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Measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine in peripheral blood mononuclear cells: Optimisation and application to samples from a case-control study on cancers of the oesophagus and cardia

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Abstract

8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is a widely used biomarker to evaluate the level of oxidative stress. This study describes in its first part the optimisation of our analytical procedure (HPLC/electrochemical detection). Particular care was exercised to avoid artefactual oxidation and in the precision of measurement, which was evaluated with blood bags from hemochromatosis patients. The best results were obtained with a DNA extraction step using the "chaotropic method" recommended by the European Standards Committee on Oxidative DNA Damage (ESCODD). Other approaches such as anion exchange columns gave ten times as much 8-oxodG as this method. Moreover, a complete DNA hydrolysis using five different enzymes allowed improved precision. The optimised protocol was applied to peripheral blood mononuclear cells (PBMC) sampled during a case-control study on cancers of the oesophagus and cardia. With 7.2 ± 2.6 8-oxodG/10^6 2′-deoxyguanosines (2′-dG) (mean $\pm$ SD), patients ($n = 17$) showed higher levels of 8-oxodG than controls (4.9 ± 1.9 8-oxodG/10^6 2′-dG, $n = 43$, Student's t-test: $p < 0.001$). This difference remained significant after technical (storage, sampling period, 2′-dG levels) and individual (age, sex, smoking, alcohol) confounding factors were taken into account ($p < 0.0001$, Generalised Linear regression Model). To our knowledge, this is the first report to demonstrate an increase of 8-oxodG in PBMCs of patients suffering from a cancer of the upper digestive tract. This elevated level of DNA damage in patients can raise interesting issues: is oxidative stress the cause or the result of the pathology? Could this biomarker be used to evaluate chemoprevention trials concerning digestive tract cancers?

Keywords: *8-OxodG, oxidative stress, lymphocytes, PBMC, oesophagus, cancer*

Introduction

With 412,000 new cases in 2000, oesophageal cancers were the eighth most common cancer type worldwide.[1] They can be classified in two major histologic types. In developed countries, the key risk factors for squamous cell carcinomas (SCC) are smoking and alcohol intake whereas patients suffering from Barrett's oesophagus or gastro-oesophageal reflux disease (GORD) are prone to adenocarcinomas (ADC). On a worldwide scale, ADC has a low incidence compared with SCC, but is increasing at a rapid rate in many western countries like the United States or the United Kingdom.[2] ADC of the cardia (tumors of the oesophago–gastric junction) exhibit the same epidemiological evolution and share molecular characteristics with ADC of the oesophagus. Some authors have even assumed that these cancers constitute the same disease.[3,4]

Reactive nitrogen and oxygen species are thought to play a role in oesophageal carcinogenesis. They could be supplied by environmental factors (smoking, alcohol metabolism, diet) or produced endogenously by inflammatory conditions (oesophagitis, GORD) and

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precancerous lesions (Barrett's oesophagus). For instance, experimentally induced reflux oesophagitis or Barrett's oesophagus in rats show increased amounts of oxidative damage to lipids (malondialdehyde)[5,6] or proteins (carbonyl contents).[7] These molecular lesions are associated with depletion in anti-oxidants such as reduced glutathione.[8] Among various biomarkers of oxidative stress, we selected 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). This lesion is one of the major forms of oxidative DNA damage and has been implicated in the induction of mutations such as G to T transversions.[9] These two interesting points and the existence of cheap analytical methods turned 8-oxodG into the most widely used marker of oxidative DNA lesions. In population studies, 8-oxodG is often determined in DNA extracted from white blood cells, peripheral blood mononuclear cells (PBMC) or lymphocytes (PBMCs being mostly lymphocytes, the distinction is rarely done in the literature). These cells are considered to be surrogate tissues and could reflect exposure of the whole organism to oxidative stress. However, some published results have highlighted the possibility of high levels of 8-oxodG which are attributable to artefactual oxidation of DNA during samples treatment.[10] Because of these difficulties, articles related to 8-oxodG are to a great extent dedicated to analytical issues, including those of the European Standards Committee on Oxidative DNA Damage (ESCODD).[11–14] The aim of this European laboratory network is to improve and harmonise 8-oxodG measurement methods.

The first part of this report deals with the optimisation of our analytical procedure (HPLC coupled with electrochemical detection (HPLC/ECD)). The second part is dedicated to the analysis of PBMC samples ($n = 60$) coming from a case-control study on cancers of the oesophagus and cardia. Moreover, volunteers recruited for this study completed a detailed questionnaire including data on smoking and alcohol consumption which permitted relationships between these data and oxidative DNA damage to be investigated.

