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Genotoxicity of aldehyde mixtures: profile of exocyclic DNA-adducts as a biomarker of exposure to tobacco smoke

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ABSTRACT

Electrophilic compounds present in humans, originating from endogenous processes or pollutant exposures, pose a risk to health though their reaction with nucleophilic sites in protein and DNA. Among this chemical class, aldehydes are mainly present in indoor air and they can also be produced by endogenous lipid peroxidation arising from oxidative stress. Known to be very reactive, aldehydes have the ability to form exocyclic adducts to DNA that, for the most if not repaired correctly, are mutagenic and by consequence potential agents involved in carcinogenesis. The aim of this work was to establish profiles of exocyclic DNA adducts induced by aldehyde mixtures, which could ultimately be considered as a genotoxic marker of endogenous and environmental aldehyde exposure. Adducts were quantified by an accurate, sensitive and validated ultra high performance liquid chromatography-electrospray ionization analytical method coupled to mass spectrometry in the tandem mode (UHPLC-ESI-MS/MS). We simultaneously measured nine exocyclic DNA adducts generated during the exposure *in vitro* of calf thymus DNA to different concentrations of each aldehyde along, as well as, to an equimolar mixture of these aldehydes. This approach has enabled us to establish dose-response relationships that allowed displaying the specific reactivity of aldehydes towards corresponding adducts formation. Profiles of these adducts determined in DNA of current smokers and non-smokers blood samples supported these findings. These first results are encouraging to explore genotoxicity induced by aldehyde mixtures and can furthermore be used as future reference for adductomic approaches.

1. Introduction

Human beings are chronically exposed to a variety of endogenous and exogenous compounds generated in response to various stresses (lifestyles, dietary, oxidative stress, inflammation, environmental, microbial, hormonal and genetic background) that can lead by their interactions to cellular and molecular modifications, predisposing in turn to the development of chronic disease including cancer. This "Whole-life" approach has been proposed by CP Wild and SM Rappaport under the name "exposome" to understand relationships between disease and life-time exposures to chemicals entering the body and internally produced (Wild, 2005; Rappaport and Smith, 2010). But today, the characterization of the exposome throughout the whole lifespan and the assessment of the causal linkages between chemicals exposures, both internally and environmental, and effect adverse remains an unresolved challenge.

Aldehydes are part of toxic contaminants that are ubiquitously present in the individual's environment and can also be endogenously generated. High levels of aldehydes have already been measured in urban and industrial pollution, biomass combustion, food, household atmosphere, and cigarette smoking (IARC, 2012). In the body, these toxic products can be also produced by lipid peroxidation, inflammation, and natural/xenobiotic metabolism (Sosa et al., 2013). Lipid peroxidation, which is one of the consequences of oxidative stress, remains the main process producing aldehydes. When the production of reactive oxygen species (ROS) exceeds natural antioxidant defense mechanisms, oxidative degradation of polyunsaturated fatty acids can occur, resulting in cell membrane damage and cell death. The final lipid

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peroxidation step is the formation of unstable hydroperoxides that in turn produce the electrophilic α,β-unsaturated highly reactive aldehydes, notably acrolein (Acro), crotonaldehyde (Croto), malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), glyoxal (Gx) and methylglyoxal (MG). With formaldehyde (FA) and acetaldehyde (AA), most of them can be found in the air with the exception of MDA and HNE that are mainly formed by the endogenous oxidative pathway and inflammation. Regarding aldehydes by airborne exposure, these products have been shown to be also capable of inducing oxidative stress through lipid peroxidation in both humans and animals (Wang et al., 2013; Lima et al., 2015). It is for this reason that they are considered as markers of lipid peroxidation and inflammation in certain physiopathological contexts (Voulgaridou et al., 2011).

The aldehydes have cytotoxic and genotoxic properties (Forman, 2010; Tudek et al., 2017). In fact, they are chemically unstable agents that possess a high electrophilic reactivity allowing them to interact directly with nucleic acids and proteins. DNA damage without efficient repair is thought to contribute to carcinogenesis through mutations, genome instability, and perturbed signaling. Several studies have reported that lipid peroxidation-derived aldehydes with their exocyclic adducts, can induce mutagenic lesions *in vitro* and in human tissues (Langouët et al., 1998; Maekawa et al., 2006; Chou et al., 2010). The level of exocyclic DNA adducts, induced by endogenous sources under normal and pathological conditions *in vivo*, is approximately 0–20 lesions per 10^8 normal nucleotides (Medeiros, 2009). These levels were found to be highly variable and to be affected by lifestyle, the dietary intake of antioxidants and persistent chronic infection or inflammation (De Bont and Van Larebeke, 2004). A high consumption of fatty acids has been shown to cause an increase in propano-, etheno- and MDA-derived adducts in human leukocyte DNA in woman but not in men (Fang et al., 1996; Nair et al., 1997).

The reaction of α,β-unsaturated aldehydes with DNA bases yields cyclic-substituted propano adducts such as 1,N^2-propano-2′-deoxyguanosine. Background levels of propano adducts have been detected in DNA from different rodent and human tissues (Chung et al., 2000; Chen, 2011).

