Age-distribution and genotype-phenotype correlation for N-acetyltransferase in Argentine children under isoniazid treatment

Guillermo Alberto Keller¹,², Lucas Fabian³, Matías Gomez³, Claudio Daniel Gonzalez¹, Roberto Alejandro Diez¹, and Guillermo di Girolamo¹

¹Pharmacovigilance Unit, Second Chair of Pharmacology, School of Medicine, Universidad de Buenos Aires. ²Emergency Department, Hospital General de Agudos Donación Francisco J. Santoianni, and ³Chair of Medicinal Chemistry, School of Pharmacy and Biochemistry, Universidad de Buenos Aires, Buenos Aires, Argentina

Abstract. Introduction: Metabolic clear-ance of isoniazid (INH) may be up to 10 times faster in individuals who are rapid acetylators compared with slow acetylators. In addition, the acetylation phenotype has been suggested to change with age. A better knowledge of the age distribution of the acetylation genotype and phenotype in children requiring INH for tuber-culosis treatment or prevention could be im-portant to optimize safety and efficacy of INH use. Objectives: The aim of the present study was to evaluate the genotype and phenotype of NAT2 in an Argentinian pediatric population from Buenos Aires. In addition, we wanted to describe genotype-phenotype correlation, as well as its distribution at different ages. Methodology: NAT2 genotyping was performed by RFLP technique, searching for common polymorphisms. Acetylisoniazid and isoniazid concentrations were measured by HPLC and NAT2 phenotype was defined from the ratio of both concentrations (Metabolic Ratio, MR). Results: Almost half of the patients (46.02%) possessed wild-type haplotype, with 17.05% of individuals having two fully functional alleles, 57.95% one fully functional allele and 25% with no fully functional allele. According to phenotype, most children (96.59%) were classified as fast acetylators, whereas 1.14% of the cases were intermediate and 2.27% slow acetylators. There was a positive association between age and MR (R = 0.52985, p < 0.000001) with a significant MR difference between age cat-eories (p < 0.001). Conclusions: We found a high proportion of rapid acetylators compared with other populations. Acetylator phenotype showed a positive correlation with age, with a significant change around the 4 th year of life.

Introduction

Arylamine N-acetyltransferase-2 (NAT2), a highly polymorphic enzyme, is in-volved in cytosolic phase II conjugation and plays a key role in the detoxification of many commonly prescribed drugs through acetyla-tion. It is also involved in the metabolism of carcinogens from environmental, industrial, and dietary sources [1]. In human popula-tions, 34 single nucleotide polymorphisms (SNP) have been identified throughout the NAT2 coding region. So far, the combination of the presence or absence of each SNP ac-counts for 73 different alleles classified in 14 main families [2]. Some of these SNP affect the activity of the enzyme, resulting in different metabolic capacities (normal for NAT2*4, *13A, *13B, *12A, *12B, *12C, and *11A alleles or decreased in the other alleles). In each individual, the combination of the two alleles determines the expected phenotype: rapid acetylators (RA, determined by the presence of two normal activity alleles), intermediate acetylators (IA, when only one normal activity allele is present) and slow acetylators (SA, when the two alleles have decreased activity) [2, 3].

Assessment of NAT2 genotype or pheno-type has been proposed as a method for pre-dicting adverse reactions to some drugs [4, 5] as well as bladder [6, 7] and liver cancer [8] risk. Among substrates of NAT2 ranks isoniazid (INH), a first-line drug used in the treatment of tuberculosis (TB), a re-emerging disease that currently is a major public health problem. The SA status has been as-sociated with INH-induced hepatitis in some populations and the RA status with impaired response to TB treatment [1, 3, 4, 5, 6, 7, 8, 9, 10, 11]. In humans, NAT2 activity can be di-rec-tly measured through molar ratio of ace-tyl-isoniazid (AcINH) to INH concentrations.
NAT2 genotype and phenotype in Argentine children

2 hours after the administration of the INH dose or predicted from the genotype of the individual.

