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DNA oxidative damage and strand breaks in young healthy individuals: A gender difference and the role of life style factors

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Abstract
The aim of this study was to analyze background levels of DNA damage in young (19–31 years) non-smoking individuals and to correlate damage to gender and life style. DNA single strand breaks (SSB) and alkali labile sites (ALS) were measured in 99 subjects living in Stockholm, Sweden. Further, oxidative DNA damage was analyzed using the DNA repair glycosylase FPG as well as HPLC-ECD for specific analysis of 8-oxo-7,8-dihydro-2′deoxyguanosine (8-oxodG). We found that males had higher ($P < 0.001$) levels of SSB + ALS than females, but no difference was seen for oxidative lesions. There was no correlation between FPG sites and 8-oxodG. For females, there was a positive correlation between FPG levels and body mass index and a negative correlation between SSB + ALS and fruit intake. We conclude that the background level of oxidative DNA damage, analyzed with improved methods, is low and that gender, fruit intake and BMI can affect DNA damage.

Keywords: *Comet assay, DNA damage, environment, oxidative stress, gender, lymphocyte*

Abbreviations: *8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ALS, alkali-labile site; BMI, body mass index; dG, 2′-deoxyguanosine; FPG, formamidopyrimidine DNA glycosylase (bacterial E. coli DNA repair endonuclease); GTC, guanidine thiocyanate; HPLC-ECD, high performance liquid chromatography with electrochemical detection; PLG, phase lock gel; ROS, reactive oxygen species; SSB, single strand-break; TEMPO, 2,2,6,6-tetramethylpiperidine-N-*oxyl

Introduction

Humans are constantly exposed to reactive chemicals and agents derived from exogenous sources such as food, air pollution, tobacco smoke or ionizing radiation, and also from endogenous sources including reactive oxygen species (ROS) formed from mitochondrial respiration, cellular metabolism and the immune defense system. By reaction with cellular biomolecules such as DNA, modifications are formed, including covalently bound adducts or oxidation products which may lead to an increased cancer risk or aging [1,2]. The assessment of damage in humans is complicated by the restriction of access to tissue, and samples are usually derived from minimally invasive sources such as cell scrapes, body fluids or muscle biopsies. Blood components, being constantly exposed to a complex mixture of chemicals from ingestion and inhalation as well as from the activity of the immune defense system [3] is the target most often used for assessment of damage in humans. DNA damage to circulating human lymphocytes has previously been reported to be increased by aging [4], exposure to air pollution particles [5], viral infection [6], various diseases [7,8], smoking [4–10], physical activity [11] and, low doses of γ-irradiation [12].

A highly sensitive and rapid method for measurement of DNA damage is the alkaline single cell gel

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electrophoresis method (the Comet assay), where cleaved DNA fragments migrate more rapidly than the non-fragmented part, forming a "tail" which is measured by light microscopy after fluorescent staining and computerized image analysis [13,14]. Single strand-breaks (SSBs) can be present due to damage or incomplete repair *in vivo*, but can also be formed *in vitro* during enzymatic or alkaline treatment. The enzyme formamidopyrimidine DNA glycosylase (FPG) can be included in the Comet assay for recognition and removal of oxidized purines including 8-oxo-7,8-dihydro-2′- deoxyguanosine (8-oxodG) and formamidopyrimidines, and also cleaves the DNA-strand at these sites [15,16], forming "FPG sites". Moreover, strong alkaline (pH $>$ 13) treatment releases adducted bases from the DNA sugar backbone but will also cleave the DNA-strand at these weakened abasic sites, forming "alkali labile sites (ALS)".

