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Duration of exposure to environmental carcinogens affects DNA-adduct level in human lymphocytes

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Abstract

Background and objective: An important issue in human biomonitoring is determining how exposure duration affects the kinetics of molecular biomarkers. In this study we compare the influence of exposure variables on DNA adducts.

Methods: DNA adducts were analysed by 32P-postlabelling in lympho/monocytes of 677 Caucasian subjects.

Results: After correction for other variables, DNA adducts increased depending on the length of occupational and smoke exposures. Higher DNA adducts were detected in workers with more than 14 years of exposure than in workers with shorter exposures (RR = 1.19, $p = 0.049$) and in smokers with more than 10 years of exposure than in smokers with shorter exposure (RR = 1.21, $p < 0.001$).

Conclusions: Exposure length is the primary factor affecting DNA-adduct level in lympho/monocytes both in smokers and in occupationally exposed subjects.

Keywords: *Human biomonitoring; DNA adducts; chronic exposure*

Introduction

Environmental exposure to genotoxic agents resulting from lifestyle, infection, natural and occupational exposures, is the primary cause of cancer in humans. However, the precise contribution of specific risk factors and their interaction with genotypes is difficult to determine. A major challenge in molecular cancer epidemiology is to improve exposure assessment by using intermediate biomarkers such as DNA adducts.

Genotoxic carcinogens exert their biological effects by inducing DNA damage. Such damage may result in several molecular DNA lesions including single- or double-strand breaks, apurinic sites and covalent nucleotide modifications, which form stable modified nucleotides called 'adducts'. Adducts are premutagenic lesions leading to errors in DNA replication if not properly repaired by cellular processes. Evaluation of DNA-adduct formation in exposed cells or tissue is a tool for monitoring the mutagenic or carcinogenic potential of an exposure to environmental carcinogens (Phillips 2005). An important research area in human biomonitoring is determining how the duration of exposure affects the kinetics of molecular biomarkers. DNA-adduct dosimetry of human exposure to carcinogens in nucleated blood cells has gained in prominence over recent years. DNA-adduct levels in accessible surrogate tissue provide a means for investigating occupational or environmental exposure to genotoxic agents such as polycyclic aromatic hydrocarbons (PAHs) in healthy individuals. High levels of lipophilic DNA adducts detected several years prior to clinical manifestation

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are reported to be significant predictors of lung cancer risk in smokers (Phillips 2005, Bak et al. 2006) and in non-smokers exposed to air pollution (Peluso et al. 2005). The recent finding that elevated PAH DNA-adduct levels are positively associated with colorectal adenoma contributes to the growing awareness that PAHs are also aetiologically relevant for colorectal carcinogenesis (Gunter et al. 2007). Recent studies have attempted to determine the involvement of environmental carcinogens and tobacco smoke in formation of lipophilic DNA adducts (Phillips 2005, Bak et al. 2006, Peluso et al. 2005). Lipophilic DNA adducts may be considered as markers of cumulative DNA damage as well as of DNA repair capability. During evolution most species have developed an adequate DNA repair mechanism to counteract genomic insults induced by environmental hazards (Hoeijmakers 2001). Most biomonitoring studies examining environmental exposures to complex mixtures use ^{32}P-postlabelling technology (Castano-Vinyals et al. 2004). This method is able to detect DNA adducts with great sensitivity using a very small amount of DNA in a large number of subjects (Georgiadis et al. 2001). DNA adducts are biomarkers of biologically effective doses of carcinogens. Carcinogen DNA adducts represent the amount of environmental carcinogen absorbed by the body, escaping detoxification, bound to cellular macromolecules, and not repaired (Perera et al. 1982). There is strong evidence that the formation of carcinogen DNA adducts reflects exposure to xenobiotics and is a key step in chemical carcinogenesis (Hemminki et al. 2000). Furthermore, DNA adducts also result from indirect pathways such as formation of lipid peroxidation products. Recent reviews on carcinogen DNA-adduct research mention epidemiological studies dealing with the association between increased adduct levels and cancer incidence (Vineis & Perera 2000). Few of these reports take into account the limitations of this research from an epidemiological point of view, due to the short half-lives of adducts, the potential effect of multiple exposures and interactions with genetic susceptibility (Phillips 2005). A factor so far not investigated but important in these studies is the effect of exposure duration on DNA-adduct levels. Typically, human exposures occur at low doses for long periods and to complex combinations of carcinogens. The commonly used method to analyse DNA adducts under these in-field conditions is ^{32}P-postlabelling (Phillips & Arlt 2007). To understand more clearly the relationship between exposure duration and DNA adducts we set up the study presented here aimed at evaluating the influence of exposure length on DNA-adduct level in lympho/monocytes compared with environmental exposure including both smoking and occupation.