However, the prediction of the metabolic phenotype from a detected genotype has several drawbacks. The detection of a particular genotype does not imply that it is expressed as encoded, first, for some uncommon alleles the actual activity of the encoded product is unknown. In addition, different events can impact the expression or activity of the enzyme. Among such variables, NAT2 activity seems to depend on the age of the subject, resulting in a correlation between activity and age and suggesting some kind of "maturation" process, that reaches a peak at the age of 4 years [12, 13, 14, 15]. Therefore, the aim of the present study was to evaluate the genotype and phenotype frequencies of NAT2 polymorphisms in a pediatric population from Buenos Aires. In addition, we wanted to describe these distributions at different ages, as well as the genotype-phenotype correspondence.

Materials and methods

Subjects

Patients aged between 0 and 15 years, with INH indication for prophylaxis of tuberculosis were included. None of the children had any gastrointestinal or hepatic dysfunction known to interfere with INH metabolism within 1 month, or INH absorption within 24 hours prior to the administration of INH. A single dose of 10 mg/kg was administered to fasting patients. Self-defined ancestry [16, 17, 18] was asked to each of the parents and registered together with anthropometric and epidemiological data of each individual.

Parents gave written informed consent for each child’s participation and the study was approved by the institutional review board of the Hospital Santojanni.

Sample collection

Three hours after the first dose for each patient, 1 mL of blood was extracted and separated in two aliquots, one tube containing 0.2 mL EDTA for NAT2 genotyping, and the second in a heparinized tube for NAT2 phenotyping through HPLC quantification of AcINH and INH concentration. Both aliquots were stored at −20 °C until subsequent processing.

NAT2 genotyping

The DNA was isolated from white blood cells from peripheral blood using Illus-tra blood genomic Prep Mini Spin Kit (GE Healthcare, Freiburg, Germany).

NAT2 genotypes were determined using a modification of a polymerase chain reaction (PCR)-restriction fragment length polymorphisms (RFLP) assay [19]. Genomic DNA (100 ng) amplification was carried out in a 25 µL-reaction volume containing 10 mmol×L⁻¹ Tris-HCl (pH 8.3), 50 mmolL⁻¹ KCl, 1.5 mmol×L⁻¹ MgCl₂, 0.2 mmol×L⁻¹ of each deoxynucleotide triphos-phate (dNTP), 0.2 µmol×L⁻¹ of each oligo-nucleotide primer: Forward, 5'-GGCTATAAGAACTCTAGGAAC-3' and Reverse, 5'-AAGGGTTTATTTTGTTCCTTTATCTAAATTTTATTTTGTTCCTTTATCTAAATTT-3', and 1.25 U of Platinum Taq DNA polymerase (Invitrogen, San Diego, CA, USA). Thermal cycling conditions for the PCR were 5 minutes at 94 °C, followed by 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes.

Following amplification, genotyping was performed using a RFLP assay to detect four different NAT2 SNPs. PCR products (895 bp) were digested separately withMspI, KpnI, TaqI, and BamHI (Fermentas, Burlington, Canada) to detect the following specific SNP: 191G>A (rs1801279), 481C>T (rs1799929), 590G>A (rs1799930), 857G>A (rs1799931), respectively. All digestions were performed according to the manufacturer’s recommendations.

Digested PCR products were separated by electrophoresis on 2% agarose gels (Pro-nadisa, Madrid, Spain) for MspI, KpnI, and BamHI (Fermentas, Burlington, Canada) to detect the following specific SNP: 191G>A (rs1801279), 481C>T (rs1799929), 590G>A (rs1799930), 857G>A (rs1799931), respectively. All digestions were performed according to the manufacturer’s recommendations.

Digested PCR products were separated by electrophoresis on 2% agarose gels (Pro-nadisa, Madrid, Spain) for MspI, KpnI, and BamHI or 10% polyacrylamide gels for TaqI (90 – 100 V for 2 hours or 6 hours, respectively) with DNA molecular size markers (Invitrogen, San Diego, CA, USA). The am-plicated products were visualized with ethid-iium bromide staining under UV light.

HPLC reagents
INH was from Sigma-Aldrich (St. Louis, MO, USA, 037K0776, purity over 99.9%) and AcINH was synthesized by the method of Fox and Gibas [20].