Chromatographic methods such as high performance liquid chromatography with electrochemical detection (HPLC-ECD)[17], liquid chromatography with tandem mass spectrometry (LC/MS/MS) [18] or gas chromatography with mass spectrometry (GC/MS) [19] have been used for assessment of oxidative DNA damage, frequently measured as 8-oxodG formed by oxidation of 2′-dG. The MS-methods are more specific but requires much more DNA and can induce oxidation during workup and analysis. Early publications using chromatographic methods frequently reported overestimation of the 8-oxodG levels. However, recent methodological improvements for HPLC method workup procedures based on increasing chemical insight [17,20–24] and inter-laboratory collaborations undertaken by European Standards Committee on Oxidative DNA Damage (ESCODD) [25–28] have significantly improved the analyses of oxidative DNA damage.

The aim of this study was to measure lymphocyte background levels of DNA damage in a young, healthy, Swedish population. The Comet assay and HPLC-ECD analysis of 8-oxodG were chosen methods to detect the DNA damage. We hypothesised that the background levels of oxidative DNA damage are low when analyzed with improved methods and that gender and life style factors can have an impact on DNA damage.

Materials and methods

Experimental design

Healthy nonsmoking volunteers, aged 19–31 years, were recruited at universities in Stockholm, Sweden. Blood samples were drawn midday on multiple occasions by authorized nurses from a superficial vein of the arm during the autumn when exposure from sun-light is negligible (a possible confounder) in Sweden. At the time point of blood collection all 99 volunteers (50 females and 49 males) signed a consent form and filled in a standardized questionnaire. The questionnaire covered information regarding: Gender, age, length, weight, physical activity, health status, place of residence, subjective experience of stress, fruit intake and smoking. Blood samples and questionnaires were coded and all data were treated as confidential. The group of 99 was selected from a larger group based on the inclusion criteria: No regular medication, no present (subjective) experience of disease and within the age of 19–31 years.

Human lymphocyte isolation

Five 8 ml sodium-heparin Vacutainer® CPT tubes (Becton Dickinson, Franklin Lakes, NJ) containing lymphocyte/monocyte separation medium were filled with venous blood from each volunteer. The tubes were immediately inverted 8–10 times, stored at room temperature, inverted again and centrifuged within 2 h at 1650*g* for 20 min at 20°C. The tubes were inverted once and the top layer containing lymphocytes/monocytes was poured off equally into two 50 ml centrifuge tubes. The CPT tubes were rinsed with RPMI 1640 medium containing L-glutamine (Gibco BRL, Paisley, UK) and the 50 ml tubes were filled up to 45 ml. After gently mixing by inverting the tubes, the cells were pelleted by centrifugation at 700*g* for 15 min at 20°C. The supernatant was carefully removed. The cells in each tube were gently re-suspended in 5 ml ice-cold medium (stored as aliquots at −20°C), consisting of nine parts heat-inactivated (56°C, 30 min) fetal bovine serum (Gibco) and one part sterile filtered DMSO, and combined into one tube. Four aliquots of 100 µl were taken to cryovials for comet analysis. The cryovials were placed in a paper box together with the 50 ml tubes. The paperbox was then placed in a closed expanded polystyrene box to ensure that the cells froze slowly at −80°C.

Analysis of SSB + ALS and FPG-sensitive sites

The lymphocytes were thawed by gentle swirling in a water bath (37°C) and centrifuged at 200*g* for 3 min at 4°C. The supernatant was removed and the cells were re-suspended in 200 µl cold RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. A suitable number of cells (40 µl) were washed in 1 ml of ice-cold PBS. The suspension was centrifuged at 200*g* for 3 min and 1 ml of the supernatant was removed. After carefully re-suspending the cells in the remaining 40 µl of solution, 15 µl was added to 100 µl 0.75% w/v low melting point agarose (38°C). Three aliquots were spread over a pre-coated (0.3% w/v agarose) microscope slide. Working on ice in the dark, the slides were positioned vertically in lysis buffer (0°C, 1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, pH 10) for 1 h and thereafter three

times for 5 min each in fresh FPG enzyme buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM Hepes, 0.2 mg/ml bovine serum albumin, pH 8) for washing and equilibration.

