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N-6-Adenine-Specific DNA Methyltransferase 1 (N6AMT1) Polymorphisms and Arsenic Methylation in Andean Women

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In humans, inorganic arsenic is metabolized to methylated metabolites mainly by arsenic (+3 oxidation state) methyltransferase (AS3MT). AS3MT polymorphisms are associated with arsenic metabolism efficiency. Recently, a putative N-6-adenine-specific DNA methyltransferase 1 (N6AMT1) was found to methylate arsenic in vitro.

**Objective:** We evaluated the role of N6AMT1 polymorphisms in arsenic methylation efficiency in humans.

**Methods:** We assessed arsenic methylation efficiency in 188 women exposed to arsenic via drinking water (~ 200 µg/L) in the Andean region. We measured the relative concentrations of arsenic metabolites in urine (inorganic arsenic, dimethylarsinic acid, and dimethylarsinic acid) by high-performance liquid chromatography coupled with hydride generation and inductively coupled plasma mass spectrometry. We performed genotyping for N6AMT1 and AS3MT polymorphisms by Taqman assays, and gene expression (in blood; n = 63) with Illumina HumanHT-12 v4.0.

**Results:** Five N6AMT1 single nucleotide polymorphisms (SNPs; rs1997605, rs2205449, rs2705671, rs16983411, and rs1048546) and two AS3MT haplotypes were significantly associated with the percentage of MMA (%MMA) in urine, even after adjusting for AS3MT haplotype. %MMA increased monotonically according to the number of alleles for each SNP (e.g., for rs1048546, mean %MMA was 7.5% for GG, 8.8% for GT, and 9.7% for TT carriers). Two SNPs were in linkage disequilibrium (R² > 0.8). Estimated associations for joint effects of N6AMT1 (haplotype 1) and AS3MT (haplotype 2) were generally consistent with expectations for additive effects of each haplotype on %MMA. Carriers of N6AMT1 genotypes associated with lower %MMA showed the lowest N6AMT1 expression, but associations were monotonic according to copy number for only one genotype and one haplotype.

**Conclusions:** N6AMT1 polymorphisms were associated with arsenic methylation in Andean women, independent of AS3MT. N6AMT1 polymorphisms may be susceptibility markers for arsenic-related toxic effects.

**Key Words:** DMA, gene × environment interactions, inorganic arsenic, methylation, MMA, polymorphisms.

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We collected blood and urine samples from each individual in the same way in 2008 and 2011. Venous blood samples were collected in K$_2$EDTA tubes (Vacutette®; Greiner Bio-One GmbH, Greiner, Germany) for DNA extraction and PAX tubes (PreAnalytix GmbH, Hombrechtikon, Switzerland) for gene expression analyses. Spot urine samples were collected in disposable urine collection cups and immediately transferred to 20-mL polyethylene bottles. We collected all the biological samples at the hospital or the local health clinics during the daytime; the project logistics did not allow for fasting before sampling. After a maximum of 24 hr at room temperature after sampling, blood and urine samples were frozen and kept at −20°C until they were transported with cooling blocks to Sweden for analyses. Analyses took place within 2 months of collection.

Both oral and written informed consents were provided by all the study participants. The study was approved by the Ministry of Health in Salta, Argentina, and the Regional Ethical Committee at Karolinska Institutet.

**Arsenic exposure and metabolism.** We assessed exposure to iAs based on the sum of the concentrations of the inorganic arsenic metabolites (iAs + MMA + DMA) in urine (UAs), and we assessed the efficiency of arsenic metabolism based on the relative proportions (percentage of the sum of urinary arsenic metabolites) of iAs metabolites (iAsIII, iAsV), MMA, and DMA in urine (Vahter 2002). Arsenic metabolites in urine (i.e., iAs, MMA, and DMA) were determined using high-performance liquid chromatography (HPLC) (Agilent 1100 series system; Agilent Technologies, Waldbronn, Germany) coupled with hydride generation (HG) and inductively coupled plasma mass spectrometry (ICP-MS: Agilent 7500ce; Agilent Technologies, Tokyo, Japan) (Concha et al. 2010). The HG system was used to introduce only the metabolites of iAs to the ICP-MS. After shaking, the urine samples, approximately 0.5 mL of each urine sample was filtered on a 0.2-µm syringe filter and transferred to the HPLC-HG-ICPMS system. For quality control, we analyzed the reference material CRM No.18 (certified DMA concentration of 36 ± 9 µg/L; National Institute for Environmental Studies, Ibaraki, Japan) along with the collected samples. We obtained 43.9 ± 4.7 µg/L (mean ± SD; n = 21), which agreed with previously reported results (Li et al. 2008). In order to compensate for variation in urine dilution, we adjusted the measured concentrations of arsenic in urine to the mean specific gravity of urine (1.020 g/mL) determined by a digital refractometer (EUROMEX RD 712 clinical refractometer; EUROMEX, Arnhem, the Netherlands).