Trichloroacetic acid, phosphoric acid, acetic anhydride, and glacial acetic acid were all of analytical grade (Merck, Darmstadt, Germany). 1-Hexanesulfonic acid sodium salt was analytical grade (Sigma-Aldrich). Acetonitrile and water were HPLC grade (Merck).

Sample preparation

Samples of heparinized plasma or aqueous calibration solutions (100 µL) were deproteinized with 50 µL 10% (w/v) trichloroacetic acid in 1.5-mL polypropylene Eppendorf tubes. The mixture was vigorously vortex-mixed for 1 minute and then centrifuged for 15 minutes at 3,000 g. The trichloroacetic acid supernatant (pH 1) was withdrawn and evaporated to dryness for 10 minutes at room temperature under a gentle nitrogen stream. The dry residue was dissolved in 100 µL of an aqueous solution of 1-hexane-sulfonic acid sodium salt (20 mM, pH 3 adjusted with phosphoric acid). This solution (20 µL) was injected for HPLC analysis.

Chromatography

Chromatographic analyses were performed using chromatograph (Thermo Separation Products, Minneapolis, MN, USA). The chromatographic system consisted of a SCM400 solvent degasser, P4000 quaternary gradient pump, AS 3500 autosampler with 100-µL sample loop, SpectraFOCUS high-speed scanning UV-2000, SN4000 system controller, and data station (Intel-Pentium 166 MMX, RAM 64 MB, HDD 2GB) with the analytical software ChromQuest 2.1 (ThermoQuest, Inc., San Jose, CA, USA).

Compounds were separated on a 250 × 4.4 mm, 5-µm particle, Alltima Alltech C18 column.

The mobile phase was a binary linear gradient prepared from an aqueous solution of 1-hexanesulfonic acid sodium salt solution (20 mM, pH 3, adjusted with phosphoric acid) and acetonitrile. The proportion of the components was initially 90 : 10 for 5 minutes. This was changed to 60 : 40 over 5 minutes, maintained for 5 minutes. Finally the proportion was changed to 90 : 10 over 2 minutes and maintained for 5 minutes for re-equilibration. The flow rate was 1 mL min⁻¹ and detection was performed at 267 nm.

Calibration

Calibration plots were constructed by analysis of appropriate working solutions of INH and AcINH prepared in water and in heparinized plasma. Concentrations of 0.0, 0.5, 1.0, 5.0, 10.0, and 20.0 µg mL⁻¹ were used for both drugs, and the samples were treated as described above.

Validation

The method was validated in accordance with published guidelines. Within-run precision and standard deviation as a measure of accuracy were examined by supplementing plasma with appropriate amounts of each of the two compounds to yield quality control (QC) samples containing 1, 4, and 12 µg mL⁻¹. The QC samples were aliquoted (n = 10), and each aliquot was analyzed as a separate sample. To avoid the risk of possible drug degradation during storage, QC samples were prepared each day from the stock solutions prepared on the first day of the study.

Inter-assay reproducibility was determined on four separate occasions by replicate analysis of each of the QC samples. Results were regarded as satisfactory if they were within ± 15% of the actual value, except for the limit of quantification, for which 20% was accepted as satisfactory.

To determine long-term freezer stability of AcINH and INH in plasma, QC solutions were analyzed in triplicate after storage of the solutions at −80 °C for 1, 2, and 30 days. The drug was regarded as stable if more than 90% was intact at the end of the study period.

