CHAPTER 4.

Physical/chemical/immunologic analytical methods

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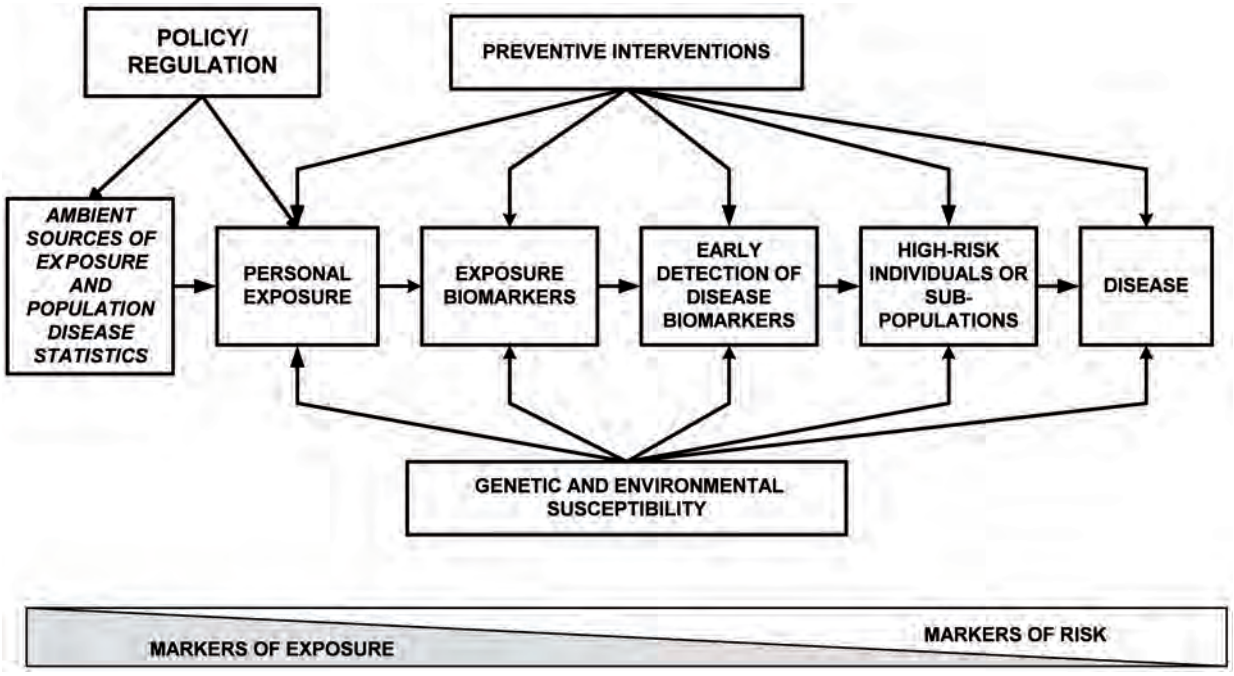
Summary

Biomarkers can be used to measure the presence of a wide variety of parent compounds and metabolites in body fluids and excreta, and serve as biomarkers of internal dose. Chemical-macromolecular adducts formed in blood and tissue or excreted in urine serve as biomarkers of exposure as well, and in many instances reflect both exposure and additional relevant biological processes. An assortment of analytical techniques have been developed to identify and measure parent compounds, metabolites, chemical-DNA and protein adducts. This chapter will discuss many analytical techniques that measure biomarkers in molecular epidemiologic studies, including biological, physical, chemical and immunological methods.

Introduction

Over the past 25 years, the development, validation and application of molecular biomarkers that reflect events from environmental exposure to the formation of clinical disease (e.g. cancer) has rapidly expanded our knowledge of the mechanisms of pathogenesis and provided opportunities for devising improved tools for disease treatment and prevention. Molecular epidemiology and its evolving paradigm refers to the use of biomarkers in epidemiological research (i.e. incorporation of molecular, cellular and other biochemical measurements into epidemiological studies of the etiology, prevention, and control of health risks faced by human populations)(1–4) (Figure 4.1). The application of validated biomarkers to traditionally descriptive epidemiological studies helps to: delineate the continuum of events between an exposure and resulting disease; identify smaller exposures to specific xenobiotics; indicate earlier events in the natural history of diseases and reduce misclassification of dependent and independent variables; enhance individual and group risk monitoring and assessments; and reveal toxicologic mechanisms by which an exposure and a disease are related (5,6). A unique feature of molecular epidemiologic studies is the interdisciplinary collaboration between population and field scientists and laboratory scientists from various disciplines, such as epidemiology, toxicology, molecular biology, genetics, immunology,

Figure 4.1. Molecular epidemiology paradigm



biochemistry, pathology and analytical chemistry. The analytic measurement of biomarkers is critical to molecular epidemiologic studies and requires special attention to the collection, handling and storage of biologic specimens, as well as development and validation of analytical methods (7).

Biomarker paradigm and validation strategies

As adapted from Perera and Weinstein (1) and the Committee on Biological Markers of the National Research Council (8), the development of disease as a result of exposure to an environmental agent or other toxicant is multistage: it starts with exposure and progresses to internal dose (e.g. deposited body dose), biologically effective dose (e.g. dose at the site of toxic action), early biological effect (e.g. at the subcellular level), altered structure or function (e.g. subclinical changes) and finally to clinical disease (Figure 4.1). Any step in this process may be modified by host-susceptibility factors including genetic traits and effect modifiers (e.g. diet or environmental exposures). Therefore, biomarkers are indicators of events for physiologic, cellular, subcellular and molecular alterations in the multistage development of specific diseases (9).

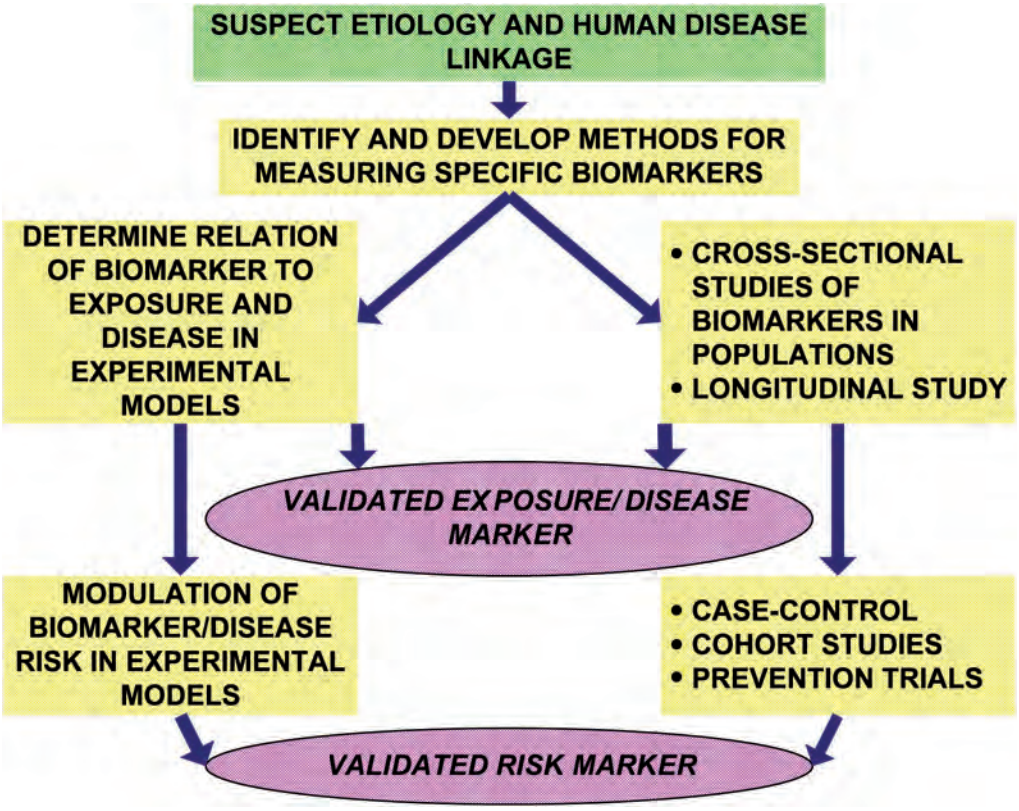
Molecular biomarkers are typically used as indicators of exposure, effect or susceptibility. A biomarker of exposure refers to measurement of the specific agent of interest, its metabolite(s), or its specific interactive products in a body compartment or fluid, which indicates the presence (and magnitude) of current and past exposure. A biomarker of effect indicates the presence (and magnitude) of a biological response to exposure to an environmental agent. Such a biomarker may be an endogenous component, a measure of the functional capacity of the system, or an altered state recognized as impairment or disease. A biomarker of susceptibility is an indicator or a metric of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance or other toxicant. Such a biomarker may be the unusual presence or absence of an endogenous component, or an abnormal functional response to an administered challenge (9). Molecular epidemiology and molecular dosimetry thus have great utility in addressing the relationships between exposure to environmental agents and development of clinical diseases, and in identifying those individuals at high risk for the diseases (2,6,10). Collectively, these data also help to inform the risk assessment process, where regulations can be tested against biological measurements of exposure to determine the efficacy of policies.

The development and application of molecular biomarkers

for environmental chemical agents should be based upon specific knowledge of their metabolism, interactive product formation and general mechanisms of action (11,12). Examples in the field are studies on the relationships between tobacco smoking and lung cancer (13–16) and between aflatoxin exposure and liver cancer (17,18). A specific application of biomarker technology to human cancer is the study of the variation in response among individuals following exposures to tobacco. For example, even in heavy tobacco smokers, less than 15% of these exposed people develop lung cancer (19); thus, intrinsic susceptibility factors must affect the time course of disease development and eventual outcome. The identification of those at highest risk for developing cancers should be facilitated by biomarker studies. Extensive efforts have been made to identify these high-risk individuals using various genetic and metabolic susceptibility markers (e.g. measurement of polymorphism of genotype and phenotype of various enzymes involved in transformative metabolic reactions of certain known carcinogens and the DNA repair process)(20–23). Although this strategy has not yet proven to be broadly applicable to many other human diseases, progress is being made for many types of cancers (24).

The validation of any biomarker-effect link requires sequential or parallel experimental and human studies (12). Following the development of the hypothesis of an exposure-disease linkage, there is the need to develop the analytical methodology necessary to measure these biological markers in human and experimental samples. Conceptually, as shown in Figure 4.2, an appropriate animal model is used to determine the associative or causal role of the biomarker in the disease or effect pathway, and to establish relations between dose and response. The putative biomarker can then be validated in pilot human studies, where sensitivity, specificity, accuracy and reliability parameters can be established. Data obtained in these studies can then be used to assess intraindividual or interindividual variability, background levels, relationship of the biomarker to external dose or to disease status, as well as feasibility for use in larger population-based studies. It is important to establish a connection between the biomarker and exposure, effect or susceptibility. To fully interpret the information that

Figure 4.2. Validation scheme for molecular biomarker research



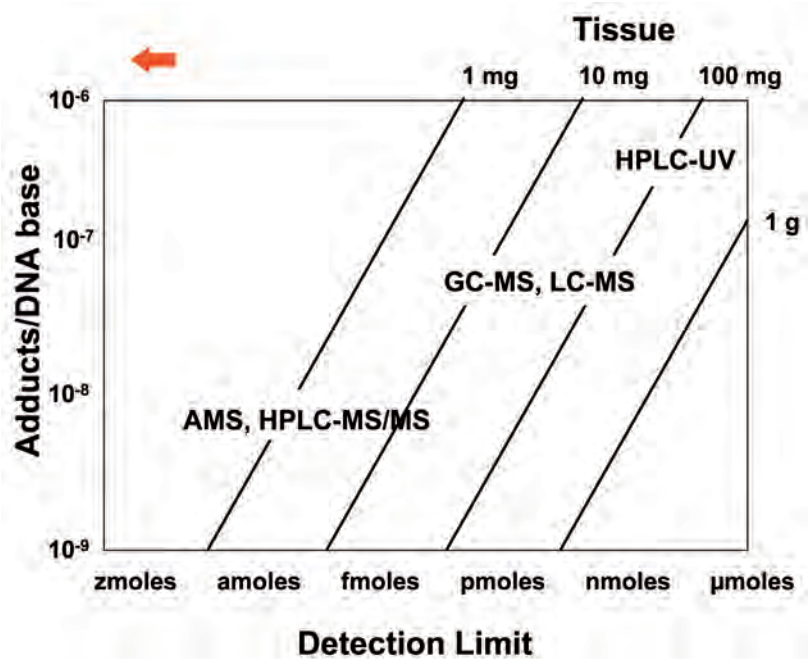
the biomarker provides, prospective epidemiological studies may be necessary to demonstrate the role that the biomarker plays in the overall pathogenesis of the disease or effect. To date, few biomarkers have been rigorously validated using this entire process.

