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Low 8-Oxo-7, 8-dihydro-2'-deoxyguanosine levels and influence of genetic background in an Andean population exposed to high levels of arsenic.

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Abstract:

Background: Arsenic (As) causes oxidative stress through generation of reactive oxygen species. 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-OxodG), a sensitive marker of oxidative DNA damage, has been associated with As exposure in some studies, but not in others, possibly due to population-specific genetic factors.

Objectives: To evaluate the association between As and 8-OxodG in urine in a population with a low urinary monomethylated As (%MMA) and high dimethylated As (%DMA), as well as the genetic impact on (a) 8-OxodG concentrations and (b) the association between As and 8-OxodG.

Materials and methods: Women (N=108) in the Argentinean Andes were interviewed and urine was analyzed for arsenic metabolites (ICPMS) and 8-OxodG (LC-MS/MS). Twenty-seven polymorphisms in genes related to oxidative stress and one in As(+III)methyltransferase (*AS3MT*) were studied.

Results: Median concentration of 8-OxodG was 4.7 nmol/L (adjusted for specific weight; range 1.6-13, corresponding to 1.7 µg/g creatinine, range 0.57-4.8) and of total urinary As metabolites (U-As) 290 µg/L (range 94-720; 380 µg/g creatinine, range 140-1100). Concentrations of 8-OxodG were positively associated with %MMA (strongest association, p=0.013), and weakly associated with U-As (positively) and %DMA (negatively). These associations were strengthened when taking ethnicity into account, possibly reflecting genetic differences in As metabolism and genes regulating oxidative stress and DNA maintenance. A genetic influence on 8-OxodG concentrations was seen for polymorphisms in apurinic/apyrimidinic endonuclease 1 (*APEX1)*, DNA-methyltransferases 1 and 3b (*DNMT1, DNMT3B*), thioredoxin reductase 1 *(TXNRD1*) and 2 (*TNXDR2*) and glutaredoxin (*GLRX*).

Conclusion: Despite high As exposure, the concentrations of 8-OxodG in this population were low compared with other As-exposed populations studied. The strongest association was

found for %MMA, stressing that some inconsistencies between As and 8-OxodG partly depend on population variations in As metabolism. We found evidence of genetic impact on 8-OxodG concentrations.

Keywords: 8-Oxo-7, 8-dihydro-2'-deoxyguanosine, Arsenic, methylation, oxidative stress, polymorphisms, *APEX1*

1. Introduction

Many millions of people around the world are exposed to high levels of arsenic (As) in drinking water. As is associated with skin, lung and bladder cancers [1,2], as well as vascular diseases, hepatotoxicity, neurotoxicity, diabetes, chronic cough and impaired fetal and child development [2-4]. One mechanism involved in various As-related toxic effects, including carcinogenesis, is the generation of reactive oxygen species (ROS) [5,6]. One of the major ROS-induced DNA base-modified products, 8-Oxo-7, 8-dihydro-2'-deoxyguanosine (8-OxodG), is a sensitive marker of oxidative DNA damage.

Associations between 8-OxodG and As have been seen in several studies [7-13]. However, other studies did not find any association among none-diseased individuals [9,14-15].

The metabolites are most toxic in their trivalent forms, i.e. inorganic arsenite (iAsIII), monomethylated As methylarsonous acid (MMAIII) and dimethylated As dimethylarsinous acid (DMAIII), while pentavalent MMA and DMA have lower body retention and are easily excreted in urine [16]. The association between the fraction of MMA in urine, probably reflecting MMA^{III} in tissues, and the risk of various health effects is well documented (see e.g. [17-20]). Concentrations of 8-OxodG in urine have also been shown to increase with increasing MMA concentrations [10,21].

The variation in As metabolite pattern is partly due to genetic polymorphisms, especially in As(+III)methyltransferase *(AS3MT)* [22-27]. Other polymorphisms that may

influence the 8-OxodG levels include genes involved in ROS detoxification and resistance. Superoxide dismutase 2 *(SOD2),* catalase *(CAT)* and glutaperoxidase 1 *(GPX1*) are involved in ROS detoxification. *GPX1* needs glutathione (GSH) to detoxify H_2O_2. GSH is also a powerful antioxidant in itself, keeping the cellular environment in a reduced state by donating electrons to ROS. The rate-limiting step in the synthesis of GSH is the formation of γ-glutamyl-cysteine by glutathione cysteine ligase (GCL), encoded by two genes (*GCLC*: glutathione cysteine ligase catalytic subunit and *GCLM:* glutathione cysteine ligase modifier subunit). The degradation of GSH is taken care of by gamma-glutamyl-transferase (*GGT1*). Glutathione reductase *(GSR)* and glutaredoxin *(GLRX)* are involved in the reduction and oxidation of GSH. Thioredoxin *(TXN)* is another potentially influential antioxidant which facilitates the reduction of other proteins by a cysteine thiol-disulfide exchange. *TXN* is reduced by thioredoxin reductases 1 and 2 *(TXNRD1, TXNRD2),* and oxidized to $TXNS_2$ by peroxiredoxin 2 (via reduction of H_2O_2). *TXN* are not only an antioxidant but also could influence arsenic related effects by its role in arsenic metabolism and apoptosis [28,29]. GSH is also a substrate in conjugation reactions to a number of different xenobiotics, thereby promoting their elimination. These reactions are facilitated by glutathione transferases (GSTs). Possibly, glutathione transferase mu *(GSTM1)* affects 8-OxodG levels [15]. Also, the DNA repair gene apurinic/apyrimidinic endonuclease *(APEX1)*, which is involved in repair of 8-OxodG, may modify 8-OxodG levels [15].

Recently, genes involved in the stability of DNA methylation have been shown to influence resistance to oxidative stress. Mishra and colleagues showed that overexpression of the DNA-methyltransferase 1 (*dnmt1*) was linked to H_2O_2 resistance in rat cells, while human knockout cells that do not express neither *DNMT1* nor *DNMT3B* were more sensitive to the cytotoxicity of H_2O_2 [30]. Polymorphisms in *DNMT1* and *DNMT3B* have been shown to

affect DNA damage of mutagenic compounds as benzo(a)pyrenediolepoxide (BPDE) among smokers [31].