Metabolic ratio (MR) and phenotype interpretation

The acetylator phenotype was assessed through the molar MR calculated as the AcINH/INH concentrations quotient for each sample. The MR was used to clas-
NAT2 genotype and phenotype in Argentine children

Table 1. Allele individuals classified by number of functional alleles at the different age groups.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Number of individuals</th>
<th>Individuals classified by number of functional alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 – 23 months</td>
<td>25</td>
<td>5   20.00% 11 44.00% 9 36.00%</td>
</tr>
<tr>
<td>2 – 3 years</td>
<td>15</td>
<td>3   20.00% 10 66.67% 2 13.33%</td>
</tr>
<tr>
<td>4 – 6 years</td>
<td>17</td>
<td>2   11.76% 10 58.82% 5 29.41%</td>
</tr>
<tr>
<td>7 – 17 years</td>
<td>31</td>
<td>5   16.13% 20 64.52% 6 19.35%</td>
</tr>
<tr>
<td>Total</td>
<td>88</td>
<td>15  17.05% 51 57.95% 22 25.00%</td>
</tr>
</tbody>
</table>

Table 2. NAT2 allele frequencies in other studies [1, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32].

<table>
<thead>
<tr>
<th>Place</th>
<th>n</th>
<th>*4</th>
<th>*5</th>
<th>*6</th>
<th>*7</th>
<th>*14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan</td>
<td>102</td>
<td>73.50%</td>
<td>1.00%</td>
<td>15.70%</td>
<td>9.80%</td>
<td>–</td>
</tr>
<tr>
<td>Panama, Ngawbe</td>
<td>210</td>
<td>72.40%</td>
<td>2.40%</td>
<td>0.00%</td>
<td>23.30%</td>
<td>–</td>
</tr>
<tr>
<td>Korea</td>
<td>288</td>
<td>61.30%</td>
<td>1.00%</td>
<td>22.40%</td>
<td>13.20%</td>
<td>0.00%</td>
</tr>
<tr>
<td>Panama, Embera</td>
<td>272</td>
<td>61.00%</td>
<td>9.90%</td>
<td>3.70%</td>
<td>22.80%</td>
<td>–</td>
</tr>
<tr>
<td>Amerindians</td>
<td>90</td>
<td>51.20%</td>
<td>25.00%</td>
<td>6.10%</td>
<td>20.10%</td>
<td>0.00%</td>
</tr>
<tr>
<td>Present study</td>
<td>88</td>
<td>46.02%</td>
<td>39.20%</td>
<td>13.64%</td>
<td>1.14%</td>
<td>0.00%</td>
</tr>
<tr>
<td>Iran</td>
<td>176</td>
<td>42.60%</td>
<td>33.00%</td>
<td>18.70%</td>
<td>5.70%</td>
<td>–</td>
</tr>
<tr>
<td>Nicaragua</td>
<td>137</td>
<td>41.60%</td>
<td>31.40%</td>
<td>16.80%</td>
<td>10.20%</td>
<td>–</td>
</tr>
<tr>
<td>Senegal</td>
<td>101</td>
<td>40.60%</td>
<td>32.20%</td>
<td>18.80%</td>
<td>0.00%</td>
<td>8.40%</td>
</tr>
<tr>
<td>Thailand</td>
<td>235</td>
<td>38.10%</td>
<td>3.80%</td>
<td>32.60%</td>
<td>20.50%</td>
<td>–</td>
</tr>
<tr>
<td>Bs As (adult population)</td>
<td>185</td>
<td>29.90%</td>
<td>37.00%</td>
<td>25.60%</td>
<td>8.00%</td>
<td>1.30%</td>
</tr>
<tr>
<td>South Africa</td>
<td>97</td>
<td>29.90%</td>
<td>36.10%</td>
<td>17.00%</td>
<td>6.70%</td>
<td>10.30%</td>
</tr>
<tr>
<td>Spain</td>
<td>258</td>
<td>25.80%</td>
<td>47.00%</td>
<td>25.00%</td>
<td>0.60%</td>
<td>0.40%</td>
</tr>
<tr>
<td>USA</td>
<td>387</td>
<td>24.20%</td>
<td>43.70%</td>
<td>26.60%</td>
<td>1.90%</td>
<td>0.10%</td>
</tr>
<tr>
<td>Germany</td>
<td>844</td>
<td>22.70%</td>
<td>42.50%</td>
<td>27.80%</td>
<td>1.30%</td>
<td>0.10%</td>
</tr>
<tr>
<td>Morocco</td>
<td>44</td>
<td>20.50%</td>
<td>51.10%</td>
<td>25.00%</td>
<td>3.40%</td>
<td>0.00%</td>
</tr>
<tr>
<td>Central Brazil</td>
<td>404</td>
<td>20.00%</td>
<td>35.00%</td>
<td>27.00%</td>
<td>4.40%</td>
<td>4.10%</td>
</tr>
<tr>
<td>Southern Brazil</td>
<td>254</td>
<td>13.80%</td>
<td>28.90%</td>
<td>10.40%</td>
<td>2.10%</td>
<td>1.40%</td>
</tr>
<tr>
<td>North Sudan</td>
<td>127</td>
<td>8.70%</td>
<td>47.20%</td>
<td>28.70%</td>
<td>3.10%</td>
<td>3.10%</td>
</tr>
</tbody>
</table>