The FPG enzyme (bacterial *E. coli* DNA repair endonuclease) [25] for detection of mainly purine oxidation products (kindly provided by Dr A. R. Collins, Rowett Research Institute, Aberdeen, Scotland, UK) was diluted 1:3000 in enzyme buffer and 30 μl diluted enzyme, or enzyme buffer only, was added to each gel on the microscope slides. Cover slips were placed on the slides and incubation was performed in a humidity chamber at 37°C for 30 min. The slides were placed in PBS containing 2 mM EDTA for 5 min and the cover slips were carefully removed. DNA unwinding was performed in 0.3 M NaOH (0°C) with 1 mM EDTA for 40 min and electrophoresis was thereafter performed in a sub-cell GT unit (BIO-RAD, Richmond, CA) for 30 min in 0.3 M NaOH with 1 mM EDTA at 25 V (≈ 0.86 V/cm). The slides were washed twice in 0.4 M Tris (pH 7.4) for 5 min followed by a water wash for 5 min, dried over night at room temperature and fixed in methanol (5 min). DNA was stained with 10 μg/ml ethidium bromide in Tris–acetate–EDTA (TAE, pH 7.8) buffer (5 min) and the slides were thereafter placed in TAE (5 min) to remove excess ethidium bromide. The comets were examined using a BH-2 fluorescence microscope (Olympus Optical Co Ltd, Tokyo, Japan) with a 20 × apochromatic objective, using the image analysis program Komet 4.0 (Kinetic Imaging Ltd, Liverpool, UK).

Three gels with FPG and three with only buffer were analyzed for each lymphocyte sample and 50 cells per gel were scored. The conversion from tail DNA (%) to 8-oxodG/10^6 dG was done based on a standard calibration curve provided by ESCODD (constructed by Dr Rudolf Stetina, Purkyné Military Medical Academy, Czech Republic, using X-ray and assuming that 1 Gray induces 0.31 breaks/109 Daltons). The net FPG level was obtained by subtracting the SSB + ALS sites obtained with no enzyme added, from the gross sites having FPG present.

Analysis of 8-oxodG

The 50 ml tubes were thawed in a 37°C water bath and ice-cold PBS was added up to 45 ml to remove the freezing medium by centrifugation at 700*g* (7 min at 4°C). From this point, the recently developed cold (0°C) high salt guanidine thiocyanate (4 M GTC) DNA extraction method was used [17]. All solutions were chelex treated to remove transition metals and all steps, except DNA hydrolysis, were performed at 0°C. Cells were lysed in 4.0 ml 0.5% v/v Tween-20, 20 mM Tris, pH 7.5 (with 1 mM freshly prepared deferoxamine mesylate) by gentle pipetting. After incubation on ice (5 min), crude nuclei were collected at 1500*g* (5 min). The supernatant (containing membranes, proteins, mitochondria and most of the RNA) was discarded and the procedure was repeated. The crude nuclei pellets were dissolved in 1.7 ml ice-cold 4 M GTC (20 mM Tris, pH 7.5, containing 1 mM freshly prepared TEMPO). After 5 min on ice and pipetting to release DNA from histones, the solutions were transferred to phase lock gel (PLG, heavy) tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany) and an equal volume of sevag (chloroform:isoamylalcohol, 24:1) was added to remove proteins and lipids by centrifugation (13,000*g*, 5 min). This procedure was repeated once. After withdrawal of the aqueous phase, an equal volume of isopropanol was added and DNA was precipitated at −20°C (15 min). DNA was collected by spinning at 20,800*g* (10 min). The DNA pellets were washed in 900 μl 70% v/v ethanol and spun down for at 20,800*g* (3 min). After removal of all liquid, the DNA was dissolved in 150 μl water containing 50 U/ml catalase (*Aspergillus niger*) and 1 mM TEMPO, as antioxidants. Hydrolysis buffer was added together with enzymes to 300 μl (final concentrations: 30 μg nuclease P1, 3 U alkaline phosphatase, 25 mM sodium acetate (pH 5.3), 0.1 mM zinc chloride, 50 U/ml catalase and 1 mM TEMPO) and the DNA was hydrolyzed (50°C, 60 min) in a 1.5 ml PLG (light) tube. The water and hydrolysis buffer were pre-incubated with 50 U/ml catalase at room temperature in the dark (4 h) to remove background hydrogen peroxide. Sevag (300 μl) was added, the tubes were briefly shaken and proteins were removed at 13,000*g* for 5 min. The upper phase was transferred to a 0.5 ml tube and stored at −80°C.

