Gender and functional *CYP2C* and *NAT2* polymorphisms determine the metabolic profile of metamizole.

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SUMMARY

Background

Metamizole has been banned in several countries because of its toxicity, but it is still used in many countries as an over the counter drug due to its effective analgesic and antispasmodic properties. Although a large variability in the biodisposition of metamizole is known, factors underlying such variability are poorly understood.

Methods.

To assessing factor related to metamizole metabolic profiles, we analyzed the urinary recovery of metabolites, as well as the association of these profiles with genetic and non-genetic factors, in a group of 362 healthy individuals.

Results.

Gender, age, drinking habits and functional polymorphisms are strongly related to metabolic profiles. The N-demethylation of the active metabolite MAA is diminished in carriers of the *CYP2C19*2* allele and in NAT2-slow acetylators. The acetylation of the secondary metabolite AA is decreased in men, in drinkers and in NAT2-slow acetylators with a differential effect of *NAT2*5* and *NAT2*6* alleles. The formylation of MAA is diminished in older subjects and in carriers of defect *CYP2C9* and *CYP2C19* alleles. Two novel arachidonoyl metabolites were identified for the first time in man. Women and *NAT2*-slow acetylators show higher concentrations, whereas the presence of the rapid *CYP2C19*17* allele is associated with lower concentrations of these metabolites. All genetic associations show a gene-dose effect.

Conclusions.

We identified for the first time genetic and non-genetic factors related to the oxidative metabolism of metamizole, as well as new active metabolites. The phenotypic and genetic factors identified in this study can be used as putative biomarkers of metamizole biotransformation and response.

Keywords

Metamizole, metabolism, CYP2C8, CYP2C19, CYP2C19, NAT2, demethylation, acetylation, phenotype, genotype. biomarkers

INTRODUCTION

Metamizole ([N-(1,5-dimethyl-3-oxo-2-phenylpyrazolin-4-yl)-N-methylamino] methanesulfonate, dipyrone, drug bank id. no. DB04817) is a non-steroidal anti-inflammatory drug (NSAID) commonly used for severe pain conditions, cancer pain and migraine as well as fever refractory to other treatments in several countries in Europe, Asia and South America. In other countries such as USA, UK and Canada, metamizole has been banned because it is associated with potentially fatal agranulocytosis [1-3]

Reported estimates of the risk of agranulocytosis associated to metamizole vary by several orders of magnitude, and are likely to be influenced by dose, duration and concomitant medication [4-5]. In addition, in the countries where it is marketed, metamizole is the most frequent drug involved in selective hypersensibility response to NSAIDs, and most patients with selective hypersensitivity to metamizole develop anaphylaxis [6-7].

Metamizole is a prodrug. After oral administration, it is rapidly hydrolyzed in the gastric juice to 4-methyl-amino-antipyrine (MAA). MAA is converted to 4-formyl-amino-antipyrine (FAA), which is an end-product, and to 4-amino-antipyrine (AA), which further metabolized to 4-acetyl-amino-antipyrine (AAA) by the polymorphic enzyme Arylamine N-acetyl-transferase 2 [8-11]. Two metamizole metabolites, MAA and AA, inhibit cyclooxygenases 1 and 2 with different potencies, MAA is roughly nine-fold more potent COX inhibitor than AA and, for both metabolites MAA and AA the potency for COX1 inhibition is about twice as that for COX2 inhibition [3]. The involvement of metamizole metabolites in adverse drug effects is not well understood and whether the adverse effects are related to the parent drug or to metamizole metabolites remains to be elucidated. Because adverse effects of metamizole seem to be related to factors that modify drug exposure or pharmacokinetics [4-5], it could be hypothesized that variability in metamizole biotransformation may modify the risk of developing adverse effects with this drug. Indirect evidence support this hypothesis, since it has been shown that many patients with hypersensitivity to metamizole are negative to skin testing and to basophil activation with metamizole [6], and that NAT2 slow-acetylation haplotypes, that modify the

ability of acetylating AA, are associated with the risk of developing infant leukemia with maternal exposure to dipyrone during pregnancy [12].

The pharmacokinetics of metamizole in man has been studied in detail. MAA, AA, FAA and AAA are detected in plasma, urine, and cerebrospinal fluid, and large interindividual variability in the metabolic ratios leading to different metabolites have been reported [8-9, 11, 13-15]. However, little information is available on factors modifying the metabolic profiles after metamizole use. The widespread prescription or over-the-counter use in many countries requires insight into factors modifying metamizole metabolic profile. In this study, we analyzed the metamizole metabolic profiles in urine, as well as the occurrence of as yet unknown metabolites, in a large group of healthy subjects. The study aims to investigate non-genetic and genetic factors influencing metamizole metabolic profiles as well as to identifying biomarkers for different metabolic profiles that may be used to investigate putative factors leading to adverse drug reactions after metamizole use.