Statistics

Values are expressed as mean ± SD (in some cases, with range); significance level was 0.05. Calculations were performed with the Statistica 6.0 and Medcalc 2009 softwares. For univariate differences Yates-corrected χ²-test, Fisher’s exact test or one-way ANOVA with post-hoc Scheffe’s test were used, depending on the data. For association between variables, Pearson’s parametric correlation, probitic analysis and Receiver Operating Characteristics (ROC) curves were employed. Outliers were identified according to Kirkwood and Sterne [25]; to avoid inappropriate exclusion, all relevant calculations were performed with and without extreme values. Based on Pearson’s correlation and the probitic analysis, a sample size of 88 individuals provided a power of 90% for an α-error of 0.05 [25].

Results

Subjects

88 subjects aged between 4 months and 14 years (25 infants between 4 and 23 months, 15 children between 2 and 3 years, 17 children between 4 and 6, and 31 children aged between 7 and 14 years) were included in this study. All children received INH for prophylaxis of tuberculosis.

The weight and height were within the normal range for age and no patient presented malnutrition or overweight. Other demo- or pharmacogenetic character-istics explaining a particular behavior were not found. INH dose was 10 mg/kg. Self-defined...
Figure 1. MR distribution histogram excluding outliers.

Table 3. NAT2 phenotype frequencies in other studies.

<table>
<thead>
<tr>
<th>Place</th>
<th>Probe</th>
<th>Slow</th>
<th>Intermediate</th>
<th>Fast</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present study</td>
<td>Isoniazid</td>
<td>2.3%</td>
<td>1.1%</td>
<td>96.6%</td>
<td>88</td>
</tr>
<tr>
<td>Serbs</td>
<td>Caffeine</td>
<td>9.0%</td>
<td>36.0%</td>
<td>55.0%</td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>Isoniazid</td>
<td>13.1%</td>
<td>86.9%</td>
<td></td>
<td>434</td>
</tr>
<tr>
<td>Panama, Embera</td>
<td>Caffeine</td>
<td>18.0%</td>
<td>82.0%</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Japan</td>
<td>Isoniazid</td>
<td>30.0%</td>
<td>40.0%</td>
<td>30.0%</td>
<td>20</td>
</tr>
<tr>
<td>Spain</td>
<td>Isoniazid</td>
<td>57.5%</td>
<td>42.5%</td>
<td></td>
<td>153</td>
</tr>
<tr>
<td>USA, Caucasian</td>
<td>Caffeine</td>
<td>58.1%</td>
<td>41.9%</td>
<td></td>
<td>255</td>
</tr>
<tr>
<td>France</td>
<td>Isoniazid</td>
<td>58.5%</td>
<td>41.5%</td>
<td></td>
<td>458</td>
</tr>
<tr>
<td>Polony</td>
<td>Isoniazid</td>
<td>60.0%</td>
<td>40.0%</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Moroccan Arabs</td>
<td>Isoniazid</td>
<td>61.8%</td>
<td>38.2%</td>
<td></td>
<td>89</td>
</tr>
<tr>
<td>Saudi Arabs</td>
<td>Sulphametazine</td>
<td>63.4%</td>
<td>36.6%</td>
<td></td>
<td>235</td>
</tr>
<tr>
<td>Libian Arabs</td>
<td>Sulphadimidine</td>
<td>65.0%</td>
<td>35.0%</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Jordania</td>
<td>Dapsona</td>
<td>67.5%</td>
<td>22.5%</td>
<td>10.0%</td>
<td>160</td>
</tr>
<tr>
<td>Swedish</td>
<td>Isoniazid</td>
<td>70.0%</td>
<td>30.0%</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>Saudi Arabs</td>
<td>Caffeine</td>
<td>72.3%</td>
<td>27.7%</td>
<td></td>
<td>296</td>
</tr>
<tr>
<td>Egyptians</td>
<td>Isoniazid</td>
<td>82.0%</td>
<td>18.0%</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>South Africa</td>
<td>Isoniazid</td>
<td>83.0%</td>
<td>17.0%</td>
<td></td>
<td>235</td>
</tr>
<tr>
<td>Saudi Arabs</td>
<td>Isoniazid</td>
<td>92.6%</td>
<td>4.4%</td>
<td></td>
<td>136</td>
</tr>
</tbody>
</table>