Techniques and strategies for measuring parent compounds and metabolites

Many analytical techniques, including biological, physical, chemical, and immunological methods, have been developed and standardized by regulating agencies (e.g. US Environmental Protection Agency) for biohazard identification and risk assessment in various exogenous settings, such as environmental and occupational arenas. For establishment of a quantitative relationship with exposure, analytical methods have been extended to measure these parent compounds in biological samples; they could serve very well as a biomarker of exposure. A good example is the measurement of various heavy metals (e.g. lead, arsenic, cadmium and mercury) in human biospecimens, such as urine, blood, hair and tissues (25). In general, most heavy metal measurements have been in environmental samples and technologies including atomic absorption (AA) (26), inductively coupled plasma-optical emission spectrometer (ICP-OES) (27) and inductively coupled plasma-mass spectrometer (ICP-MS)(28). In addition to the measurement of environmental sources of heavy metals, methods such as X-ray fluorescence (XRF) permit the assessment of body burden of lead through its deposition in bone (29). Of importance to molecular epidemiology is the need to speciate the heavy metal compound, since toxicity can vary by both charge state (e.g. tri- and penta-valent state of arsenic) (30) and methylation (e.g. methyl mercury) (31). Measurement of parent organic compounds in biological samples, although still in practice, is less favoured because most organic toxic/carcinogenic compounds undergo metabolism and exert their toxicologic/carcinogenic effects through metabolic activation (22). Collectively, the problem faced in these investigations is the relationship between exposure and dose. Unlike most organic compounds, metals' measurements often reflect both exposure and dose. Figure 4.3 illustrates a nomograph relating the increase in analytical sensitivity by various instrument methods of the past 10–15 years. As these technologies have advanced, the number of non-detects in human epidemiological studies has dramatically decreased.

Incorporation of biomarkers in molecular epidemiology can provide critical information on interindividual variation in dose–response of environmental agents, the central focus in risk assessment. While biomarkers have been most widely applied in the area of clinical pharmacology, they are increasingly being used to document interindividual variation in the context of the much lower exposures found in the environment (32,33). There are many examples of variation in the pharmacologic action of drugs in people; in general, the response varies about 10-fold in the general population for most pharmaceutics. At issue with the extrapolation of these findings to environmentally occurring toxicants is the recognition that a limited variance in response is selected for during drug development, whereas in an environmental setting, there may be a much wider range of toxicologic outcomes. This is further complicated by the age, gender and health status of the general population when compared to people being treated

Figure 4.3. Nomograph for analytical methods



for a clinically diagnosed disease. Further, exposures to drugs tend to be of shorter duration then the lifetime exposures to environmental compounds that can have wide day-to-day variations in dose. While the ability of environmental exposures to reach the level where K_m of an enzyme becomes limiting is debatable, the effective concentration of an agent might well exceed this level at the cellular site of action (34).

A very significant consideration in the design of the molecular epidemiologic investigation is the balance between the analytical sensitivity of the chosen method and the operational sample throughput per day. Our infrastructure capacity to collect, annotate and store large numbers of samples far outpaces the number of samples that can be analysed. Most environmental and occupational toxicants of concern exist at trace levels in human samples. Thus, while the biological impact may be substantial, both the sample size available and analytical technology employed conspire to reduce the number of tests that can be run in a given day. Further, as analytical sensitivity increases, the contribution of noise to an analysis also increases, necessitating more extensive clean-up methods to maintain an appropriate signal-to-noise ratio. For most quantitative analyses, reliable measurements can be made only with signal-to-noise ratios that exceed 3:1. Unfortunately, judgement about the amount of material that will be used for an analysis results in many samples being at this borderline level of detection. Since the background from a urine (or other biological) sample can be very heterogeneous from person-to-person, many pilot studies have inadvertently generated false-positives when they do not employ multiple confirmatory analytical techniques and other quality control methods. Each sample matrix poses varied challenges for clean-up. Thus, as the complexity of sample clean-up increases, the effective number of samples that can be analysed in a given day diminishes. The largest automated, robotically driven clean-up strategy, The National Report on Human Exposure to Environmental Chemicals (http://www.cdc.gov/exposurereport/), is based upon the NHANES survey samples. This repository has been used to explore many environmental exposures to low molecular weight chemicals (35,36). However, most individual laboratories lack this type of infrastructure for large-scale sample preparation for chemical analysis; hence, these operational considerations impinge upon the size and scope of the molecular epidemiology study.

A variety of metabolites of toxicants/carcinogens found in body fluids and excreta (e.g. blood, urine, feces, hair and milk) have the potential for use as biomarkers of internal dose. These measures could provide information about the actual concentration of toxicants/carcinogens that have been absorbed and distributed in the body. Measurement of these metabolites has been incorporated into several human epidemiologic studies. For example, excretion of aflatoxin M_1 (AFM_1), one of the major metabolites of aflatoxin B_1 (AFB_1), has been used as a biomarker for the evaluation of human exposure to aflatoxin and was found to be associated with the risk of liver cancer (37,38). Reflecting its complex pharmacokinetics, this metabolite has also been measured in both human urine and milk samples (39). Specific metabolites of one of the tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent chemical carcinogen, have been detected and quantified in the urine of smokers; these metabolites were not found in the urine of non-smokers (40). Intraindividual and interindividual variations in these metabolites of NNK in smokers' urine were noted and might prove to be important in disease risk (41). Two metabolites of NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and NNAL-β-O-D-glucosiduronic acid (NNAL-Gluc), excreted in the urine of smokeless tobacco users, were found to be associated with the presence of oral leukoplakia (42). Other examples of internal dose markers include the measurement of blood and serum levels of DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)-ethylene), the major metabolite of DDT (2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane), which have been used as biomarkers in breast cancer studies in women (43,44).

An example of the use of agent-specific biomarkers to identify etiologic factors in human cancer is the study of aristolochic acid (45,46). This compound has been associated with Balken endemic nephropathy (BEN), a chronic renal tubulointerstitial disease that often is accompanied by upper urinary tract urothelial cancer. Using ^{32}P-postlabelling/PAGE and authentic standards, both adenine and guanine aristolactam DNA adducts were detected in the renal cortex of patients with BEN, but not in patients with other chronic renal diseases. In addition, urothelial cancer tissue was obtained from residents of endemic villages with upper urinary tract malignancies. The AmpliChip p53 microarray was then used to sequence exons 2–11 of the *p53* gene where 19 base substitutions were identified. Mutations at A:T pairs accounted for 89% of all *p53* mutations, with 78% of

these being A:T→T:A transversions. It was concluded that DNA adducts derived from aristolochic acid are present in renal tissues of patients with documented BEN. These adducts can be detected in transitional cell cancers, and A:T→T:A transversions dominate the *p53* mutational spectrum in the upper urinary tract malignancies found in this population.

The process of toxicant/carcinogen metabolism produces a variety of reactive electrophilic intermediates that constitute biologically effective forms of the ultimate chemicals, such as aflatoxin B_1-8,9-epoxide, benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE), and N-acetoxy-2-acetylaminofluorene (47–49). The actual concentration of the products formed by these ultimate forms of toxicants/carcinogens in the human body or target tissues should serve as biomarkers for biologically effective dose. Great efforts have been made over many years to develop methods to identify and detect these active metabolic products. These ultimate toxicants/carcinogens can be directly monitored in various *in vitro* models and *in vivo* animal systems by a variety of sensitive analytical techniques. These approaches are often difficult to apply in human populations simply because of the extremely short half-lives of these ultimate toxicants/carcinogens, and the high background of interfering substances. Alternative methods for measuring the formation of DNA and protein adducts in human blood and tissues include measuring their further metabolites, such as diols, conjugates (including glucuronide and mercapturic acids) or nucleic acid base adducts in human urine.

As discussed previously, clean-up methods are required to lower the noise and enhance the signal for the detection of any of the chemical-specific biomarkers in biological samples. Many of the studies to measure these biomarkers employ single or multiple chromatographic step(s) to facilitate biomarker detection. In a preparative mode, this chromatography usually consists of high-capacity chromatographic columns using reverse phase, normal phase and ion-exchange resins. These columns are generally gravity or low-pressure devices and provide a crude first stage enrichment method. Analytical chromatography is used as a low-capacity, but highly-selective, technology to separate many of the compounds in a complex mixture. Liquid chromatography is the most common mode for separation, but many compounds, if they are intrinsically or derivatisable to a volatile agent, can be used in gas chromatography (GC). GC has a much higher capacity to resolve different chemical species. All chromatography methods are coupled with a form of spectroscopy for selective detection. For example, GC and high performance liquid chromatography (HPLC) coupled online with UV, fluorescence and electron-capture detectors and mass spectrometry (MS), are necessary techniques in these studies. In general, modern analytical instrumentation can lead to a limit of detection of chemical biomarkers in the femptomole (fmol) to low picomole (pmol) range. As a caution, many studies report sensitivity and limits of detection using standards; however, the operational limits of detection are between 10 to 100-fold higher when measuring real biological samples.

Immunoassays, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and immunoaffinity chromatography (IAC), are also used in biomarker analysis (50,51) for both preparative methods and analytical measurement. Further, several investigations have employed hybrid methods using HPLC separation with the collection of fractions followed by analysis using immunoassays (52). An obvious advantage of clean-up using chromatography before an immunoassay is the removal of potential cross-reactive materials that contribute to a false-positive result. It is also important to note that any clean-up before an analytical measurement lowers the number of samples per day and potentially introduces other artefacts, such as cross-contamination of samples and stability of the biomarker.

Internal standard development is an area of considerable importance that has received far less attention than it should. All quantitative measurements require the use of an internal standard to account for sample-to-sample recovery variations. In the case of mass spectrometry, internal standards generally employ an isotopically labelled material that is physicochemically identical to the chemical that is being measured. Obtaining such isotopically labelled materials does require chemical synthesis (if they are not commercially available), which has impeded the application of internal standards in many studies. In the case of immunoassays internal standards pose a different challenge, since the addition of an internal standard that is recognized by an antibody results in a positive value contribution. The dynamic range is usually less than 100 in immunoassays; therefore great care must be taken to spike a sample with an internal standard to obtain a valid result (53). In contrast, for example, most chromatographic methods result in dynamic ranges of analyses that can be over a 10 000-fold range of levels.

Techniques for measuring DNA adducts

The metabolically activated ultimate form of carcinogens can covalently interact with cellular DNA, which is a critical step in the process of carcinogenesis (23,54–56). Measurement of carcinogen-DNA adducts has an important role in human biomonitoring and molecular epidemiologic studies, as they are specific biomarkers that provide a way to measure human exposure to chemical carcinogens and provide information about a specific dose to a carcinogen target site (DNA). Moreover, it has been possible to establish a correlation between tumour incidence and exposure by measuring the level of these adducts (see (57) as an example).