METHODS

Participants.

Three hundred and sixty two unrelated healthy volunteers (131 men, 231 women), all self-reported as Spanish of Caucasian descent, with ages ranging from 19 to 30 years were included in this study following informed consent. All participants were questioned about previous diseases, type and amount of alcohol ingested, smoking habits and consumption of drugs. Table 1 summarizes such information. Subjects with present or previous hepatic disease were excluded. Subjects taking any drug, including oral contraceptives, 2 weeks prior to the administration of metamizole were also excluded. The inclusion of healthy individuals with Caucasian descent only, as well as the narrow age range, was intended to minimize the effect of potential confounders related to liver disorders, ethnicity or age. The protocol of this study was approved by the ethics committee of the University of Extremadura, Badajoz, Spain.

Analysis of metamizole and metabolites in urine.

After overnight fasting to avoid absorption variability [16] the participants were instructed to empty their bladders, and thereafter metamizole magnesium salt (Nolotil, Europharma S.A., Madrid, Spain) was administered as a single oral dose of 575 mg. Total urine was collected during 24h thereafter according previously described procedures [13-14]. Urine aliquots (20 mL) were stored in sterile plastic containers at - 80°C until analysis. Urine (1 mL) was alkalinized, and metamizole and its metabolites were chloroform-extracted. The organic phase was evaporated to dryness under a nitrogen stream. The residues were reconstituted in 500 µL mobile phase, and 20 µL aliquots were analyzed by HPLC. A Spherisorb ODS 5 µm particle size column (250 X 4.6 mm; Sugelabor, Madrid, Spain) was used. The mobile phase was water: methanol: triethylamine: acetic acid (70.9: 27.7: 0.9: 0.5), and the flow rate was 1 mL/min. Column effluents were monitored at 254 nm. Details of these procedures are described elsewhere [14]. Pure samples of methyl aminoantipyrine (MAA), formyl aminoantipyrine (FAA), acetyl aminoantipyrine (AAA), aminoantipyrine

(AA), and the internal standard isopropyl aminoantipyrine were kindly provided by Drs Bremer and Eekert (Hoesehst Aktiengesellsehaft, Radiochemistry Laboratory, D-6230 Frankfurt am Main 80, Germany). The metabolic ratios were calculated on the basis of the molar ratios by dividing the amount of metabolites by the amount of unchanged drug or metabolite, as follows: N-demethylation (AA+AAA/MAA); Formylation: FAA/MAA; Acetylation: AAA/AA. Additional ratios for these three reactions were calculated by dividing the amount of metabolites corresponding to each metabolic step by the amount of all the remaining metabolites.

Identification of new metabolites: Ultra Performance Liquid Chromatography (UPLC) was carried out on an ACQUITY UPLC system (Waters Corp.) with a conditioned autosampler at 4 °C. The separation was accomplished on an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm), which was maintained at 40 °C. The analysis was performed using methanol and water (50 :50 v/v containing 0.01% formic acid) as the mobile phase with a flow rate of 0.5 mL/min and an injection volume of 3µL. The Waters ACQUITY™ XevoQToF Spectrometer (Waters Corp.) was connected to the UPLC system via an electrospray ionization interface. This source was operated in positive ionization mode at 100 °C with the capillary voltage at 3.0 kV and a temperature of desolvation of 300 °C. The cone and desolvation gas flows were 40 and 800 L/h, respectively. The collision gas flow applied was 0.2 mL / min. All data collected in Centroid mode were acquired using Masslynx™ software (Waters Corp.). Leucine-enkephalin was used at a concentration of 250pg/µL as the lock mass generating an [M+H]+ ion (m/z 556.2771) and fragment at m/z 120.0813 with a flow rate of 50 µL/min to ensure accuracy during the MS analysis.

Genotyping study.

Genomic DNA was obtained from peripheral leukocytes and purified according to standard procedures. The target SNPs were selected according functional impact and allele frequency in the population studied. We analyzed the SNPs which constitute the signature for the alleles *CYP2C8*3* (rs11572080), *CYP2C8*4* (rs1058930), *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *CYP2C19*2* (rs4244285), *CYP2C19*3* (rs4986893), *CYP2C19*17*

(rs12248560), *NAT2*5* (rs1801280), *NAT2*6* (rs1799930), *NAT2*7* (rs1799931), *NAT2*14* (rs1801279). All SNPs were tested by means of pre-designed TaqMan Assays (Life Sciences, Alcobendas, Madrid, Spain) designed to detect the above mentioned SNPs (see supplemental Table S1 for details).