The aim of this study was to elucidate the interaction between As exposure, As metabolism and genetic factors, for the induction of DNA damage, as assessed by 8-OxodG in urine. Therefore, we phenotyped and genotyped inhabitants from a rural Andean village in northern Argentina exposed to high levels of As in drinking water. This population, mainly consisting of indigenous Andean people, shows an unusual As metabolism characterized by low %MMA and a high %DMA in urine [32].

2. Materials and Methods

2.1 Study areas and populations

Participants were women living in San Antonio de los Cobres (SAC), a village with about 5000 inhabitants in the northern Argentinean Andes (3800 m above sea level). All individuals have drinking water from the same source with a concentration of about 200 μg As/L [23], with a fairly small variation over time [33]. This is a poor population. The local economy is based on rudimentary agriculture and breeding of llamas, goats and sheep. About 26% of the population is illiterate. Most people live in houses of clay that are heated by stoves. The climate is characterized by cold and dry winters and hot summers. Spot urine and venous blood samples [23] were collected from a total of 111 women in 2004 and 2005, of which 108 individuals had successful 8-OxodG data. Interviews revealed that individuals from SAC were mainly of indigenous (Atacameño) origin, with varying Spanish origin. Participants were not first-degree relatives.

2.2 As analysis

Speciation of As metabolites (inorganic As (iAs), MMA and DMA) in urine for assessment of the metabolite pattern was performed using HPLC-HG-ICPMS (Agilent 1100 series system; Agilent 7500ce; Agilent Technologies, Japan and Germany) employing adequate quality control [23,34]. To compensate for variations in dilution of urine, concentrations were adjusted to the mean specific gravity (SG) of 1.019 g/ml, measured by a hand refractometer (Atago, Japan).

2.3 8-OxodG analysis

The samples haves been stored in -20 °C for 2-3 years before analysis, and during this storage condition 8oxodG has been reported to be stable [35]. Concentrations of 8-OxodG in urine were measured using LC/MS/MS, with prior purification using solid-phase extraction according to Ref. [36] with some modifications. 5 pmol of the internal standard, $^{15}N_5$-8-OxodG [37], was added to aliquots of 200 µl urine and ddH_2O was added up to a volume of 1 ml. An external standard in urine of a total of 0, 500, 1000, 2500 and 5000 fmol 8-OxodG (Sigma-Aldrich, Germany) per sample was used as a calibration standard for all samples. Oasis HLB columns, 1cc, 30mg (Waters, Milford, MA, USA) were used for the solid-phase extraction. Samples were dried in N_2 and dissolved in 50 µl ultrapure H_2O. The analysis of the samples was performed using LC/MS/MS with electrospray ionization (API 3000, Applied Biosystems, Foster City, CA, USA) coupled to a liquid chromatography system from Perkin Elmer (Norwalk, CT, USA). The MS analyses were carried out using selected reaction monitoring (SRM) in the positive ion mode according to the method published by Teichert et al. [36].

A 10 µl aliquot of the purified sample was injected into a GraceSmart RP C18 183u 50mm × 2.1mm column (Grace, Deerfield, IL, USA) and eluated with 0.5% HAc in H_2O and 0.5% HAc in MeOH. The flow rate was 0.3 ml/min. Initially, the mobile flow was 95% water

for 0.5 min isocratically, and then a linear gradient was applied to 95% methanol in 2 min. The column was equilibrated at 95% water for 5 min. The temperature of the auxiliary gas was set to 350 °C and the ion spray voltage was 5000 V. Nitrogen was used as the curtain, turbo ion spray, collision and nebulizer gas in the system. All samples were above the limit of detection (0.5nmol/L).

All data acquisition and processing were performed using the Analyst 1.4.2 software (Applied Biosystems, Foster City, CA, USA). Quantification of 8-OxodG in each urine sample was determined from the ratio of the peak area of 8-OxodG to that of the internal standard in the same sample, calibrated on the standard curve. The samples were prepared and analyzed in two different analytical sample sets on different days and each sample was measured twice in the same set. The coefficient of variance was 8%.

The 8-OxodG concentration was calculated as the mean value of the two runs. Samples that had a difference in mean values of over 20% for two determinations were rerun. Samples from two individuals failed to show a difference in mean values lower than 20%, in spite of a number of reruns, and were thus excluded. Concentrations were adjusted to the mean specific gravity (SG) of 1.019 g/ml. Also, in order to make comparisons with other studies, the data was corrected by urinary creatinine, where the ratio of 8-OxodG (μg/L) to creatinine content (g/L) was evaluated. The creatinine levels were analyzed using an enzymatic method described by Mazzachi et al. [38].

2.4 Genotyping

DNA was isolated from either whole blood or buccal cells according to Schläwicke Engström et al. [23]. In our previous studies regarding association between genotype and As metabolite pattern [23,24], forty-nine SNPs were genotyped using Sequenom™ (San Diego, CA, USA) technology, multiplex PCR, restriction fragment length polymorphism analysis (RFLP) and

Taqman allelic discrimination assay (Applied Biosystems). Among these, twenty-four SNPs were in genes considered relevant for this study. Additionally, *CAT* rs1001179, *SOD2* rs4880, *GPX1* rs1050450 and *APEX1* rs1130409 were genotyped. Sequences of primers and probes are available upon request. The *CAT* rs1001179 and *APEX1* rs1130409 were genotyped using the Taqman allelic discrimination assay (ABI 7000 instrument; Applied Biosystems) with a reaction volume of 25 μl and 10 ng/reaction DNA, as template. For *GPX1*, 2 unit *Dde*1 (New England Biolabs Inc., Ipswich, MA, USA) were used on 14 μl purified template in a reaction volume of 16.5 μl and incubated in a heat oven at 37 °C for 4h. For *SOD2*, 0.5 unit *BsaW*1 (New England Biolabs Inc.) was used on 15 μl purified template in a reaction volume of 20 μl and incubated in a heat oven at 60 °C for 2 hours. The RFLP products were analyzed with agarose gel electrophoresis (3%). Controls for each genotype were included in each run, and genotyping was repeated on 5% of the samples.