Materials and methods

Study design and subjects

The examined population consisted of 677 Caucasian subjects, all resident in Italy, 75% male, recruited from three cross-sectional studies. These studies include: (1) 71 floriculturists exposed to pesticides, 94 traffic officers exposed to air pollutants and 107 control subjects living in the Ligurian region (Merlo et al. 1997, Peluso et al. 1996); (2) subjects living in the province of Taranto including 123 subjects living near a steel plant exposed to airborne PAHs and 173 unexposed controls living in two small rural towns (Alberobello and Locorotondo) (Ferri et al. 2003); (3) 109 control subjects from a lung cancer case–control study (Peluso et al. 2005).

The overall characteristics of the whole examined population are given in Table 1. Recruited subjects were informed about the research aims and signed a written informed consent. Occupational exposure and cigarette smoking habits were assessed using a standardized questionnaire that included items on lifetime occupational history and smoking habits (i.e. number of cigarettes smoked per day, age at start of smoking, years since quitting). The questionnaire form was filled in at the time of blood sample collection.

Lipophilic DNA adducts were measured in DNA from lympho/monocytes (LMF) on blind coded DNA samples using ^{32}P-postlabelling analysis. Samples were processed in two different laboratories (University of Genoa and ISPO Florence) according to the same protocol (Peluso et al. 2005, Merlo et al. 1997, Peluso et al. 1996, Ferri et al. 2003). This study was approved by the Ethics Committee of the National Cancer Research Institute of Genoa, Italy.

Evaluation of exposure to environmental genotoxic agents

Exposure to environmental genotoxic agents, with particular reference to PAHs, was evaluated by questionnaire. Occupational exposures and their duration were divided into four categories: unexposed; exposed for ≤8 years; exposed for >8 and ≤14 years; and exposed for >14 years. Smoking habits were expressed as number of cigarettes per day. Current smokers were defined as subjects who had not stopped smoking within 1 month prior to enrolment in the study. Years of smoking were divided into five decade classes: <10, 10–19, 20–29, 30–39 and ≥40 years. Former smokers were defined as subjects who had stopped smoking more than 1 month before blood sampling.

Lympho/monocyte isolation and DNA extraction

LMF were isolated within 4 h of blood collection. DNA was isolated from cell pellets by enzymatic digestion of RNA and

Table 1. DNA-adduct levels (adducts/10^8 nucleotides): descriptive statistics by study covariates.

		DNA adducts/10^8 nucleotides			
Covariates	*n* (%)	Mean	SD	Median	Range (min–max)
Gender					
Male	508 (75.0)	1.81	2.09	1.29	0.01–13.38
Female	169 (25.0)	1.60	1.73	1.00	0.01–8.16
Age at blood sampling					
≤34 years	146 (21.6)	1.37	1.50	0.93	0.01–7.09
35–38 years	126 (18.6)	2.04	2.03	1.58	0.01–13.38
39–44 years	132 (19.5)	2.10	2.23	1.48	0.01–10.62
45–51 years	134 (19.8)	1.98	1.89	1.75	0.01– 9.99
>51 years	139 (20.5)	1.35	2.23	0.45	0.01–13.10
Occupational exposure					
Unexposed	305 (45.1)	1.56	2.09	0.97	0.01–13.10
≤8 years	89 (13.2)	2.07	1.96	1.72	0.01–9.99
9–14 years	85 (13.6)	1.69	1.38	1.51	0.01–5.01
>14 years	89 (13.2)	1.75	2.25	1.17	0.01– 9.99
Unknown	109 (16.1)	2.11	1.97	1.64	0.01–13.38
Smoking habits					
Never smoker	271 (40.0)	1.62	2.07	0.97	0.01–13.38
Former smoker	174 (25.7)	1.52	1.78	1.06	0.01–12.90
Current smoker	232 (34.3)	2.10	2.06	1.81	0.01–13.10
Whole group	677	1.76	2.01	1.20	0.01–13.38

proteins followed by solvent extraction (Peluso et al. 1996, Ferri et al. 2003). DNA purity was evaluated by spectrophotometric analysis obtaining 260/230 nm and 260/280 nm absorbance ratios around 2.3 and 1.7, respectively.

