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Regulatory factors of basal F_2-isoprostane formation: Population, age, gender and smoking habits in humans

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Abstract

Oxidative stress is assumed to be the key underlying factor in the pathogenesis of many common diseases. This study describes the basal levels of 8-iso-$PGF_{2\alpha}$, a major F_2-isoprostane and an *in vivo* oxidative stress biomarker in healthy subjects from three countries, namely Italy, Poland and Sweden, in relation to their smoking habits, age and gender. It studied urinary 8-iso-$PGF_{2\alpha}$ in 588 subjects from Sweden ($n = 220$), Italy ($n = 203$) and Poland ($n = 165$). Polish subjects had the highest levels of F_2-isoprostanes followed by the Swedish and Italians when adjusted for smoking, age, sex and creatinine and the inter-country differences were statistically significant. Smokers had significantly higher levels of 8-iso-$PGF_{2\alpha}$ compared to non-smokers in all countries and there was a moderate decrease with age. Women had only slightly lower 8-iso-$PGF_{2\alpha}$ than men. There is a difference in F_2-isoprostane levels *in vivo* between countries. Smoking, age and gender affect isoprostane formation and should be taken into consideration in clinical studies of oxidative stress.

Keywords: *Isoprostanes, oxidative stress, variation, population, age, gender, demography, humans*

Introduction

Oxidative stress has been postulated to be the key underlying feature in the pathogenesis of many of our common diseases, such as cancer, neurological diseases, diabetes, cardiovascular diseases and obesity [1]. It is a state of serious imbalance between production of free radicals and anti-oxidative defences *in vivo*. A number of endogenous and exogenous factors are believed to be involved in the basic mechanism of catalysing this process that promote disease initiation and its further development and it also is involved in the normal ageing process in humans [2]. Oxidative stress might account for progressive damage to lipids, proteins and/or DNA. It is apparently taking place in our body continuously during the slow progression of atherosclerotic and ageing processes or in an accelerated manner during a severe episode of an acute inflammatory response. It is also becoming recognized that the free radicals comprise properties that are not only destructive in character in certain states but also essential in cell signalling to instigate assorted biochemical events necessary to maintain customary physiological functions [3,4].

Nevertheless, studies on oxidative stress in large populations have mainly been hampered by the methodological difficulties to assess lipid peroxidation

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in vivo consistently [5]. The prostaglandin-like F_2-isoprostanes are formed *in vivo* through free-radical catalysed peroxidation of arachidonic acid and are considered to be reliable indicators of oxidative stress [6,7]. F_2-isoprostanes are chemically stable bioactive compounds and are implicated in a large number of human diseases and appear to have a role in acute and sub-clinical chronic inflammation [2,4,8]. This may be linked to subtle differences in the levels of the oxidants or their end products in the typical basal state and to further pronounced elevated levels in disease circumstances They are, however, also found in a noticeable quantity in a range of tissues and body fluids yet at the normal basal state with a broad inter- and intra-individual variation [8]. The causes of this natural variation are not well understood. However, dietary factors in concert with low-grade oxidative changes and together with anti-oxidative status in the body might be involved in this observed variation.

Exceedingly little is recognized on the basal levels of isoprostanes in various populations. Neither has any study described the associations between isoprostanes and age, gender and smoking habits in different countries, except for a few reports within a population [8]. The measurement of oxidative stress by quantifying free urinary F_2-isoprostanes in large human populations allows us to study the extent of low-grade oxidative stress that might contribute as an additional risk factor to the development of several diseases [2,9]. This study describes the levels of a key F_2-isoprostane, 8-iso-$PGF_{2\alpha}$ in healthy human populations from three countries, namely Italy, Poland and Sweden, in relation to their age, gender and smoking practice.

Materials and methods

Subjects

As part of the European Union funded project to study exposure to mercury in people living close to certain industries in Sweden, Italy and Poland, subjects aged 17–66 were recruited among those living in the vicinity of these industries and also from reference areas [10,11]. Subjects were contacted by mail or personal visits to their home. Since no increased mercury exposure or other impact could be shown, the present study is based on the general population close to the industrial sites, as well as from the reference areas. The subjects were randomly selected from population registries. None of the areas were located in a large city or in a remote rural district. The Italian sites were coastal, while the Swedish and Polish sites were located inland along rivers. The only exclusion criterion was work in the afore-mentioned mercury-emitting industries. In total 681 subjects agreed to answer a questionnaire and deliver a first morning urine sample (Table I). The participation rate was 67% in Sweden, 68% in Italy and 96% in Poland. The questionnaire included questions on whether they were suffering from diabetes, hypertension or kidney disease. There were no questions on cardiovascular disease (other than hypertension) or on body weight. The study was approved by the respective Ethics committees in the three countries. However, our main analyses are based on a sub-group of 588 individuals free from self-reported diabetes, hypertension and having creatinine concentrations within the range of 0.3–4 g/L. It is known that for many biomarkers, adjustment for creatinine does not work very well in extremely diluted or concentrated urine samples collected from study subjects [12].