Many different analytical techniques have been developed to identify and measure carcinogen-DNA adducts, including: immunoassays: ELISA, RIA, IAC, and immunohistochemical staining assay (IHC); radiometric postlabelling methods: ^{32}P-post-labelling; and various physicochemical methods: GC, HPLC, GC-MS, LC-MS, electrochemical detection (ECD), fluorescence and phosphorescence spectroscopy, or a combination of these methods (4,10,20,50,58–63). Capillary electrophoresis and other new separation techniques have improved the sensitivity and specificity of these methods. Nuclear magnetic resonance (NMR) spectrometry has also been used to determine stereospecificity and three-dimensional structure (64,65).

The ^{32}P-post-labelling assay, which radioactively labels adducts digested from sample DNA, has been widely applied because of its high sensitivity and the requirement for only microgram amounts of DNA. This assay has been especially useful for detection of adducts in single exposure experimental systems and as a means of elucidating the metabolic activation of previously uninvestigated potential carcinogens. ^{32}P-post-labelling can give an impression of total adduct burden, but it is rarely possible to quantify specific adducts accurately in human samples. Advances may lie in the use of better chemical standards, more advanced preparative techniques, and in connection with MS techniques (3,22). Carcinogen-DNA adduct detection by fluorescence has been applied to compounds that lead to either highly fluorescent products or adducts that can subsequently be derived to highly fluorescent chemical species. Physicochemical methods, including MS, offer the advantage of high chemical specificity. Major improvements in sensitivity have allowed the measurement of increasingly smaller amounts of adducted species in biological matrices. The sensitivities of individual methods vary and often depend on the amount of DNA that can be analysed. Detection limits for quantitative assays are typically in the range of one adduct in 10^7 or 10^9 nucleotides. However, accelerator mass spectrometry (AMS), which is highly sophisticated and involved in use of low levels of ^{3}H- or ^{14}C-labelled compound, has a detection limit of one adduct in 10^{12} nucleotides (66,67). A recent application of this technology has been in the identification of the fate of a variety of alkylanilines in experimental models (68). In this investigation, the ^{14}C-labelled 2,6-dimethyl- (2,6-DMA), 3,5-dimethyl- (3,5-DMA), and 3-ethylaniline (3-EA) compounds, associated with human bladder cancer (69), were administered to C57BL/6 mice, which were subsequently sacrificed 2, 4, 8, 16 and 24 hours post-dosing. Bladder, colon, kidney, liver, lung and pancreas were harvested from each animal, and DNA was isolated from each tissue. Adducts were detectable in the bladder and liver DNA samples from every animal at every time point, at levels that ranged from three per 10^9 to 1.5 per 10^7 nucleotides. Adduct levels were highest in animals given 3,5-DMA and lowest in those given 3-EA. Taken together, the results strongly suggest that these three alkylanilines are metabolized *in vivo* to electrophilic intermediates that covalently bind to DNA, and that adducts are formed in the DNA of bladder, which is a putative target organ for these alkylanilines (68).

Many analytical techniques have been used to measure composite and specific DNA adducts in cellular DNA isolated from peripheral lymphocytes, bladder, breast, lung and colonic tissues, as well as excreted DNA adducts in urine (3,50,60). These techniques have also been applied in the clinical setting to examine carcinogen-macromolecular adducts of people undergoing chemotherapy with alkylating agents, in an attempt to associate adduct levels with clinical outcome (70,71). Recently, these methods have also been applied to human clinical trials to validate various intervention tools for the assessment of chemopreventive agents in modulating various intermediate biomarkers (17,72).

Measurement of DNA adducts for studying complex mixtures of carcinogen exposure

Many studies have used DNA adducts to assess potential sources of carcinogen exposure. One classic study has examined a spectrum of molecular biomarkers to assess human exposure to complex mixtures of environmental pollution in Poland

(73). Measurement of genotoxic damage in peripheral blood samples from residents of high-exposure regions indicated that environmental pollution is associated with significant increases in carcinogen-DNA adducts (polynuclear aromatic hydrocarbon (PAH)-DNA and other aromatic adducts), sister chromatic exchanges, chromosomal aberrations and frequency of increased *ras* oncogene expression. The presence of aromatic adducts on DNA was found to be significantly correlated with chromosomal mutation, providing a possible link between environmental exposure and genetic alterations relevant to disease.

Tobacco smoke, the primary cause of lung cancer, contains several types of known carcinogens. The most abundant of these are PAHs, arylamines and the tobacco-specific nitrosamines, including the lung-specific carcinogen NNK. These carcinogens are metabolically activated to reactive species which form specific DNA adducts. Smokers are usually found to have significantly elevated levels of aromatic and/or hydrophobic adducts as compared with non-smokers, and some studies found that DNA-adduct levels are linearly related to total smoking exposure (74). One investigation measured the level of bulky, hydrophobic DNA adducts in lung parenchyma of smokers and ex-smokers by the ^{32}P-postlabelling method. Smokers had five-fold higher levels of DNA adducts than did ex-smokers. A positive linear correlation between bulky adduct levels and *CYP1A1* (AHH) activity was found in smokers. A statistically significant correlation was determined comparing pulmonary microsomal AHH activity and the level of BPDE-DNA adducts ($r = 0.91$; $P < 0.01$) (71). Additionally, BPDE-DNA adducts have been detected in oral mucosa cells of smokers and non-smokers. Levels of DNA damage were elevated in each of 16 smokers compared to 16 age-, race-, and sex-matched non-smokers. There was about a three-fold range between smokers and non-smokers (75).

Measurement of carcinogen-DNA adducts in target tissues can offer useful information related to the mechanism of carcinogenesis; however, the limitation of availability of these specimens in humans is an impediment to extensive studies. An alternative method is to use the DNA isolated from peripheral white blood cells (WBC). One example is the detection of PAH-DNA adducts in specific subsets of WBC. It was observed that DNA combined from lymphocyte and monocyte fractions of smokers, exhibited detectable levels of DNA adducts with a mean of 4.38 ± 4.29 adducts/10^8 nucleotides, while the corresponding values were 1.35 ± 0.78/10^8 ($P < 0.001$) in non-smokers (76). The elevated levels of PAH-DNA adducts in DNA obtained from WBC of smokers compared to non-smokers suggested that only certain subsets of WBC are a valid, readily accessible source for monitoring genotoxicity from cigarette smoke.

The decline of PAH-DNA and 4-aminobiphenyl-haemoglobin (4-ABP-Hb) adducts in peripheral blood following smoking cessation in serial samples from 40 heavy smokers (≥ 1 pack/day for ≥ 1 year) was described (77). The substantial reduction (50–75%) of PAH-DNA and 4-ABP-Hb adduct levels after quitting indicates that they are reflective of smoking exposure, which is essential information in the validation of biomarkers (77). The estimated half-life of the PAH-DNA adducts in leukocytes was 9–13 weeks; for 4-ABP-Hb adducts, it was 7–9 weeks. Women had higher levels of 4-ABP-Hb adducts at baseline and after smoking cessation. These results show that PAH-DNA and 4-ABP-Hb adducts can be useful as intermediate biomarkers by verifying smoking cessation and possibly identifying persons who are at increased risk of cancer from exposure to cigarette smoke, due to high levels of carcinogen binding.

In other reports, anti-BPDE-DNA adducts were detected in four of seven colon mucosa samples, but not in any of 11 human pancreas samples from smokers and non-smokers. Adduct levels in human colon samples varied between 0.2–1.0 adducts/10^8 nucleotides (78). DNA adducts have also been detected in biopsy samples of human urinary bladder tissue. Total PAH-DNA adduct levels, and the average levels of several specific adducts, were significantly elevated in samples from current smokers, compared to never-smokers and ex-smokers who had abstained from smoking for at least five years (79). Putative aromatic amine adducts were detected, one of which displayed chromatographic behaviour identical to the predominant adduct induced by the human urinary bladder carcinogen, 4-ABP, which is present in cigarette smoke. Immunohistochemical quantitation of 4-ABP-DNA adducts and *p53* nuclear overexpression in T1 bladder cancer of smokers and non-smokers was described (80). Mean relative staining intensity for 4-ABP-DNA adducts was significantly higher in current smokers compared to non-smokers. There was a linear relationship between mean level of relative staining and number of cigarettes smoked, with lower levels in the 1–19 cig/day group, compared to the 20–40 and the >40 cig/day groups. Nuclear overexpression of

p53 was observed in 27 (59%) of the 45 stage T1 tumours analysed. Nuclear staining of *p53* was correlated with smoking status, cig/day, and 4-ABP-DNA adducts. In another study, 4-ABP-DNA adducts in 11 human lung and eight urinary bladder mucosa specimens were analysed by alkaline hydrolysis and negative chemical ionization GC-MS. Adduct levels were found to be 0.32–49.5 adducts/10^8 nucleotides in the lung and 0.32–3.94 adducts/10^8 nucleotides in the bladder samples (81).

Carcinogen-DNA adducts in human breast tissue samples have been reported (82). A total of 31 breast tissue samples, which included tumour and tumour-adjacent tissues from 15 women with breast cancer and normal tissue samples from four women undergoing breast reduction, were analysed. Among the breast cancer cases, the mean aromatic/hydrophobic-DNA adduct level assayed was 5.3 ± 2.4 adducts/10^8 nucleotides, compared to 2.3 ± 1.5/10^8 nucleotides from the non-cancer patients. Five of 15 tissues from the cases displayed a pattern of adducts associated with tobacco smoke exposure; all of these positive samples were from current smokers. Tissue samples from the eight non-smoking cases did not exhibit this pattern. This study indicated that biomarkers may be useful in investigating specific environmental exposures that could contribute to breast cancer. In another study, BPDE-DNA and other adducts were also found in the smooth muscle layer of atherosclerotic lesions in abdominal aorta specimens by various analytical methods (83).

Alkylating agents, such as N-nitroso compounds, are potential human carcinogens. Humans are known to be exposed to N-nitrosoamines from diet, workplace, cigarette smoke, and through endogenous formation. These compounds alkylate DNA, leading to formation of various types of DNA adducts, such as 7-alkyl-2'-deoxyguanosine (dG) (e.g. 7-methyl-dGp and 7-ethyl-dGp). Several investigations have focused on the levels of 7-methyl-dG adducts in human lung tissue, where higher levels have been found in smokers compared to non-smokers (84–87). Separately, 7-methyl-dG levels in lung tissues have been associated with cytochrome P4502D6 and 2E1 genetic polymorphisms (84). One study analysed N^7-alkylguanine adduct levels in DNA in a group of 46 patients with larynx tumours by the ^{32}P-postlabelling method. The average level of N^7-alkylguanines was 26.2/10^7 nucleotides in tumour cells, 22.7/10^7 in non-tumour cells, and 13.1/10^7 in blood leukocytes. Males and smokers had significantly higher levels of adducts than females and non-smokers (88). In another study, 7-alkyl-2'-deoxyguanosine adducts were measured in eight separate lung segments of 10 autopsy specimens (89). Levels of 7-methyl-dGp were detected in all eight samples (ranging from 0.3–11.5 adducts/10^7 dG; mean 2.5 ± 2.3). In all but five of the samples, 7-ethyl-dGp levels were detected (ranging from <0.1–7.1 adducts/10^7 dG; mean 1.6 ± 1.7). 7-methyl-dG levels were approximately 1.5-fold higher than 7-ethyl-dG, and were positively correlated with each other in most individuals. There was no consistent pattern of adduct distribution in the different lung lobar segments (90).