Ancestry was “white” for both parents in 76 cases (86.36%), and mixed (one was defined as “white” whereas the other was self-defined as Native American) for 12 cases (13.64%).

**NAT2 genotypes**

The 176 alleles (haplotypes) were distributed as follows: 81 wild-type (46.02%), 69 M1 (39.20%), 24 M2 (13.64%) and 2 M3 (1.14%); no M4 haplotypes were detected. No cases of two mutations in one allele were found (Table 2).

The allelic combinations were 27 WT/M1 (30.68%), 22 WT/M2 (25.00%), 20 M1/M1 (22.73%), 15 WT/WT (17.05%), 2 WT/M3 (2.27%) and 2 M1/M2 (2.27%). M1/M3, M2/M2 and M2/M3 combinations were not found. Table 1 presents the distribution of these combinations according to the age group. 15 subjects (17.05%) displayed two fully functional alleles, 51 (57.95%) only one fully functional allele (WT/WT, WT/M1, WT/M2 or WT/M3), and 22 (25%) had no fully functional allele.

**Chromatographic technique validation**

In separate runs with the standards of INH and AcINH, the retention times of these compounds were 7.03 minutes and 6.50 minutes, respectively. The relative retention time for INH was set to 1 and for AcINH was calculated as 0.924. Calibration curves proved to be linear over the concentration range studied. The efficacy of the extraction method for INH and AcINH was > 80%. The equation describing the curves was INH = 340807X – 3736.3 ($r^2 = 0.9997$), and AcINH = 311491X + 12196 ($r^2 = 0.9997$). The lower limit of quantification for both INH and AcINH was 0.1 µg/ml. The efficacy of the extraction method for INH and AcINH was superior to 80%.

**INH and AcINH concentrations**

INH and AcINH concentrations were determined for all subjects. INH and AcINH concentrations were 0.364 ± 0.208, range 0.021 – 1.246, and 1.231 ± 1.360, 0.010 – 7.905 µg/mL, respectively. The resulting metabolic ratio was 3.324 ± 2.250 (0.171 – 13.267). According to their pheno-type, subjects were classified fast acetylators (n = 85, 96.59%), intermediate acetylators, (n = 1, 1.14%), and slow acetylators (n = 2, 2.27%).

Four individuals from group 3 (4 – 6 years old), whose MR were 8.11, 9.58, 10.01, and 10.23, respectively, belonging to the same family, complied with criteria for outliers and accordingly, were excluded from the statistical analysis. These MR values were
NAT2 genotype and phenotype in Argentine children

Figure 2. Distribution in relationship to predefined age categories.

Figure 3. Pearson correlation between age (months) and MR.

Figure 4. Association between MR and genotype.

Figure 5. ROC curve of MR for discrimination of rapid and slow genotype. Area under the ROC curve (AUC) = 0.725; standard error = 0.0572; 95% confidence interval = 0.620 – 0.815; Z statistic = 3.937; significance level p (Area = 0.5) = 0.0001.

significantly higher than those of their class, even doubling the highest values obtained in the entire sample.