Measurement of 8-iso-$PGF_{2\alpha}$ (oxidative stress indicator)

The urinary samples were analysed for free 8-iso-$PGF_{2\alpha}$ by radioimmunoassay (RIA) at our laboratory as described elsewhere [13]. In brief, unextracted urine samples were used in the assay. The cross-reactivity of the 8-iso-$PGF_{2\alpha}$ antibody with 15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, TXB_2, 11β-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$ and 8-iso-$PGF_{3\alpha}$, respectively, was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6%. The Italian and Swedish samples were analysed on one occasion and the Polish samples on another. Since the internal-standard showed 14% higher values when the Polish samples were analysed, these values were multiplied by 0.86. The levels of 8-iso-$PGF_{2\alpha}$ were corrected for urinary creatinine concentrations. Creatinine levels in urine were measured by a colorimetric method (IL Test creatinine 181672-00) in a Monarch®

Table I. Characteristics of all study subjects and the sub-group (stratified by country) after exclusion of subjects with self-reported diabetes, hypertension or kidney disease or with extreme creatinine levels (<0.3 g/L or >4 g/L; 1 g = 8.84 mmol).

Group	*n*	Gender % female	Age, years mean (range)	Smokers %	Urinary creatinine, mmol/L mean (range)	Urinary 8-iso-$PGF_{2\alpha}$ (nmol/L) mean (range)
All subjects	681	46	34 (17–66)	29	13.7 (2.2–44.9)	2.81 (0.24–25)
Sub-group	588	47	33 (18–64)	29	13.8 (2.7–34.9)	2.89 (0.58–25)
Swedish	220	40	27 (18–60)	27	15.9 (3.5–34.9)	3.36 (0.72–25)
Italian	203	42	37 (18–63)	29	12.4 (2.7–32.1)	2.31 (0.58–15)
Polish	165	62	35 (18–64)	33	12.8 (4.0–31.9)	2.96 (0.71–8.2)

2000 centrifugal analyser (Instrumentation Laboratories, Lexington, MA).

Statistical analysis

The variable 8-iso-$PGF_{2\alpha}$ had a skewed distribution and was log-transformed to reach homoscedasticity and a normal distribution. Differences in 8-iso-$PGF_{2\alpha}$ between Swedish, Italian and Polish subjects and effects of creatinine concentration, age, gender and smoking were assessed by analysis of covariance. In addition, the data was tested for an interaction between country and smoking. Associations between isoprostane and creatinine concentrations were evaluated using the Pearson correlation coefficient. *P*-values < 0.05 were statistically significant. Calculations were performed with Stata 8.2 (Stata Corporation, College Station, TX) and SAS 9.1 (SAS Institute Inc., Cary, NC).

Results

The characteristics of the subjects are described in Table I.

F_2-isoprostanes, demography and creatinine

The urinary levels of 8-iso-$PGF_{2\alpha}$ in Sweden, Italy and Poland are shown in Table II. When adjusted for age, gender, smoking and creatinine concentrations, the differences between countries were all highly statistically significant ($p < 0.0001$). Figure 1 shows the age and creatinine-adjusted levels stratified for smoking status. The somewhat higher F_2-isoprostane levels in Sweden compared to those in Italy are not revealed in the unadjusted results in Table II, since the mean age of the Swedish subjects was lower and the creatinine levels higher, both factors associated with decreasing F_2-isoprostane levels, see below. As could be expected creatinine concentrations were higher in men than in women (medians 15.1 and 11.5 mmol/L, respectively).

