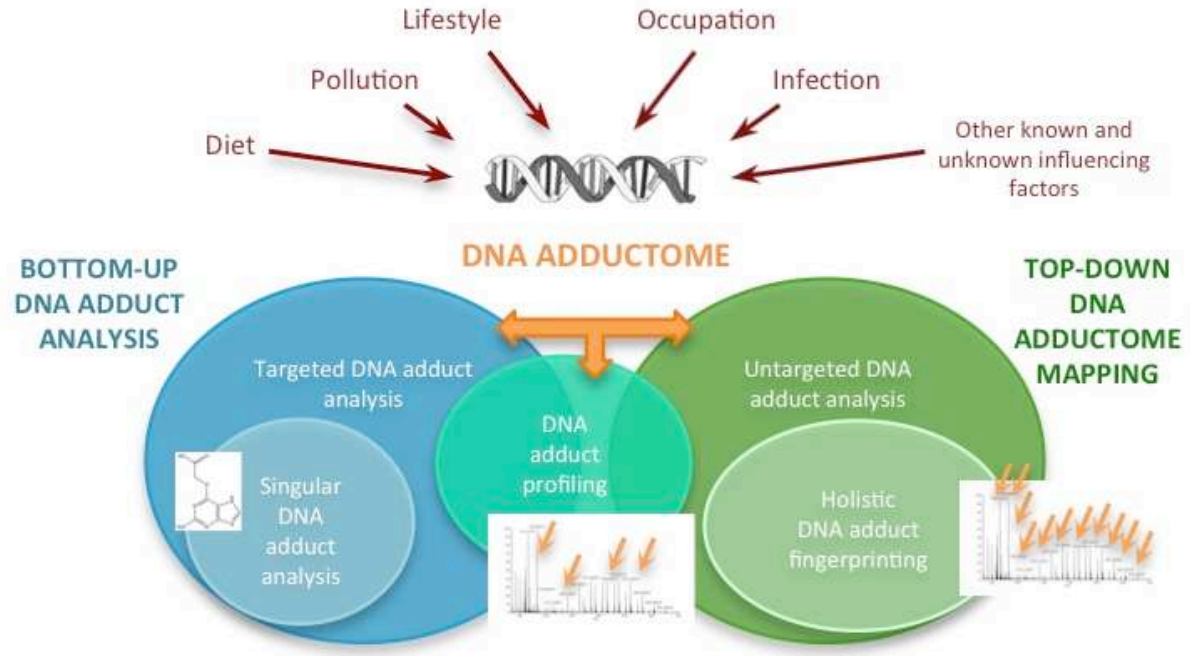
Lifestyle
Occupation
Pollution
Infection
Diet
Other known and unknown influencing factors
DNA ADDUCTOME
BOTTOM-UP DNA ADDUCT ANALYSIS
TOP-DOWN DNA ADDUCTOME MAPPING
Targeted DNA adduct analysis
Singular DNA adduct analysis
DNA adduct profiling
Untargeted DNA adduct analysis
Holistic DNA adduct fingerprinting