Materials and methods

Patients and controls

Following ethical approval (Comité Consultatif pour la Protection des Personnes en Recherche Biomédicale/Basse-Normandie), the consenting patients and controls were recruited between 1996 and 2000 within the context of a case-control study meant to identify biomarkers suitable for molecular epidemiology of oesophageal cancers.[15] The control group ($n = 43$) included two patients recruited at the University Hospital of Caen and 41 donors from the Ligue Nationale Conter le Cancer. The 17 patients suffering from oesophageal cancer were recruited at the University Hospital of Caen. Diagnosis was performed by the hepato-gastroenterology department (University Hospital of Caen) and by the anatomopathology department of the François Baclesse Centre. Nine patients presented with SCC, seven with ADC (oesophagus: 1, cardia: 6) and one with leiomyoma (rare histology). The distinction between ADC of the oesophagus and ADC of the cardia was performed according to Siewert criteria.[16] Interviews including a detailed questionnaire on tobacco and alcohol consumption were conducted by trained dieticians for 36 control subjects and ten cases. These data are summarized in Table I.

Optimisation of the analytical procedure

Artefactual oxidation and precision (intra and inter-day variabilities) have been critical criteria to control, especially concerning DNA extraction and hydrolysis

Table I. Description of technical and individual data.

	Controls				Cases			
	Mean	Standard Deviation	Extreme values	*n*	Mean	Standard Deviation	Extreme values	*n*
Technical variables								
Storage (days)	1666	105	1453–1869	43	1694	432	1110–2626	17
[2′-dG] (mM)	0.42	0.18	0.11–0.97	43	0.33	0.17	0.15–0.67	17
Individual variables								
8-oxodG/10^6 2′-dG	4.9	1.9	2.0–12.5	43	7.2	2.6	3.4–12.4	17
Smoking (cig/week)	52	79	0–240*	36	113	97	0–325†	10
Smoking (years)	10	13	0–42	36	30	14	0–45	10
Alcohol (g/week)	143	140	0–620	36	289	258	27–700	10
Wine (ml/week)	960	1240	0–5250	36	1510	1560	5–4550	10
Beer (ml/week)	170	360	0–1800	36	1440	2490	0–6125	10
Cider (ml/week)	550	1090	0–4200	36	1430	2060	0–7000	10
Spirit (ml/week)	27	34	0–120	36	17	28	0–90	10
Age (years)	64	8	39–87	43	63	9	50–75	17
Sex (men/women)		27/16		43		14/3		17

* Nineteen non-smokers. † One non-smoker.

stages. The interday and intraday variabilities of the measurement protocol were first evaluated using 8-oxodG standards and calf thymus DNA. Then, we used blood bags taken at the "Etablissement Français du Sang de Normandie" (blood transfusion centre) after informed consent from patients suffering from hemochromatosis. This disease indeed requires blood removals which allowed us to isolate a sufficient amount of PBMCs collected the same day and from the same patient. The interday experimental variability was assessed by including a control PBMC sample taken from the same blood bag in each measurement series of PBMCs from the case-control study.

DNA isolation protocols evaluated included home-made and commercial kits with various procedures (Table II): (i) NaCl precipitation, (ii) anion exchange (Nucleobond AX, Macherey-Nagel), (iii) "chaotropic method": initial isolation of nuclei and NaI precipitation (home-made and DNA extractor WB kit, Wako). Both commercial kits were used according to manufacturers' instructions. Concerning the "NaCl protocol", 3 ml of extraction buffer (20 mM Tris, 20 mM NaCl, 10 mM EDTA, 0.1 mM desferrioxamine, pH 8) and 0.2 ml of SDS were added to PBMC pellets. After dispersion of the pellet and addition of 50 µl protease (20 mg/ml, Qiagen), samples were incubated 1 h at 37°C. DNA was precipitated with 1 ml of saturated NaCl (6 M) and 8 ml of 100% ice-cold ethanol. A rinsing with 4 ml of 100% ice-cold ethanol was performed. After removal of ethanol, 1 ml of RNAse buffer (10 mM Tris, 1 mM EDTA, 0.1 mM desferrioxamine, pH 7.4), 100 µl of ribonuclease (RNase) A (1 mg/ml, Sigma-Aldrich) and 10 µl of RNase T1 (1 U/µl, Sigma-Aldrich) were added. This mixture was incubated 1 h at 37°C. DNA was precipitated with 33 µl of saturated NaCl (6 M) and 2.5 ml of 100% ice-cold ethanol. A further rinsing with 2.5 ml of 100% ice-cold ethanol was performed. The "chaotropic home-made" method selected for samples of the case-control study is described in a following paragraph.

DNA hydrolysis was tested with the commonly employed enzymes nuclease P1 (NP1; Roche Diagnostics) and alkaline phosphatase (AP; Roche Diagnostics), and with a more complete mixture made of NP1, AP, deoxyribonuclease I (DNase I; Roche Diagnostics) and phosphodiesterases (PDE) I (Amersham) and II (Calbiochem). Data obtained with blood bag PBMCs and samples from cases and controls allowed information on the influence of storage in liquid nitrogen, storage in autosampler, sampling period and 2′-deoxyguanosine (2′-dG) concentration to be assessed. Finally selected conditions are detailed in the next paragraph.