HPLC-ECD analysis. Simultaneous analysis of 8-oxodG and dG by HPLC-ECD was performed as previously described [17]. Triplicate injections of 100 μl in succession was performed and the amount of 8-oxodG and dG were calculated from calibration curves (commonly $r = 0.95\ -1$) made from four injections of each standard on a daily basis. The method is validated within ESCODD and gave very low background levels [25].

Statistical analysis

For populations comparisons the Student's *t*-test was used. Linear regression using STATISTICA (StatSoft Inc., Tulsa, OK) was used for tests of correlation (variables were assumed to weakly correlate if the correlation coefficient $r > 0.3$ and to strongly correlate if $r > 0.5$). The statistical significance level was set at $P < 0.05$. Male and female data were separately analyzed against lifestyle and physical factors.

Results

Background levels of DNA damage

As shown in Figure 1(A)–(C), the background levels of SSB + ALS, net FPG and 8-oxodG in the human lymphocytes were normally (Gaussian) distributed. For Comet analysis the means were 0.387 ± 0.081 (SSB +ALS)/10^6 dG ($n = 99$) and 0.236 ± 0.076 net FPG/10^6 dG ($n = 99$). For 8-oxodG the mean background level in DNA from lymphocytes was 0.917 ± 0.418 8-oxodG/10^6 dG ($n = 94$; female $n = 46$, male $n = 48$). The three parameters of DNA damage differed significantly ($P < 0.001$).