Measurement of DNA adducts for studying occupational carcinogen exposure

Occupational exposure to many chemical carcinogens has been well documented. Since the initial reports in aniline dye workers, many laboratories have applied technologies for measuring these carcinogens and their metabolites to confirm exposure in the workplace (56). The occurrence of DNA adducts in exfoliated urothelial cells of a worker exposed to the aromatic amine 4,4'-methylene-bis(2-chloroaniline) (MOCA), an agent that induces lung and liver tumours in rodents and urinary bladder tumours in dogs, was reported (91). ^{32}P-postlabelling analysis revealed the presence of a single, major DNA adduct that cochromatographed with the major N-hydroxy-MOCA-DNA adduct, N-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol, formed *in vitro.* PAH-DNA adducts in WBC and 1-hydroxypyrene in urine were examined in a group of 105 workers from a primary aluminum plant with different PAH exposures (92). Exposure was measured by personal monitoring and ranged from 0.4–150 fg/m^3. High exposure to PAH in the work atmosphere was associated with increased concentration of 1-hydroxypyrene in the urine. PAH-DNA adducts were detected in 93% of the worker samples. Workers with a high PAH exposure had significantly higher adduct levels than did those with a low PAH exposure. A good correlation was found between PAH exposure and the average PAH-adduct values in blood. A statistically significant correlation was also found between the average adduct values and the concentration of 1-hydroxypyrene in the urine of smokers.

The lymphocyte bulky PAH-DNA adduct levels have been examined in workers exposed to ambient air pollution (93). A significantly higher adduct level was found in bus drivers working in central Copenhagen compared with those driving in suburban areas. The urban drivers

had higher adduct levels than rural controls. There was no observation of significant influence on adduct level by potential confounders including smoking and diet, *GSTM1*, or *NAT2*. A separate study measured BPDE-DNA adducts in 39 coke oven workers (exposed to PAH) and 39 non-exposed controls (each group consisting of smokers and non-smokers) (94). Adducts were detected in 51% of workers and in 18% of controls. The mean level in workers ($15.7x10^8$ nucleotides) was 15 times higher than in non-exposed controls. Although large interindividual variations were noted, smoking workers had 3.5 times more adducts than non-smokers.

Measurement of DNA adducts in excretion for studying carcinogen exposure

In addition to monitoring carcinogen-DNA adducts *in situ* in DNA, the excised products of these adducts can be determined in urine samples. These urinary biomarkers have been especially amenable to comprehensive validation studies (95). One example is the examination of the dose-dependent excretion of urinary aflatoxin biomarkers in the rat following a single exposure to AFB_1 (96). The relationship between AFB_1 dose and the excretion of the major nucleic acid adduct, AFB_1-N^7-Guanine (AFB-N^7-Gua), over the initial 24-hour period following exposure, showed an excellent linear correlation between dose and excretion in urine. In contrast, other oxidative metabolites, such as aflatoxin P_1 (AFP_1), revealed no linear excretion characteristic.

One approach for the development and validation of aflatoxin adduct biomarkers has entailed parallel experiments in animal models with the systematic evaluation of these molecular biomarkers in humans. The urinary excretion of aflatoxin metabolites in an area of China with a high incidence of liver cancer was studied (97). Total 24-hour urine samples were collected and analysed by an IAC-HPLC analysis to determine individual aflatoxins in the urine samples. The aflatoxins most commonly detected were AFB-N^7-Gua, AFM_1, AFP_1 and AFB_1; however, only AFB-N^7-Gua and AFM_1 showed a dose-dependent relationship between aflatoxin intake and urinary levels, which indicates that these two metabolites might be useful biomarkers of exposure. Interestingly, these studies also demonstrated that the kinetics of formation and excretion of AFB-N^7-Gua in urine is almost identical in the F344 rat and humans, thereby enhancing the value of rodent studies for assessing risk to humans.

Modification of N-3 at the position of adenine is a major route of DNA adduct formation for many alkylating carcinogens. The resulting 3-alkyldeoxyadenosines are unstable, rapidly depurinating to give the corresponding 3-alkyladenines (3-alkAde) that are excreted in urine. These can then be quantitated by immunochemical and/or GC-MS methods. In a study of cancer patients receiving methylnitrosourea (MNU) as part of combination chemotherapy, 24-hour urine samples were collected. An analysis of urinary 3-MeAde in smokers showed increased excretion of this biomarker. Overall, a dose-dependent excretion of 3-MeAde was observed (98).

Measurement for endogenous DNA damage

Endogenous oxidative DNA damage may play an important role in the formation of chronic degenerative diseases, including cancer (99). Among the many oxidatively damaged DNA bases formed, 8-hydroxy-2'-deoxyguanosine, or 8-oxo-7, 8-dihydro-2'-deoxyguanosine (oxo8dG) is a lesion that can be sensitively measured. Several techniques have been developed and applied to determine this damage product in biofluids and tissue samples from animals and humans. These methods include HPLC-EC, GC/MS, immunoassay, fluorescence postlabelling, ^{3}H-postlabelling and ^{32}P-postlabelling (100). IA column methods have also been described for the analysis of oxidative damage products of nucleic acids excreted in urine (101). Quantitative analysis of these adducts in the urine of rats fed a nucleic acid-free diet suggests that 8-oxo-7, 8-dihydroguanine is the principal repair product from oxo8dG in DNA. In addition to these reports, excretion of oxidative DNA damage products in urine has also been correlated with dietary antioxidant consumption in humans (102,103). Thus, these markers may eventually be used to assess protection status as well as risk of disease in people.

Malonaldehyde (MA) is the major reactive aldehyde resulting from the peroxidation of polyunsaturated fatty acids (PUFA) constituents of biological membranes, and is a by-product of prostaglandin biosynthesis (104). MA has been proven to be carcinogenic in rats, mutagenic in several bacterial and mammalian mutation assays, and readily reacts with DNA to produce several adducts. Relatively high endogenous levels of MA-DNA adducts have been detected in healthy individuals (1–10 adducts/10^7 nucleotides). Thus, MA is considered an important endogenously produced genotoxic agent that may contribute to the development of some human cancers, particularly to

the carcinogenic effects associated with high dietary fat intake. Recent reviews on this field can be found in (105,106).

The effect of dietary fatty acid composition on the endogenous formation of MA-DNA adducts was investigated in a group of 59 healthy men and women (107). They were initially fed a milk fat-based diet (rich in saturated fatty acids) for two weeks to induce a homogeneous dietary background. Following this period, the subjects were randomly divided into two subgroups: 30 people were given a sunflower oil-based (SO) diet (rich in polyunsaturated fatty acid), and the remaining 29 people were fed a low erucic acid rapeseed oil-based (RO) diet (rich in monounsaturated fatty acid) for 25 days. At the end of the study, the fatty acid composition of plasma lipids and the level of MA-DNA adduct in total WBC were determined. A higher concentration of PUFA in plasma triglycerides and higher levels of MA-DNA adducts were found in the subjects of the SO diet group as compared with those in the RO diet group. The average adduct level (7.4 ± 8.7 adducts/10^7 nucleotides) in the SO group was 3.6-fold higher than that in the RO group, although large interindividual variation was noted.

Malondialdehyde (MDA)-DNA adducts were analysed in surgical specimens of normal breast tissues of 51 breast cancer patients, while normal breast tissue samples from 28 non-cancer patients served as controls (108). Two previously characterized MDA-deoxyadenosine (dA) and one MDA-deoxyguanosine (dG) adducts were detected in all tissue samples examined. Normal breast tissues from cancer patients exhibited significantly higher levels than those found in non-cancer controls. Ten of the 51 cancer patients and one of the 28 controls were found to contain the MDA-DNA adducts at the level of >1/10^7 nucleotides. Age and body mass did not significantly influence the levels of these adducts. However, the presence of a previously detected BP-DNA adduct in the breast tissues was associated with higher levels of the MDA-dA adducts in cancer patients. Interestingly, the level of MA-dA adducts was significantly lower in smokers and ex-smokers compared to non-smokers. Tumor tissues (n = 11) also displayed significantly lower levels of MA adducts than their corresponding normal adjacent tissues. These results suggest that lipid peroxidation products can accumulate in human breast tissues and reach relatively high levels in the breast tissues of women with breast cancer.

Estrogen is also known to be a major risk factor in breast cancer, and its biological effects are mediated by both receptor and metabolism (109). Estrogen can form DNA adducts, which have been measured in human samples (110). Formation of the 4-hydroxyestradiol-*N7*-guanine (4-OHE2-*N7*-guanine) adduct from the reaction of estradiol-3,4-quinone with DNA and its detection *in vivo,* has been established. Therefore, the development and application of methods to measure estrogen-guanine adducts will, in the future, explore the biological implications of these compounds to determine their contribution to estrogen toxicology.

Techniques for measuring carcinogen-protein adducts

Formation of carcinogen-protein adducts is considered to be a valuable surrogate for DNA adducts, since many chemical carcinogens bind to both DNA and protein in blood with similar dose–response kinetics (3,111). Haemoglobin and serum albumin are the proteins of choice, although efforts have been made to validate histone and collagen adducts, because they are readily accessible, more abundant than DNA, and have known rates of turnover. The lifespan of haemoglobin is ~60 days in rodents and 120 days in humans, and the half-life of serum albumin in humans is 23 days. Because protein adducts are stable and are not removed by active repair processes, they constitute a much more precise dosimetry tool when compared with DNA adducts. Interaction of a carcinogen with a protein typically occurs by substitution at a nucleophilic amino acid. For alkylating agents, the most commonly substituted amino acid is cysteine, but modifications for other carcinogens have been reported at lysine, aspartate, glutamate, tryptophan, histidine and valine (3,112).

Formation of haemoglobin or serum albumin adducts has been reported in experimental animals and humans for many categories of carcinogens, including AFB_1, aromatic amines, B(a)P, benzene, dimethylnitrosamine, ethylene oxide, 2-amino-3-methylimidazo(4,5-f) quinoline, methylmethane sulfonate, NNK, propylene oxide, styrene, and workplace and medicinal (psoriasis) PAHs (3,50,113). Techniques for measuring carcinogen-protein adducts include immunoassays (ELISA, RIA, and IAC) and analytical chemical methods (GC, GC-MS, HPLC, LC-MS, and AMS). Several combinative methods, such as IAC-HPLC with fluorescent detection and isotope dilution MS, have been applied to measure protein adducts (114). Sensitivity of these methods typically can be within the pmol and fmol range. For detection of haemoglobin or albumin adducts in humans, samples must

be enriched for adducts, or adducts must be removed from the protein, before analysis (3,60,111). This is accomplished by either chemical or enzymatic release of the adduct or carcinogen from the protein or digestion of the protein into peptides and amino acids. Solvent extraction or IAC purification may then be used for partial purification before undergoing analysis with GC-MS, HPLC, or LC-MS.

Measurement of haemoglobin adducts for studying carcinogen exposure

A wide variety of aromatic amines and PAHs have been found to bind at high levels to haemoglobin (115). A brief summary showing the range of the different chemical haemoglobin adducts that have been detected in human non-smokers is found in Table 4.1. One carcinogen-Hb adduct that has been well characterized is formed by the potent urinary bladder carcinogen 4-ABP, and has been reported in human blood specimens (113). It has been concluded that 4-ABP-Hb adduct is closely associated with three major risk factors for bladder cancer: cigarette smoking, the type of tobacco smoked and acetylator phenotype. The relation between exposure to environmental tobacco smoke (ETS) and levels of 4-ABP-Hb adducts in non-smoking pregnant women compared to adduct levels in those women who smoked during pregnancy has been reported (116). A questionnaire on smoking and exposure to ETS was administered to pregnant women. Samples of maternal blood and cord blood were collected during delivery and analysed for 4-ABP-Hb adducts by GC-MS. The mean adduct level in smokers was approximately nine-fold higher than that in non-smokers. Among non-smokers, the levels of 4-ABP-Hb adducts increased with increasing ETS level. This relationship between ETS exposure and 4-ABP-Hb adduct levels supports the concept that ETS is a probable hazard during pregnancy.