2.5 Statistical analyses

In order to evaluate the association between As and 8-OxodG (dependent variable) in urine, univariate regression analyses were performed. The following As markers were evaluated: U-As (total urinary metabolites of As), %iAs, %MMA and %DMA. Furthermore, for this population there was information about the following potentially influential variables: weight, BMI, age, hemoglobin and chewing of coca leaves; each variable was tested and included in the multivariate regression analyses if it had a p-value below 0.2 in the association with 8-OxodG. There were no smokers and all in the study population lived in areas with low air pollution.

The genetic impact on 8-OxodG concentrations was analyzed by analysis of variance (ANOVA), followed by multivariate regression. ANOVA was first performed with each SNP grouped into three genotypes (reference homozygotes, heterozygotes and variant

homozygotes). Since no SNP showed any evident per-allele effect on 8-OxodG outcome, further analyses were conducted with genotypes combined into a dichotomous variable, based on the results from the analysis with three genotypes. Genotypes were also combined into haplotypes by PHASE [39]. In the extended multivariate analyses – adding genetic variables – genotype/haplotype was included as a dichotomous variable.

The multivariate analysis was first performed with an interaction term between genotype and As exposure variable in order to evaluate the influence of genetic variation on the association between As and the concentration of 8-OxodG (effect modification). When no significant interaction was present, a model without an interaction term was employed to explore main effects. All analyses were also conducted separately for a subset of individuals with a documented indigenous ancestry (based on interviews) in order to evaluate variations by ethnicity.

In order to evaluate if a genotype associated with lower levels of 8-OxodG was more frequent in SAC compared to the HapMap CEU population (CEU HapMap population (CEPH, Utah residents with ancestry from northern and western Europe; N=60, website: www.hapmap.org), Fischer's Exact test was employed (a statistically significant difference in genotype frequencies for p-values below 0.05). Deviations from Hardy-Weinberg equilibrium were tested using chi-square analysis. Linkage disequilibrium (LD) analysis was performed using Haploview [40].

All statistical analyses were performed using SPSS (Version 15; SPSS, Chicago, IL, USA).

3. Results

Demographic information of the study population is presented in Table 1.

3.1 Association between 8-OxodG and As exposure markers

The median 8-OxodG concentrations, adjusted to the average specific weight of 1.019 g/ml, was 4.7 nmol/L (mean 4.8, range 1.6-13), corresponding to 1.7 μg/g creatinine (mean 1.8, range 0.57-4.8). The median arsenic exposure was 278 μg/L, range 94-724, corresponding to 380 μg/g creatinine, range 140-1100. There was no difference in 8-OxodG in relation to ethnicity (data not shown). The characteristics of the groups subdivided into Indigenous and Spanish ancestry were similar to each other and to that of the whole study population, except for that a statistically significant difference in %MMA between Indigenous (median = 7%, N=77) and Spanish (median = 9%, N=31) was seen (p=0.015, Mann-Whitney test).

In the analysis on As exposure and 8-OxodG for the total population, the strongest association was seen for %MMA, i.e. with increasing fraction of %MMA, higher concentrations of 8-OxodG were observed ($\beta = 0.25$, $p = 0.013$). This association was somewhat stronger for individuals with indigenous ancestry ($N = 77$, $\beta = 0.31$, $p = 0.010$). β denotes the regression coefficient. There was no clear association with 8-OxodG for the other metabolites (%iAs $\beta = 0.049$, $p = 0.56$; %DMA $\beta = -0.69$, $p = 0.10$; U-As $\beta = 0.13$, $p = 0.29$) in the total population, or for individuals with indigenous ancestry (%iAs $\beta = 0.12$, $p = 0.35$, DMA $\beta = -1.1$, $p = 0.10$; U-As $\beta = 0.16$, $p = 0.25$) (Figures 1 and 2).

Among potentially influential variables, coca chewing was strongly associated with lower levels of 8-OxodG ($\beta = -0.23$, $p = 0.008$), and included in the multivariate analysis. The association between %MMA and 8-OxodG remained significant after inclusion of coca ($\beta = 0.22$, $p=0.024$) in the analysis. All associations grew stronger when only individuals with indigenous ancestry was included in the analyses (for %MMA $\beta = 0.29$, $p = 0.014$; where also %DMA ($\beta = -1.1$, $p = 0.076$) and U-As ($\beta = -0.42$, $p = 0.096$) were weakly associated with 8-OxodG.

Among other potentially influential variables, hemoglobin (Hb, $\beta = 0.009$, $p = 0.001$) and body mass index (BMI, $\beta = 0.19$, $p = 0.016$) were positively associated with 8-

OxodG levels. However, since Hb was negatively associated with U-As (β= -12.8, p = 0.010) and positively associated with 8-OxodG, we did not include Hb in the multivariate model; also we had only data for 83 women. The positive association between 8-OxodG and BMI was strongly outlier-dependent; after removal of an extreme outlier (BMI = 38.0 kg/m^2) β was 0.008 and the p-value was 0.54. Also, we only had data for 90 women.

3.2 Genetic impact on 8-OxodG levels

A list of all genotyped SNPs and their allele frequencies is shown in Table 2. All SNPs were in Hardy-Weinberg equilibrium.

The ANOVA analysis (Table 3) showed that individuals with the CC genotype for *TXNRD1* rs11111979 had near-significantly higher concentrations of 8-OxodG than individuals with at least one G-allele (p = 0.059; Table 3). However, there were few individuals with this genotype (five in total, only of indigenous ancestry). An effect of a SNP in the other thioredoxin reductase, *TXNRD2* (rs5746847), was also seen. However, this effect was only clear among the sub-population with indigenous ancestry. A significant effect was seen for *APEX1* Asp148Glu, where individuals with the Glu-allele had higher concentrations of 8-OxodG compared with individuals with the AspAsp genotype (p = 0.012; Table 3). There were larger differences between the genotypes when ethnicity was considered. Individuals with the *DNMT3B* rs2424932 GA+AA genotype had near-significantly higher 8-OxodG than individuals with the GG genotype (p = 0.052; Table 3). The effect was also valid for *DNMT3B* rs2424913 and *DNMT3B* 6087990, which were in complete LD (R^2 = 1) with *DNMT3B* rs2424932.

The multivariate regression analysis of the association between genotypes and 8oxodG, taking As exposure and coca chewing into account, resulted in weaker associations

compared to the ANOVA analysis (Table 3) for *TXNRD1* rs11111979 and *DNMT3B* rs2424932. For *TXNRD2* rs5746847 adjusted values showed a stronger effect, but an effect was still only seen among individuals with indigenous ancestry. Also, *APEX1* showed a stronger effect than in the ANOVA.