**Genotyping and gene expression analysis.** DNA was isolated from peripheral blood with the Qiagen DNA Blood Mini kit (QIAGEN, Hilden, Germany). We genotyped five N6AMT1 single nucleotide polymorphisms (SNPs in 5'-3' order: rs1997605, rs2205449, rs2705671, rs16983411, and rs1048546) by Tagman® SNP genotyping assays on a fast real-time PCR System (7900HT; Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instructions. Blanks and controls for each genotype were included in each run, and genotyping was repeated on 3% of the samples.

Because none of the N6AMT1 SNPs have previously been analyzed in relation to functional impact on gene expression or protein activity, we selected SNPs that tag genetic variation within the gene due to strong linkage disequilibrium with other SNPs in the same region (tagSNPs) (Table 1). TagSNPs for N6AMT1 were selected using Haploview (version 4.1; Barrett et al. 2005) on the HapMap (http://hapmap.ncbi.nlm.nih.gov) CEU population (Utah residents with ancestry from northern and western Europe). We inferred haplotypes from N6AMT1 rs1997605, rs2205449, rs2705671, and rs1048546 by PHASE software (Stephens and Donnelly 2003). We did not include rs16983411 in the final haplotype analysis because the frequency was very low (minor allele frequency of 1%).

**Genotyping for AS3MT SNPs associated with arsenic metabolism was performed as described previously (Engström et al. 2011). Briefly, eight SNPs were genotyped using Sequenom™ (Sequenom, San Diego, CA, USA) technology. We inferred haplotypes**
from the AS3MT SNPs by PHASE software. In this population we previously found that the major AS3MT haplotype (referred to here as haplotype 2; haplotype frequency = 70%) was GCCATCAG [5′−3′ order of AS3MT SNPs: rs7085104, rs3740400, rs3740393, rs3740390, rs11191439, rs11191453, rs10748835, and rs1046778 (Engström et al. 2011)]. This haplotype was associated with low %MMA and high %DMA, consistent with more efficient arsenic metabolism (Engström et al. 2011). In the 34 women recruited in 2011, we genotyped AS3MT rs3740400, rs3740393, rs11191439, and rs1046778 with Taqman® SNP genotyping assays according to the manufacturer’s instructions, and inferred haplotype 2 based on the four SNPs only, as we have shown that we obtain very similar inferred AS3MT haplotypes with fewer SNPs compared with a larger number of SNPs (Schiebusch et al. 2013).

To examine gene expression we extracted RNA with the PAXgene Blood RNA kit (PreAnalytiX) and stored the samples at −80°C. We evaluated RNA concentration and purity using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and the results confirmed high-quality RNA (RNA integrity number > 7.5, where 1 is the worst and 10 the best). For the gene expression analysis, we selected participants who had the best quality and highest quantity of RNA for array analyses. These individuals, with a wide range of urinary arsenic concentrations (10–1,251 µg/L), were classified into low/high U-As groups based on median values, and then frequency-matched for age, weight, and BMI so that there were no major differences between the two groups (p > 0.05 for age, weight/BMI), resulting in a total of 63 individuals for the gene expression analyses. For the whole genome gene expression analysis, we used DirectHyb HumanHT-12, version 4.0 (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions, and the analysis was performed at the Sgewgene Center for Integrative Biology at Lund University. Background signals were filtered by BioArray Software Environment (BASE) (Vallon-Christersson et al. 2009) and results are presented in relative fluorescence units. There were two N6AMT1 transcripts in the HumanHT-12, version 4.0 array: ILMN_2315569, corresponding to mRNA NM_182749.2, which encodes the longer isoform [National Center of Biotechnology Information (NCBI) Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore)], and ILMN_1754988, corresponding to NM_013240.3, which lacks an alternate in-frame exon, resulting in a shorter protein.