DNA-adduct analysis by 32P-postlabelling

Lipophilic DNA adducts were measured in LMF by the nuclease P1-enhanced ^{32}P-postlabelling method as previously detailed (Peluso et al. 2005, Merlo et al. 1997, Peluso et al. 1996, Ferri et al. 2003).

Briefly, DNA (5 μg) was depolymerized for 3.5 h at 37°C by incubation with micrococcal nuclease (0.04 U μg^{-1} DNA) and spleen phosphodiesterase (1 mU μg^{-1} DNA). Adducts were enriched by nuclease P1 digestion and ^{32}P-postlabelled by incubation for 40 min at 24°C with polynucleotide kinase (8 U) and 80 μCi of carrier-free [γ-^{32}P]-ATP with a specific activity ≥7000 Ci $mmol^{-1}$. Thin-layer chromatography was carried out according to the standard procedure (Peluso et al. 1996, Ferri et al. 2003, Gupta 1995, Izzotti et al. 1995) using urea 7.5 M in D3 and D4 developments.

Autoradiography was performed by using a ^{32}P InstantImager electronic autoradiographic system equipped with the InstantQuant software (model A2024; Packard, Meriden, CT, USA). The relative adduct levels were calculated for each sample and expressed as DNA adducts/10^8 nucleotides. The total DNA adduct amount resulting from the sum of both individual adducts and diagonal radioactive zone was calculated. Each DNA sample was tested by ^{32}P-postlabelling at least twice and the average value calculated. A benzo(*a*)-pyrene diolepoxide-N_2-dGp reference standard (National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO, USA) was used as a positive control in each labelling experiment. DNA free samples were used as negative controls.

Statistical analysis

Adduct levels were treated in the analyses both as continuous and categorical variables (positive or non-detectable). Positive subjects were those with adduct levels >1 adducts/10^8 nucleotides. Multivariate regression analysis was used to assess the influence on DNA adducts of the selected variables including smoking habits, gender, age, occupational exposure and exposure length. Multivariate regression analysis was performed using negative binomial distribution. The results were expressed as rate ratios (RR), i.e. the ratio of the median level of DNA adducts in the variable category relative to those observed in the reference category, as adjusted for all variables included in the statistical model. The estimated RR was tested for significance using the Wald test.

All statistical analyses were performed using Stata Statistical Software (Stata Statistical Software 7.0, Stata Corporation, College Station, Texas, USA).

Results

Table 1 shows the distribution of DNA-adduct levels in the examined population (n = 677) by sex, age, smoking habits

and duration of occupational exposure. The median level of lipophilic DNA adducts was 1.87-fold higher in current smokers (median 1.81 adducts/10^8 nucleotides) than in subjects who never smoked (median 0.97 adducts/10^8 nucleotides). DNA adduct level was similar in former smokers (median 1.06 adducts/10^8 nucleotides) compared with never smokers. The median adduct level measured in subjects occupationally exposed (independent of duration) was 1.26 adducts/10^8 nucleotides (range 0.01–9.99) (data not shown) and 0.97 adducts/10^8 nucleotides (range 0.01–13.1) in unexposed subjects (Table 1). No effect of sex and age on DNA-adduct levels was observed. Data concerning the influence of specific exposures other than cigarette smoke on DNA adducts for each exposure conditions can be inferred from previously published studies (i.e. Merlo et al. 1997, Peluso et al. 1996 for floriculturists and traffic officers; Ferri et al. 2003 for residents in the nearby of a steel plant).

Table 2 shows the DNA-adduct RR estimated by multivariate regression analysis by independent covariates. The increase in DNA-adduct levels was related to the length of occupational exposure; a significantly increased rate ratio (RR = 1.19, 95% confidence interval (CI) 1.01–1.42) was observed in the group of workers with occupational exposure longer than 14 years, compared with unexposed subjects. The estimated RR for subjects with unknown duration of exposure ($n = 109$, data not shown) was 1.15 (95% CI 0.86–1.61).