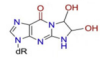
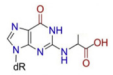
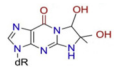
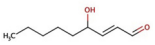
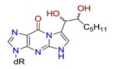
In the context of carcinogenetic risk exposure, the analysis of exocyclic DNA adducts may contribute to understanding the oxidative stress-related cancer process but also may be relevant as exposure/effect markers for the studies related to aldehydes exposures (Nair et al., 2007).

In order to assess exposure to a carcinogenic risk and in the context of biomonitoring, the quantification of aldehydes in biological matrices is difficult because of their high reactivity and short half-lives, hence the interest in analysis of stable exocyclic DNA adducts.

Current advances are given to the adductomic approach to detect and identify DNA damage in human (Kanaly et al., 2006; Carlsson and Törnqvist, 2017). Several exocyclic DNA adducts have been already identified by this strategy in human tissues (Chou et al., 2010; Matsuda et al., 2013; Li et al., 2017). They were identified and measured by ultra high performance liquid chromatography-electrospray ionization coupled with tandem mass spectrometry (UHPLC-ESI-MS/MS) an approach that has made a great advance in detection of multiple DNA adducts simultaneously using addductomic approach. Currently researchers have been able to identify various lipid peroxidation-derived DNA adducts in human tissues and show a relationship between amounts/profile and the risk of cancer.

In our approach we opted for a pre-selection of DNA adducts induced by the aldehydes mainly present in tobacco smoke and those induced endogenously by lipid peroxidation. All the aldehydes presented in Table 1 can induce a set of potentially mutagenic exocyclic DNA adducts (Maekawa et al., 2006; Nair et al., 2007). Thus, our recently validated UHPLC-ESI-MS/MS method aimed for the simultaneous detection and quantification of nine selected exocyclic DNA adducts (Alamil et al., 2020). Our analytical strategy was designed to establish exocyclic DNA adducts profiles for smokers and non-smokers in a preliminary study following comparison to the variations of expression of this profile in the context of an exposure *in vitro* to a single aldehyde or an equimolar mixture of aldehydes known to be produced by lipid peroxidation.

Table 1
Structures and names of eight aldehydes and their corresponding adducts.

Aldehyde	Aldehyde structure	Adduct	Adduct structure*
Malondialdehyde (MDA)		Reduced MDAdG	
Glyoxal (Gx)		GxdG	
Methylglyoxal (MG)		CEdG	
		cMGdG	
Formaldehyde (FA)		Reduced FAdG	
Acetaldehyde (AA)		Reduced AAdG	
Crotonaldehyde (Croto)		CrotodG	
Acrolein (Acro)		AcrodG	
4-Hydroxy-2-nonenal (HNE)		HNEdG	

* dR: deoxyribose.

2. Experimental Procedures

2.1. Chemicals and solutions

Acrolein, crotonaldehyde, acetaldehyde, formaldehyde (37% in water), glyoxal solution (40% in water), pyruvaldehyde solution (40% in water), 4-hydroxynonenal-dimethyl acetal (HNE-DMA), 1,1,3,3-tetraethoxypropane, sodium cyanoborohydride ($NaBH_3CN$) and calf thymus DNA were all purchased from Sigma-Aldrich (Steinheim, Germany). Potassium phosphate monobasic (KH_2PO_4) was procured from Fluka (Steinheim, Germany). The DNA extraction kit Nucleobond® CB 100 was purchased from Macherey-Nagel (Duren, Germany). All solvents were HPLC MS grade obtained from VWR (Kelsterbach, Germany). 4-Hydroxy-2-noneal solution was prepared from HNE-DMA following the instructions of the supplier company (Sigma-Aldrich). Malondialdehyde solution was obtained by hydrolysis of 1,1,3,3-tetraethoxypropane. DNA calibration standards and quality control samples were spiked as described in our previous work (Alamil et al., 2020) with nine stable adducts standards: AcrodG, CrotodG, reduced AAdG, reduced FAdG, reduced MDAdG, HNEdG, GxdG, CEdG and cMGdG (structures represented in Table 1) and, their isotopically labeled homologues prepared by reaction of each aldehyde with 2′-Deoxyganosine (dG) and [$^{13}C_{10}$,$^{15}N_5$] dG.

2.2. Human samples

Human blood samples, from five smokers before smoking cessation and five non-smokers, were obtained from Tabacology Unit at CHU UCL Namur asbl, Belgium. Ethical approval for the study was obtained from the ethical committee of CHU UCL Namur Godinne (NUB: B039201316167). Venous blood was collected in a BD Vacutainer® spray-coated K_2EDTA tube. Immediately after collection, the tubes were

Table 2
Different conditions of adducts induced by *in vitro* exposure of ctDNA to aldehydes.