Metabolic polymorphism, both in *NAT* and in *CYP1A2*, is also expected to affect the formation of 4-ABP-DNA- and Hb-adducts. Levels of DNA adducts in bladder cells and 4-ABP-Hb adducts in 79 individuals, together with the acetylator phenotype and genotype, were determined (117). Among the slow acetylators, levels of 4-ABP-Hb adducts were significantly higher compared to those present in rapid acetylators. This study indicated that clearance of low-dose carcinogens is decreased in the slow acetylator phenotype. Since the highest levels of adducts were found in individuals with rapid N-oxidation (*CYP1A2*) and slow N-acetylation (*NAT2*) phenotype, determination of phenotypes and genotypes may provide a better prediction and assessment of human cancer risk.

It was found that mean 3- and 4-ABP-Hb adduct levels in 151 subjects were statistically significantly higher in cigarette smokers compared to non-smokers, and that the level increased with increasing number of cigarettes smoked per day (118). Again, slow acetylators consistently exhibited higher mean levels of ABP-Hb adducts compared to rapid acetylators. The mean level of 4-ABP-Hb adduct was higher in subjects possessing the *GSTM1*-null versus *GSTM1*-non-null genotype (46.5 versus 36.0 pg/g Hb; P = 0.037). In another study, a polymorphic distribution of the *CYP1A2* and *NAT2* phenotypes was examined in relation to ABP-Hb adduct formation in 97 healthy males (119). Rapid oxidizers and subjects with the combined slow acetylator-rapid oxidizer phenotype showed the highest ABP-Hb adduct levels at

Table 4.1. Haemoglobin adducts in human non-smokers

Compound	fmole/g Haemoglobin
HPB from NNK	29.3 ± 25.9
2-Aminonapthalene	40 ± 20
4-ethylaniline	99 ± 10
2,6-dimethylaniline	157 ± 50
4-Aminobiphenyl	166 ± 77
3,5-dimethylaniline	220 ± 20
o-Toluidine	320 ± 90
p-Toluidine	640 ± 370
m-Toluidine	6400 ± 1900
N-(2-carbamoylethyl)valine	19000 ± 12000
Aniline	41000 ± 22000
N-(2-Hydroxyethyl)valine	58000 ± 25000

Table compiled from (69,115,120).

a low smoking dose. However, in a subset of 45 available samples, no association was seen between the ABP-Hb adduct levels and *GSTM1* genotype.

A wide variety of aromatic amines and PAHs have been found to bind at high levels to haemoglobin (115). Tobacco-specific nitrosamine binding to haemoglobin from pyridyloxobutylation has been detected at 29.3 ± 25.9 fmole/g haemoglobin (54). 2-Aminonapthalene, 4-ethylaniline, 2,6-dimethylaniline, 4-Aminobiphenyl,3,5-dimethylaniline, o-Toluidine, p-Toluidine, m-Toluidine, N-(2-carbamoylethyl)valine, Aniline, and N-(2-Hydroxyethyl)valine have been measured at 40 ± 20, 99 ± 10, 157 ± 50, 166 ± 77, 220 ± 20, 320 ± 90, 640 ± 370, 6400 ± 1900, 19 000 ± 12 000, 41 000 ± 22 000, and 58 000 ± 25 000 fmole/g haemoglobin, respectively (69,115,120). One of the carcinogen-Hb adducts that has been well characterized is formed by 4-ABP, the potent urinary bladder carcinogen; 4-ABP-Hb adducts in human blood specimens have been reported (113). The results indicate that the 4-ABP-Hb adduct is closely associated with three major risk factors for bladder cancer: cigarette smoking, the type of tobacco smoked, and acetylator phenotype.

The role of aromatic amines in the development of bladder cancer in non-smokers in Los Angeles, USA was explored in a population-based case–control study involving 298 case subjects with bladder cancer and 308 controls. To assess arylamine exposure, levels of arylamine-haemoglobin adducts of nine selected alkylanilines (2,3-dimethylaniline (2,3-DMA), 2,4-DMA, 2,5-DMA, 2,6-DMA, 3,4-DMA, 3,5-DMA, 2-ethylaniline (2-EA), 3-EA, and 4-EA) were measured in peripheral blood collected from study subjects. Levels of all arylamine-haemoglobin adducts, with the exception of 2,6-DMA, were higher in smokers than in non-smokers, and levels of all arylamine-haemoglobin adducts were higher in cases than in controls. Arylamine-haemoglobin adducts of 2,6-DMA, 3,5-DMA, and 3-EA were all independently statistically significantly (all $P < 0.001$) associated with bladder cancer risk after adjusting for cigarette smoking at the time of blood collection, lifetime smoking history and other potential risk factors. These adducts were also independently associated with bladder cancer risk when only non-smokers at the time of blood draw were considered (highest quartile versus lowest quartile: 2,6-DMA, relative risk (RR) of bladder cancer = 8.1, 95% confidence interval (CI) = 3.6–18.0; 3,5-DMA, RR = 2.7, 95% CI = 1.2–6.0; 3-EA, RR = 4.3, 95% CI = 1.6–11.6). Thus, diverse arylamine exposures are strongly associated with bladder cancer risk among non-smokers (69).

Measurement of albumin adducts for studying carcinogen exposure

In addition to carcinogen-Hb adducts, carcinogen-albumin adducts have also been investigated, particularly for AFB_1 exposure (113,121). There are four analytical techniques currently available for measuring AFB_1-albumin adducts in human blood: ELISA, RIA, IAC-HPLC with fluorescence detection, and isotope dilution MS (IDMS) (50,114). Using RIA, levels of aflatoxin-serum albumin adducts in serum samples from residents of Guangxi, China were monitored; a highly significant association between AFB_1-albumin adduct level and AFB_1 intake was found (122). Further, it was calculated that about 2% of the ingested AFB_1 became covalently bound to serum albumin, a value very similar to that observed when rats were administered AFB_1. When the data for AFB-N^7-Gua adduct excretion in urine and serum albumin were compared, a statistically significant relationship was seen with a correlation coefficient of 0.73 (123). Using ELISA, AFB_1-albumin adducts in human sera from several regions of the world were investigated (124). It was found that 12–100% of serum samples from children and adults of various African countries contained AFB_1-albumin adducts, with levels up to 350 pg AFB_1-lysine equivalent/mg albumin. In studies conducted in the Gambia, West Africa, a strong dose–response relationship between aflatoxin exposure and AFB_1-albumin adducts was also seen (125), similar to that previously reported in China (122). From a practical perspective pertinent to epidemiologic studies, the measurement of serum AFB_1-albumin adduct offers a rapid, facile approach that can be used to screen very large numbers of people (17).

Three methods for AFB_1-albumin adduct measurement were compared using serum samples from highly exposed residents of Qidong (n=88), Fushui (n=65), and Chongming Island (n=115), China, and The Gambia (n=29). Although the average levels of AFB_1-albumin adducts were similar among these regions, great individual variations were found, as evidenced by the detectable level ranging from 0.124–25.925 pmol aflatoxin/mg albumin in the 297 samples. High correlations across the methods (r = 0.80–0.90) were obtained by comparing samples with each of the different analytical methods. Moreover, some of these samples can be stored for over 10 years with insignificant losses of albumin adduct levels (126). The ELISA and IDMS methods were compared in measurement of 20

human serum samples collected in Guinea, West Africa; high correlation between these two methods was found (r = 0.856, $P < 0.0001$) (127). In an experimental study, the level of AFB_1-albumin adducts formed as a function of a single dose of AFB_1 in rodents was compared to data from humans exposed to AFB_1. This comparison yielded a value for the three rat strains (Fischer 344, Wistar, and Sprague-Dawley) of between 0.3–0.51 pg AFB_1-lysine/mg albumin/1 µg AFB_1/kg body weight and a value for the mouse (C57BL) of <0.025. The best estimate for people from the Gambia and southern China was 1.56 pg/mg albumin for the same exposure. These data suggest that humans exposed to AFB_1 form amounts of albumin adducts closer to those observed in AFB_1-sensitive species and 1–2 orders of magnitude higher levels than the AFB_1-resistant species (128).

Measurement of DNA and protein adducts for studying cancer risk

DNA and protein adducts not only serve as biomarkers for exposure, but as biomarkers for cancer risk. A nested case-control study initiated in 1986 in Shanghai, examined the relationship between biomarkers for aflatoxin and hepatitis B virus (HBV) and the development of liver cancer (129,130). In this study, over 18 000 urine samples were collected from healthy males between the ages of 45 and 64. In the subsequent seven years, 50 of these individuals developed liver cancer. The urine samples for cases were age-matched and residence-matched with controls and analysed for both aflatoxin biomarkers and HBsAg status. A highly significant increase in the RR of 3.5 was observed for those liver cancer cases where urinary aflatoxin biomarkers (AFB-N^7-Gua and other AFB_1 metabolites) were detected. The RR for people who tested positive for the HBsAg was about eight, but individuals with both urinary aflatoxin biomarkers and positive HBsAg status had a RR for developing liver cancer of 57. These results show, for the first time, a relationship between the presence of carcinogen-specific biomarkers and cancer risk. Moreover, these findings provided the first demonstration of a multiplicative interaction between these two major risk factors for liver cancer. Further, when individual aflatoxin metabolites were stratified for liver cancer outcome, the presence of the AFB-N^7-Gua in urine always resulted in a two- to three-fold elevation in risk of developing liver cancer (130).

A case–control study measured BPDE-DNA adducts in DNA samples from WBC of lung cancer patients and healthy controls. High levels of adducts were found in WBC from lung cancer patients, with a range of 65–533 adducts/10^8 nucleotides. In WBC-DNA samples from healthy controls (smokers, non-smokers), the presence of adducts was detected only in smokers, but at a lower level than in lung cancer patients (74). PAH-DNA adducts in peripheral leukocytes were investigated from 119 non-small cell lung cancer patients and 98 controls (131). Among them, 31 cases had adduct measurements in leukocytes, lung tumour and non-tumour specimens collected at surgery, and 34 had paired leukocyte and tumour specimens. After adjustment for age, gender, ethnicity, season and smoking, DNA adducts in leukocytes were significantly higher in cases than controls; the odds ratio was 7.7 (95% CI = 1.7–34; $P<0.01$). DNA adducts in leukocytes were increased significantly in smokers and ex-smokers compared to non-smokers among cases and controls (separately and combined) after adjusting for age, gender, ethnicity, and season.

Aflatoxin-albumin adducts have been used as biomarkers for liver cancer risk. A nested case-control study carried out in Taiwan, China, followed a cohort of 8068 men for three years (132,133). Twenty-seven cases of hepatocellular carcinoma (HCC) were identified and matched with 120 healthy controls. Serum samples were analysed for AFB_1-albumin adducts by ELISA. The proportion of subjects with a detectable serum AFB_1-albumin adduct level was higher for HCC cases (74%) than matched controls (66%), giving an odds ratio of 1.5. There was a statistically significant association between detectable level of AFB_1-albumin adduct and HCC risk among men younger than 52 years old, showing a multivariate-adjusted odds ratio of 5.3, although no association was observed between AFB_1-albumin adduct level and HBsAg carrier status. Another prospective, nested case–control study (134) was carried out in Qidong, China in 1991. Serum samples from 804 healthy HBsAg-positive individuals (728 male, 76 female) aged 30–65 were obtained and stored frozen. Between the years 1993–95, 38 individuals developed liver cancer. The serum samples for 34 of these cases were matched by age, gender, residence and time of sampling to 170 controls. Serum AFB_1-albumin adduct levels were determined by RIA. The RR for HCC among AFB_1-albumin positive individuals was 2.4 (95% CI = 1.2–4.7).