For all polymorphisms, the effect (β-coefficient) was similar for adjustments for U-As as well as %MMA (Table 3). All associations were stronger when selecting individuals with indigenous ancestry only (plots for *APEX1* and *DNMT3B* are shown in Figures 3 and 4), except for *TXNRD1* rs11111979, where the results were similar compared to the whole study population. No significant results were seen for the other genotypes or in the haplotype analyses.

We also analyzed genetic effect modification on the association between As exposure and 8-OxodG concentrations (Table 4). Genetic effect modification was mainly seen for U-As, but also for %DMA. The strongest effect modifier was *DNMT1* rs8111085, where individuals with the TT genotype had a steeper slope, i.e. increasing 8-OxodG levels with increasing U-As, compared with individuals with the TC+CC genotype. Individuals with the *DNMT1* rs7253062 GA genotype had lower 8-OxodG with increasing U-As, compared to individuals with the GG genotype. For *GLRX* rs3822751, individuals with the GG genotype had lower 8-OxodG with increasing U-As, compared to individuals with the CC+CG genotype.

The strongest genetic effect modifier for the association between %DMA and 8-OxodG was *DNMT1* rs7253062. Individuals with the *GLRX* rs4561 TT genotype had almost significantly steeper regression slope than individuals with the TC genotype. For *GLRX* rs3822751, *DNMT3b* rs2424932 and *TXNDR1* rs11111979, genotypes associated with lower levels of 8-OxodG were more frequent (a statistically significant difference in genotype frequencies) in SAC compared to the HapMap CEU population.

4. Discussion

We found that the concentrations of 8-OxodG in this female population were low compared with other As-exposed populations. This population was highly exposed to As via drinking water (median As was 278 µg/L, compared to the WHO drinking water guideline value of 10 µg/L), but had an efficient methylation. However, 8-OxodG concentrations increased with increasing U-As. When taking the metabolites into account, the strongest association was found between 8-OxodG and %MMA, supporting that this metabolite is associated with increased risk of oxidative damage, which may lead to malignant transformation. The association between %MMA and 8-OxodG, as well as between other As markers (%DMA and U-As) and 8-OxodG, grew stronger when stratifying for ethnicity. This is, to our knowledge, the first study evaluating the genetic impact on the association between As metabolites and 8-OxodG concentrations. We found evidence that variations in genes involved in the defense against oxidative stress or DNA maintenance influenced the 8-OxodG levels.

An association between MMA concentrations and 8-OxodG in urine has earlier been reported for individuals exposed to As from drinking water [10] and workers exposed to As in the semiconductor industry [21]. Taking ethnicity into account in the present study strengthened the association between %MMA and 8-OxodG. We have in previous studies showed that this indigenous population has a genetic set-up associated with a more efficient As metabolism, where the *AS3MT* genotype is the main genetic determinant of metabolite pattern [23,24]. In the present study, there was no effect of the *AS3MT* genotype on 8-OxodG concentrations. Due to its strong impact on metabolite pattern the effect of *AS3MT* is already taken into account when evaluating the impact on 8-OxodG concentrations. The ethnicity also

influenced the genetic associations of *TXNRD2*, *APEX1,* and *DNMT3B* on 8-OxodG, strengthening the idea that the role of ethnicity reflects different genetic backgrounds of indigenous and Spanish descendants, rather than differences in socioeconomic factors.

Interestingly, a strong effect on 8-OxodG concentrations was seen for coca chewing, with users having lower concentrations of 8-OxodG. Coca has traditionally been used as a treatment against disorders associated with increased oxidative stress, such as ulcers [41], but to our knowledge the information about health-associated effects of coca usage is very sparse.

We also found that the hemoglobin (Hb) concentrations, which were high with a mean level of 150 g/L, due to the altitude, were positively associated with 8-OxodG. Hemoglobin can spontaneously oxidize to methemoglobin and superoxide radicals are formed [42]. Subsequently, such radicals can form DNA-reactive hydroxyl radicals by the catalytic action of ferric ions. On the other hand, there was a negative association between Hb and U-As, indicating as an inhibiting effect of arsenic on the heme synthesis. This is an interesting result, considering that a recent study from Bangladesh showed a negative association for As on Hb levels, but only at Hb levels below 100 g/L [43]. Similarly, a recent study from Chile observed that As exposed women had a larger decrease in Hb with advancing pregnancy [44]. We did not see any effects for the other potentially influential (age, weight, U-As) when they were included in the metabolite models.

The 8-OxodG levels detected were low compared to the other studies of As-exposed populations with similar exposure range [8-11]. It is important to note that the high 8-OxodG levels detected in other studies [8, 10-15] are partly due to the methods used; especially ELISA, the most commonly used method for 8oxodG determination, has been shown to overestimate 8-OxodG levels, whereas mass spectrometric techniques appear to be more robust [45]. In a study by Cooke et al. [45], levels of urinary 8-OxodG determined by the JICA ELISA kit gave fourfold higher values compared with a LC/MS-MS assay, similar to

that used in our study, with a preceding solid-phase extraction. Still in two studies analyzing As exposure and 8oxodG where they have employed similar LC/MS-MS methods higher 8oxodG levels were found: Fujino et al [9] reported mean 8-OxodG levels of 5.5 μg/g creatinine among subjects without As dermatosis and mean U-As levels of 500 μg/g creatinine; Hu et al. [21] reported 8-OxodG levels of 5.6 μg/g creatinine among exposed semiconductor workers with U-As levels of 33 μg/g cre. Thus, the method used in the present study cannot explain the low concentrations of 8-OxodG in this population. For comparison, we also analyzed samples from 13 healthy Swedish individuals not exposed to As (unpublished data, study subjects were adults, both sexes) that had been frozen for 5 years (a similar time period as for the samples from Argentina), and these individuals had higher levels of 8-OxodG than the Argentinean population (median 2.9 μg/g creatinine compared to 1.8 μg/g creatinine for the Argentinean population).

Populations living in the Andes have been exposed to As through drinking water for thousands of years and live at a high altitude, which is associated with increased oxidative stress [46,47]. However, this rural environment is also lacking a number of factors associated with oxidative stress; e.g. few industries, few cars, no female smokers and low alcohol consumption [32], which may partly explain the low levels of 8-OxodG.