Aldehyde	Aldehyde concentrations (mM)	PB concentration and pH	References
Acro	0.005; 0.05; 0.5; 1	25 mM, pH 7	Kawai et al., 2003
Croto	0.005; 0.05; 0.5; 1	25 mM, pH 7	Chung et al., 1989; Budiawan et al., 2000
AA	0.005; 0.05; 0.5; 1	25 mM, pH 7	Wang et al., 2000; Singh et al., 2009
FA	0.005; 0.05; 0.5; 1	25 mM, pH 7	Arif et al., 2006
MDA	0.005; 0.01; 0.05; 0.1; 0.5; 1	100 mM, pH 4.3	Vaca et al., 1992
HNE	0.005; 0.05; 0.5; 1	25 mM, pH 7	Douki and Ames, 1994
Gx	0.005; 0.05; 0.5; 1	100 mM, pH 7	Kasai et al., 1998; Olsen et al., 2005
MG	0.005; 0.05; 0.5; 1	50 mM, pH 9.5	Dennehy and Loeppky, 2005; Shuck et al., 2018

placed and then stored at -80 °C until the DNA was extracted using Macherey-Nagel kit (Macherey-Nagel, Nucleobond® CB 100). Along with the blood samples, four tobacco smoking-related markers were collected as follows: number of cigarettes/day, delay of the last cigarette smoked, measurement of CO level in exhaled air by electrochemistry (Smokerlyser Micro +, SineFuma, Breda, The Netherlands), and urinary cotinine concentration measured by UHPLC with a photodiode array detector (Waters Acquity UPLC H-Class system, Waters Association) and a data acquisition and processing module (Empower 2 Software, Waters Association, Milford, MA, USA).

2.3. Treatment of calf Thymus DNA with aldehydes

In 600 µL Eppendorf® tubes, calf thymus DNA (ctDNA, 1 mg/mL) was modified by reaction with different concentrations ranging from 0.005 to 1 mM of each aldehyde in phosphate buffer (PB) at 37 °C for 24 h under constant stirring. The different experimental conditions chosen according to previous studies are described in Table 2. Similarly, ctDNA (1 mg/mL) was also incubated with an equimolar mixture (1 mM) of the eight aldehydes in 25 mM PB (pH 7). The modified DNA was precipitated by adding sodium chloride (5 M) and cold ethanol then centrifuging at 10,000 rpm for 10 min at 4 °C. The DNA was washed with 70% ethanol and centrifuged once again. Following the supernatant discarding, the pellet was evaporated to dryness on Speedvac®.

2.4. Adducts analysis by liquid chromatography tandem mass spectrometry

DNA extracted from 10 human blood samples and ctDNA modified by the aldehydes taken separately or in a mixture were treated then analyzed on UHPLC-ESI-MS/MS as described in our previous work (Alamil et al., 2020). Briefly, the DNA was dissolved in adjusted volume of ultrapure water to obtain a final concentration of 1 mg/mL, reduced by addition of $NaBH_3CN$ then, enzymatically hydrolyzed in the presence of isotopically labeled internal standards. Following hydrolysis, modified nucleosides and samples were analyzed on UHPLC-ESI-MS/MS according to our recently developed and validated method. The analytical column used for analytes separation was a reversed phase Acquity C_{18} UPLC® HSS C18 SB 1.0 mm × 150 mm, 1.8 µm (Waters, Ireland). The separation system interfaces with a triple quadrupole MS (LCMS–8030Plus, Shimadzu). The electrospray ionization source (ESI) was set in the positive ion mode and mass spectrometer parameters were the same as described in our previous study. MRM mode was carried out to detect and quantify adducts. The amount of each DNA adduct was quantified according to a matrix-matched calibration curve obtained by plotting peak area ratio of each analyte to its corresponding labeled IS *versus* standard concentrations.

The level of DNA adducts was calculated using the following formula (Li et al., 2017):

$$\text{Relative adduct level} = \frac{(\text{DNA adduct Concentration / MW of DNA adduct})}{(\text{DNA Concentration / mean MW of bases})}$$

The results obtained were multiplied by 10^7 and the levels of adducts were expressed in adducts per 10^7 normal nucleotides.

2.5. Statistical data analysis for human blood DNA

Statistical evaluation of human blood DNA data was performed using Student *t*-test or Wilcoxon test (SAS software version 9.4) for comparison between adducts levels in smokers and non-smokers DNA. The level of statistical significance was $p < 0.05$. Pearson and Spearman correlations were used for establishing correlations between adducts levels and the tobacco smoking-related markers.

3. Results and discussion

One strategy to address identification of smokers who have higher cancer risk is to establish and validate smoking-related biomarkers associated with tobacco exposure and cancer risk. The aim of this work is to assess profiles of exocyclic DNA adducts induced by aldehyde mixtures which could ultimately be considered as genotoxic markers of aldehydes exposure from tobacco smoke as well as endogenous sources. We applied our previously developed UHPLC-ESI-MS/MS method (Alamil et al., 2020) to detect and quantify profiles of exocyclic DNA adducts in ctDNA modified by aldehydes *in vitro*, as well as in genomic DNA isolated from smokers and non-smokers blood samples.