**Statistical analysis.** We analyzed deviations from Hardy-Weinberg equilibrium using chi-square analysis, and estimated linkage disequilibrium using Haploview (Barrett 2009).

We estimated associations between genotypes or haplotypes (independent variables) and proportions of individual metabolites (%iAs, %MMA, or %DMA as dependent variables) in multivariable-adjusted regression models. All models were adjusted for natural log–transformed total urinary arsenic concentration (lnU-As) because the level of arsenic exposure in itself influences metabolism (Vahter 2002). Genotypes and haplotypes were modeled as categorical variables (for genotypes: zero, one, or two alleles; and for haplotypes: zero, one, or two copies), with the genotype or haplotype associated with low MMA fractions as the reference group for each variant in all models. When the frequency of a homozygote genotype included < 3% of individuals, this group was pooled with the heterozygotes. In the multivariable-adjusted analyses, we considered the AS3MT haplotype to be a potential confounder. We first modeled associations between each SNP genotype or haplotype and each outcome (%iAs, %DMA, or %MMA) in separate models adjusted for lnU-As (model 1), then fit models that also were adjusted for AS3MT haplotype (model 2). We also modeled associations between AS3MT haplotype 2 and arsenic metabolites adjusted for each N6AMT1 variant in individual models. In addition, we modeled the joint effects of the most common N6AMT1 haplotype and AS3MT haplotype 2 by estimating associations for each possible combination of genotypes based on the two haplotypes (i.e., with two copies of each haplotype used as the reference category, two copies of the N6AMT1 haplotype and one copy of the AS3MT haplotype, etc., for a total of eight possible combinations), with adjustment for lnU-As.

We analyzed correlations between total arsenic or fraction of MMA in urine and gene expression data using the Spearman correlation coefficient (r). Gene expression data were normally distributed and, therefore, relations between N6AMT1 SNPs/haplotypes and gene expression for N6AMT1 transcripts were analyzed by analysis of variance (ANOVA). All calculations were made using IBM SPSS® Statistics, version 20.0 (IBM, Chicago, IL, USA). Statistical significance was determined as p < 0.05 (two-tailed).

**Results**

**General characteristics.** The characteristics of the study population are presented in Table 2. The studied women (n = 188) were on average 34 years of age with a median U-As concentration of 210 µg/L (median urinary fraction of iAs, 12%; MMA, 8.1%; and DMA, 80%).

The N6AMT1 SNPs were situated within 12,451 base pairs (distance between SNP rs1997605 in intron 1 and rs1048546 in 3′ UTR). Genotypes of all SNPs were in Hardy Weinberg equilibrium (Table 1). The allelic frequencies for the N6AMT1 SNPs varied between the Andean population and other reference populations from the Hapmap study (Table 1). Rs1048546 was in linkage disequilibrium (LD) with rs2705671 (R² = 0.80) and rs1997605 (R² = 0.94), and the LD between rs2705671 and rs1997605 was R² = 0.85 [see Supplemental Material, Figure S1 (http://dx.doi.org/10.1289/ehp.1206003)]. Rs2205449 was in weaker LD with these three SNPs (R² between 0.42 and 0.51). Rs16983411 showed a very low minor allele frequency (1%) in Andeans, and it was not in LD with any of the other SNPs.