PBMC collection, DNA isolation and hydrolysis

Blood samples (12 ml) were collected before surgery, radio or chemotherapy concerning cases. Indeed, these treatments are susceptible to induce oxidative DNA damage.[17,18] PBMCs were isolated using Unisep Maxi tubes (Novamed). They were stored in liquid nitrogen until DNA isolation. This step was performed using the "protocol G" described by Ravanat *et al.*[19] with modifications. Throughout the protocol, tubes were put on ice and centrifugations performed at +4°C. PBMC pellets were transferred to 2 ml tubes and centrifuged for 2 min at 5000*g*. Supernatant was removed and 1 ml lysis solution was added (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris, 0.1 mM desferrioxamine, 1% Triton x-100, pH 7.5). After dispersion of the pellet, tubes were centrifuged 20 s at 16,000*g*. The supernatant was removed and the latter step repeated with 1.8 ml lysis solution. Then, 350 µl of the second lysis solution were added (10 mM Tris, 5 mM EDTA–Na_2, 0.15 mM desferrioxamine, sarcosyl 1% w/v, pH 8), samples were mixed and supplemented with 10 µl RNase A (1 mg/ml, Sigma-Aldrich) and 3 µl RNase T_1 (1 U/µl, Sigma-Aldrich). Samples were incubated for 15 min at 50°C, then 50 µl protease (20 mg/ml, Qiagen) were added. The digestion lasted 1 h 30 min at 37°C under gentle shaking. DNA was precipitated with 700 µl of NaI solution (7.6 M NaI, 40 mM Tris, 20 mM EDTA, 0.3 mM desferrioxamine, pH 8) and 850 µl ice-cold 100% isopropanol. After gently mixing by inversion, tubes were centrifuged for 1 min at 16,000*g*. Two similar rinsings were performed with 1.8 ml 40% ice-cold isopropanol and 1.8 ml 70% ice-cold ethanol.

Table II. Results of DNA extraction and hydrolysis evaluations performed with blood bags from hemochromatosis patients (intraday variability).

Blood bags	DNA extraction	Hydrolysis*	*n*	8-oxodG/10^6 2′-dG Mean ± SD	CV (%)
1	"NaCl"	2 enzymes	10	17.0 ± 10.7	63
2	Ion exchange	2 enzymes	4	55.4 ± 41.2	74
2	Chaotropic	2 enzymes	10	5.5 ± 1.3	24
3	Chaotropic† without Rnase	2 enzymes	10	15.4 ± 4.5	29
4	Chaotropic†	5 enzymes	10	8.0 ± 1.3	16

The four blood bags were taken from four different hemochromatosis patients. They were aliquoted in tubes containing each 12 ml of blood. The experimental intraday variability was evaluated by measuring 8-oxodG the same day in *n* tubes coming from the same bag.* Two enzymes: NP1 + AP; five enzymes: NP1 + AP + DNase I + PDE I + PDE II.† Home-made protocol.

After the last centrifugation, ethanol was removed and the pellet suspended into 90 µl of sterilized water for injections. Ten microlitres of NP1 buffer (300 mM sodium acetate, 1 mM $ZnSO_4$, 0.1 mM desferrioxamine, pH 5.3) and 10 µl of NP1 (1 U/µl) were added to this solution which was then incubated for 1 h at 37°C under gentle shaking. A second step of DNA digestion used 3 µl of DNase I (5 U/µl), 1 µl of AP (1 U/µl), 2 µl of PDE I (0.002 U/µl), 1 µl of PDE II (2 mg/ml), 3 µl of $MgCl_2$ 0.5 M and 11 µl of buffer (500 mM Tris, pH 8). The incubation lasted 2 h at 37°C under gentle shaking. Then, the solution was centrifuged for 30 min at 12,000*g* on Microcon YM-10 devices (Millipore).

HPLC-ECD analysis

The mobile phase (pH 4.6) was prepared with analytical grade reagents and passed through a 0.22 µm filter before use. It was constituted of sterilized water for injections, methanol (7.5%) and potassium dihydrogen phosphate (10 mM). The flow rate was set at 0.6 ml/min (Esa 580 pump, Esa). An Esa 5020 guard cell (potential: +500 mV) preceded the Midas 830 autosampler where DNA hydrolysates were stored at +5°C. Nucleosides separation was performed by a Supelcosil reversed-phase C18 HPLC column (150 × 3 mm, 5 µm-Supelco). 8-oxodG was detected with an Esa Coulochem 5100A detector and an Esa 5011 analytical cell (electrode 1: +50 mV, electrode 2: +350 mV, potentials determined by a voltammogram performed with 8-oxodG standards, data not shown). 2′-dG was detected at 290 nm with a Pharmacia LKB VWM 2141 detector situated after the ECD cell. This supra-optimal wavelength lowered UV signals but allowed the injection of the whole hydrolysate which was essential for the ECD of 8-oxodG. We checked the linearity of the signal at 290 nm with 8-oxodG standards. The short duration of each run (15 min) allowed limited storage of hydrolysates in the autosampler. Representative chromatograms are shown in Figure 1. The external calibration curves were established with 0.1, 0.5 and 1.5 mM 2′-dG solutions and with 1, 5 and 10 nM 8-oxodG solutions. According to recommendations of Wood *et al.* [20] these standards were not prepared by weighing but by adjustment after measurement of the absorbance with a recently calibrated UV detector.