One other possibility for the low 8-OxodG levels is that this population has a phenotype that protects them from oxidative damage. As mentioned above, the low 8-OxodG phenotype could be the result of a more efficient metabolism of As, with low %MMA and high %DMA, which were associated with lower 8-OxodG concentrations. We also found that this was partly explained by polymorphisms in *DNMT3B*, *TXNDR1*, *TXNDR2* and *APEX1*. Several genotypes associated with lower concentrations of 8-OxodG are more common in SAC. The functional impacts of these SNPs are yet not known. The effect for *APEX1* Asp148Glu was in the opposite direction compared with a recent study in Bangladesh [15], i.e. the variant was

associated with higher 8-OxodG in urine. We have at present no explanation for this inconsistency. One major difference between the two studies is that the As levels were a lot lower in Bretons' study compared to this study (mean U-As was 39 μg/g creatinine compared to 380 μg/g creatinine). We also evaluated genetic effect modification on the association between As and 8-OxodG. Two SNPs in *DNMT1* influenced 8-OxodG levels. There was also some evidence of effect modification for *GLRX*. However, it is important to note that a larger study is needed in order to detect genetic effect modification and the results presented are preliminary. To conclude, we found some indications that this population may have certain genotypes giving lower levels of 8-OxodG, that could be interpreted as protective.

Little is known about the prevalence of As-associated health effects in this population. We did not find any clinical signs of As-induced hyperkeratosis on the palms of the hands in any of the women studied, although a previous study showed an increased frequency of micronuclei and of trisomy in lymphocytes from exposed children and women compared with non-exposed controls [48]. Although it is clear that several environmental exposures result in increased levels of 8-OxodG [49], the relation between levels of urinary 8-OxodG and future risk of health effects, such as cancer, is not well characterized. There is one Danish prospective study on 8-OxodG and lung cancer showing that increasing levels of 8oxodG were associated with increased risk of cancer among non-smokers [35].

In conclusion, this study showed that the population from San Antonio de los Cobres, in the Argentinean Andes, had low concentrations of 8-OxodG, in spite of a high exposure to As in drinking water. Still, it is important to stress that there was an increase in oxidative stress with increasing As exposure, and that this increase may be sufficient for causing mutations. The low 8-OxodG levels are largely explained by the efficient methylation; thus, in further studies, %MMA should be taken into account when evaluating associations between As and 8-OxodG. Also, ethnicity was shown to be an important factor in determining the association

between As and 8-OxodG levels, reflecting genetic differences in As metabolism as well as genes regulating oxidative stress and DNA maintenance. This study is the first to show that genetic variants in the *DNMT*s exhibit an effect modification on As toxicity.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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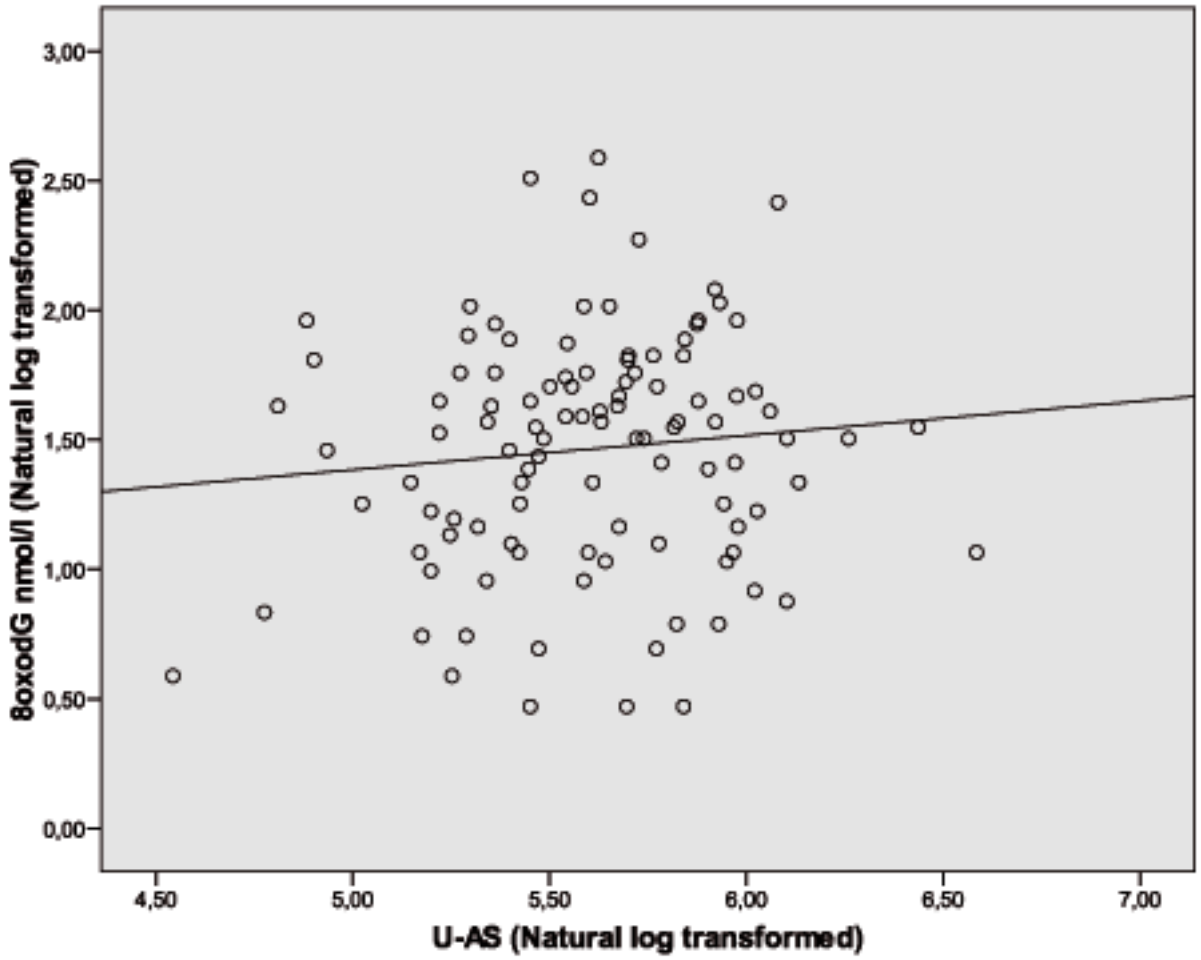
Figure legends