Except for country, the creatinine concentration was the strongest predictor for creatinine-adjusted 8-iso-$PGF_{2\alpha}$ ($p < 0.0001$). As shown in Figure 2A, the concentrations of unadjusted 8-iso-$PGF_{2\alpha}$ increased with creatinine concentration, as could be expected (the theoretical basis for creatinine adjustment). The Pearson correlation coefficient between concentrations was 0.45 ($p < 0.0001$). However, levels expressed per mmol creatinine caused a certain amount of 'over-adjusment' (Figure 2B). The negative association was weaker ($r_p = -0.27$), but highly significant ($p < 0.0001$). The effect size corresponded to a decrease of $\sim 22\%$ for an increase in creatinine concentration of 1 g/L (8.84 mmol/L) in a model including country, age, smoking and gender.

F_2-isoprostanes, smoking, age and gender

Results for urinary F_2-isoprostane stratified by country, smoking and gender are shown in Table II. The urinary level of 8-iso-$PGF_{2\alpha}$ was on average 20% higher in smokers than in non-smokers ($p < 0.0001$, adjusted for country, smoking, age and gender). The effect of age in the model was relatively small ($\sim 5\%$ decrease for every 10 years), but statistically significant ($p < 0.0006$). Women had significantly lower ($\sim 8\%$) levels of 8-iso-$PGF_{2\alpha}$ than men in the multivariate model ($p = 0.02$).

In sub-analyses by country, the effects of creatinine, smoking, age and gender were similar to in the total data set, although, the effects of age and gender in some cases did not reach the 5% significance level. Although the interaction between country and smoking was not statistically significant, analyses stratified by country showed that the effect size of smoking was strongest in Sweden (28% higher), intermediate in Italy (20% increase) and smallest in Poland (13% increase). Note, however, also that we did not have individual quantitative data on smoking in all countries.

Discussion

This study presents variation in basal urinary F_2-isoprostane levels *in vivo* across countries, namely Italy, Poland and Sweden. Smokers have higher basal

Table II. F_2-isoprostanes (pmol/mmol creatinine) stratified by country, smoking habits and gender (median, range and *n*) after exclusion of subjects with self-reported diabetes, hypertension or kidney disease or with extreme creatinine levels (<0.3 g/L or >4 g/L).

	Men		Women		
	Non-smokers	Smokers	Non-smokers	Smokers	All
All	182 (64–1235), $n=217$	213 (70–677), $n=89$	204 (68–449), $n=195$	245 (116–752), $n=81$	200 (64–1235), $n=588$
Swedish subjects	169 (64–1094), $n=101$	210 (70–543), $n=28$	170 (79–377), $n=59$	238 (125–752), $n=30$	179 (64–1094), $n=220$
Italian subjects	176 (74–1235), $n=75$	198 (106–677), $n=40$	181 (68–407), $n=67$	222 (116–352), $n=17$	189 (68–1235), $n=203$
Polish subjects	221 (107–525), $n=41$	230 (143–436), $n=21$	235 (133–449), $n=69$	281 (145–415), $n=34$	243 (107–525), $n=165$

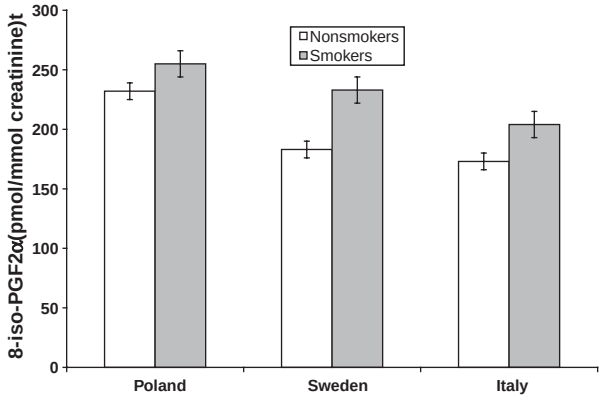

Figure 1. Mean 8-iso-$PGF_{2\alpha}$ (pmol/nmol creatinine) and SEM in 306 healthy non-smoking (217) and smoking (89) Swedish, Italian and Polish men. F_2-isoprostane levels were adjusted for age and creatinine concentrations.

urinary F_2-isoprostane levels. There were also modest effects of age and gender.

We have found a negative correlation between creatinine-adjusted 8-iso-$PGF_{2\alpha}$ and creatinine concentration. The reason for the slight negative association between creatinine-adjusted 8-iso-$PGF_{2\alpha}$ and creatinine concentration could be that high urinary flow rate, resulting in 'diluted' urine and a low creatinine concentration, increases the excretion rate of 8-iso-$PGF_{2\alpha}$. Another factor to consider is body weight. It is well known that a large muscle mass increases creatinine excretion. However, since the excretion of isoprostane increases with body mass index [14], this would have an effect in the opposite direction compared to that shown in Figure 2B, and could not explain the negative association between creatinine-adjusted 8-iso-$PGF_{2\alpha}$ and creatinine concentration.