Statistical analysis

Variables were tested for normal distribution by the Shapiro–Wilk test and the Fisher's *F*-test was used to compare variances. When appropriate, distributions

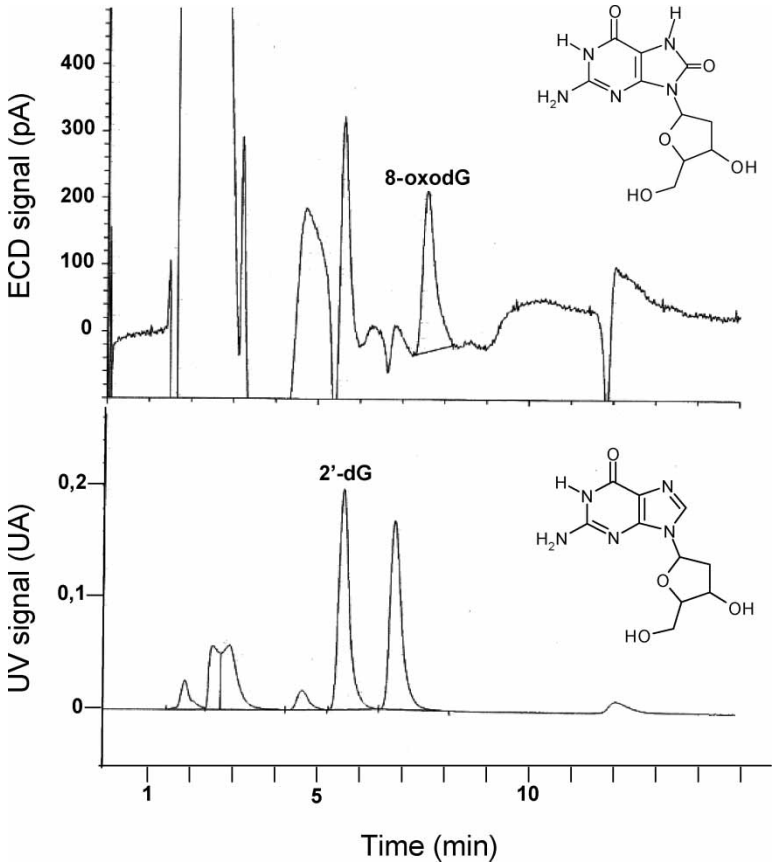

Figure 1. Representative chromatograms of 8-oxodG (top) and 2′-dG (bottom) from a sample of human PBMCs.

were normalised by logarithmic transformations and monovariate analyses were performed by the Dunnett test (one-sided, $\alpha = 0.05$, impact of sampling period), Student's t-test (two-sided, $\alpha = 0.05$) or Pearson correlation test (two-sided, $\alpha = 0.05$). Multivariate analysis (Generalised Linear regression Model (GLM)) was conducted to assess the influence of each variable on 8-oxodG levels. Programs XLSTAT 6.1.9 and SAS 8.2 were used for these statistical analyses.

Results

Optimisation of 8-oxodG analysis using blood bags

Intra and interday variability ($n = 10$) in 8-oxodG analysis were first investigated with standard solutions of 8-oxodG and calf-thymus DNA. Coefficients of variation (CV) below 10% were achieved (data not shown). However, results were far less satisfactory when evaluating precision with PBMCs from blood bags with CVs reaching more than 70% (Table II). Ravanat *et al.*[19] showed that withdrawal of RNases does not seem to affect 8-oxodG levels. As each part of an analytical protocol is a potential source of imprecision and/or inaccuracy, we tried to eliminate RNase treatment. Moreover, this alternative avoided a 50°C heating step which could stimulate an artefactual oxidation. But this omission was not conclusive and did not improve the precision. An excessive RNA contamination could interfere with DNA enzymatic digestion and with ECD and UV signals.[21] The best intraday and interday performances were CVs of 16% ($n = 10$) and 17% ($n = 7$), respectively. They were obtained with sodium iodide ("chaotropic method") and the five enzyme digestion method (Table II). As we did not perform an evaluation of the commercial "chaotropic method" with the five enzyme digestion, our data do not allow us to differentiate the precision of the home-made protocol from that of the commercial kit.

Values measured with the "chaotropic method" were also the lowest. Notably, two commercial kits, one using the "chaotropic method" and the other the anion exchange method, were tested with PBMCs from the same bag (blood bag 2). This comparison revealed levels of 8-oxodG 10 times higher with the anion exchange approach. Replicate samples of PBMCs taken from the same blood bag allowed us to examine the possibility of a potential artefactual oxidation during storage in the autosampler. The waiting time between the first and last runs of DNA hydrolysates ($n = 10$) lasted 4 h 30 min, including standards. This parameter was not associated with the 8-oxodG/2′-dG ratio (Pearson correlation test, two-sided, $p = 0.948$).