3.1. Analysis of ctDNA treated with single aldehyde

UHPLC-MS/MS analysis of untreated ctDNA revealed the presence of a basal level of adducts induced by Acro, AA, FA and HNE. Therefore, levels of AcrodG, HNEdG, as well as both reduced AAdG and FAdG, could only be measured starting at incubation concentrations of 0.05 mM. Whereas, for the other aldehydes (Croto, MDA, Gx and MG), the increase in the level of induced adducts was noticed from the incubation concentration of 0.005 mM.

In vitro treatment of ctDNA with different concentrations of aldehydes (0.005–1 mM) for 24 h in phosphate buffer maintained at 37 °C gave rise to linear dose-response curves for the formation of their stable adducts (*Supplementary Data* Fig. 1–9). The concentrations were chosen taking into account the physiological concentrations already reported in humans.

For HNE, mainly produced endogenously, it was found at concentrations about 0.05 − 0.15 µM physiologically in many tissues as well as in serum (Esterbauer et al., 1991). In many pathological situations and close to the lipid peroxidation sites, its concentration can be greatly increased to more than 100 µM (Smathers et al., 2012). In the literature, it is often mentioned that at these concentrations the risk of adduction to DNA - the promutagenic activity of HNE – is more frequent (Dalleau et al., 2013).

The endogenous concentration of FA in the human blood of unexposed subjects was reported around 0.1 mM (IARC, 2006). In our study, this concentration showed the formation of adducts during the reaction of ctDNA with the FA. Likewise, the AA concentration measured in the whole blood of four normal fasting human subjects (IARC, 1985) stood at 1.30 µM. In our *in vitro* approach, we could not reach

Table 3
Tobacco smoking-related markers and adducts levels in smokers blood DNA per 10^7 normal nucleotides with the standard deviations for n = 3.

Human blood DNA sample no.	S1		S2		S3		S4		S5	
Sex	M		F		M		F		M	
Age	40		42		61		55		40	
Smoking status	Smoker		Smoker		Smoker		Smoker		Smoker	
Number of cig/day	20		15		25		30		13	
Timing from last cig smoked (h)	2		1		3		2.5		2.25	
CO (ppm)	11		19		12		16		32	
Cotinine/Creatinine	1202.53		2686.98		487.18		3003.04		2314.56	
AcrodG	2.68	± 0.54	6.90	± 1.02	4.79	± 0.48	4.13	± 0.99	11.36	± 3.33
CrotodG	48.71	± 3.35	47.51	± 7.38	47.97	± 2.58	76.90	± 2.29	39.33	± 5.66
Reduced AAdG	3.94	± 0.44	5.96	± 1.51	19.75	± 7.01	11.30	± 0.68	9.15	± 1.43
Reduced FAdG	12.66	± 0.53	21.06	± 2.78	12.60	± 2.64	25.25	± 1.55	17.42	± 2.56
Reduced MDAdG	7.38	± 1.54	10.61	± 3.09	25.26	± 7.87	26.21	± 2.87	12.98	± 2.09
HNEdG	1.24	± 0.21	1.43	± 0.01	n.q.		1.73	± 0.23	1.08	± 0.07
GxdG	108.13	± 17.53	163.78	± 15.64	83.55	± 3.21	224.25	± 16.41	345.42	± 22.20
CEdG	148.56	± 18.93	194.00	± 17.84	88.47	± 4.92	254.76	± 26.32	127.86	± 33.73
cMGdG	501.12	± 47.68	506.27	± 80.32	381.23	± 41.76	649.19	± 43.40	547.32	± 52.85

n.q.: not quantifiable.

such a low concentration, since our experimental conditions did not allow it. Other authors have attempted to measure levels of reduced AAdG, by a LC–MS/MS system equipped with an online column switching valve, after reaction of ctDNA with 0.001–100 mM of AA. They observed a similar linear response only starting at 0.5 mM of AA thus, less sensitivity comparing to our method (Singh et al., 2009).

Regarding Gx and MG adducts, the results are consistent with previous studies that showed ascending dose-response for adducts formation after treatment of 1 mg/mL of ctDNA with rising concentrations up to 100 mM of Gx and MG for 15 h (Dennehy and Loeppky, 2005; Thornalley et al., 2010).

In the literature, MDA-induced adduct was rather studied in its unreduced form M_1dG than the reduced form. We verified that the reduction does not influence the type of response. In fact, we had the same type of curve with or without reduction (data not shown). MDA was found at concentrations of 3.42 ± 0.94 µM in the men's serum and 3.10 ± 0.62 µM in the women's serum (IARC, 1985). Even if these concentrations are slightly lower than the MDA concentrations studied in our cell-free model, they remain very close.

These first results allowed us to show that our experimental conditions maintain a good sensitivity which is likely to detect low levels of endogenously induced adducts. Thus, these findings enable us to study exocyclic adducts which were formed *in vitro* at levels close to those detected in human tissues at physiological and toxic conditions. Even if, overall, an increase in the genotoxic burden of these products is observed with ascending concentrations, the type of aldehyde and the exposure conditions (aldehyde concentration, incubation time…) remain the parameters which strongly influence the adducts levels.