The detection was carried out by qPCR in an Eppendorf realplex thermocycler by using fluorescent probes. The amplification conditions were as follows: After a denaturation time of 10 min at 96uC, 45 cycles of 92uC 15 sec 60uC 90 sec were carried out and fluorescence was measured at the end of every cycle and at endpoint. All samples were determined by triplicate and genotypes were assigned both, by the gene identification software (RealPlex 2.0, Eppendorf) and by analysis of the reference cycle number for each fluorescence curve, calculated by the use of CalQPlex algorithm (Eppendorf). For technical validation purposes, the amplified fragments for twenty individuals carrying every genotype, when available, were sequenced. For some SNPs the number of individuals homozygous for the minor allele did not permit to sequence twenty individuals (see below) and in such cases the amplified fragments corresponding to all homozygous individuals identified were sequenced.

Statistical analyses

A test for normality was applied to examine whether the continuous variables were normally distributed (see supplemental Table S2). When values were dichotomic (gender, drinking, smoking) the intergroup comparisons were performed by means of the two-tailed Student's t-test, except when the ratios studied had a non-normal distribution. For the rest of non-genetic comparisons, the non-parametric Spearman's correlation was used. The variables with a p value < 0.1 in the univariate analysis were included in a multivariate analysis based on a logistic regression model by exact methods (maximum likelihood tests) to identify which were independently related to the result. To test the association of genotypes with the metabolic recoveries we used the non-parametric K test. The statistical analysis was carried out by using the statistical software SPSS 17.0 for Windows (SPSS Inc. Chicago, Illinois, USA). Putative departures of Hardy-Weinberg equilibrium were calculated by using the software Haploview 4.2. Phenotype inference based on genetic analyses was

carried out according to standard procedures, assuming as loss of function or slow alleles *CYP2C8*3*, *CYP2C8*4*, *CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2, CYP2C19*3, NAT2*5*, *NAT2*6*, *NAT2*7* and *NAT2*14*, and as gain of function or rapid the allele *CYP2C19*17*. For recent reviews on phenotype inference, see [17-18]. According to standard procedures, a functional phenotype was inferred in the absence of these variant alleles. Haplotype reconstruction was carried out separately for *CYP2C* and for *NAT2* alleles as follows: All possible haplotypes combining the SNPs analyzed in this study were constructed by using PHASE [19]. The reconstructed haplotypes were used for the analyses of the putative effect of haplotypes on metamizole metabolic profile. In addition, phased *NAT2* haplotypes were used for phenotype inference in combination with the *NAT2* haplotype table described elsewhere [20].

RESULTS

The four major metamizole metabolites were detected in the urine of all the subjects studied, albeit with a high interindividual variability. The major metabolite recovered was AAA, followed by FAA, MAA and AA, and no unchanged metamizole was detected in agreement with previous studies [9, 11, 13-15, 21-23]. Table 2 shows the influence of non-genetic factors on the metamizole metabolic profile. Gender was significantly related to the acetylation capacity, with women showing about 20% higher AAA concentration and about 40% higher acetylation ratio (AAA/AA) than men. The recoveries of the rest of major metabolites and the rest of metabolic ratios did not significantly differ between women and men. A statistically significant correlation of age with the recovery of the metabolites MAA and FAA, as well as the FAA/MAA ratio was observed (Table 2), with older individuals showing higher recovery of MAA, lower recovery of FAA and lower FAA/MAA ratio. Other parameters such as height, weight, body mass index, urine volume in 24 hours displayed statistically significant associations with the recovery of AA and AAA, as well as the ratio AAA/AA. However, this association may be due to the strong association of gender with the rest of the parameters (supplemental information Table S3 shows such association). Smoking was statistically associated with an increase of roughly 40% in the demethylation ratio as well as a decrease of roughly 20% in the recovery of MAA and FAA among smokers as compared to non-smokers (Table 2). In addition, the analysis of the bivariate correlation of the number of cigarettes per day was related to the increase in the demethylation ratio, and to a decrease in the recovery of FAA (see Table 2). Drinking habits were associated with a significant decrease in the acetylation ratio of about 30%, and with a significant increase of about 20% in the recovery of AA. In addition, the number of drinks per week inversely correlated with the acetylation ratio (Table 2). Because many of the previously mentioned non-genetic factors are associated (see supplemental table S3), we performed multivariate analyses to elucidate which factors are actually associated with the recovery of major metamizole metabolites and with the metabolic ratios by using multiple regression. These analyses confirmed the influence of gender in the acetylation ratio ($p = 0.005$), the influence of age in the formylation ratio ($p = 0.001$), as well

as the inverse correlation of the number of drinks per week in the recovery of AAA (p<0.001). The rest of putative associations were discarded in the multivariate analysis.