Excluding outliers, the distribution of the values of MR ratio in the total sample is shown in Figure 1 and compared with previous results in Table 3. The distribution of MR ratios as a function of the age groups were: 1.699393 ± 0.713810 (25 infants between 4 and 23 months of age), 2.396179 ± 1.034111 (14 children aged between 2 and 3 years), 3.520949 ± 1.648952 (14 children between 4 and 6 years), and 4.169255 ± 2.028842 (31 children aged between 7 and 14 years of age) and are represented in Figure 2.

The Pearson correlation showed a positive association between age and MR ratio (Figure 3), with an R of 0.52985 (p < 0.000001).

A t-test comparing the obtained MR ratio as a function of genotypes (average MR for slow genotype 0.522280; and 1.152434 for rapid genotype) yields a significant value (T = –4.06005; p = 0.000108) (Figure 4). At the probistic analysis, there was an association between MR and genotype when adjusted for age (p = 0.01142, T(80) – 2.58942, SE 0.00684).

ANOVA analysis (Figure 2), shows a significant MR difference between age categories (Scheffe’s test) showed significant differences between groups 1 and 3, 1 and 4, and 2 and 4.
Figure 6. ROC curve of acetyl-isoniazid (left) and isoniazid (right) concentrations for the differentiation of rapid and slow genotype.

Through the use of ROC curves, we established the cutoff value that best discriminates between fast and slow genotypes, setting this value (cutoff point) to 1.82 (Figure 5).

The concentration of Ac-INH was significantly, albeit borderline, correlated with the genotype (Figure 6). This was not the case for INH.

Discussion

This is the first study to show the distribution of NAT2 genotype and phenotype in the pediatric population of different ages from the city of Buenos Aires, analyzing the correlation between genotype and phenotype, as well as the effect of maturation. Our study found a 46.02% of wild-type haplotype with 17.05% of individuals having two fully functional alleles, 57.95% one fully functional allele and 25% having no fully functional allele. Strikingly, according to their phenotype, 96.59% were classified as fast acetylators, 1.14% as intermediate acetylators, and 2.27% as slow acetylators. There was a positive association between age and MR with a significant MR increase with age.

Potential limitations of this study include the use of a genotyping technique that only detects 4 SNP called: M1 (481C>T), M2 (590G>A), M3 (857G>A), and M4 (191G>A). The combination of 34 different SNP (including the 4 we analyzed) in 1 individual leads to the 73 different known alleles. Using the present technique, we can obtain different output groups including all known alleles [2, 15, 16, 38, 41].

In previous genotype studies from other populations, roughly 50% of white individuals were classified as slow acetylators with highly variable results among countries [1, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45]. Our results are consistent with previous reports on adult patients from Buenos Aires [46], with an allele distribution intermediate between other populations studied elsewhere.

The *4 allele (wild -type) frequency exceeds the value reported in white population, but...
is less frequent than in Amerindians. The opposite phenomenon is observed for alleles *5 and *6. No cases of allele *14 were found, probably because of the lack of individuals of African origin. The frequency of allele *7 was much lower than that observed in Native American populations, and similar to that reported in other populations of Caucasian origin. These results were expected, since the current population of Buenos Aires is a mixture of different origins, including large migratory flows from Europe in the early years of the XX century (mainly Spanish and Italian people, though other groups also came to Argentina), together with the descendents of the colonial inhabitants and Amerindians.

M1, M2, M3, and M4 alleles are considered to be linked to the slow acetylation profile, whereas the absence of these alleles is described within the fast acetylation genotype. Among the 73 known alleles, using the technique we selected, only 3 rapid alleles can be erroneously detected as a slow genotype (*11A, *11B, *12C) and 7 slow alleles as rapid genotype (*5C, *5D, *5K, *10, *12D, *17, *19). The prevalence of those alleles is very low [47], except in African population (which is not represented in our study) [1]. Discarding the presence of genotypes that do not match the ethnic group analyzed (alleles from African origin * 11A, * 11B and * 12) and those alleles that have been described in isolated cases and have a very low expected frequency (* 5E, * 5J, * 6O), analyzed mutations can be translated to the current nomenclature as: *4 (wt), *5 (M1), *6 (M2), *7 (M3), *14 (M4).