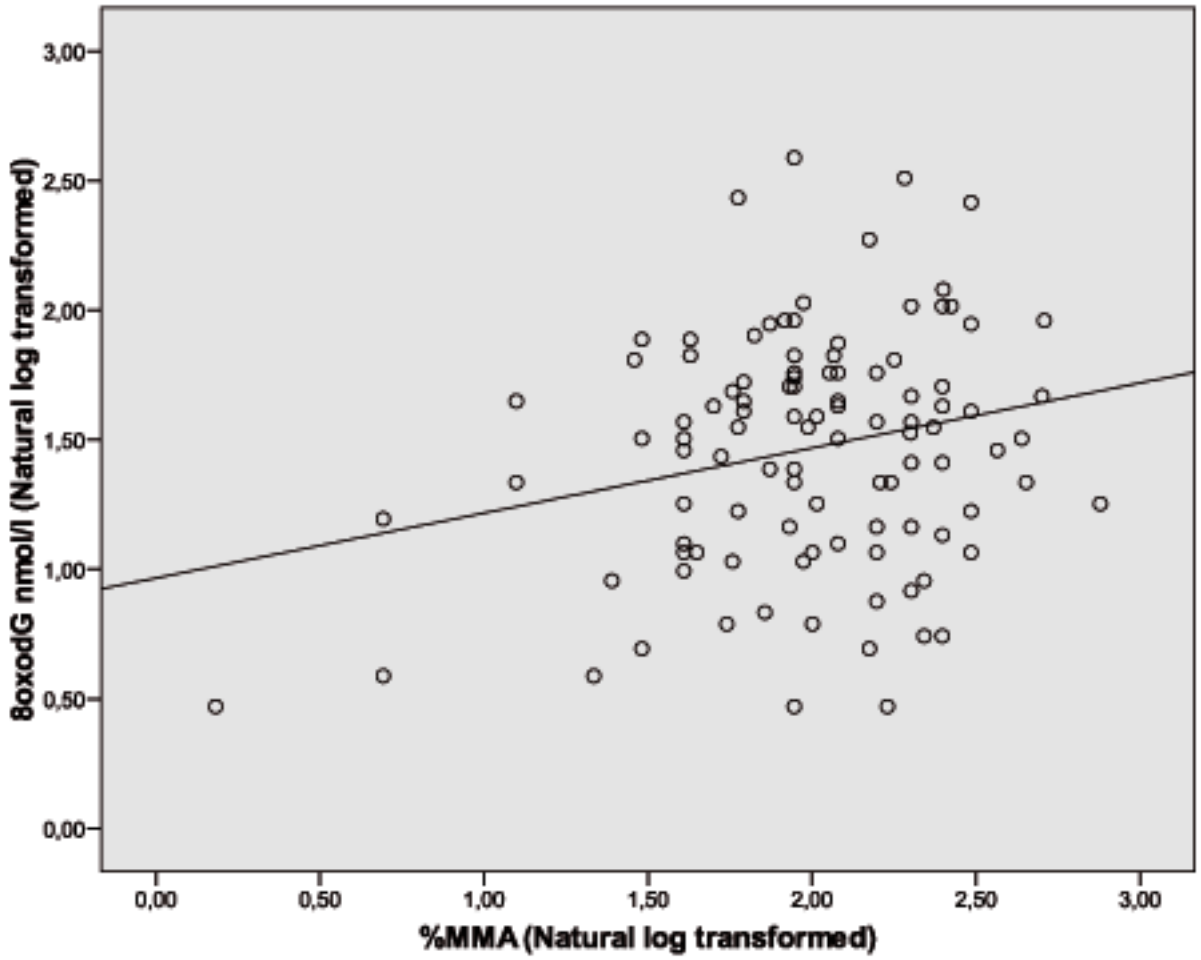
Figure 1. Scatterplot of the association between total urinary arsenic (U-As, natural log transformed) and concentrations of 8-OxodG (natural log transformed). The simple linear regression line is also shown.

Figure 2. Scatterplot of the association between %MMA (natural log transformed) and concentrations of 8-OxodG (natural log transformed). The simple linear regression line is also shown.

Figure 3. Scatterplot of concentrations of 8-OxodG (natural log transformed) for different *APEX1* rs1130409 genotypes in the indigenous subgroup, with %MMA (natural log transformed) as exposure marker. The simple linear regression line, in each subgroup, is also shown.

Figure 4. Scatterplot of concentrations of 8-OxodG (natural log transformed) for different *DNMT3B* rs2424932 genotypes in the indigenous subgroup, with %MMA (natural log transformed) as exposure marker. The simple linear regression line, in each subgroup, is also shown.


3,00
2,50
2,00
1,50
1,00
0,50
0,00
8oxodG nmol/l (Natural log transformed)
4,50
5,00
5,50
6,00
6,50
7,00
U-AS (Natural log transformed)


3,00
2,50
2,00
1,50
1,00
0,50
0,00
8oxodG nmol/l (Natural log transformed)
0,00
0,50
1,00
1,50
2,00
2,50
3,00
%MMA (Natural log transformed)