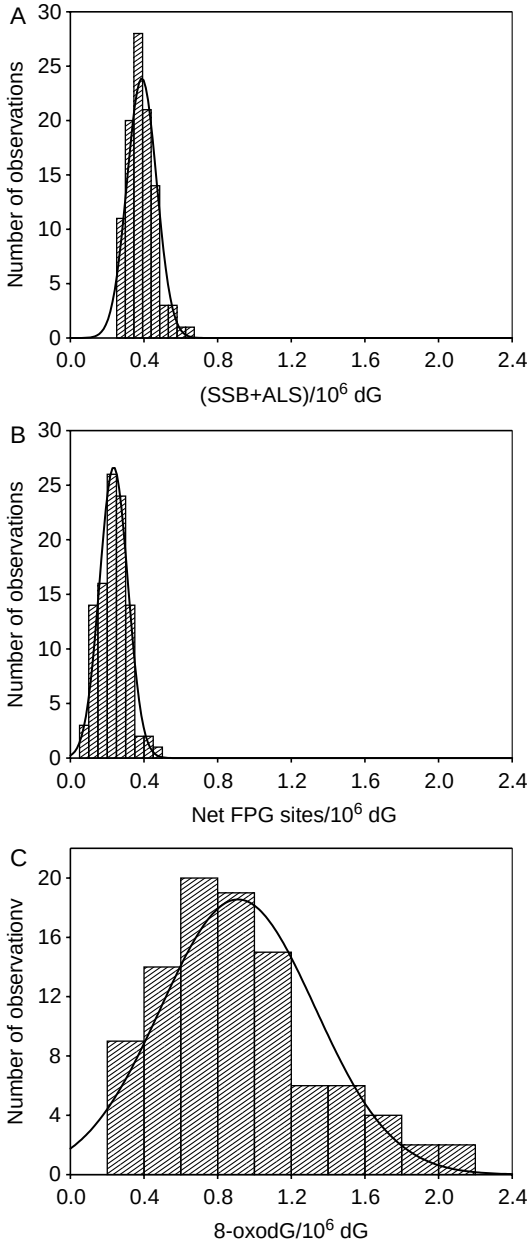

Figure 1. Normal distribution profiles of background DNA damage in lymphocytes from persons aged 19.31 years. (A) Comet SSB + ALS (no enzyme present) with a mean of 0.387 ± 0.081/10^6 dG ($n = 99$); (B) comet net FPG sites with a mean of 0.236 ± 0.076/10^6 dG ($n = 99$); (C) HPLC-ECD analysis of 8-oxodG with a mean of 0.917 ± 0.418 8-oxodG/10^6 dG ($n = 94$). The three parameters differ significantly ($P < 0.001$).

Gender comparison of DNA damage

For gender comparisons (Table I and Figure 2) of SSB + ALS damage, males (0.416 ± 0.080 (SSB + ALS)/10^6 dG, $n = 49$) had significantly ($P < 0.001$) higher levels of DNA damage than females (0.358 ± 0.071 (SSB +ALS)/10^6 dG, $n = 50$). Also the individual Comet parameters; olive tail moment (arbitrary units), tail DNA (%) (Figure 2) and tail length (μm) were significantly higher in males (Table I). However, for oxidative damages (measured by net FPG or 8-oxodG), no gender differences were found.

Relationship between the biomarkers for DNA damage

As shown in Figure 3(A), no correlation between the background oxidative DNA damage parameters 8- oxodG and net FPG data could be found (trend-line: $Y = -0.183X + 0.962$, $r = 0.03$). Also for 8-oxodG versus (SSB + ALS) (trend-line: $Y = -0.344X + 1.05$, $r = 0.07$), or net FPG versus (SSB + ALS) (trendline: $Y = 0.0564X + 0.214$, $r = 0.06$), no correlation was observed (graphs not shown).

Correlation tests for physical or lifestyle factors

Correlation tests for physical and lifestyle factors are shown in Table II and Figures 3 and 4. For age, a slight non-significant increase in DNA damage for the three investigated parameters was noticed using trend-lines (Figure 3(B) and (C)). This observation was seen for both males and females in the narrow age range (19–31 years of age) of the population.

For fruit intake, a significant correlation ($P < 0.01$) for an observed decrease in DNA damage (SSB + ALS) levels with increasing fruit intake for females was observed (Table II). Females reported a significantly ($P < 0.01$) higher average daily fruit-intake (2.1 ± 1.4 times/day) when compared to males (1.3 ± 1.2),

Table I. Effect of gender on various parameters using the Comet assay or 8-oxodG-analysis.

Parameter	Female (n=50)	Male (n=49)
(SSB + ALS)/10^6 dG	0.358 ± 0.071⋆	0.416 ± 0.080
Tail DNA (%)	6.67 ± 1.30⋆	7.90 ± 1.44
Olive tail moment (a.u.)	1.43 ± 0.28⋆	1.75 ± 0.54
Tail length (μm)	34.57 ± 4.29†	38.24 ± 8.25
Net FPG/10^6 dG	0.225 ± 0.073	0.247 ± 0.078
8-oxodG/10^6 dG	0.930 ± 0.384	0.905 ± 0.452

All values are mean ± S.D. in each group. ⋆$P < 0.001$, when compared to males. †$P < 0.01$, when compared to males.