DNA adducts significantly increased with smoking in subjects who had smoked for 10 or more years (Table 2). RRs estimates, adjusted for the number of cigarettes smoked per day and age at start of smoking, indicated a progressive DNA-adduct increase with increasing the years of smoking. No significant effect of smoking status (apart from duration, as above reported) on DNA-adduct formation was observed in current smokers compared with former and never smokers (RR = 1.12, 95% CI 0.86–1.48) and no relationship was detected between the number of cigarettes smoked per day or age at start of smoking (Table 2).

Tables 3 and 4 report RRs for current and former smokers, respectively. This analysis evaluates the effect on DNA adducts of smoke-related variables including the number of cigarettes smoked per day, age at start of smoking, years since quitting smoking (for former smokers only) and smoking duration. Duration of smoking was the main factor affecting DNA-adduct levels in both current and former smokers. The detected relationship between years of smoking and DNA-adduct levels in current and former smokers was statistically significant with RR increasing with the increasing number of years of smoking. A dose–response relationship was observed between DNA-adduct formation and years of smoking after adjustment for number of cigarettes smoked per day and age at start.

Years since quitting had an effect on the DNA-adduct decrease, although to a borderline statistically significant level (RR = 0.98, 95% CI 0.93–1.00, $p = 0.07$).

Discussion

Results of this study provide evidence that DNA-adduct levels in blood LMF are related to both duration of cigarette smoking and length of occupational exposure with a stronger significant association with smoking duration.

Table 2. DNA-adduct rate ratio (RR)[a] estimated by multiple regression analysis (whole group).

Covariates	RR	95% CI	WT	*p*-Value
Occupational exposure				
Unexposed	1.00	Reference		
Exposed ≤8 years	1.14	0.82–1.58	0.62	0.430
Exposed 9–14 years	1.03	0.93–1.15	0.34	0.559
Exposed >14 years	1.19	1.01–1.42	3.85	0.049
Smoking habits				
Former/never smokers	1.00	Reference		
Current smokers	1.12	0.86–1.48	0.71	0.403
No. of cigarettes per day	1.00	0.98–1.02	0.03	0.860
Age at start smoking	1.01	0.99–1.04	0.77	0.378
Years of smoking				
<10 years	1.00	Reference	18.06	<0.001
10–19 years	1.21	1.12-1.32		
20–29 years	1.48	1.24-1.77		
30–39years	1.81	1.38-2.37		
≥40 years	2.21	1.54-3.17		
Mean deviance = 0.994 (df = 656)				

[a]Adjusted by sex, age at blood sample, study population and seasonality.
RR, DNA-adduct rate ratio; 95% CI, 95% confidence interval of the RR;
WT, Wald test; Reference, reference category; Mean deviance, model χ^2/df; df, degrees of freedom of the regression model.

Table 3. DNA-adduct rate ratio (RR)[a] estimated by multiple regression analysis among the subgroups of current and never smokers ($n = 503$).

Covariates	RR	95% CI	WT	*p*-Value
Smoking habits				
Never smokers	1	Reference		
Current smokers	1.11	0.80–1.56	0.42	0.515
No. of cigarettes per day	1.00	0.98–1.02	0.06	0.800
Age at initiation	1.02	0.99–1.04	2.10	0.146
Years of smoking				
<10 years	1	Reference	16.08	<0.001
10–19 years	1.25	1.13–1.41		
20–29 years	1.57	1.27–2.00		
30–39 years	1.98	1.43–2.80		
≥40 years	2.48	1.61–3.95		

[a]Adjusted by sex, age at blood sample, study population and seasonality.
WT, Wald test; Reference, reference category.

Table 4. DNA-adduct rate ratio (RR)[a] estimated by multiple regression analysis among the subgroups of former and never smokers ($n = 445$).

Covariates	RR	95% CI	WT	*p*-Value
Smoking habits				
Never smokers	1	Reference		
Former smokers	1.03	0.88–1.19	0.10	0.751
No. of cigarettes per day	0.98	0.95–1.02	0.76	0.383
Age at initiation	1.01	0.96–1.07	0.27	0.606
Years since quitting	0.98	0.93–1.00	3.28	0.070
Years of smoking				
<10 years	1	Reference	6.15	0.013
10–19 years	1.20	1.03–1.37		
20–29 years	1.43	1.06–1.88		
30–39 years	1.71	1.09–2.57		
≥40 years	2.04	1.13–3.53		

[a]Adjusted by sex, age at blood sample, study population and seasonality.
WT, Wald test; Reference, reference category.