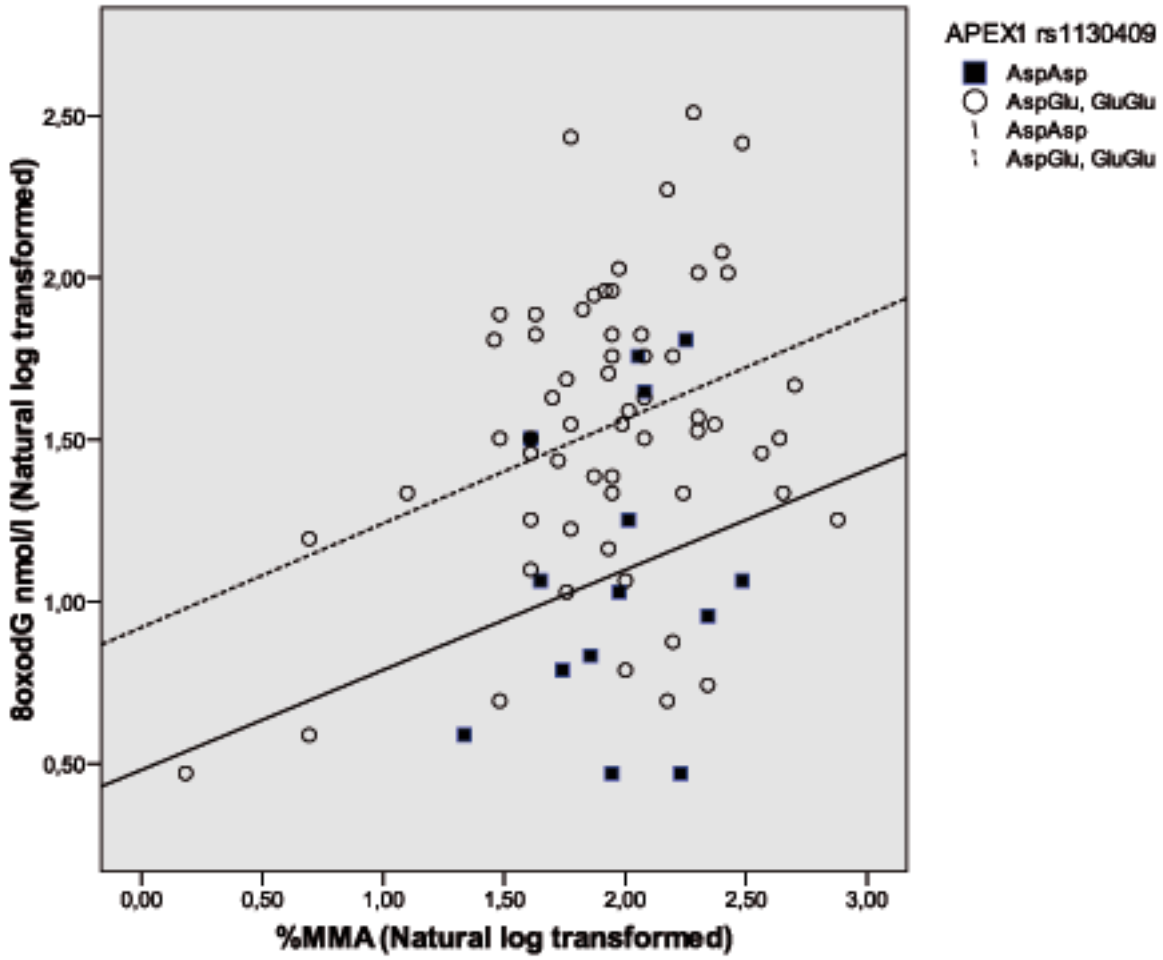

APEX1 rs1130409
AspAsp
AspGlu, GluGlu
AspAsp
AspGlu, GluGlu
8oxodG nmol/l (Natural log transformed)
2,50
2,00
1,50
1,00
0,50
0,00
0,50
1,00
1,50
2,00
2,50
3,00
%MMA (Natural log transformed)

Table 1. Characteristics of the study population.

	Median	Range	(N)
Age (years)	32	15-76	(107)
Weight (kg)	57	40-88	(90)
Height (cm)	151	134-163	(90)
BMI (kg/m^2)	25	17-38	(90)
Hb (g/L)	153	94-188	(82)
Coca users (%)	51		(108)
Tobacco users (%)	0		(108)
Alcohol users (%)	0		(108)
Parity	3	0-12	(101)
U-As (μg/L) [a]	278	94-724	(108)
iAs (%)	12	1-48	(108)
MMA(%)[b]	7.3	1.2-18	(108)
DMA (%)	80	47-93	(108)
8-OxodG (nmol/L) [a]	4.7	1.6-13	(108)

[a] 8-OxodG concentrations and U-As were adjusted to the mean specific gravity of 1.019 g/ml.

[b] A statistically significant difference in %MMA was seen between individuals with Indigenous and Spanish ancestry (p=0.015, Mann-Whitney test). No other statistically significant differences were seen between individuals with Indigenous and Spanish ancestry.

Table 2. Genes and polymorphisms successfully genotyped.

Gene	Unigene Nr [a]	Rs-nr[b]	SNP type[c]	SNP position[d]	Allele frequencies SAC	Allele frequencies[e] European population
**AS3MT*[f]	Hs.34492	rs7085104	Intron A/G	23377399, NT_0300592	25/75	68/32
APEX1	Hs.73722	rs1130409	p.D148E g.T>G	1924994, NT_026437	47/53	49/51
CAT	Hs.502302	rs1001179	5' near gene g.G>A	33247472, NT_009237	96/4	73/27
**DNMT1*	Hs.202672	rs16999593	p.H97R, g.T>C	1553983, NT_011295	93/7	100/0
**DNMT1*	Hs.202672	rs8111085	p.I311V, g.T>C	1536174, NT_011295	63/37	96/4
**DNMT1*	Hs.202672	rs7253062	Intron, g.G>A	1557926, NT_011295	92/8	56/44
**DNMT3b*	Hs.655708	rs2424913	Intron, g.C>T	1570351, NT_028392	6/94	66/34
**DNMT3b*	Hs.655708	rs6087990	5' near gene, g.T>C	1546000, NT_028392	6/94	71/29
**DNMT3b*	Hs.655708	rs2424932	3'UTR, g.G>A	1592628, NT_028392	94/6	52/48
**GCLM*	Hs.315562	rs41303970	Promotor, g.C>T	64347228, NT_032977	48/52	84/16[g]
**GGT1*	Hs.645535	rs2236626	5' near gene, g.C>T	4370016, NT_011520	32/68	21/79[h]
**GLRX*	Hs.28988	rs3822751	Intron, g.G>C	3468082, NT_023148	51/49	30/70
**GLRX*	Hs.28988	rs4561	Synonymous, g.C>T	3466185, NT_023148	7/93	41/59
**GSR*	Hs.271510	rs2253409	Intron, g.G>C	867740, NT_007995	86/14	74/26
**GSR*	Hs.271510	rs2978296	Intron, g.G>C	895714, NT_007995	76/24	83/17