The NAT2 phenotype has been measured in Argentina and in others countries using a varied list of pharmacological probes, including isoniazid, caffeine, sulphadimidine, sulphamethoxazol, and dapsone. The use of different probes, or different sampling time makes it difficult to differentiate the intermediate and fast groups in some reports, in which the denomination ‘fast Acetylator’ currently should be understood as the sum of the fast and intermediate groups [28, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63]. Interestingly our results show a very high frequency (the highest ever reported) of rapid acetylator phenotype. These frequencies are only comparable to those obtained in ethnically different populations like some Amerindians (Embera people in Panama) or Japanese, or in a predominantly Caucasian population, among Serbs. However, the parents’ self-defined ethnicity of our patients does not support the presence of such association: in only 12 cases one of the parents was of Native American origin. Therefore further explanation is required, since our genotype results are similar to previous studies. As a hypothesis, we propose that the high proportion of fast phenotype should be attributed to non-genetic factors, like illness, diet [64], unreported traditional medications [65], or other epigenetic influences [66]. In fact, other studies have shown increased NAT2 phenotype in populations that do not differ in NAT2 genotype (Koreans display higher NAT2 activity than Swedish people, regardless of NAT2 genotype) [67], or found rapid acetylators frequencies higher than classically reported, even close to this study [62]. Concomitant illness modifies the acetylation phenotype, as demonstrated for the discor-dance between caffeine or dapsone pheno-type and genotype, emphasizing the need of caution in the use of caffeine as a probe for NAT2 in acutely ill patients [68]. INH might exhibit similar behavior.

Our results show a high correlation between the two methods of estimating acetyl-ator status. When outliers are removed there is MR (adjusted for age) and genotype association. But our study shows a discrepancy in the values obtained from MR. These values are usually associated with a fast phenotype, lacking in the present study of a possible explanation to obtain such high values. Howev-er, the apparent discrepancy of finding high metabolic rates in slow genotype patients has been previously reported in the pediat-ric population [14]. MR values also have a right relationship with genotype. So, using ROC curves, we established the cutoff value to discriminate between MR of fast and slow genotypes.

The positive association between age and MR and the MR difference between age is consistent with earlier reports of NAT2 ac-tivity maturation, after the age of 18 months [15] and with a marked point of change (but not a terminal plateau) at 4 years [12]. We observed a tendency to increase metabolic activity after 4 years of age, which may re-
reflect a maturation process after this age, less intense and no evidenced by previous studies, probably because those studies did not focus on ages greater than 4 years).

NAT2 activity maturation has been previously scarcely studied. A previous study (458 children receiving isoniazid) postulated a decrease of the apparent distribution volume with age, as well as differences in clearance and half-life between slow and rapid acetylators [48]. Another group (using caffeine as probe for NAT2 activity) failed to reach the plateau in the recruited individuals (who were < 15 months old) [15], and later (with pediatric individuals receiving isoniazid) [12] found an increase with age of the cumulative frequency of fast acetylators, with a plateau reached around 4 years including a MR category change in 30 slow acetylators (12 became fast acetylators, and 10 showed a variable phenotyping at different ages). A third group observed a trend of change of variable phenotyping at different ages). A previous study (458 children receiving isoniazid) [12] found that the frequency of fast acetylators, with a plateau reached around 4 years including a MR category change in 30 slow acetylators (12 became fast acetylators, and 10 showed a variable phenotyping at different ages). A third group observed a trend of change of variable phenotyping at different ages).

Conclusions

This describes distribution of NAT2 genotype and phenotype, its correlation and the presence of a maturation process in pediatric patients receiving isoniazid in the City of Buenos Aires. All these findings offer new insights for the investigation of NAT2 gene in mixed populations.

Declaration of funding

The present study was supported by a “Ramón Carrillo – Arturo Oñativia” Grant (2008), Comisión Salud Investig., Ministerio de Salud de la Nación Argentina.

Declaration of financial/other relationships

There are no relationships to be declared for any of the authors.

References


NAT2 genotype and phenotype in Argentine children