Analysis of samples from the case-control study

The "chaotropic method" of DNA isolation with five enzyme hydrolysis (see above) were used for the analysis of 8-oxodG in PBMCs from the case-control study.

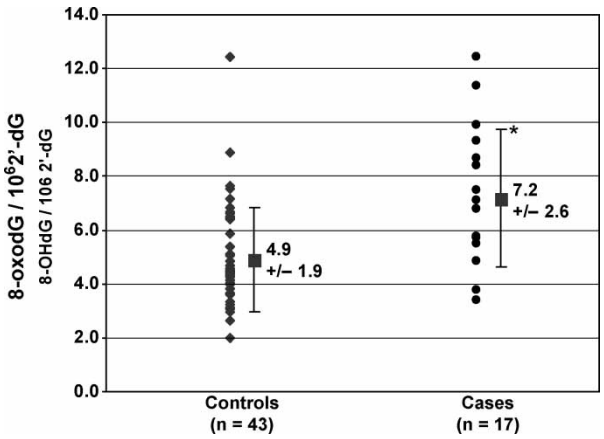

Figure 2. Levels of 8-oxodG in PBMCs from cases and controls. Vertical bars: ±SD. $\star p < 0.001$ after logarithmic transformation (Student' t-test).

Patients suffering from cancer of the oesophagus or cardia showed higher oxidative DNA damage in comparison with controls (7.2 8-oxodG/10^6 2′-dG vs. 4.9 8-oxodG/10^6 2′-dG, Student's t-test, $p < 0.001$, Figure 2). Monovariate analyses were performed to test whether sampling period, duration of PBMC storage in liquid nitrogen and 2′-dG concentration could have confounded this result. For control samples, an increase of 8-oxodG levels was associated with increasing time of storage in liquid nitrogen (Pearson correlation test, two-sided, $p = 0.001$, Figure 3). Oxidative DNA damage was higher in PBMCs of control subjects obtained in June (Dunnett test, $p < 0.05$, Figure 3). Moreover, cases and controls combined, a negative association between the amount of 2′-dG and 8-oxodG levels emerged (Pearson correlation test, two-sided, $p < 0.0001$, Figure 3).

In order to verify that the difference between cases and controls could not be accounted for these potentially confounding factors, we analysed data using a multivariate approach (GLM, Table III). Sex and age of individuals were included in the models. Model 1 included the 60 subjects enrolled in the study. Models 2 and 3 contained additional variables related to smoking and alcohol consumption (collected for 46 subjects, it can be pointed out that cancer patients presented unsurprisingly higher levels of tobacco and alcohol consumptions). Models 4 and 5 included only data of control samples and allowed us to evaluate the role of smoking and alcohol on oxidative damage in subjects without cancer. The main conclusions after these adjustments were (i) a confirmed higher level of 8-oxodG in cancer patients ($p < 0.0001$), (ii) a significant role of technical variables (sampling period, storage, 2′-dG concentration) on levels of 8-oxodG and (iii) no association of 8-oxodG with sex, age, smoking and alcohol excepting a positive correlation with cider consumption ($p = 0.0166$) when cases and controls were included in the same model.