**Table 2. Characteristics of the study population.**

| Characteristic                  | Median, %, or n | 5th–95th percentiles |
|---------------------------------|-----------------|----------------------|
| Age [years (median)]            | 34              | 19–64                |
| Time of residency [years (median)] | 25              | 3.0–53               |
| Town of residency (SAC/other village (n)) | 154/24         | 19–35                |
| BMI [kg/m² (median)]           | 25              |                      |
| Tobacco users (%)              | 53              |                      |
| Alcohol users (%)              | 3.7             |                      |
| Total U-As [µg/L (median)]     | 210             | 33–502               |
| iAs (%)                        | 12              | 4.8–23               |
| MMA (%)                        | 8.1             | 4.2–15               |
| DMA (%)                        | 80              | 65–89                |
| N6AMT1 haplotypes (n)          |                 |                      |
| Haplotype 1: AATG (2/1/0 copies) | 30/81/77       |                      |
| Haplotype 2: ATTG (2/1/0 copies) | 5/46/137       |                      |
| N6AMT1 haplotypes (n)          |                 |                      |
| AS3MT haplotype 2: GCCATCAC (2/1/0 copies) | 72/73/43       |                      |

*SAC: San Antonio de los Cobres; other villages: Santa Rosa de los Pastos Grandes, Pocitos, Olacapato, Cobres, Rosario de Lerma. **Total U-As was adjusted to the mean specific gravity of 1.020 g/mL. *Number of copies associated with lower %MMA are denoted first. Number of copies that have previously been shown to have an association with lower %MMA are denoted second (Engström et al. 2011).
Genotype patterns in relation to genotype/haplotype. At least one genotype of each \(\text{N6AMT1} \) SNP was significantly associated with %MMA in urine based on the multivariable linear regression analysis (Table 3). With the exception of rs16983411, which was modeled as a dichotomous genotype because of small numbers of variant alleles, associations with %MMA increased monotonically according to the number of alleles for all SNPs [see Supplemental Material, Figure S2A–D (http://dx.doi.org/10.1289/ehp.1206003)].

For example, for rs1048546, the mean %MMA was 7.5% (95% confidence interval (CI): 6.7, 8.4) for GG, 8.8% (95% CI: 7.9, 9.4) for GT, and 9.7% (95% CI: 8.9, 10.7) for TT carriers. After adjusting for multiple comparisons (Bonferroni-corrected significance level of 0.01), associations of rs2205449, rs2705671, and rs1048546 with %MMA remained statistically significant. The estimated differences in mean %MMA decreased somewhat after adjusting for \(\text{AS3MT} \) haplotype (model 2, Table 3).

For each SNP, the direction of associations with %iAs and %DMA were in the opposite direction from associations from %MMA, but were closer to the null and not statistically significant (Table 3). In most cases, the \(\text{N6AMT1} \) genotypes associated with the lowest %MMA were associated with the highest %iAs and %DMA. However, the associations with iAs% and %DMA did not reach statistical significance (Table 3). In general, further adjustment for \(\text{AS3MT} \) increased the estimates for iAs%, which became similar in size to those of %MMA but in the opposite direction, whereas the \(\text{AS3MT} \)-adjustment tended to decrease the estimates for %DMA.

\(\text{AS3MT} \) haplotype 2 was significantly associated with percentages of iAs, MMA, and DMA when adjusted for individual \(\text{N6AMT1} \) SNPs [see Supplemental Material, Table S3 (http://dx.doi.org/10.1289/ehp.1206003)]. Differences in estimated mean %MMA were smaller in association with \(\text{N6AMT1} \) SNPs than in association with \(\text{AS3MT} \) haplotype 2: for example, mean %MMA increased (\(\beta = 2.3 \); 95% CI: 1.1, 3.5) in carriers of rs1048546 TT relative to GG carriers (\(\beta = 1.8 \); 95% CI: 0.61, 2.9) when adjusted for \(\text{AS3MT} \) haplotype (Table 3), whereas %MMA in women with no copies of \(\text{AS3MT} \) haplotype 2 was increased (\(\beta = 3.2 \); 95% CI: 1.7, 4.7) relative to women with two copies (\(\beta = 2.8 \); 95% CI: 1.3, 4.2) when adjusted for \(\text{N6AMT1} \) rs1048546 (see Supplemental Material, Table S3).