Demography

The current study shows that there are demographic differences in this population-based study. Polish people have the uppermost levels of isoprostanes followed by the Swedish and Italian subjects when adjusted for smoking, age and gender. To our knowledge, this is the first population-based study using alike methods, showing such dissimilarity in the basal levels of lipid peroxidation amongst countries. The causes might be, for instance, involvement of diet, antioxidants or metabolic factors, endothelial function and redox potential of the body. It has earlier

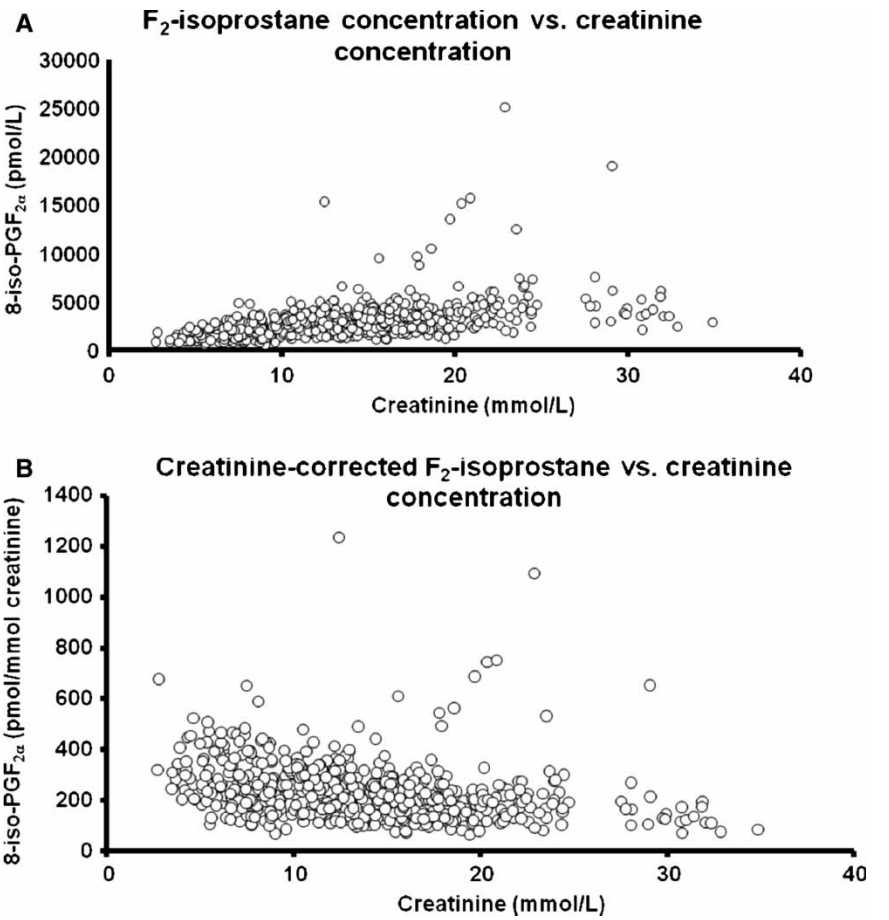

Figure 2. Excretion of 8-iso-$PGF_{2\alpha}$ in 588 healthy subjects from Sweden, Italy and Poland. (A) The positive association between uncorrected urinary F_2-isoprostane concentrations and the creatinine concentrations. (B) After adjustment for creatinine, the adjusted F_2-isoprostane excretion is somewhat negatively associated with the creatinine concentration, indicating a slight 'over-adjustment'.