The relationship between cigarette smoking and DNA adducts in LMF has previously been explored by other studies using a limited numbers of subjects (Phillips 2002). Lipophilic DNA adducts are biomarkers of exposure to aromatic compounds and of the individual ability to metabolically activate carcinogens and to repair DNA damage. In a recent meta-analysis a weakly but statistically significant increase in lung cancer risk was evident for subjects bearing high DNA-adduct level in LMF, this association being evident only in current smokers but lacking in former and never smokers (Veglia et al. 2008).

DNA damage primarily reflects exposure to carcinogens but is also modulated by inherited and acquired susceptibilities. Age, sex, physical exercise, consumption of charcoal-broiled food and intake of fresh fruits and vegetables have been reported to influence DNA-adduct levels (Peluso et al. 2001, Vineis et al. 2000, Palli et al. 2004). DNA damage may be repaired and DNA adducts removed, a high interindividual variability affecting the rate of this event (Berwick & Vineis 2000, Matullo et al. 2003).

Many factors contribute to formation of DNA adducts as detected by ^{32}P-postlabelling, which is a very sensitive but unspecific methodology. Arif et al. (2006) demonstrated that the adducts determined in a diagonal radioactive zone are mostly from an endogenous origin. Accordingly, the most realistic explanation for the exposure length-related DNA-adduct increase we observed is that with increasing time of exposure to cigarette smoke or to unhealthy workplace conditions adduct repair becomes less efficient than in controls thus leading to higher adducts levels. In fact, it is highly unrealistic that the small percentage of long-living lymphocytes which accumulate DNA adducts would lead to an overall increase in the whole LMF fraction analysed in this study.

Tobacco smoking is recognized as the primary preventable cause of human cancer. Therefore, many studies have explored the influence of smoking on DNA-adduct formation in nucleated blood cells in order to identify an early and sensitive biomarker of effective intake of tobacco carcinogens (Phillips 2002). However, these emerging data are sometimes difficult to decode because of differences in DNA-adduct measurement techniques and analysed cell types as well as data collected under conditions of high occupational

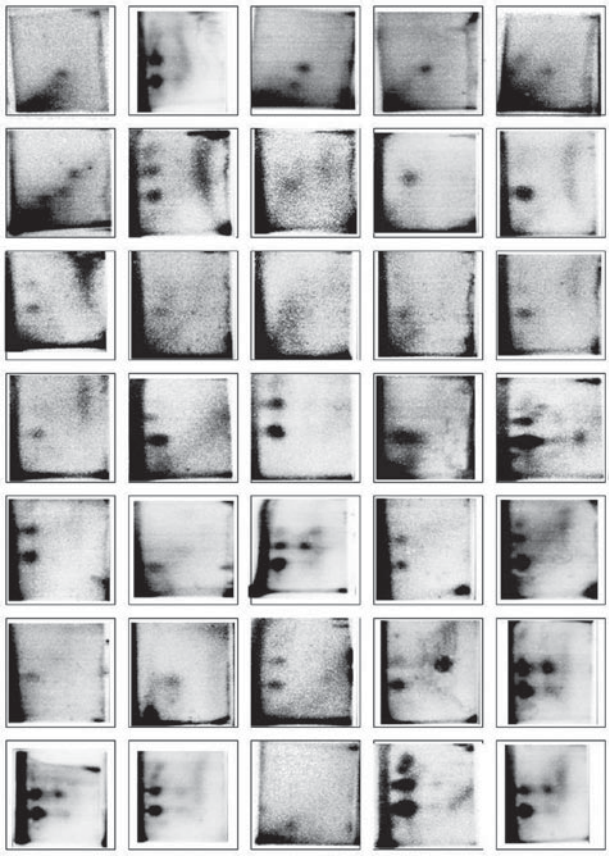

Figure 1. Examples of DNA adducts as detected by ^{32}P-postlabelling in 35 lymphocyte samples. Reported autoradiograms were obtained from residents in the nearby of a steel plant (study 2, see Ferri et al. 2003 for details).

exposure or with small sample sizes, often fewer than 50 subjects. Two independent prospective studies (Peluso et al. 2005, Tang et al. 2001) have indicated that DNA-adduct dosimetry in nucleated blood cells can identify individuals with a high probability of developing cancer. However, a review study (Phillips 2002) reported both positive and no association of adduct dosimetry with developing cancer. High DNA-adduct levels in nucleated blood cells are reported to be predictors of lung cancer risk in current smokers (Perera et al. 2002) and, more recently, also in non-smokers exposed to air pollution (Husgafvel-Pursiainen 2004).