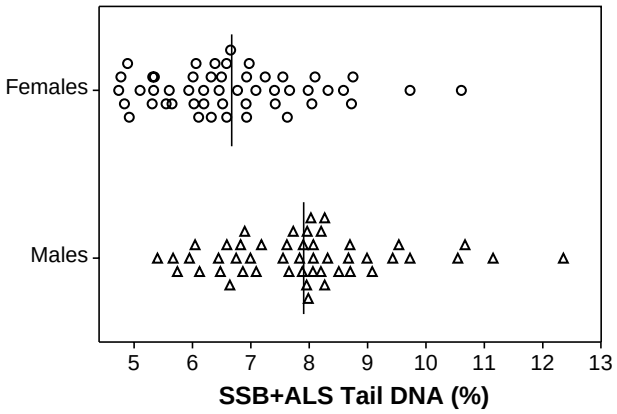

Figure 2. Gender comparison of background Comet SSB + ALS tail DNA (%) in human lymphocytes. Males (7.90 ± 1.44; $n = 50$) had significantly higher levels ($P < 0.001$) than females (6.67 ± 1.30; $n = 49$). Scatter plots with lines at means.

being the largest observed differing factor for the two sexes.

A correlation between increased BMI and increased oxidative stress (net FPG) levels was observed in the female part of the population ($P < 0.05$, Figure 4). BMI (kg/m^2) indicates the degree of obesity (>30.0), overweight (>25.0) or underweight (<18.5) for adults over 20 years of age [29]. The studied group was within the normal BMI range with an average of 22.1 and 23.5 for females and males, respectively.

For physical activity (ranged from 0 (no activity) to 10 (heavy intense activity every day)), subjective experience of stress (ranged from 0 (never) to 10 (constantly)), and number of colds experienced in recent years (ranged from 0–10 times per year), no significant correlation ($r < 0.3$ and $P > 0.05$) to DNA damage was observed.

The participant's place of residence in Stockholm was geographically divided into central (down-town, 30.3%), suburb (66.7%) or countryside (3.0%), respectively. No difference was found for any of the residence parameters measured in relation to DNA damage.

Discussion

The study population represented a selected healthy and young group with a normal BMI and without any known diseases or medication, living in Sweden. Therefore, it could be assumed that all data would be within a narrow range.

The three parameters measured for human lymphocyte DNA damage were normally distributed (Figure 1). The comet data (Figure 1(A) and (B)) showed a considerable narrower span when compared to the 8-oxodG data (Figure 1(C)). Further, the FPG (oxidative lesions) level was lower when compared to the total level of strand breakes indicating that other exposures led to DNA damage.