Summary and perspectives for the future

Over the past 25 years, the development and application of

molecular biomarkers reflecting events from exposure to formation of clinical disease has rapidly expanded our knowledge of the mechanisms of disease pathogenesis. These biomarkers will have increasing potential for early detection, treatment, interventions and prevention. Biomarkers derived from toxicant/carcinogen metabolism include a variety of parent compounds and metabolites in body fluids and excreta, which serve as biomarkers of internal dose. Carcinogen-macromolecular adducts, such as DNA and protein adducts formed in blood and tissue or excreted in urine, serve as biomarkers for: exposure to the complex mixture of occupational carcinogens; biologically effective dose or early biological effect, to measure the actual dose to the carcinogen target site; and for risk assessment between carcinogen exposure and eventual cancer formation.

Many different analytical techniques have been developed to identify and measure parent compounds, metabolites, carcinogen-DNA and -protein adducts. These include immunoassays (ELISA, RIA, IHC, and IA), radiometric postlabelling methods (^{32}P-post-labelling) and various physicochemical methods (GC, HPLC, GC-MS, LC-MS, ECD, fluorescence and phosphorescence spectroscopy, or a combination of these methods). Capillary electrophoresis and other new separation techniques have improved sensitivity and specificity of these strategies. NMR spectrometry has also been used to determine stereospecificity and three-dimensional structure. Molecular epidemiologic studies that employ carcinogen-macromolecular adduct measurements are likely to be widely applied in the future. They have the potential to generate hypotheses regarding underlying basic biologic mechanisms that subsequently can be tested in the laboratory. The complex nature of gene–environment and chemical–biological interactions will be better understood through the use of advanced techniques, such as rapidly developing metabolomics and proteomics, which include NMR-MS and matrix-assisted laser desorption/ionization (MALDI)-MS, and incorporation of validated biomarkers into large-scale studies. In the future, integration of data for these biomarkers, together with other environmental and host susceptibility factors in molecular epidemiologic studies of human cancer, will assist in the elucidation of human cancer risk.

The molecular epidemiology investigations of aflatoxins probably represent one of the most extensive data sets in the field, and may serve as a template for future studies of other environmental agents. The development of aflatoxin biomarkers has been based upon the knowledge of their biochemistry and toxicology gleaned from both experimental and human studies. These biomarkers have subsequently been used in experimental models to provide data on the modulation of these markers under different situations of disease risk. This systematic approach provides encouragement for preventive interventions and should serve as a template for the development, validation and application of other biomarkers to cancer or other chronic diseases.

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References

1. Perera FP, Weinstein IB (1982). Molecular epidemiology and carcinogen-DNA adduct detection: new approaches to studies of human cancer causation. *J Chronic Dis,* 35:581–600.doi:10.1016/0021-9681(82)90078-9 PMID:6282919

2. Hulka BS (1991). ASPO Distinguished Achievement Award Lecture. Epidemiological studies using biological markers: issues for epidemiologists. *Cancer Epidemiol Biomarkers Prev,* 1:13–19. PMID:1845163

3. Poirier MC, Santella RM, Weston A (2000). Carcinogen macromolecular adducts and their measurement. *Carcinogenesis*, 21:353–359.doi:10.1093/carcin/21.3.353 PMID:10688855

4. Wogan GN (1992). Molecular epidemiology in cancer risk assessment and prevention: recent progress and avenues for future research. *Environ Health Perspect,* 98:167–178. PMID:1486846

5. Schulte PA. A conceptual and historical framework for molecular epidemiology. In: Schulte PA, Perera FP, editors. Molecular epidemiology: principles and practices. San Diego (CA): Academic Press Inc; 1993. p. 3–44.

6. Perera FP (2000). Molecular epidemiology: on the path to prevention? *J Natl Cancer Inst,* 92:602–612.doi:10.1093/jnci/92.8.602 PMID:10772677

7. Hattis D, Silver K. Use of biomarkers in risk assessment. In: Schulte PA, Perera FP, editors. Molecular epidemiology: principles and practices. San Diego (CA): Academic Press Inc; 1993. p. 251–273.

8. Committee on Biological Markers of the National Research Council (1987). Biological markers in environmental health research. *Environ Health Perspect,* 74:3–9. PMID:3691432

9. Wang JS, Links JM, Groopman JD. Molecular epidemiology and biomarkers. In: Choy WN, editor. Genetic toxicology and cancer risk assessment. New York (NY): Marcel Dekker; 2001. p. 269–296.

10. Wogan GN (1989). Markers of exposure to carcinogens. *Environ Health Perspect*, 81:9–17. PMID:2667991

11. Groopman JD, Kensler TW (1993). Molecular biomarkers for human chemical carcinogen exposures. *Chem Res Toxicol,* 6:764–770.doi:10.1021/tx00036a004 PMID: 8117914

12. Groopman JD, Kensler TW (1999). The light at the end of the tunnel for chemical-specific biomarkers: daylight or headlight? *Carcinogenesis*, 20:1–11.doi:10.1093/carcin/20.1.1 PMID:9934843

13. Hecht SS (1999). Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst,* 91:1194–1210.doi:10.1093/jnci/91.14.1194 PMID:10413421

14. Hecht SS, Carmella SG, Foiles PG, Murphy SE (1994). Biomarkers for human uptake and metabolic activation of tobacco-specific nitrosamines. *Cancer Res,* 54 Suppl;1912s–1917s. PMID:8137311

15. Tang D, Phillips DH, Stampfer M *et al.* (2001). Association between carcinogen-DNA adducts in white blood cells and subsequent risk of lung cancer in the Physicians' Health Study. *Cancer Res,* 61:6708–6712. PMID:11559540

16. Hecht SS (2003). Tobacco carcinogens, their biomarkers and tobacco-induced cancer. *Nat Rev Cancer,* 3:733–744.doi:10.1038/nrc1190 PMID:14570033

17. Groopman JD, Wogan GN, Roebuck BD, Kensler TW (1994). Molecular biomarkers for aflatoxins and their application to human cancer prevention. *Cancer Res,* 54 Suppl;1907s–1911s. PMID:8137310

18. Kensler TW, Qian GS, Chen JG, Groopman JD (2003). Translational strategies for cancer prevention in liver. *Nat Rev Cancer,* 3:321–329.doi:10.1038/nrc1076 PMID:12724730

19. Mattson ME, Pollack ES, Cullen JW (1987). What are the odds that smoking will kill you? *Am J Public Health,* 77:425–431. doi:10.2105/AJPH.77.4.425 PMID:3826460

20. Kaderlik RK, Lin DX, Lang NP, Kadlubar FF (1992). Advantages and limitations of laboratory methods for measurement of carcinogen-DNA adducts for epidemiological studies. *Toxicol Lett,* 64–65:469–475.

21. Sugimura T, Inoue R, Ohgaki H, *et al.* (1995). Genetic polymorphisms and susceptibility to cancer development. *Pharmacogenetics*, 5:S161–S165.

22. Guengerich FP (2000). Metabolism of chemical carcinogens. Carcinogenesis, 21:345–351.doi:10.1093/carcin/21.3.345 PMID:10688854

23. Perera FP, Poirier MC, Yuspa SH *et al.* (1982). A pilot project in molecular cancer epidemiology: determination of benzo[a] pyrene–DNA adducts in animal and human tissues by immunoassays. *Carcinogenesis*, 3:1405–1410.doi:10.1093/carcin/3.12.1405 PMID:6295657

24. Wogan GN, Hecht SS, Felton JS *et al.* (2004). Environmental and chemical carcinogenesis. *Semin Cancer Biol,* 14:473–486.doi:10.1016/j.semcancer.2004.06.010 PMID:15489140

25. Perera FP, Weinstein IB (2000). Molecular epidemiology: recent advances and future directions. *Carcinogenesis*, 21:517–524. doi:10.1093/carcin/21.3.517 PMID:10688872

26. Nordberg GF, Jin T, Hong F *et al.* (2005). Biomarkers of cadmium and arsenic interactions. *Toxicol Appl Pharmacol,* 206:191–197.doi:10.1016/j.taap.2004.11.028 PMID:15967208

27. Savory J, Herman MM (1999). Advances in instrumental methods for the measurement and speciation of trace metals. *Ann Clin Lab Sci,* 29:118–126. PMID:10219699

28. Sengoku S, Wagatsuma K (2006). Comparative studies of spectrochemical characteristics between axial and radial observations in inductively coupled plasma optical emission spectrometry. *Anal Sci,* 22:245–248.doi:10.2116/analsci.22.245 PMID:16512416

29. Lobiński R, Schaumlöffel D, Szpunar J (2006). Mass spectrometry in bioinorganic analytical chemistry. *Mass Spectrom Rev,* 25:255–289.doi:10.1002/mas.20069 PMID:16273552

30. Hu H, Milder FL, Burger DE (1989). X-ray fluorescence: issues surrounding the application of a new tool for measuring burden of lead. *Environ Res,* 49:295–317.doi:10.1016/S0013-9351(89)80074-X PMID:2753011

31. Aposhian HV, Aposhian MM (2006). Arsenic toxicology: five questions. *Chem Res Toxicol,* 19:1–15.doi:10.1021/tx050106d PMID:16411650

32. Dopp E, Hartmann LM, Florea AM *et al.* (2004). Environmental distribution, analysis, and toxicity of organometal(loid) compounds. *Crit Rev Toxicol,* 34:301–333.doi:10.1080/10408440490270160 PMID:15239389

33. Guengerich FP (2008). Cytochrome p450 and chemical toxicology. *Chem Res Toxicol,* 21:70–83.doi:10.1021/tx700079z PMID:18052394

34. Guengerich FP (2000). Metabolism of chemical carcinogens. *Carcinogenesis*, 21:345–351.doi:10.1093/carcin/21.3.345 PMID:10688854

35. Liebler DC, Guengerich FP (2005). Elucidating mechanisms of drug-induced toxicity. *Nat Rev Drug Discov*, 4:410–420. doi:10.1038/nrd1720 PMID:15864270

36. Calafat AM, Kuklenyik Z, Reidy JA *et al.* (2007). Serum concentrations of 11 polyfluoroalkyl compounds in the U.S. population: data from the National Health and Nutrition Examination Survey (NHANES). *Environ Sci Technol,* 41:2237–2242. doi:10.1021/es062686m PMID:17438769

37. Muntner P, Menke A, DeSalvo KB *et al.* (2005). Continued decline in blood lead levels among adults in the United States: the National Health and Nutrition Examination Surveys. *Arch Intern Med*, 165:2155–2161.doi:10.1001/archinte.165.18.2155 PMID:16217007

38. Groopman JD, Donahue PR, Zhu JQ *et al.* (1985). Aflatoxin metabolism in humans: detection of metabolites and nucleic acid adducts in urine by affinity chromatography. *Proc Natl Acad Sci USA*, 82:6492–6496. doi:10.1073/pnas.82.19.6492 PMID:3931076

39. Zhu JQ, Zhang LS, Hu X *et al.* (1987). Correlation of dietary aflatoxin B1 levels with excretion of aflatoxin M1 in human urine. *Cancer Res,* 47:1848–1852. PMID:3102051

40. Zarba A, Wild CP, Hall AJ *et al.* (1992). Aflatoxin M1 in human breast milk from The Gambia, west Africa, quantified by combined monoclonal antibody immunoaffinity chromatography and HPLC. *Carcinogenesis*, 13:891–894.doi:10.1093/carcin/13.5.891 PMID:1587004

41. Carmella SG, Akerkar S, Hecht SS (1993). Metabolites of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in smokers' urine. *Cancer Res*, 53:721–724. PMID:8428352