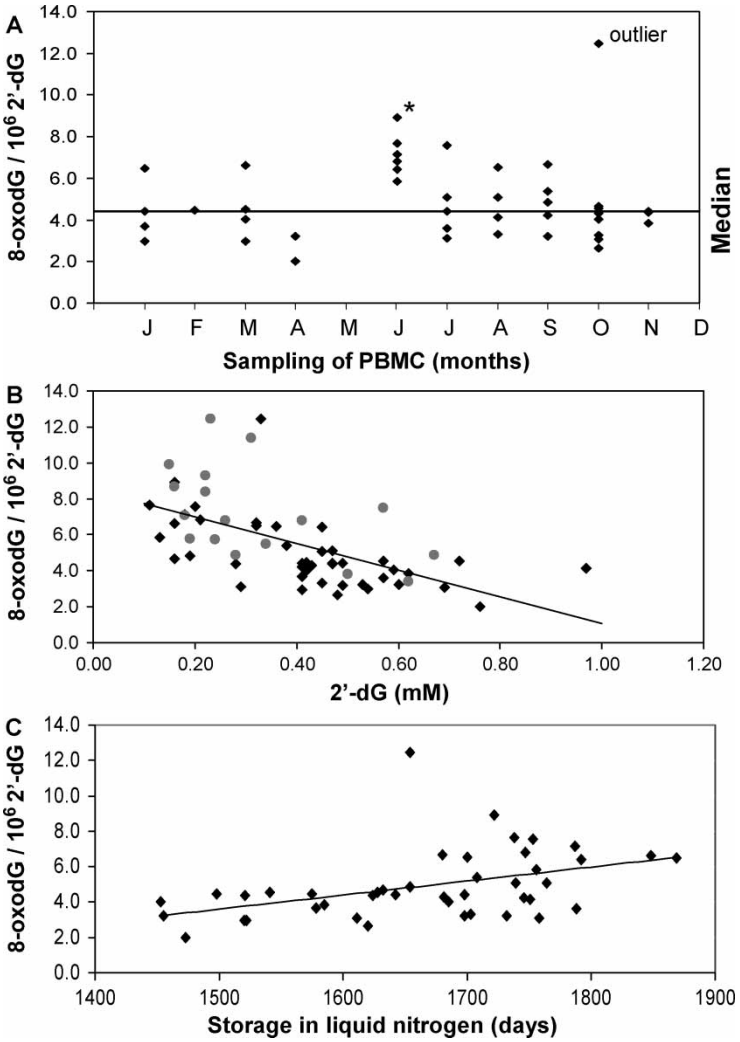

Figure 3. Influence of technical variables on 8-oxodG levels. (A) Sampling period (controls), *oxidative DNA damage was higher in PBMCs obtained in June (Dunnett test, $p < 0.05$, difference statistically significant when excluding the outlier) (B) Concentrations of 2′-dG in DNA hydrolysate (• cases and ◆ controls combined, $p < 0.0001$, Pearson correlation test). (C) Storage of PBMCs in liquid nitrogen (controls). An increase of 8-oxodG level was associated with storage duration ($p = 0.001$, Pearson correlation test).

Discussion

To our knowledge, this study is the first to use PBMCs from blood bags to optimise 8-oxodG measurement. This permitted replicate measurements to be made from blood taken a single day from the same patient. Moreover, this approach provided samples biologically very close to those collected in the case-control study. Previously published works have performed interday and/or intraday variabilities evaluations with 8-oxodG standards, calf thymus DNA or cultured cells. These models are therefore quite different compared with human samples. Moreover, determinations of CV were often performed with a limited number of consecutive measurements ($n = 2$ or 3). In some extreme cases, "Materials and methods" sections did not mention any validation data. In this study, we obtained low CVs with 8-oxodG standards and calf thymus DNA, however, results were less satisfactory when measuring precision of 8-oxodG analysis with PBMCs. This confirmed that one of the most critical parameters to achieve reliable precision of 8-oxodG measurement is DNA extraction. CVs of 16% (intraday) and 17% (interday) were obtained adopting the chaotropic extraction protocol in conjunction with a DNA digestion using NP1, AP, DNase I, PDE I and PDE II. This result is in accordance with the evaluations of Huang *et al.* [21] who showed the importance of a complete release of nucleosides in minimising variability of 8-oxodG determinations. We did not succeed in reaching CV lower than 15%. Combined with a small sample size ($n = 60$), this could prevent us from demonstrating small differences of 8-oxodG levels between 2 groups of individuals.

In the field of 8-oxodG analysis, another technical difficulty is to avoid artefactual oxidation of nucleosides. To achieve this goal, we minimised time delays and temperatures. We used also well-known adaptations like the addition of desferrioxamine (a transition metal chelator) to extraction and digestion buffers, or NaI precipitation. An underestimation of 8-oxodG levels has

Table III. Factors affecting 8-oxodG levels in PBMCs: p values of adjustments by multivariate linear regression models.

	Cases and controls			Controls	
	Model 1 n=60	Model 2 n=46	Model 3 n=46	Model 4 n=36	Model 5 n=36
Technical variables					
Storage duration	0.8630	0.9185	0.9126	0.0279*	0.0283*
[2'-dG]	<0.0001*	0.0028*	0.0016*	0.0430*	0.0434*
Sampling in June	0.0002*	0.0004*	0.0002*	0.0001*	0.0001*
Individual variables					
Cases/controls	<0.0001*	<0.0001*	<0.0001*	–	–
Smoking (cig/week)	–	0.5456	0.5167	0.8879	0.8876
Smoking (years)	–	0.3344	0.3009	0.9875	0.9875
Alcohol (total intake)	–	0.3731	–	0.9443	–
Wine	–	–	0.5197	–	0.3913
Beer	–	–	0.1256	–	0.3229
Cider	–	–	0.0166*	–	0.3537
Spirit	–	–	0.6392	–	0.4648
Age	0.6678	0.5204	0.4905	0.9841	0.9841
Sex	0.4365	0.6437	0.6197	0.8228	0.8223

The dependent variable is log (8-oxodG/10^6 2′-dG). Model 1: all the 60 subjects (cases and controls) enrolled in the study. Model 2: interviewed subjects, no distinction between alcoholic drinks. Model 3: interviewed subjects, distinction between alcoholic drinks. Model 4: interviewed controls, no distinction between alcoholic drinks. Model 5: interviewed controls, distinction between alcoholic drinks. *$p < 0.05$.

been attributed to NaI which could decompose the oxidised nucleoside.[22] However more recent studies demonstrate by re-extracting DNA with NaI [23] or by using an isotope-labelled nucleoside [19], that this salt does not seem to destroy 8-oxodG.