Wiencke et al. (1999) reported that smoking during adolescence produces physiological changes leading to increased DNA-adduct persistence and individuals who begin smoking early in life tend to be heavier smokers. An epidemiological study reported that duration is more important than intensity of cigarette smoking in predicting lung cancer risk (Perera et al. 1982). Our results provide evidence of this phenomenon at the molecular level thus shedding light on the mechanisms involved in the aetiology of smoking-related cancers. Lipophilic DNA adducts result from genotoxic absorption, activation and detoxification, as well as from DNA repair and tissue turnover.

In our study DNA-adduct increase was detected in subjects with 14 or more years of occupational exposure and 10 or more years of cigarette smoking. Accordingly, exposure duration appears to be more important than intensity in affecting DNA-adduct levels. Increase of DNA adducts were detected after 10 years for smoking and 14 years for occupational exposure. This difference is probably related to the fact that occupational exposure occurs at lower doses than cigarette smoke exposure.

The higher levels of lipophilic DNA adducts in cancer cases compared with controls have been related to the accumulation of unrepaired DNA damage overwhelming the repair processes (Wild & Pisani 1998, Veglia et al. 2008). Therefore, lipophilic DNA adducts result both from exposure and metabolism of genotoxic compounds, as well as from failure in DNA repair. This interpretation is supported by *in vitro* observations reporting that DNA damage in peripheral blood leukocytes collected from subjects exposed to styrene oxide, is affected by the duration of occupational exposure (Godderis et al. 2004). Furthermore, this interpretation is consistent with the current knowledge on the importance of exposure duration in the aetiology of chemically induced cancers (Shuck et al. 2008, Doll & Peto 1978).

The observed lack of difference between DNA-adduct levels in current smokers and non-smokers supports the evidence that DNA adducts in LMF are not a highly sensitive biomarker for monitoring exposures to cigarette smoke. In fact, DNA adducts significantly increase only after 10 years of smoking or 14 years of occupational exposure. Thus, on the whole, LMF DNA adducts progressively increase with the duration of both occupational and smoking exposures. Years since quitting smoking are associated with a progressive decrease in LMF DNA adducts.

DNA-adduct kinetics are affected by the lifespan of adduct-bearing cells. In chronic exposures to genotoxic agents, such as smoking and occupational exposures, the long lifespan of lymphocytes results in progressive DNA-adduct accumulation. A plateau, representing a dynamic equilibrium between DNA-adduct formation and removal, is ultimately reached, as demonstrated in various organs of rodents exposed to cigarette smoke (Hemminki et al. 1990, Izzotti et al. 1999). In this study, DNA-adduct increase was observed only after more than 10–14 years of exposure, a time window exceeding the lifespan of LMF cells analysed in our study. Accordingly, it is conceivable that some mechanisms involved in DNA-adduct removal are altered as a consequence of long-lasting exposures, such as a progressive deficiency in DNA repair pathways. Chronic exposure to toxins may progressively damage DNA repair capacities by inducing a progressive accumulation of mutations in genes

encoding for DNA repair activities. Therefore, the analysis of DNA adducts in blood lymphocytes is not only an indicator of exposure but also an indicator of DNA repair capability (Hemminki et al. 1990). This interpretation is supported by experimental animal studies demonstrating that the kinetic of DNA-adduct removal is affected by exposure duration. In fact, 1 week after smoking cessation DNA adducts decreased in rodents exposed for 1 month (Izzotti et al. 1999) while no decrease was detected in rodents exposed for 1 year (Izzotti et al. 2006).

Cigarette smoke is known to induce progressive damage in tumour suppressor genes involved in DNA repair activities, as specifically demonstrated for p53 (Izzotti et al. 2004). In addition, not only DNA repair but general defence mechanisms against radicals become lower with age leading to an increase of both endogenous and exogenous DNA adducts resulting from environmental exposures.

In conclusion, the present study highlights that duration of the exposure is a crucial factor increasing DNA-adduct level in LMF. These findings help to explain why lung cancer risk increases linearly with the number of smoked cigarettes and exponentially with smoking duration.

Declaration of interest

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