3.2. Effect of aldehyde mixtures on the formation of ctDNA-adducts

The effect of aldehyde mixtures on the formation of dG-adducts was further investigated at an equimolar concentration of 1 mM. Mostly and as expected, the concentrations of the adducts formed by the equimolar mixture prepared at 1 mM, were either much lower than those induced by each aldehyde taken separately or even absent (*Supplementary Data* Table 1). This finding could be explained by an inhibitory effect of DNA adduction when the aldehydes are mixed. We suggest that the aldehydes reacted with each other to form a new product with weaker or even absent electrophilicity towards DNA, or on the contrary, an electrophilic activity deported to other bases of DNA which were not sought in our study. Aldehydes have also been shown to be able to bind covalently with adenine, cytosine and thymine (Kawai et al., 2003; Fu et al., 2014). The absence of FA-induced dG adducts *in vitro* can be justified by the fact that the FA is more likely to form cross-links when it is incubated with ctDNA in the presence of AA (Cheng et al., 2003; Yu et al., 2015). Likewise, the very low concentration of reduced MDAdG tends to suggest strong competition between aldehydes towards dG. Esterbauer et al. (1991) reported a decrease in the reactivity of MDA compared to α,β-unsaturated aldehydes under physiological conditions. In parallel, a reaction between aldehydes might be possible to form other adducts, not studied in our approach (Nielsen and Houlihan, 1968; Voulgaridou et al., 2011).

These first results *in vitro* reveal that the aldehyde mixture in liquid medium preserves an electrophilic reactivity towards DNA and in particular dG. This means that in a biological matrix, such as blood or other tissues, if these products are not degraded, they can interact with each other, but also directly with other macromolecules present in the cell, especially the human genomic DNA. The next step was to explore the genotoxic potential of aldehydes in DNA extracted from the blood of few individuals that were exposed to aldehydes in a mixture: smokers were chosen. It is well-known that the cigarette smoke contains a majority of these aldehydes in mixture and that itself can induce DNA damage.

3.3. Analysis of human blood DNA

The levels of adducts detected in human blood DNA samples from smokers and non-smokers are shown in Tables 3 and 4, respectively.

For the first time, we were able to detect simultaneously nine adducts in all DNA samples except for AcrodG which was absent in non-smokers DNA. When the quantification was possible, significant differences were noticed in adduct mean levels between smokers and non-smokers with the exception of reduced MDAdG as shown in Table 5. The average level of reduced MDAdG was just slightly higher in smokers DNA (16.49 ± 4.34 adducts per 10^7 normal nucleotides) comparing to its average level in non-smokers DNA (13.31 ± 2.73 adducts per 10^7 normal nucleotides). This is consistent with previous studies which have reported no significant relationship with smoking, but rather with factors such as diet, lifestyle and chronic inflammation (Everett et al., 2001; Leuratti et al., 2002). The average level of AcrodG for all DNA samples from smokers was 5.97 ± 1.69 adducts per 10^7 normal nucleotides. Whereas, this adduct was neither quantifiable nor detectable in the five DNA samples of non-smokers. This difference in AcrodG levels was statistically significant between smokers and non smokers ($p = 0.0079$). The mean CrotodG level of all smokers DNA samples (52.08 ± 7.19 adducts per 10^7 normal nucleotides) was significantly higher than that found in non-smokers DNA (2.8 ± 0.77 adducts per 10^7 normal nucleotides) with $p = 0.0079$. Similarly, the averages levels of both reduced AAdG and FAdG were significantly increased in smokers DNA ($p = 0.0238$ and 0.0079, respectively).

However Gx-induced DNA adduct, just like HNEdG, was not

Table 4
Adducts levels in non-smokers blood DNA per 10^7 normal nucleotides and the standard deviations for n = 3.

Human blood DNA sample no.	NS1		NS2		NS3		NS4		NS5	
Sex	M		F		F		M		F	
Age	29		38		53		32		54	
Smoking status	Non-smoker		Non-smoker		Non-smoker		Non-smoker		Non-smoker	
AcrodG	n.d.		n.d.		n.d.		n.d.		n.d.	
CrotodG	n.d.		4.12	± 2.24	3.18	± 1.86	n.d.		1.10	± 0.15
Reduced AAdG	1.80	± 1.10	n.d.		n.d.		7.00	± 3.07	n.d.	
Reduced FAdG	3.32	± 0.80	n.d.		n.d.		4.86	± 2.95	n.d.	
Reduced MDAdG	12.34	± 1.05	17.30	± 4.51	13.91	± 2.64	18.40	± 5.83	4.58	± 0.91
HNEdG	n.d.		n.d.		n.d.		n.q.		n.q.	
GxdG	n.q.		n.q.		n.d.		n.q.		n.d.	
CEdG	66.63	± 12.15	n.q.		138.16	± 37.81	n.d.		39.91	± 7.10
cMGdG	472.35	± 182.70	85.41	± 22.06	55.78	± 22.19	n.d.		25.61	± 11.22

n.d.: not detectable; n.q.: not quantifiable.

Table 5
The mean of adducts levels in smokers and nonsmokers DNA per 10^7 normal nucleotides.