Nine \(\text{N6AMT1} \) haplotypes were inferred from rs1997605, rs2205449, rs2705671, and rs1048546: a) AATT, b) AAGG, c) ATGG, d) ATTG, e) GAGT, f) GTGG, g) GTTT, h) GTGG, and i) GTGG; of which only haplotypes 1, 3, and 9 were common enough (36%, 16%, and 42%, respectively, Table 2) for analyses. %MMA was significantly higher among women with no copies (vs. two copies) of haplotype 1 (\(\beta = 2.1 \); 95% CI: 0.75, 3.5) and among women with two copies (vs. no copies) of haplotype 9 (\(\beta = 1.9 \); 95% CI: 0.72, 3.2) (Table 4). %MMA was also higher among women with no copies (vs. two copies) of haplotype 3, although the association was not statistically significant. The association between %MMA and haplotype 1 remained significant after adjusting for multiple comparisons (Bonferroni-corrected significance level of 0.01). Associations between \(\text{N6AMT1} \) haplotypes and %iAs and %DMA were not statistically significant (Table 4).

There were no first-degree relatives but two pairs of second-degree relatives in our study population. In a sensitivity analysis we randomly excluded one in each pair of second-degree relatives and estimated associations between arsenic metabolites and \(\text{N6AMT1} \) SNPs and haplotypes, but results were similar to those reported (data not shown).

Mean %MMA estimated for women with two copies of \(\text{N6AMT1} \) haplotype 1 and two copies of \(\text{AS3MT} \) haplotype 2 was 6.2% (95% CI: 4.6, 7.8), compared with 10.4% (95% CI: 8.7, 12.0) for women with no copies of either haplotype (\(\beta = 4.2 \); 95% CI: 1.9, 6.5) for no copies vs. two copies of each haplotype (Table 5). This estimated joint

| Table 3. Multivariable regression analyses of the influence of \(\text{N6AMT1} \) genotypes on fractions of arsenic metabolites. |
|---------------------------------------------------------------|
| **Metabolite/SNP** | **Genotype** | **n** | **Mean (95% CI)** | **Model 1** | **Model 2** |
|---------------------------------------------------------------|
| **iAs** | | | | | |
| rs1997605 | AA | 63 | 13.1 (11.6, 14.6) | | |
| rs2205449 | AA | 30 | 13.8 (11.7, 16.0) | | |
| rs2705671 | TT | 77 | 12.5 (11.1, 13.9) | | |
| rs16983411 | AA | 181 | 12.8 (11.4, 14.2) | | |
| rs1048546 | GG | 12.6 (10.9, 14.3) | | |
| **MMA** | | | | | |
| rs1997605 | AA | 63 | 7.7 (7.0, 8.6) | | |
| rs2205449 | AA | 30 | 7.2 (6.3, 8.3) | | |
| rs2705671 | TT | 71 | 7.7 (7.0, 8.5) | | |
| rs16983411 | AA | 181 | 8.1 (7.8, 8.5) | | |
| rs1048546 | GG | 60 | 8.1 (7.8, 8.5) | | |
| **DMA** | | | | | |
| rs1997605 | AA | 63 | 7.2 (7.3, 8.0) | | |
| rs2205449 | AA | 30 | 7.0 (6.3, 8.1) | | |
| rs2705671 | TT | 71 | 7.2 (6.7, 7.8) | | |
| rs16983411 | AA | 181 | 7.8 (7.7, 7.9) | | |
| **Notes:** Model 1: Arsenic metabolite \(\alpha + \beta \times \text{genotype} + \gamma \times \text{U-As} \) (In transformed). Model 2: Arsenic metabolite \(\alpha + \beta \times \text{genotype} + \gamma \times \text{U-As} \) (ln) + \(\lambda \times \text{AS3MT} \) haplotype. Allele genotypes associated with lower %MMA are denoted first. Mean values and 95% CIs are adjusted values based on model 1.
association is generally consistent with additive effects of each haplotype, i.e., $\beta = 2.1$; 95% CI: 0.75, 3.5 for 3 copies versus two copies of N6AMT1 haplotype 1 (Table 4), and $\beta = 3.3$; 95% CI: 1.8, 4.7 for no copies versus two copies of AS3MT haplotype 2 [see Supplemental Material, Table S3 (http://dx.doi.org/10.1289/ehp.1206003)]. In general, mean %MMA increased as the number of copies of AS3MT haplotype 2 copies increased and as copies of N6AMT1 haplotype 1 decreased, and vice versa, although %MMA was highest among 8 women with either one or two copies of N6AMT1 haplotype 1 and no copies of AS3MT haplotype 2 (11.1; 95% CI: 9.0, 13.2). This pattern of associations was only seen for %MMA.