been reported that the levels of F_2-isoprostanes are not affected by the lipid content of the diet [15], but are regulated by exogenous antioxidants among smokers and in the animal models of oxidant stress [16–19]. A differential intake or endogenous status of antioxidants might be a major regulating factor when considering the oxidative status among different populations. Consumption of a Mediterranean diet or other long-term beneficial diets that contains high levels of antioxidants could exert a free radical scavenging effect that may be reflected in the lower levels of F_2-isoprostanes among the Italian subjects. Nonetheless, a 4-week Mediterranean-inspired diet in healthy humans did not change the urinary F_2-isoprostane levels compared to the normal Swedish diet [20]. This might be related to the short-term use of the diet in that study. Another factor to consider is body weight. High BMI has been shown to increase F_2-isoprostane levels [14,21]. In the WHO MONICA study the mean BMI in the mid-1990s was 26 in Italy, 27 in Poland and 26 in Sweden. The percentage of energy intake from fat was lower in Poland than in Italy and Sweden [22]. Nevertheless, since we do not have such data on individual levels in our study groups, we cannot exclude that anthropometric differences could partly explain the differences between countries in F_2-isoprotane levels. Apart from diet, exposure to air pollution is another causative factor that differs between the three countries. For example, coal burning for heating is still common in Poland and environmental tobacco smoke may also be more common in this country. A more detailed study of the effect on isoprostane levels of dietary and environmental factors within and between countries would be valuable. Both oxidative stress and low-grade inflammation have been hypothesized to be the central underlying factors in the development of cardiovascular diseases. The observed heterogeneity in the basal levels of isoprostanes among various populations could further contribute to different patterns of risk factors of cardiovascular disease.

Age

The role of free radicals and accumulation of oxidative damage in ageing processes is recognized [23]. Health consequences of free radicals increase with advancing age and this might be the result of either the increased levels of free radicals reactions or a deficiency in antioxidant status within the organism. Age of the subjects in our study ranged from 17–66 years. A weak decrease of F_2-isoprostanes excretion with age was found. However, this was mainly seen among non-smokers. The reason for this could be that the pro-oxidant effect of smoking outweighs the effect of age. Another reason could be lower muscle mass or oxygen consumption perhaps associated with age. In sub-analyses the effect of age was similar in all three countries. Results from the Framingham cohort showed that 8-iso-$PGF_{2\alpha}$ was negatively correlated with age [14], which is in accordance with the present study. However, most reports involving adults show a lack of relationship between age and 8-iso-$PGF_{2\alpha}$ levels [24,25] or even a positive association exists [26,27]. Since the effect of age is relatively modest, large studies, like the present one, may be needed to demonstrate the effects of age.

Gender

Women had significantly (point estimate 8%) lower levels of F_2-isoprostanes, when creatinine and other background factors were taken into account. It should be noted that women generally have lower lean body mass and consequently lower levels of creatinine. The creatinine-adjusted F_2-isoprostanes levels in women should therefore be considered as results 'adjusted' for the fact that women on average are somewhat smaller than men. However, since we also included creatinine concentrations in our model, the interpretation of the slight gender difference is complicated. The crude analyses (Table II) showed similar F_2-isoprostanes in non-smoking women and men and even somewhat higher levels in smoking women. Several studies have found higher levels in women, at least in the elderly [14,24,28,29]. Thus, the impact of gender in population studies should be further studied.

Smoking habits

Cigarette smoking is universal worldwide and is associated with accelerating atherosclerosis and cardiovascular diseases [30]. Cigarette smoke contains free radicals and an assortment of toxins which contribute to the induction of free radical generation [31]. The underlying mechanisms by which smoking induces cardiovascular diseases seem to be gradual development of chronic inflammation and oxidative stress. Increased levels of F_2-isoprostanes and prostaglandins, that are potent vasoconstrictive compounds, have earlier been demonstrated among smokers in several studies [32,33]. In this study, we have revealed that smokers have higher levels of F_2-isoprostanes compared to the non-smokers also in models adjusted for age, gender, country and urinary creatinine levels in the multiple regression analysis. The present study supports previous findings and shows that both male and female smokers of varying age have elevated levels of oxidative stress across populations in different countries.

This study is a population-based cross-sectional study concerning basal F_2-isoprostane levels in men and women from three countries with varying dietary habits. Smoking and to some extent age and gender were associated with F_2-isoprostane formation in this study. Since the findings were similar across countries

the results should be valid at least for Europe and for subjects of the age range of the populations (17–66 years). Lack of data on dietary and exercise habits are the possible limitations of the study.

In conclusion, this study presents variation in basal F_2-isoprostane formation *in vivo* across countries. Smokers have higher basal urinary F_2-isoprostane levels. There were also modest effects of age and gender. These factors should be taken into consideration in oxidative stress-related clinical studies.

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At the time of the study, D. Jarosinska was with the Institute of Occupational Medicine and Environmental Health, Poland; she is now the national expert in environment and health at the European Environment Agency, Copenhagen, seconded from the Regional Board of Water Management, Gliwice. The findings and views expressed in this paper are solely of the authors and not of the European Environment Agency or its Management Board.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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