	Means of adducts levels per 10^7 normal nucleotides in				*p* value
	Five smokers		Five non-smokers		
AcrodG	5.97	± 1.69	n.d.		0.0079
CrotodG	52.08	± 7.19	2.80	± 0.77	0.0079
Reduced AAdG	10.02	± 3.07	4.40	± 1.84	0.0238
Reduced FAdG	17.80	± 2.74	4.09	± 0.54	0.0079
Reduced MDAdG	16.49	± 4.34	13.31	± 2.73	> 0.05
HNEdG	1.37	± 0.14	n.q.		0.0052
GxdG	185.03	± 52.40	n.q.		0.0196
CEdG	162.73	± 31.99	81.57	± 25.40	0.0184
cMGdG	517.03	± 48.21	159.79	± 104.90	0.0159

n.d.: not detectable; n.q.: not quantifiable.

quantifiable in non-smokers DNA, making the difference statistically significant ($p = 0.0196$ and 0.0052 for GxdG and HNEdG, respectively). Concerning MG-induced adducts, CEdG and cMGdG were the most abundant in all samples. The calculated averages of MG-induced adducts in smokers DNA were significantly higher than those found in non-smokers DNA samples ($p = 0.0184$ and 0.0159 for CEdG and cMGdG, respectively).

The major findings of this study were marked by a significant difference in the profiles of exocyclic adducts of DNA extracted from the blood cells of smoking and non-smoking subjects. This increase strongly suggests the contribution of genotoxic effect of aldehydes, which are certainly partly released by tobacco. In fact, tobacco smoke consists of solid particles and toxic mixture of more than 7000 chemicals and compounds that cause immediate damage to the body. The compounds reach the lungs quickly every time smokers inhale the smoke and the blood carries toxicants to different organs. Volatile organic compounds (VOCs) contain mainly the aldehydes that are targeted in our study. The latter aldehydes were found at much higher levels than polycyclic aromatic hydrocarbons (PAHs) and nitrosamines (Carmella et al., 2009; Sampson et al., 2014). The literature reports that these aldehydes damage DNA by reaction with the exocyclic amino group of bases in various animal and human tissues. By this mechanism, they can cause the formation of promutagenic lesions rising the risk of developing cancer (for review, Voulgaridou et al., 2011; Sapkota and Wyatt, 2015; Hecht, 2017). In mice, recent obtained data suggested that damages caused by FA, AA, Croto and Acro in the lung and bladder may be preponderant than those induced by other smoke carcinogens (Weng et al., 2018). In fact, the authors' hypothesis was based on the mechanisms referring to cellular events that initiate tumor development. They focused on the presence of exocyclic DNA adducts in these tissues coupled to the study of inhibitions of DNA repair and metabolic activation of other tobacco procarcinogens such as PAHs. Also, many studies have observed different variations between smokers and non-smokers adducts levels in various tissues. DNA exocyclic adducts induced by α,β-unsaturated aldehydes have already been detected at significantly high levels in bronchoalveolar cells as well as in bronchial and pulmonary epithelia of smokers (Hecht et al., 2016). In other studies, these adducts were also detected in the lung tissue, but without significant differences between the subjects exposed or not to tobacco smoke (Singh et al., 2009; Weng et al., 2018). Yet, these studies have a lack of data concerning the smoking status for each individual. In smoking exposure conditions, the levels of reduced FA-derived DNA adduct were detected by LC-ESI-MS/MS, in human smoker saliva samples at the average levels of 99.6 ± 75.7 adducts per 10^7 normal nucleotides (Li et al., 2017). These levels are much higher than those measured in smoker leukocytes in our study (17.80 ± 2.74 per 10^7 normal nucleotides), this can be due to the fact that in respiratory tracts, the buccal cell is the first cell line that is exposed to cigarette smoke, followed by bronchial epithelial cell and macrophage in the lung. In another study with the same analytical method, CrotodG was detected in saliva smokers samples (n = 16) at 26 ± 21 adduct per 10^7 normal nucleotides (Yang et al., 2019). Weng et al. (2018) detected, by an immunochemical approach, CrotodG in human buccal cells ($p < 0.0001$) and sputum ($p < 0.05$) at significantly higher levels in smokers (n = 33 and n = 22, respectively) comparing to non-smokers (n = 17 and n = 8, respectively). Concerning adducts derived from Acro, these authors obtained also a significant increase in smokers comparing to non-smokers samples, both in saliva ($p < 0.0001$) and in sputum ($p = 0.0093$). Similarly, in our study, the levels of CrotodG and AcrodG obtained in blood cells are significantly higher in smokers than non-smokers. However, other studies detected AcrodG in saliva and in human leukocytes DNA without significant variation between the subjects exposed or not to tobacco smoke (Zhang et al., 2011; Li et al., 2017). To explain this contradiction, we suggest that parameters such as the delay of last cigarette smoked, the duration and the intensity of exposure before sampling should be always considered. In our study, smokers were exposed to tobacco few hours before sampling as indicated by the smoking-related markers: the cigarette intake before the consultation within short time period (2.15 ± 0.74 h), and the measure of CO (18.00 ± 8.46 ppm) in exhaled air, an immediate and non-invasive method., along with the number of daily-consumed cigarettes and the concentration of cotinine, the main metabolite of nicotine in the urine (Hecht, 2003; Deveci et al., 2004; Kim, 2016). Our results provided cotinine levels in smoker urines that are maintained below 100 µg/g of creatinine (cutoff between smoker and non-smoker) with a median cotinine concentrations of 2315 µg/g of creatinine (Goniewicz et al., 2011; Paci et al., 2018). This biomarker can be related to the number of cigarettes that remains elevated for each smoke user (median 20 cig./day, 13–30 cig./day). Taking into consideration the number of daily consumed cigarettes and estimating the compounds amounts