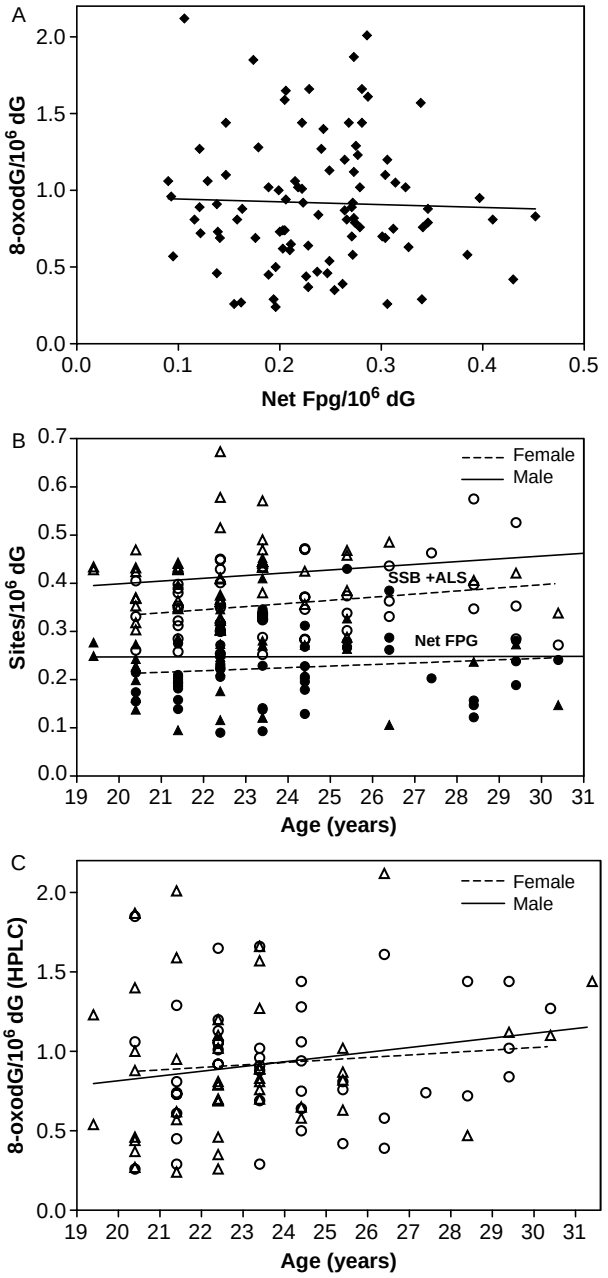

Figure 3. Correlation tests in human lymphocytes for DNA damage, measured by the Comet assay ($n = 99$) and HPLC-ECD ($n = 94$), respectively. (A) Test for correlation of oxidative DNA damage in human lymphocytes for 8-oxodG (HPLC-ECD) versus the enzymatic method (Comets, net FPG). No significant correlation between the 8-oxodG and the net FPG data could be found in a narrow span (19.31 years) of healthy individuals (trend-line: $Y = -0.183X + 0.962$, $r = 0.03$, $n = 94$); (B) and (C) show tests for correlation between age and DNA damage (see Table II for tests and *r*-values of other factors). Five of the six curves had a trend of increasing damage over the narrow age span of 19–31 years of age in males and females. Circles (○) indicate females and triangles (△) males, respectively. Open symbols represent SSB + ALS, and filled symbols represent net FPG.

Using sensitive methodology with improved workup procedures, the presented data is lower and shows less deviation in comparison to most other published

Table II. Correlation test between lifestyle or physical factors and DNA damage using the coefficient *r*.

Factor (span)	Sex	Average ($x \pm$ SD)	SSB + ALS (*r*)	Net FPG (*r*)	8-oxodG (*r*)
Age (19–31 years)	f	24.0 ± 2.7	0.24	0.12	0.11
	m	23.4 ± 3.3	0.19	0.00	0.18
BMI (17.5–30 kg/m^2)	f	22.1 ± 2.2	0.01	**0.30**†	0.02
	m	23.5 ± 2.5	0.18	−0.15	0.15
Physical activity (0–10)	f	5.5 ± 1.7	−0.05	0.15	−0.25
	m	5.0 ± 2.2	0.04	0.03	−0.01
Fruit intake (0–7 times/day)	f	2.1 ± 1.4*	**−0.37***	0.20	0.21
	m	1.3 ± 1.2	−0.05	0.00	0.01
Stress (0–10)	f	4.7 ± 2.0	0.09	0.04	−0.02
	m	4.5 ± 2.3	0.01	−0.03	−0.23
Colds (0–10 times/year)	f	2.7 ± 2.0	0.08	0.05	0.01
	m	2.9 ± 1.7	−0.18	−0.01	−0.07

$^\star P < 0.01$; $^\dagger P < 0.05$

values for lymphocyte levels of SSB + ALS [30], net FPG sites [31] and 8-oxodG [31–33], thus representing a thorough analysis of background levels of DNA damage in a healthy young population. When compared to double blind inter laboratory trials the methods applied in this paper represent the lowest levels (i.e. a minimum of work-up generated oxidation) as reported by ESCODD [25]. The authors of this paper represented laboratory "15" in that paper [25].