### Discussion

In our study population of Andean women, variation in the relative amount of MMA in urine was associated with genetic variation in N6AMT1 in an allele–dose dependent manner, a finding that supports the hypothesis that N6AMT1 is involved in human arsenic metabolism. Associations between %MMA and N6AMT1 variants persisted when adjusted for a common AS3MT haplotype.

### Table 4. Multivariable regression analyses of the influence of N6AMT1 haplotypes on fractions of arsenic metabolites.

| Metabolite/AS3MT haplotype (sequence) | n | Copy n$^a$ | Mean (95% CI)$^b$ | Model 1 |  | Model 2 |  |
|---|---|---|---|---|---|---|---|
| tAs | | | | | | | |
| Haplotype 1 (AATT) | 30 | 2 | 13.8 (11.7, 16.0) | | | |
| | 61 | 1 | 12.5 (11.2, 13.8) | $-1.3\text{(3)}\text{,} 1.2$ | 0.30 | $-1.2\text{(3)}\text{,} 1.3$ | 0.34 |
| | 77 | 0 | 12.5 (11.1, 13.9) | $-1.3\text{(3)}\text{,} 1.2$ | 0.30 | $-1.7\text{(4)}\text{,} 0.79$ | 0.18 |
| Haplotype 3 (ATTG) | 52 | 2 | 16.2 (11.0, 21.6) | | | |
| | 46 | 1 | 11.7 (10.0, 13.5) | $-4.6\text{(2)}\text{,} 0.96$ | 0.10 | $-4.4\text{(1)}\text{,} 8.1$ | 0.18 |
| | 137 | 0 | 12.9 (11.9, 13.9) | $-3.4\text{(2)}\text{,} 8.0$ | 0.21 | $-3.5\text{(2)}\text{,} 8.1$ | 0.19 |
| Haplotype 9 (GTGT) | 72 | 0 | 12.7 (11.4, 14.2) | | | |
| | 73 | 1 | 13.0 (11.6, 14.4) | $0.97\text{(4)}\text{,} 1.3$ | 0.79 | 0.020 $\text{(-1)}\text{,} 19.2$ | 0.98 |
| | 43 | 2 | 12.1 (10.3, 13.9) | $0.98\text{(4)}\text{,} 1.3$ | 0.55 | $-1.3\text{(-3)}\text{,} 0.98$ | 0.27 |

### Table 5. Multivariable regression analyses of haplotype × haplotype interaction between AS3MT (haplotype 2) and N6AMT1 (haplotype 1) and fractions of arsenic metabolites.

| Metabolite | AS3MT haplotype 2 × N6AMT1 haplotype$^a$ | n | Mean (95% CI)$^b$ | $\beta$ (95% CI) | p-Value | $\beta$ (95% CI) | p-Value |
|---|---|---|---|---|---|---|---|
| tAs | | | | | | | |
| 2 copies AS3MT + 2 copies N6AMT1 | 15 | 12.1 (8.9, 15.1) | | | | | |
| 2 copies AS3MT + 1 copy N6AMT1 | 44 | 11.7 (9.9, 13.5) | $-0.42\text{(-3)}\text{,} 3.9$ | 0.32 | | | |
| 2 copies AS3MT + 0 copies N6AMT1 | 31 | 10.6 (8.5, 12.7) | $-1.5\text{(-5)}\text{,} 2.2$ | 0.43 | | | |
| 1 copy AS3MT + 2 copies N6AMT1 | 12 | 13.7 (10.4, 17.1) | $1.2\text{(-9)}\text{,} 6.1$ | 0.48 | | | |
| 1 copy AS3MT + 1 copy N6AMT1 | 30 | 13.7 (11.6, 15.9) | $1.6\text{(-1)}\text{,} 5.3$ | 0.38 | | | |
| 1 copy AS3MT + 0 copies N6AMT1 | 33 | 13.2 (11.1, 15.2) | $1.0\text{(-6)}\text{,} 4.7$ | 0.57 | | | |
| 0 copies AS3MT + 1 and 2 copies N6AMT1 | 8 | 15.6 (11.5, 19.8) | $3.5\text{(-1)}\text{,} 6.7$ | 0.18 | | | |
| 0 copies AS3MT + 0 copies N6AMT1 | 13 | 15.1 (11.9, 18.4) | $3.0\text{(-1)}\text{,} 4.7$ | 0.14 | | | |