already found in tobacco smoke condensates provided by IARC, smokers in our study are certainly exposed to high concentrations ranging from µg to mg of aldehydes including FA, AA, Croto and Acro (IARC, 1986). It is worth noting that dietary or environmental exposures can contribute a little to the body burden of these biomarkers (Paci et al., 2018).

No significant relationship between a specific smoking-related marker and levels of all aldehydes-induced DNA adducts was observed among smokers in our study. However, even if it is misleading to establish a relationship between an exocyclic DNA adduct and one marker of active smoking exposure, preliminary correlations were observed between consumed cigarettes number and CrotodG levels ($r = 0.9000$, $p = 0.0374$) and between urinary cotinine concentrations and each of the following adducts: reduced FAdG ($r = 0.93053$, $p = 0.0218$), HNEdG ($r = 0.9000$, $p = 0.0374$), CEdG ($r = 0.9000$, $p = 0.0374$) and cMGdG ($r = 0.9000$, $p = 0.0374$). The CO level correlated with GxdG ($r = 0.92904$, $p = 0.0224$) and AcrodG ($r = 0.96560$, $p = 0.0076$). Reduced AAdG correlated with the delay from the last cigarette smoked ($r = 0.9000$, $p = 0.0374$). These correlations must be considered with much caution for several reasons: *i)* data are available for a small population impairing the power test that was too low *ii)* kinetic clearance parameters of each adduct are unknown for the most samples and, *iii)* the epidemiologic data concerning diet and endogenous oxidative stress are unknown and consequently interactions between all agents are not integrated. The fact that we don't have a significant relationship to all tobacco-related biomarkers tends to rule out that one biomarker is not sufficient. The availability of a pattern of exposure biomarkers would be necessary to integrate a set of simultaneous exposures.

It is well known that excessive intake of dietary sugars might interfere with lipid metabolism (Aragno and Mastrocola, 2017). Glycation is initiated by a nucleophilic addition reaction between the free amino group from a protein, lipid or nucleic acid and the carbonyl group of monosaccharides (Aragno and Mastrocola, 2017). Notably, in animals, the metabolic outcomes of a caloric restriction were attenuated by the diet enrichment with MG, demonstrating that dietary advanced glycation end products (AGEs) can induce oxidative stress (Cai et al., 2008). GxdG significantly differed between current and non-smokers. Gx and MG levels were observed in plasma of diabetic (Lapolla et al., 2003). It is well-known that nicotine influences the metabolism of glucose by enhancing the production of cortisol that is a hyperglycemic hormone, in blood (Tweed et al., 2012). Smoking is a risk factor for diabetes of type 2. The high level of GxdG might be indirectly related with the hyperglycemic activity of tobacco nicotine.

The low number of samples of this approach greatly limited this analysis. To improve the statistical power, it would be necessary to continue this study on larger cohorts with a detailed analysis of biological samples in connection with clinical data and tobacco smoke-related markers.

3.4. Comparison between the profiles obtained in vitro and in human DNA

We compared the adducts profiles in the two groups, smokers and non-smokers, to those obtained after exposure of ctDNA to the equimolar mixture of aldehydes at 1 mM (Fig. 1). GxdG and CEdG were presented separately from the others because of their relatively high levels (Fig. 2). In terms of reactivity, we observed that all the adducts induced by aldehydes were found in the modified ctDNA and in the human samples at different levels except for cMGdG and reduced FAdG absent *in vitro*. Knowing that 1 mM remains a toxicologically relevant concentration, the comparison between the three profiles shows that current smokers have been exposed to relatively high levels of aldehydes and therefore, unlike non-smokers, they are exposed to genotoxic and potentially carcinogenic risks.

The human system is a dynamic microenvironment in which aldehydes can react with proteins or other biological molecules. The content of nine DNA adducts represents the sum of their formation and repair in

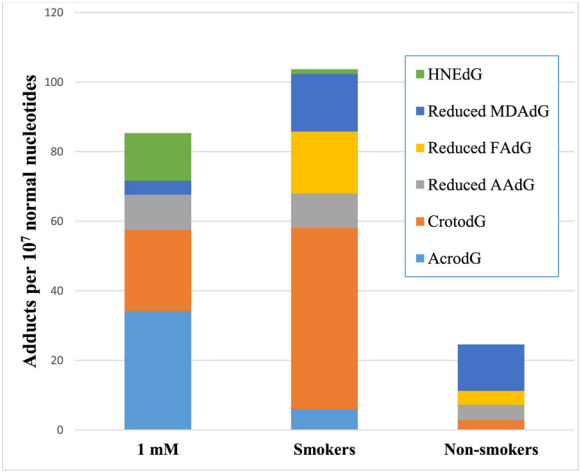


Fig. 1. Comparison of the average levels of six adducts in the blood DNA of the five smokers and five non-smokers with those obtained following the exposure of ctDNA in equimolar mixture (1 mM) of aldehydes.