Moreover, the finally selected extraction protocol lowered oxidation during treatment steps by lysing first cellular membrane before DNA isolation from nuclei. This method avoided contact between DNA and oxidative compounds from the cytosol. Applied to blood bag PBMCs, this protocol allowed us to measure 8-oxodG levels of 5.5 ± 1.3 and 8.0 ± 1.3 lesions/10^6 2′-dG (Table II). It may be interesting to point out that these values are quite low in comparison with those collected in recently published studies using HPLC/ECD.[22,24–30] (Figure 4). These low levels were observed although blood bags originated from patients suffering from hemochromatosis, an iron overload disease which has been associated with oxidative stress in some studies.[31] In addition, control samples exhibited 4.9 ± 1.9 8-oxodG/10^6 2′-dG (mean ± SD), levels which closely adhere to the median reported by the latest ESCODD trial[32] of about 4.2 8-oxodG/10^6 2′-dG in lymphocytes from healthy young men (measurement method: HPLC/ECD). Aims of this European group of laboratories are to evaluate measurement methods of oxidative DNA markers and to establish a consensus on background levels of these damages. This constitutes a quite challenging task taking into account discrepancies between the different analytical methods (HPLC/ECD, comet assay, HPLC/mass spectrometry (MS), gas chromatography/MS) and even between different laboratories using the same technique. This observation is especially true for HPLC/ECD and it is worth pointing out that recent studies reported a background level of 0.5 8-oxodG/10^6 2′-dG in lymphocytes with this technique, which is eight times lower than values mentioned above.[24] This low background level is close to those measured with enzymic approaches (comet assay, alkaline elution or alkaline unwinding) using bacterial endonucleases involved in the excision of oxidative DNA damage. Indeed, the median value obtained with these methods in lymphocytes from young non-smoking subjects during the last ESCODD trial was close to 0.3 8-oxodG/10^6 2′-dG.[32] These discrepancies are still a matter of debate: they could be attributed to an overestimation by HPLC/ECD in spite of specific procedures to prevent experimental oxidation. Moreover, even if the specificity of HPLC/ECD towards 8-oxodG is provided by the ECD potential and by

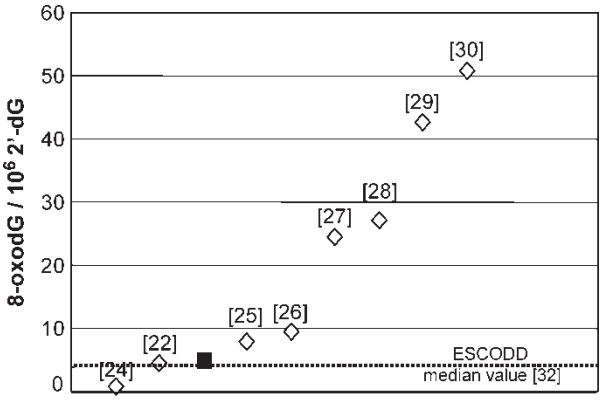

Figure 4. Selection of recently measured background 8-oxodG levels by HPLC/ECD in lymphocytes. Selected populations: pre-intervention [25,27], healthy controls [26,28–30], healthy volunteers [22,24]. The ESCODD median value has been obtained with young healthy non-smokers [32]. ■ Present study.

elution time, there is no structural evidence that the peak identified as 8-oxodG contains only this oxidised nucleoside. On the other hand, improvements should be provided to enzymic approaches concerning calibration steps and the efficiency of excision. Furthermore, specificity of the endonuclease fpg (formamido pyrimidine glycosylase) towards oxidative damage has been reported to be questionable.[33]

Data of control subjects seemed to indicate a higher level of oxidative DNA damage when sampling was performed in June. This information needs to be confirmed by carefully designed studies including a larger number of subjects. Nevertheless, this observation corroborated former studies mentioning elevated levels of oxidative DNA damage during summer months.[34] A potential role for UV radiations has been assumed. It can be underlined that UV radiations have been implicated in seasonal variation of hOGG1 (human 8-OxoGuanineGlycosylase 1) RNA levels[35] (hOGG1 is a DNA repair enzyme involved in the excision of 8-hydroxyguanine).

Duration of storage in liquid nitrogen (between 4 and 5 years) seemed to elevate DNA oxidation rates in control samples. In addition, an inverse relationship was found between 8-oxodG and 2′-dG concentrations. This has already been observed and attributed to a dilution of artefactual 8-oxodG at higher 2′-dG levels.[24] Even if this confounding factor did not affect the significance of our results (multivariate analysis, Table III), this point should be considered in future studies. Variation in 2′-dG levels may be linked to variability of PBMC concentrations among individuals, but they also depend on the efficiency of DNA extraction and hydrolysis.