### Table 6. Allele–dose effect of the expression of N6AMT1 was observed for some SNPs/ haplotypes (Table 6).
that was also associated with %MMA. When homozygous carriers of variants of each gene were compared, associations between N6AMT1 variants and %MMA were not as strong as associations with the AS3MT haplotype. When estimated according to combined copy numbers of N6AMT1 and AS3MT haplotypes associated with low %MMA, associations were consistent with an additive effect of variants in the two genes, such that women with two copies of the two haplotypes had the lowest mean %MMA (6.2; 95% CI: 4.6, 7.8), in contrast with a mean of 10.4 (95% CI: 8.7, 12.0) (β = 4.2; 95% CI: 1.9, 6.5) among women with no copies of either haplotype. Although there were few individuals in some of the combined haplotype groups, the differences observed could be sufficient to increase the risk of arsenic-related disease, as higher %MMA in urine [mostly MMA(V)] is related to increasing risk of several adverse health effects (Chung et al. 2002; IARC 2004; Leonardii et al. 2012; Avalos et al. 2005; Meliker et al. 2007; Navas-Acien et al. 2005; Valter 2002). All N6AMT1 SNPs except one (rs16983411) were common in the study population (minor allele frequencies > 40%) and in HapMap reference populations (Thornton et al. 2005), and apart from rs2205449, more than half of the participants were carriers of alleles that were associated with lower %MMA. For AS3MT, we previously showed a strong overrepresentation of the haplotype associated with low MMA and high DNA (i.e., an efficient and less toxic metabolism) in this Andean population, compared with all other studied populations worldwide (Schlawicke Engström et al. 2007).

The gene N6AMT1 was recently identified in humans (Ratel et al. 2006) and is located on 21q12.1 [NCBI HomoloGene database (http://www.ncbi.nlm.nih.gov/homologene)]. It is conserved in chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, fruit fly, mosquito, Caenohabditis elegans, Saccharomyces pombe, Arabidopsis thaliana, and rice (NCBI HomoloGene database). The mouse homolog of N6AMT1 has been shown to methylate glutamine in the translation termination factor eRF1 and to be crucial for embryological development (Liu et al. 2009). N6AMT1 has about 25% amino acid sequence similarity with AS3MT in the S-adenosylmethionine binding domain class I (Ajees et al. 2012; see also NCBI Conserved Domains database (http://www.ncbi.nlm.nih.gov/ccd)), a structural fold shared by most methyltransferases (Schubert et al. 2003). However, the structural similarity between N6AMT1 and AS3MT does not include the three cysteine residues (C72, C174, and C224) that bind inorganic AsIII and MMAIII and are necessary for the arsenic methylation steps performed by AS3MT (Marapakala et al. 2012). Thus, other amino acid residues are probably important for the arsenic methylating capacity of N6AMT1. In accordance with the previous experimental studies on N6AMT1 and arsenic metabolism (Ren et al. 2011), our results suggest that %MMA may be influenced by N6AMT1. Although the associations with %As or %DNA were not statistically significant, the consistent opposite direction of the associations with iAs and MMA, which is in contrast to the findings for AS3MT (Engström et al. 2011), suggests different effects of N6AMT1 on the two methylation steps. Findings for AS3MT, on the other hand, suggest that it may affect both steps to about the same extent (Engström et al. 2011).