42. Carmella SG, Akerkar SA, Richie JP Jr, Hecht SS (1995). Intraindividual and interindividual differences in metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokers' urine. *Cancer Epidemiol Biomarkers Prev,* 4:635–642. PMID:8547830

43. Kresty LA, Carmella SG, Borukhova A *et al.* (1996). Metabolites of a tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), in the urine of smokeless tobacco users: relationship between urinary biomarkers and oral leukoplakia. *Cancer Epidemiol Biomarkers Prev,* 5:521–525. PMID:8827356

44. Wolff MS, Toniolo PG, Lee EW *et al.* (1993). Blood levels of organochlorine residues and risk of breast cancer. *J Natl Cancer Inst,* 85:648–652.doi:10.1093/jnci/85.8.648 PMID:8468722

45. Krieger N, Wolff MS, Hiatt RA *et al.* (1994). Breast cancer and serum organochlorines: a prospective study among white, black, and Asian women. *J Natl Cancer Inst,* 86:589–599.doi:10.1093/jnci/86.8.589 PMID:8145274

46. Nedelko T, Arlt VM, Phillips DH, Hollstein M (2009). TP53 mutation signature supports involvement of aristolochic acid in the aetiology of endemic nephropathy-associated tumours. *Int J Cancer,* 124:987–990.doi:10.1002/ijc.24006 PMID:19030178

47. Grollman AP, Jelaković B (2007). Role of environmental toxins in endemic (Balkan) nephropathy. October 2006, Zagreb, Croatia. *J Am Soc Nephrol,* 18:2817–2823.doi:10.1681/ASN.2007050537 PMID:17942951

48. Essigmann JM, Croy RG, Nadzan AM *et al.* (1977). Structural identification of the major DNA adduct formed by aflatoxin B1 *in vitro*. *Proc Natl Acad Sci USA*, 74:1870–1874. doi:10.1073/pnas.74.5.1870 PMID:266709

49. Conney AH (1982). Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. *Cancer Res,* 42:4875–4917. PMID:6814745

50. Miller EC (1978). Some current perspectives on chemical carcinogenesis in humans and experimental animals: Presidential Address. *Cancer Res,* 38:1479–1496. PMID:348302

51. Wang JS, Groopman JD. Biomarkers for carcinogen exposure: tumor initiation. In: Wallace K, Puga A, editors. Molecular biology of the toxic response. Washington (DC): Taylor & Francis; 1998. p. 145–66.

52. Groopman JD, Skipper PL, editors. Molecular dosimetry and human cancer: analytical, epidemiological and social consideration. Boca Raton (FL): CRC Press, Inc; 1991.

53. Wild CP, Chapot B, Scherer E *et al.* Application of antibody methods to the detection of aflatoxin in human body fluids. In: Bartsch H, Hemminki K, O'Neill K, editors. Methods for detecting DNA damaging agents in humans: applications in cancer epidemiology and prevention. Lyon: IARC Scientific Publication; 1988(89). p. 67–74.

54. Gange SJ, Muñoz A, Wang JS, Groopman JD (1996). Variability of molecular biomarker measurements from nonlinear calibration curves. *Cancer Epidemiol Biomarkers Prev,* 5:57–61. PMID:8770468

55. Harris CC (1991). Chemical and physical carcinogenesis: advances and perspectives for the 1990s. *Cancer Res*, 51 Suppl;5023s–5044s. PMID:1884379

56. Dipple A (1995). DNA adducts of chemical carcinogens. *Carcinogenesis*, 16:437–441. doi:10.1093/carcin/16.3.437 PMID:7697795

57. Pitot HC 3rd, Dragan Y. Chemical carcinogenesis. In: Klaassen CD, editor. Casarett and Doull's toxicology: the basic science of poisons. 5th ed. New York: McGraw-Hill; 1996. p. 201–267.

58. Hecht SS (1998). Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem Res Toxicol,* 11:559–603.doi:10.1021/tx980005y PMID:9625726

59. Weston A (1993). Physical methods for the detection of carcinogen-DNA adducts in humans. *Mutat Res*, 288:19–29. PMID:7686262

60. Strickland PT, Routledge MN, Dipple A (1993). Methodologies for measuring carcinogen adducts in humans. *Cancer Epidemiol Biomarkers Prev,* 2:607–619. PMID:8268781

61. Santella RM (1999). Immunological methods for detection of carcinogen-DNA damage in humans. *Cancer Epidemiol Biomarkers Prev,* 8:733–739. PMID:10498391

62. Randerath K, Reddy MV, Gupta RC (1981). 32P-labeling test for DNA damage. *Proc Natl Acad Sci USA*, 78:6126–6129.doi:10.1073/pnas.78.10.6126 PMID:7031643

63. Randerath K, Randerath E (1994). 32P-postlabeling methods for DNA adduct detection: overview and critical evaluation. *Drug Metab Rev,* 26:67–85.doi:10.3109/03602539409029785 PMID:8082582

64. Phillips DH, Farmer PB, Beland FA *et al.* (2000). Methods of DNA adduct determination and their application to testing compounds for genotoxicity. *Environ Mol Mutagen,* 35:222–233.doi:10.1002/(SICI)1098-2280(2000)35:3<222::AID-EM9>3.0.CO;2-E PMID:10737957

65. Patel DJ, Mao B, Gu Z *et al.* (1998). Nuclear magnetic resonance solution structures of covalent aromatic amine-DNA adducts and their mutagenic relevance. *Chem Res Toxicol,* 11:391–407.doi:10.1021/tx9702143 PMID:9585469

66. Geacintov NE, Cosman M, Hingerty BE *et al.* (1997). NMR solution structures of stereoisometric covalent polycyclic aromatic carcinogen-DNA adduct: principles, patterns, and diversity. *Chem Res Toxicol,* 10:111–146. doi:10.1021/tx9601418 PMID:9049424

67. Dingley KH, Curtis KD, Nowell S *et al.* (1999). DNA and protein adduct formation in the colon and blood of humans after exposure to a dietary-relevant dose of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Cancer Epidemiol Biomarkers Prev,* 8:507–512. PMID:10385140

68. Vogel JS, Turteltaub KW, Finkel R, Nelson DE (1995). Accelerator mass spectrometry. *Anal Chem,* 67:353A–359A.doi:10.1021/ac00107a001 PMID:9306729

69. Skipper PL, Trudel LJ, Kensler TW *et al.* (2006). DNA adduct formation by 2,6-dimethyl-, 3,5-dimethyl-, and 3-ethylaniline in vivo in mice. *Chem Res Toxicol,* 19:1086–1090.doi:10.1021/tx060082q PMID:16918249

70. Gan J, Skipper PL, Gago-Dominguez M *et al.* (2004). Alkylaniline-hemoglobin adducts and risk of non-smoking-related bladder cancer. *J Natl Cancer Inst,* 96:1425–1431. doi:10.1093/jnci/djh274 PMID:15467031

71. Poirier MC, Beland FA (1992). DNA adduct measurements and tumor incidence during chronic carcinogen exposure in animal models: implications for DNA adduct-based human cancer risk assessment. *Chem Res Toxicol,* 5:749–755.doi:10.1021/tx00030a003 PMID:1489923

72. Poirier MC, Reed E, Litterst CL *et al.* (1992). Persistence of platinum-ammine-DNA adducts in gonads and kidneys of rats and multiple tissues from cancer patients. *Cancer Res,* 52:149–153. PMID:1727376

73. Kensler TW, Egner PA, Wang JB *et al.* (2004). Chemoprevention of hepatocellular carcinoma in aflatoxin endemic areas. *Gastroenterology*, 127 Suppl 1;S310–S318. doi:10.1053/j.gastro.2004.09.046 PMID:15508099

74. Perera FP, Hemminki K, Gryzbowska E *et al.* (1992). Molecular and genetic damage in humans from environmental pollution in Poland. *Nature*, 360:256–258.doi:10.1038/360256a0 PMID:1436106

75. Bartsch H (1996). DNA adducts in human carcinogenesis: etiological relevance and structure-activity relationship. *Mutat Res,* 340:67–79. PMID:8692183

76. Zhang YJ, Hsu TM, Santella RM (1995). Immunoperoxidase detection of polycyclic aromatic hydrocarbon-DNA adducts in oral mucosa cells of smokers and nonsmokers. *Cancer Epidemiol Biomarkers Prev,* 4:133–138. PMID:7537994

77. Santella RM, Grinberg-Funes RA, Young TL *et al.* (1992). Cigarette smoking related polycyclic aromatic hydrocarbon-DNA adducts in peripheral mononuclear cells. *Carcinogenesis*, 13:2041–2045.doi:10.1093/carcin/13.11.2041 PMID:1423873

78. Mooney LA, Santella RM, Covey L *et al.* (1995). Decline of DNA damage and other biomarkers in peripheral blood following smoking cessation. *Cancer Epidemiol Biomarkers Prev,* 4:627–634. PMID:8547829

79. Alexandrov K, Rojas M, Kadlubar FF *et al.* (1996). Evidence of anti-benzo[a] pyrene diolepoxide-DNA adduct formation in human colon mucosa. *Carcinogenesis*, 17:2081–2083.doi:10.1093/carcin/17.9.2081 PMID:8824539

80. Talaska G, al-Juburi AZ, Kadlubar FF (1991). Smoking related carcinogen-DNA adducts in biopsy samples of human urinary bladder: identification of N-(deoxyguanosin-8-yl)-4-aminobiphenyl as a major adduct. *Proc Natl Acad Sci USA,* 88:5350–5354. doi:10.1073/pnas.88.12.5350 PMID:2052611

81. Curigliano G, Zhang YJ, Wang LY *et al.* (1996). Immunohistochemical quantitation of 4-aminobiphenyl-DNA adducts and p53 nuclear overexpression in T1 bladder cancer of smokers and nonsmokers. *Carcinogenesis*, 17:911–916.doi:10.1093/carcin/17.5.911 PMID:8640937

82. Lin D, Lay JO Jr, Bryant MS *et al.* (1994). Analysis of 4-aminobiphenyl-DNA adducts in human urinary bladder and lung by alkaline hydrolysis and negative ion gas chromatography-mass spectrometry. *Environ Health Perspect,* 102 Suppl 6;11–16.doi:10.2307/3432144 PMID:7889831

83. Perera FP, Estabrook A, Hewer A *et al.* (1995). Carcinogen-DNA adducts in human breast tissue. *Cancer Epidemiol Biomarkers Prev,* 4:233–238. PMID:7606197

84. Izzotti A, De Flora S, Petrilli GL *et al.* (1995). Cancer biomarkers in human atherosclerotic lesions: detection of DNA adducts. *Cancer Epidemiol Biomarkers Prev,* 4:105–110. PMID:7742716

85. Kato S, Onda M, Matsukura N *et al.* (1995). Cytochrome P4502E1 (CYP2E1) genetic polymorphism in a case-control study of gastric cancer and liver disease. *Pharmacogenetics*, 5 Spec No;S141–S144.doi:10.1097/00008571-199512001-00016 PMID:7581484

86. Shields PG, Povey AC, Wilson VL *et al.* (1990). Combined high-performance liquid chromatography/32P-postlabeling assay of N7-methyldeoxyguanosine. *Cancer Res*, 50:6580–6584. PMID:2208119

87. Mustonen R, Schoket B, Hemminki K (1993). Smoking-related DNA adducts: 32P-postlabeling analysis of 7-methylguanine in human bronchial and lymphocyte DNA. *Carcinogenesis*, 14:151–154.doi:10.1093/carcin/14.1.151 PMID:8425264

88. Kato S, Petruzzelli S, Bowman ED *et al.* (1993). 7-Alkyldeoxyguanosine adduct detection by two-step HPLC and the 32P-postlabeling assay. *Carcinogenesis*, 14:545–550. doi:10.1093/carcin/14.4.545 PMID:8386066