In addition to these technical data, we examined the effect of individual variables on 8-oxodG levels. Because of marked discrepancies between published reports, there is no consensus on the role of age, sex and smoking on oxidative DNA damage measured in PBMCs, lymphocytes or leukocytes[30,34,36,37]. In this report, none of these three factors influenced 8-oxodG concentrations. However, this work was designed to study oesophageal cancers and affected therefore a narrow age group. Besides tobacco, alcohol is another exogenous factor often associated with oxidative stress and cancers of the oesophagus.[38] Concerning alcohol consumption, total intake has not been associated with increased DNA damage in our study. The positive association found with cider should be interpreted with caution because of the limited number of subjects included in this work. This linkage was only significant when pooling controls and cases, these latter presenting the highest consumptions. Unlike Bianchini *et al.* who found an inverse correlation between wine and 8-oxodG in lymphocytes from women with low intakes,[39] we did not observe any influence of wine on the levels of 8-oxodG. Further studies recruiting heavy drinkers could bring interesting data on subjects prone to SCC of the oesophagus, especially in our area (Lower-Normandy). Indeed, North-Western France has been for a long time the major area in Europe for the incidence of oesophageal cancers. This over-incidence has been attributed to an elevated consumption of alcoholic beverages like wine or local apple-based products (e.g. cider, calvados). As previously mentioned, we cannot exclude that small variations of 8-oxodG related to smoking, alcohol, sex or age are hindered because of our small sample size and to the precision of our analysis protocol.

The main result of this study is probably the significantly higher level of oxidative DNA damage in PBMCs from patients suffering from oesophageal cancer after adjustment for confounding technical factors. Oxidative DNA damage has frequently been reported in cancer tissues[40] or in pre-malignant lesions such as Barrett's mucosa.[41] Moreover, studies comparing controls and cancer patients show in most cases an increased level of oxidative lesions in these latter subjects. This has already been noticed in solid tissues[42,43] and in urine.[44] This is the first published work to show this relationship in circulating blood cells in the context of an upper digestive tract cancer but similar observations have been raised for lung cancer[45], leukaemia[29,46] and colorectal carcinoma.[26]

The origins of this oxidative stress and whether it is a cause or a result of the disease constitute interesting queries.[47] These DNA lesions could result from exposure to environmental oxidative compounds. Our study did not reveal a marked influence of smoking and alcohol. On the contrary, Vulimiri *et al.* [45] found a positive correlation between 8-oxodG rates and smoking in control subjects and lung cancer patients. These findings are not sufficient to prove the direct involvement of reactive oxygen species generated by tobacco in the induction of cancers. Indeed, some studies showed background levels several times higher than those recommended by ESCODD and should therefore be interpreted cautiously. Secondly, PBMCs are surrogate cells and do not always reflect the extent of oxidative lesions in the target tissue.[48] Finally, the half-life of 8-oxodG in PBMCs is not well characterised at the present time owing to different lifetimes of various PBMC sub-populations and to analytical discrepancies.[49] These data are essential to evaluate the period of exposure to environmental factors reflected by DNA adduct levels, especially for cancer patients who are prone to modify their way of life after diagnosis. Apart from exogenous factors, the oxidative stress detected in patient blood cells could be generated by the disease itself. We can assume that inflammatory and tumoral cells are able to produce reactive oxygen species or pro-oxidant mediators maintaining a persistent oxidative stress in the whole organism. Another source of oxidative DNA damage in PBMCs from patients presenting an oesophageal cancer could be provided by drastic modifications of their nutritional status. Finally, we can mention

the role of 8-oxodG repair deficiencies. Xing *et al.* found a genetic susceptibility toward SCC of the oesophagus linked to the Ser326Cys polymorphism in the *hOGG1* gene.[50] This polymorphism has been reported to reduce the repair activity of the enzyme.[51]

In conclusion, our optimised protocol of HPLC/ECD allowed us to measure 8-oxodG in PBMCs of subjects from a case-control study dedicated to cancers of the oesophagus and cardia. After adjustment for technical variables, age, sex, smoking and alcohol, we found that patients presented an elevated level of oxidative DNA damage in comparison with controls. This result requires to be confirmed in separate and larger series of ADC and SCC patients. In addition, a further confirmation could be provided by complementary measurements of 8-oxodG levels using methods such as the comet assay or MS. This last approach could offer the advantage of a structural evidence for 8-oxodG. Furthermore, it could allow the simultaneous detection of other relevant oxidative DNA damage products.[52] The elevated level of oxidative DNA lesions could be related to exogenous or endogenous factors. It may be interesting to explore the repercussions of this generalised oxidative stress for the patient. Moreover, we could wonder if 8-oxodG in PBMCs could provide a useful biomarker to evaluate consequences and efficiency of chemoprevention studies in the context of digestive tract cancers (e.g. with anti-inflammatory drugs or dietary anti-oxidants). This strategy could be particularly helpful for pre-cancerous and inflammatory lesions such as chronic oesophagitis or Barrett's oesophagus.

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