A limited range of expression of N6AMT1 in blood may be one of the reasons why we did not find any association between N6AMT1 expression in whole blood and total arsenic in urine; alternatively, the expression of N6AMT1 may not be induced by arsenic. Genotypes associated with low %MMA were associated with the lowest N6AMT1 expression, although in most cases expression did not change monotonically according to allele or haplotype copy numbers. The direction in expression is similar to observations for AS3MT: For carriers of the haplotype associated with more proficient arsenic metabolism and less MMA in urine, reduced AS3MT expression was found in blood (Engström et al. 2011). Still, the relation between genotype–gene expression and metabolic pattern should be further explored, ideally in tissues where N6AMT1 is highly expressed, such as the adrenal and parathyroid glands and the kidneys (http://www.proteinatlas.org) (Ren et al. 2011; Uhlen et al. 2005).

Apart from AS3MT and N6AMT1, we have previously reported evidence that genetic variation in DNA methyltransferases DNM1T and DNM3T8B influences arsenic metabolism efficiency, and similar to N6AMT1, associations with these methyltransferases were weaker than associations with AS3MT variants (Engström et al. 2011; Gardner et al. 2012). These findings suggest that AS3MT is the major arsenic methyltransferase but that other, probably less specific methyltransferases, can also methylate arsenic. In addition, although part of the differences in arsenic metabolism efficiency is genetically determined, the phenotype appears to be polygenic.

### Conclusions

Polymorphisms in N6AMT1 significantly predicted the %MMA in urine in a population of women from the Argentinean Andes, suggesting additional pathways and methyltransferases involved in the metabolism of arsenic. This emphasizes the need to consider N6AMT1 in future studies of populations exposed to arsenic.

#### Table 6. N6AMT1 gene expression as expressed relative fluorescence units, stratified by N6AMT1 SNPs and haplotypes.

| N6AMT1 SNP or haplotype | Genotype or no. of copies | ILMN_2315669 (Mean 95% CI) | ILMN_1765088 (Mean 95% CI) |
|-------------------------|-------------------------|-----------------------------|-----------------------------|
| rs16983411              | AA                      | 11.7 (111.3, 123.0)         | 11.0 (108.0, 114.0)         |
|                         | AG                      | 12.6 (119.8, 133.9)         | 11.4 (117.1, 119.8)         |
|                         | GG                      | 122.9 (122.6, 133.2)        | 115.2 (113.3, 119.1)        |
| rs2205449               | AA                      | 11.4 (107.3, 122.1)         | 113.9 (107.8, 119.1)        |
|                         | TT                      | 120.3 (114.4, 126.3)        | 112.3 (109.0, 114.7)        |
|                         | GT                      | 123.5 (120.0, 135.1)        | 114.6 (111.7, 117.8)        |
|                         | GG                      | 119.6 (107.7, 131.8)        | 115.7 (111.8, 119.6)        |
| rs1048546               | CG                      | 115.6 (109.3, 121.8)        | 111.0 (107.5, 114.6)        |
|                         | GT                      | 126.1 (120.3, 132.9)        | 114.3 (111.8, 117.0)        |
|                         | TT                      | 122.7 (111.6, 133.8)        | 115.5 (113.3, 119.6)        |
| Haplotype 1             | 2 copies                | 114.7 (107.3, 122.1)        | 113.9 (108.7, 119.1)        |
|                         | 1 copy                  | 120.3 (114.4, 126.3)        | 112.3 (109.0, 114.7)        |
|                         | 0 copies                | 123.3 (119.5, 134.2)        | 114.2 (111.2, 117.5)        |
| Haplotype 3             | 1 and 2 copies          | 125.7 (117.6, 133.7)        | 111.8 (108.2, 115.4)        |
|                         | 0 copies                | 120.5 (115.6, 125.4)        | 114.3 (112.2, 116.4)        |
| Haplotype 9             | 0 copies                | 117.2 (111.9, 121.5)        | 111.7 (108.8, 114.7)        |
|                         | 1 copy                  | 128.1 (121.9, 135.5)        | 114.1 (111.3, 117.2)        |
|                         | 2 copies                | 118.1 (105.7, 132.4)        | 116.1 (118.9, 120.4)        |

*p-Values from ANOVA.

[^1]: The genotype/haplotype associated with lower %MMA is denoted first and used as reference group. rs16883411 was excluded from the analysis because there were so few carriers with variant genotypes (n = 3). Due to low frequency of carriers of two copies of haplotype 3, carriers of one and two copies were merged.
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