Because it has been previously shown that the acetylation ratio is strongly related to *NAT2* polymorphisms [24], we analyzed the inferred *NAT2* genotypes according to gender and the results indicate similar frequencies in women and men, for slow, intermediate and rapid acetylator inferred phenotypes (see supplemental information Table S4), thus indicating that the gender-related differences observed in the acetylation ratio are independent of *NAT2* polymorphisms.

The SNP frequencies observed in the study group are consistent with those previously described in healthy Spaniards subjects [20, 25-29] and are at Hardy-Weinberg's equilibrium (see supplemental information Table S5). Table 3 shows the effect of inferred phenotypes on the major metamizole metabolites and metabolic ratios. Details about the influence of every SNP are summarized in supplemental information Table S6. Common functional *CYP2C8* polymorphisms did not show any influence in the recoveries of metamizole metabolites or in the metabolic ratios. The inferred CYP2C9 phenotype was related to a nearly-significant decrease of the formylated metabolite, as well as a significant decrease in the formylation ratio, among carriers of loss-of function *CYP2C9* alleles. Most of this effect is attributable to the variant allele *CYP2C9*3*, whereas the presence of *CYP2C9*2* was related to a weaker effect (see supplemental information Table S6), which is consistent with the weaker impairment in drug metabolism *in vivo* caused by *CYP2C9*2* as compared to *CYP2C9*3* [17, 30-31].

CYP2C19 loss of function alleles markedly influenced the demethylation ratio as shown in Table 3. The reduction in the demethylation ratio is consistent with a reduction in the recovery of downstream metabolites AA and AAA among slow CYP2C19 metabolizers. This effect is due to the *CYP2C19*2* allele since no carriers of the loss of function *CYP2C19*3* alleles were identified in the study group. The gain-of-function *CYP2C19*17* allele did not significantly increase the demethylation ratio or the concentration of downstream metabolites.

Because *CYP2C* SNPs are frequently at linkage disequilibrium [17], *CYP2C* haplotypes were reconstructed from genotyping data. The commonest haplotype was that lacking functional SNPs at the *CYP2C9* locus, with a frequency of 45.7% (see supplemental information Figure S7). When participants were stratified according such haplotypes, correlation with metamizole metabolite recoveries and metabolic ratios was lower than that obtained by using phenotype inference (see supplemental information Table S8). This indicates that, for metamizole metabolism, *CYP2C* haplotype analyses do not improve the phenotype inference obtained by genotyping because the most of the association is due to the *CYP2C19* gene only.

The inferred slow *NAT2* phenotype is strongly associated with a decrease in the acetylation ratio and in the recovery of AAA (Table 3). This effect is associated to all *NAT2* loss of function alleles analyzed (see supplemental information Table S6). Interestingly, the decrease in the acetylation ratio is accompanied by a significant increase of the recovery of the upstream metabolite MAA. Slow acetylator individuals show almost double concentrations of MAA and less than half of the concentrations of AAA as compared to non carriers of loss of function *NAT2* alleles, in agreement with previous reports [32]. The increase in MAA concentration is likely to underlie the association of the inferred slow *NAT2* phenotype with significantly lower ratios in the metabolic steps related to MAA (formylation and demethylation) shown in Table 3, because the recoveries of FAA and AA are not significantly affected by the inferred *NAT2* phenotype. Because a differential functional effect *in vivo* of *NAT2* loss of function alleles has been described by using caffeine as a substrate [33], we tested whether such differential effect was observed on the acetylation of AA. The comparison of the recoveries of MAA, AAA and the acetylation ratio between carriers of *NAT2*5* and *NAT2*6* in heterozygosity and in homozygosity is shown in supplemental Table S9. Individuals who were homozygous for the *NAT2*6* allele displayed a significantly lower AAA recovery and lower acetylation ratio as compared to individuals homozygous for the *NAT2*5* allele, thus indicating a differential effect of these variant alleles, which is consistent with that reported with caffeine [33]. The differential effect of *NAT2*7* could not be disclosed because only two individuals carried the *NAT2*7* allele in combination with

NAT2*4 and no homozygous individuals for NAT2*7 were identified in the study group.