**GSTA1*	Hs.446309	rs3957356	Promotor, g.C>T	43526901, NT_007592	7/93	61/39[i]
**GSTM1*	Hs.301961	deletion	null allele	(allele present denoted first)	55/45	21/79[i]
**GSTT1*	Hs.268573	deletion	null allele	(allele present denoted first)	83/16	71/29[i]
**GSTP1*	Hs.523836	rs1695	p.I105V, g.A>G	12658484, NT_033903	27/73	61/39
GPX1	Hs.76686	rs1050450	p.P200L	49334838, NT_022517	97/3	88/12
**PRDX2*	Hs.695971	rs10427027	3' near gene, g.T>C	12772285, NT_011295	88/12	95/5 [h]
**PRDX2*	Hs.695971	rs12151144	Intron, g.A>C	4175198, NT_011295	88/12	95/5 [h]
SOD2	Hs.487046	rs4880	p.V16A, g.T>C	2401213, NT_007422	19/81	56/44
**TXNRD1*	Hs.696144	rs11111979	5'UTR, g.C>G	28162972, NT_019546	26/74	49/51
**TXNRD1*	Hs.696144	rs6539137	Intron, g.T>A	28189370, NT_019546	93/7	86/14
**TXNRD2*	Hs.443430	rs5746847	Intron, g.C>T	3073153, NT_011519	45/55	57/43
**TXNRD2*	Hs.443430	rs5992495	p.S229R, g.T>G	3035134, NT_011519	92/8	85/15
**TXNRD2*	Hs.443430	rs5748469	p.A66S, g.C>A	3059249, NT_011519	91/9	65/35

[a] Unigene accession number from NCBI (National Center for Biotechnology Information) Unigene Database (website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene).

[b] Rs numbers for the SNPs from NCBI SNP Database (website: http://www.ncbi.nlm.nih.gov/SNP).

[c] When applicable, amino acid position/gene region is denoted. Ancestral allele, according to NCBI SNP Database is denoted first when known.

[d] Contig accession numbers (NT_) and positions from NCBI Nucleotide database (website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

[e] From the CEU HapMap population (CEPH, Utah residents with ancestry from northern and western Europe; N=60, website: www.hapmap.org).

[f] (*) Denotes SNPs where allele frequencies for SAC have been presented earlier [24].

[g] Allele frequencies from a Danish/Swiss population (Submitter handle CNPSCZ at NCBI SNP Database).

[h] Allele frequencies from Coriell Cell Repository at NCBI SNP Database.

[i] Allele frequencies from the Caucasian population in the NCI SNP500Cancer Database (website: http://snp500cancer.nci.nih.gov).

Table 3. Genetic impact on 8-OxodG concentrations (geometric mean). Only genotypes with a p-value below 0.1 in any of the analyses are presented.

		ANOVA					**Multivariate regression**					
		All individuals			Indigenous ancestry		All individuals				Indigenous ancestry			
							U-As [a]		%MMA [b]		U-As [a]		%MMA [b]	
Gene	Rs-nr	8-OxodG[c]	N	p-value	8-OxodG[c]	N	p-value	$ß_1$	P-value	$ß_1$	p-value	$ß_1$	P-value	$ß_1$	p-value
Genotype[d]															
TXNDR1	**rs11111979**														
GG+GC[d]		4.2	96	0.059	4.0	65	0.054	-0.35	0.081	-0.35	0.075	-0.38	0.078	-0.37	0.082
CC		6.2	5		6.2	5									
TXNDR2	**rs5746847**														
CC+CT		4.2	86	0.59	4.0	60	0.079	-0.48	0.41	-0.069	0.56	-0.28	0.065	-0.30	0.043
TT		4.5	16		5.2	11									
APEX1	**rs1130409**														
AspAsp		3.5	22	0.012	3.0	14	0.001	-0.34	0.001	-0.34	0.001	-0.45	0.001	-0.47	<0.001
GluAsp+GluGlu:		4.6	84		4.7	61									
DNMT3b	**rs2424932** [e]														

GG [d]	4.1	90	0.052	4.0	63	0.055	-0.23	0.081	-0.22	0.080	-0.33	0.054	-0.33	0.055
GA+AA	5.4	12		5.6	8									

[a] The model is as follows: 8-OxodG (natural log transformed) = intercept + β_1*genotype + β_2*U-As (natural log transformed) + β_3*coca.

[b] The model is as follows: 8-OxodG (natural log transformed) = intercept + β_1*genotype + β_2*MMA (natural log transformed) + β_3*coca.

[c] Values are unadjusted.

[d] The genotype(s) associated with lower levels of 8-OxodG is more frequent in SAC compared to the HapMap CEU population (a statistically significant difference in genotype frequencies, p-value<0.05, Fischer's Exact test). Genotype frequencies for the HapMap CEU population are derived from www.hapmap.org

[e] Rs2424932 is in complete LD with the *DNMT3b* SNPs rs2424913 and rs6087990.

Table 4. Influence of genetic variation on the association between As and the concentration of 8-OxodG [a].

Metabolite	Genotype	Effect modification β_4 [b]	p-value
%DMA [c]			
	DNMT1 rs7253062	4.1	0.004
	GG (86) vs. AG (15)		
	GLRX rs4561	2.3	0.052
	TT (85) vs. TC (16)		
U-As [d]			
	DMNT1 rs8111085	0.61	0.021
	TT (44) vs.TC + CC(57)		
	DNMT1 rs7253062	1.1	0.031
	GG (86) vs. GA (15)		
	GLRX rs3822751 [e]	-0.55	0.049
	GG (27) vs. CG + CC (72)		

[a] Genotypes are dichotomized and referents are denoted last.

[b] The term β_4 denotes the difference between the inclinations of the regression slopes for 8-OxodG on %DMA or U-As.

[c] According to the interaction term in the following model: 8-OxodG (natural log transformed) = intercept + β_1*genotype + β_2*%DMA (natural log transformed) + β_3*coca + β_4 (Genotype*%DMA).

[d] According to the interaction term in the following model: 8-OxodG (natural log transformed) = intercept + β_1*genotype + β_2*%U-As (natural log transformed) + β_3*coca + β_4(genotype*%U-As).

[e] The genotype associated with lower levels of 8-OxodG is more frequent in SAC compared to the HapMap CEU population (a statistically significant difference in genotype frequencies, p-value<0.05, Fischer's Exact test).