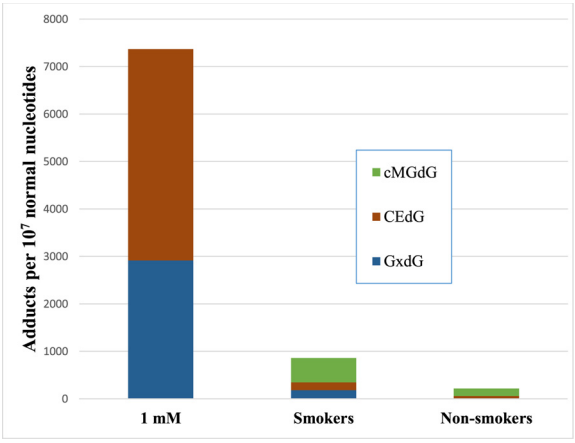


Fig. 2. Comparison of the average levels of three adducts in the blood DNA of the five smokers and five non-smokers with those obtained following the exposure of ctDNA in equimolar mixture (1 mM) of aldehydes.

the lifespan of white blood cells of several days. This means that the distribution of any induced-adduct in human reflects a DNA profile which is unique to each individual and consequently, completely different from a profile obtained in an acellular assay. The general descending order of carbonyl compounds in tobacco smoke is: AA (1110–2101 µg/cig) > Acro (238–468 µg/cig) > FA (87–243 µg/cig) > Croto (40–50 µg/cig) > MDA (18.9–36 µg/cig), MG (13.4–59.6 µg/cig) > Gx (1.93–6.98 µg/cig) (Fujioka and Shibamoto, 2006). With regard to the main aldehydes present in cigarette smoke, Acro and Croto, we found that contrarily to our *in vitro* results, CrotodG prevailed mostly over all of the others in the smokers DNA. In the context of chronic exposure to both humans and animals, previous studies reported that adducts induced by Acro predominate. Weng et al. (2018) observed that adducts induced by Acro are predominant compared to those induced by Croto in the lungs of mice. The *TP53* tumor suppressor gene is frequently mutated in smokers' lung tumors. Feng et al. (2006) showed *in vitro* that the distribution of AcrodG on the *TP53* gene was similar to that described in human lung tumors. To explain our diverging results of AcrodG from those of other studies, we suggest that the electrophilicity of Acro has shifted to other bases of DNA, which may explain the low levels of AcrodG. In fact, adducts of Acro to dA and dC have already been found both in the A549 cell line and in human leukocytes, some of which may be present at levels comparable to those of the predominant AcrodG (Yin et al., 2013). Another

explanation to consider in modifications of exocyclic DNA adducts may be the exposure of smokers to additional factors and sources of aldehydes such as exposure to air pollutants, diet, oxidative stress, disease status and occupational exposures.

Otherwise, earlier evidence indicates the involvement of antioxidant factors and DNA damage repair mechanisms in eukaryotic cells, particularly NER and BER, which are absent in acellular models. This greatly contributes to the formation of adducts (Minko et al., 2009; Tudek et al., 2017; Bukowska and Karwowski, 2018). HNEdG remained at low levels in human samples, whether smokers or not. A frequently advanced explanation in the literature is that HNE is highly toxic to the cell. It is extremely reactive, mainly towards plasma proteins with which it can bind covalently, especially if it is not consumed first by the reaction with cytosolic glutathione (GSH). Under these conditions, there are few HNE molecules that reach the nucleus, which may explain the little damage caused to DNA (Xie et al., 2016). In the same way, the relatively high levels of both reduced AAdG and FAdG could be due to the proportion of the corresponding aldehydes in tobacco smoke but also to the low reactivity of AA with cytosolic GSH and proteins and to moderate FA cytotoxicity. This low reactivity with cytoplasmic elements allows them to diffuse in the nucleus, inducing substantial damage to DNA (Xie et al., 2016).

4. Conclusion

To conclude, our acellular model based on analysis of multiple DNA exocyclic adducts induced *in vitro* represents relatively a simple method of choice to assess and compare genotoxic potentials of aldehyde mixtures. For the first time, we were able to detect simultaneously nine exocyclic adducts in human DNA leukocytes with significant differences noticed in all adducts levels between smokers and non-smokers. Due to the weak sampling level, these findings must be confirmed on a larger cohort. It will be important to consolidate the use of these profiles of exocyclic DNA adducts as specific biomarkers of systemic exposure to aldehydes present in various environments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2020.05.010.

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