89. Szyfter K, Hemminki K, Szyfter W *et al.* (1996). Tobacco smoke-associated N7-alkylguanine in DNA of larynx tissue and leucocytes. *Carcinogenesis*, 17:501–506.doi:10.1093/carcin/17.3.501 PMID:8631136

90. Kato S, Bowman ED, Harrington AM *et al.* (1995). Human lung carcinogen-DNA adduct levels mediated by genetic polymorphisms in vivo. *J Natl Cancer Inst,* 87:902–907. doi:10.1093/jnci/87.12.902 PMID:7666479

91. Blömeke B, Greenblatt MJ, Doan VD *et al.* (1996). Distribution of 7-alkyl-2′-deoxyguanosine adduct levels in human lung. *Carcinogenesis*, 17:741–748.doi:10.1093/carcin/17.4.741 PMID:8625485

92. Kaderlik KR, Talaska G, DeBord DG *et al.* (1993). 4,4′-Methylene-bis(2-chloroaniline)-DNA adduct analysis in human exfoliated urothelial cells by 32P-postlabeling. *Cancer Epidemiol Biomarkers Prev,* 2:63–69. PMID:8420614

93. van Schooten FJ, Jongeneelen FJ, Hillebrand MJ *et al.* (1995). Polycyclic aromatic hydrocarbon-DNA adducts in white blood cell DNA and 1-hydroxypyrene in the urine from aluminum workers: relation with job category and synergistic effect of smoking. *Cancer Epidemiol Biomarkers Prev,* 4:69–77. PMID:7894326

94. Nielsen PS, de Pater N, Okkels H, Autrup H (1996). Environmental air pollution and DNA adducts in Copenhagen bus drivers–effect of GSTM1 and NAT2 genotypes on adduct levels. *Carcinogenesis*, 17:1021–1027. doi:10.1093/carcin/17.5.1021 PMID:8640907

95. Rojas M, Alexandrov K, Auburtin G *et al.* (1995). Anti-benzo[a]pyrene diolepoxide–DNA adduct levels in peripheral mononuclear cells from coke oven workers and the enhancing effect of smoking. *Carcinogenesis*, 16:1373–1376.doi:10.1093/carcin/16.6.1373 PMID:7788857

96. Shuker DE, Farmer PB (1992). Relevance of urinary DNA adducts as markers of carcinogen exposure. *Chem Res Toxicol,* 5:450–460. doi:10.1021/tx00028a001 PMID:1391611

97. Groopman JD, Roebuck BD, Kensler TW (1992). Molecular dosimetry of aflatoxin DNA adducts in humans and experimental rat models. *Prog Clin Biol Res,* 374:139–155. PMID:1320272

98. Groopman JD, Zhu JQ, Donahue PR *et al.* (1992). Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in Guangxi Autonomous Region, People's Republic of China. *Cancer Res,* 52:45–52. PMID:1727385

99. Prevost V, Shuker DE, Friesen MD *et al.* (1993). Immunoaffinity purification and gas chromatography-mass spectrometric quantification of 3-alkyladenines in urine: metabolism studies and basal excretion levels in man. *Carcinogenesis*, 14:199–204.doi:10.1093/carcin/14.2.199 PMID:8435861

100. Ames BN, Gold LS (1991). Endogenous mutagens and the causes of aging and cancer. *Mutat Res,* 250:3–16. PMID:1944345

101. Marnett LJ, Burcham PC (1993). Endogenous DNA adducts: potential and paradox. *Chem Res Toxicol,* 6:771–785.doi:10.1021/tx00036a005 PMID:8117915

102. Park EM, Shigenaga MK, Degan P *et al.* (1992). Assay of excised oxidative DNA lesions: isolation of 8-oxoguanine and its nucleoside derivatives from biological fluids with a monoclonal antibody column. *Proc Natl Acad Sci USA*, 89:3375–3379.doi:10.1073/pnas.89.8.3375 PMID:1565629

103. Simic MG, Bergtold DS (1991). Dietary modulation of DNA damage in human. *Mutat Res,* 250:17–24. PMID:1944333

104. Simic MG (1992). Urinary biomarkers and the rate of DNA damage in carcinogenesis and anticarcinogenesis. *Mutat Res,* 267:277–290. PMID:1376430

105. Vaca CE, Wilhelm J, Harms-Ringdahl M (1988). Interaction of lipid peroxidation products with DNA. A review. *Mutat Res,* 195:137–149. PMID:3277035

106. Marnett LJ, Riggins JN, West JD (2003). Endogenous generation of reactive oxidants and electrophiles and their reactions with DNA and protein. *J Clin Invest,* 111:583–593. PMID:12618510

107. Marnett LJ (2002). Oxy radicals, lipid peroxidation and DNA damage. *Toxicology*, 181-182:219–222.doi:10.1016/S0300-483X(02)00448-1 PMID:12505314

108. Fang JL, Vaca CE, Valsta LM, Mutanen M (1996). Determination of DNA adducts of malonaldehyde in humans: effects of dietary fatty acid composition. *Carcinogenesis*, 17:1035–1040.doi:10.1093/carcin/17.5.1035 PMID:8640909

109. Wang M, Dhingra K, Hittelman WN *et al.* (1996). Lipid peroxidation-induced putative malondialdehyde-DNA adducts in human breast tissues. *Cancer Epidemiol Biomarkers Prev,* 5:705–710. PMID:8877062

110. Yager JD, Davidson NE (2006). Estrogen carcinogenesis in breast cancer. *N Engl J Med,* 354:270–282.doi:10.1056/NEJMra050776 PMID:16421368

111. Rogan EG, Cavalieri EL (2004). Estrogen metabolites, conjugates, and DNA adducts: possible biomarkers for risk of breast, prostate, and other human cancers. *Adv Clin Chem,* 38:135–149.doi:10.1016/S0065-2423(04)38005-4 PMID:15521191

112. Skipper PL, Peng X, Soohoo CK, Tannenbaum SR (1994). Protein adducts as biomarkers of human carcinogen exposure. *Drug Metab Rev,* 26:111–124.doi:10.3109/03602539409029787 PMID:8082561

113. Skipper PL, Tannenbaum SR (1990). Protein adducts in the molecular dosimetry of chemical carcinogens. *Carcinogenesis*, 11:507–518.doi:10.1093/carcin/11.4.507 PMID:2182215

114. Skipper PL, Tannenbaum SR (1994). Molecular dosimetry of aromatic amines in human populations. *Environ Health Perspect,* 102 Suppl 6;17–21.doi:10.2307/3432145 PMID:7889842

115. McCoy LF, Scholl PF, Schleicher RL *et al.* (2005). Analysis of aflatoxin B1-lysine adduct in serum using isotope-dilution liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom,* 19:2203–2210.doi:10.1002/rcm.2045 PMID:16015671

116. Tannenbaum SR (1990). Hemoglobin-carcinogen adducts as molecular biomarkers in epidemiology. *Princess Takamatsu Symp*, 21:351–360. PMID:2134688

117. Hammond SK, Coghlin J, Gann PH *et al.* (1993). Relationship between environmental tobacco smoke exposure and carcinogen-hemoglobin adduct levels in nonsmokers. *J Natl Cancer Inst,* 85:474–478.doi:10.1093/jnci/85.6.474 PMID:8445675

118. Vineis P, Bartsch H, Caporaso NE *et al.* (1994). Genetically based N-acetyltransferase metabolic polymorphism and low-level environmental exposure to carcinogens. *Nature*, 369:154–156.doi:10.1038/369154a0 PMID:7909916

119. Yu MC, Ross RK, Chan KK *et al.* (1995). Glutathione S-transferase M1 genotype affects aminobiphenyl-hemoglobin adduct levels in white, black and Asian smokers and nonsmokers. *Cancer Epidemiol Biomarkers Prev,* 4:861–864. PMID:8634658

120. Landi MT, Zocchetti C, Bernucci I *et al.* (1996). Cytochrome P4501A2: enzyme induction and genetic control in determining 4-aminobiphenyl-hemoglobin adduct levels. *Cancer Epidemiol Biomarkers Prev,* 5:693–698. PMID:8877060

121. Schettgen T, Rossbach B, Kütting B *et al.* (2004). Determination of haemoglobin adducts of acrylamide and glycidamide in smoking and non-smoking persons of the general population. *Int J Hyg Environ Health,* 207:531–539.doi:10.1078/1438-4639-00324 PMID:15729833

122. Sabbioni G, Skipper PL, Büchi G, Tannenbaum SR (1987). Isolation and characterization of the major serum albumin adduct formed by aflatoxin B1 in vivo in rats. *Carcinogenesis*, 8:819–824.doi:10.1093/carcin/8.6.819 PMID:3111739

123. Gan LS, Skipper PL, Peng XC *et al.* (1988). Serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis: correlation with aflatoxin B1 intake and urinary excretion of aflatoxin M1. *Carcinogenesis*, 9:1323–1325.doi:10.1093/carcin/9.7.1323 PMID:3133131

124. Groopman JD, Hasler JA, Trudel LJ *et al.* (1992). Molecular dosimetry in rat urine of aflatoxin-N7-guanine and other aflatoxin metabolites by multiple monoclonal antibody affinity chromatography and immunoaffinity/high performance liquid chromatography. *Cancer Res,* 52:267–274. PMID:1728400

125. Wild CP, Jiang YZ, Allen SJ *et al.* (1990). Aflatoxin-albumin adducts in human sera from different regions of the world. *Carcinogenesis*, 11:2271–2274.doi:10.1093/carcin/11.12.2271 PMID:2265478

126. Wild CP, Hudson GJ, Sabbioni G *et al.* (1992). Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in The Gambia, West Africa. *Cancer Epidemiol Biomarkers Prev,* 1:229–234. PMID:1339083

127. Wang LY, Hatch M, Chen CJ *et al.* (1996). Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan. *Int J Cancer,* 67:620–625.doi:10.1002/(SICI)1097-0215(19960904)67:5<620::AID-IJC5>3.0.CO;2-W PMID:8782648

128. Scholl PF, Turner PC, Sutcliffe AE *et al.* (2006). Quantitative comparison of aflatoxin B1 serum albumin adducts in humans by isotope dilution mass spectrometry and ELISA. *Cancer Epidemiol Biomarkers Prev,* 15:823–826.doi:10.1158/1055-9965.EPI-05-0890 PMID:16614131

129. Wild CP, Hasegawa R, Barraud L *et al.* (1996). Aflatoxin-albumin adducts: a basis for comparative carcinogenesis between animals and humans. *Cancer Epidemiol Biomarkers Prev,* 5:179–189. PMID:8833618

130. Ross RK, Yuan JM, Yu MC *et al.* (1992). Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet*, 339:943–946.doi:10.1016/0140-6736(92)91528-G PMID:1348796

131. Qian GS, Ross RK, Yu MC *et al.* (1994). A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. *Cancer Epidemiol Biomarkers Prev,* 3:3–10. PMID:8118382

132. Tang D, Santella RM, Blackwood AM *et al.* (1995). A molecular epidemiological case-control study of lung cancer. *Cancer Epidemiol Biomarkers Prev,* 4:341–346. PMID:7655328

133. Wang LY, Hatch M, Chen CJ *et al.* (1996). Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan. *Int J Cancer,* 67:620–625.doi:10.1002/(SICI)1097-0215(19960904)67:5<620::AID-IJC5>3.0.CO;2-W PMID:8782648

134. Kuang SY, Frang X, Lu PX *et al.* (1996). Aflatoxin-albumin adducts and risk for hepatocellular carcinoma in residents of Qidong, People's Republic of China. [abstract]. *Proc Am Assoc Cancer Res,* 371:A1216.