Notably, for oxidative lesions the measured average level of 8-oxodG was 3.9 times higher using chemical detection by HPLC-ECD than for the enzymatic detection of net FPG sites using the Comet assay (Figure 1(B) and (C)), and no correlation between these parameters was observed (Figure 3(A)). This is in agreement with a recent lymphocyte study by Gedik et al. [34]. The main reason for this is probably that the methods do not measure the same damage. In addition to 8-oxodG, FPG is known to also recognize ring-opened purines [35] and there have also been studies reporting the recognition of 5-hydroxycytosine [36] as well as alkylation damage [37]. However, this does not explain the lower levels of FPG sites compared with 8-oxodG. It is possible that the measured 8-oxodG levels by HPLC-ECD are still too high due to artifactual oxidation but is it also possible that the FPG enzyme underestimated the damage [38]. HPLC-ECD has, however, recently been found to be equally effective as the Comet assay using FPG in detecting oxidative DNA damage induced in HeLa cells by visible light and photosensitizer in direct comparison [34]. In this study the lymphocyte background level of 8-oxodG in a young healthy population was found to be approximately 0.9 ± 0.4 (8-oxodG/10^6 dG).

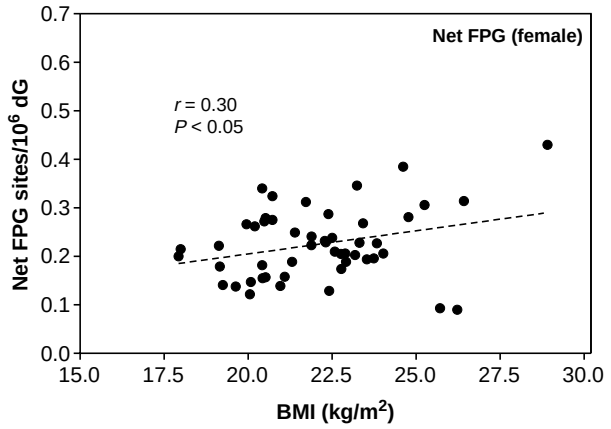

Figure 4. Correlation test between female BMI and DNA damage measured by the Comet assay. With increasing BMI followed a significant increase in the net FPG levels ($P < 0.05$, $n = 50$).

The finding herein that lymphocytes from males had significantly elevated background levels of DNA damage (SSB + ALS) compared to females in a healthy young control group is worthy of note. This finding is supported by other recent studies measuring SSB + ALS in non-smokers using the alkaline Comet assay: Baypayee et al. [30] found statistically significant ($P < 0.001 - 0.05$ depending on Comet parameter, $n = 206$) higher levels of DNA damage in healthy Indian urban male subjects aged 20–30 years when compared to women. Lam et al. [9] found significantly ($P < 0.001$, $n = 255$) higher levels for non-smoking male Chinese workers, and Wojewódzka et al. [12] reported significant ($P < 0.02 - 0.05$ depending on test, $n = 40$) higher levels for males in a control group in comparison with women. Further, Betti et al. [4], with inclusion of 30% smokers, reported borderline significant ($P = 0.053$, $n = 200$) higher levels in Italian males, and Giovanelli et al. [39] reported non-significant higher levels ($P = 0.20$, $n = 71$) in male Florencians (Italy) versus females. No support for the opposite (females higher than males) could be found reported. This gender difference could possibly be explained by different food habits, differences in metabolism, hormonal effects, and/or DNA repair efficiency.

From the present study, the higher levels of SSB + ALS in males could best be explained by the significantly lower fruit intake reported by males, but could also be due to non-investigated factors. Fruit

contains protective phenolic acids, flavonoids and antioxidants [40]. Other studies of the Swedish population have shown that males generally consume less fruit than females [41,42]. Noroozi et al. [43] found that flavonoids, when directly incubated with human lymphocytes, strongly and dosedependently protected from H_2O_2-induced DNA damage measured by the Comet assay (SSB + ALS).

In conclusion, we suggest that the background levels of DNA damage in a young healthy population is normally distributed for the three investigated parameters of DNA damage (SSB + ALS sites, net FPG sites and 8-oxodG) without showing any mutual correlation. Further, there is a sex-difference with higher DNA damage (SSB + ALS) in males. Factors that can affect DNA damage in a young healthy population are fruit intake (high intake; lower damage) and BMI (high BMI; higher damage). The lymphocyte background levels of 8-oxodG in a young healthy Swedish population is approximately 0.9 ± 0.4 8-oxodG/10^6 dG.

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