In order to identify additional metamizole metabolites, we analyzed a subgroup of 55 participants selected according their genotypes. HPLC-mass spectrometry analyses revealed the presence of two additional metabolites, namely arachidonoyl (N)-methylamide (designated as ARA-NMA) and arachidonoyl methylamide (ARA-MA). Besides these, no additional metabolites were identified. The mean ± SD recoveries were 0.68 ± 1.17 mg and 1.03 ± 2.20 mg, respectively. However, large interindividual variability was observed, with some individuals displaying high concentrations. The maximum recovery among the 55 individuals analyzed was equal to 7.23 mg for ARA-NMA and 12.93 mg for ARA-MA. The occurrence of these arachidonoyl amides have been previously reported to occur in mice, and it has been shown that these metabolites have pharmacological activity [34]. The recoveries of both ARA-NMA and ARA-MA strongly correlated with the recovery of the precursor MAA ($p < 0.001$) and ARA-MA correlated with FAA at lesser extent ($p = 0.014$) (see supplemental Table S10). In addition the recovery of ARA-NMA strongly correlated with initial metabolic ratios (formylation and demethylation; $p = 0.002$ and $p =< 0.001$, respectively) whereas the correlation with the acetylation ratio was weaker ($p = 0.028$). The recovery of ARA-MA correlated with the formylation ($p = 0.001$) and demethylation ratios ($p < 0.001$), but the correlation of ARA-MA with the acetylation ratio did not reach statistical significance ($p = 0.053$). Women displayed about 40% higher mean concentrations of both arachidonoyl metabolites as compared to men, although the differences were not statistically significant in the univariate analysis. The non-genetic factor with the strongest association to the recovery of arachidonoyl metabolites was drinking with concentrations of ARA-NMA being 70% higher ($p = 0.039$) and concentrations of ARA-MA being over twice ($p = 0.001$) in drinkers as compared to non-drinkers. However, no correlation was observed with the number of drinks per week. Supplemental Table S11 summarizes all comparisons. Multivariate analyses confirmed the influence of gender ($p = 0.002$) and height ($p = 0.012$) in the recovery of ARA-NMA. Similar associations for gender ($p = 0.016$) and height ($p = 0.007$) in the recovery of ARA-MA were observed, whereas the rest

of non-genetic factors were discarded in the multiple comparison analysis. Putative associations of arachidonoyl metabolites with other phenotypic parameters as observed in the univariate analyses (see supplemental Table S11) are likely to be due to the strong association of gender with the rest of the parameters (supplemental information Table S3 shows such association).

Regarding genetic factors, Table 4 shows that, besides a weak effect of the *CYP2C9* genotype in the recovery of ARA-NMA, individuals who were homozygous for the gain of function *CYP2C19*17* allele display a marked reduction in the recovery of both arachidonoyl metabolites as compared with individuals with non-mutated *CYP2C19* genes. By turn, individuals homozygous for *NAT2* slow alleles show the highest mean concentration for both arachidonoyl metabolites. For both genotypes (*CYP2C19* and *NAT2*) a gene-dose effect on the recovery of both metamizole metabolites can be observed (Table 4).

DISCUSSION

Metamizole is a commonly used analgesic which is prescribed or used over-the-counter use in many countries. This, together with the severity of some of the adverse reactions secondary to the use of metamizole justifies studies focusing on factors underlying the variability of metamizole biotransformation. The understanding of factors modifying metamizole biodisposition is surprisingly low. The association of metamizole acetylation with the acetylator phenotype and genotype is well known [10-11, 32, 35-36]. In contrast, little is known about the enzymes involved in the initial (oxidative) metamizole biodisposition in vivo. These enzymes have not been identified in man, although *in vitro* evidences supports a role for CYP2C19 and other CYP enzymes in the N-demethylation of aminopyrine, a closely related compound that follows the same metabolic route [37]. The enzyme involved in the formylation of MAA remains unknown, but It has been shown that the metabolic ratios of MAA demethylation and formylation *in vivo* strongly correlate between themselves [36]. The influence of *CYP2C19* and other gene variants on the oxidative biotransformation of metamizole in vivo remains to be elucidated. This study is aimed to elucidate the putative role of polymorphic drug metabolism as well as phenotypic factors in metamizole metabolism in vivo.

It is well known that the main pharmacological effects of metamizole are due to the primary metabolite MAA. After the oral administration of 500 mg dipyrone, MAA plasma concentration is above the IC50 concentration for the inhibition of COX-1 and COX-2 for over 8 hours, whereas the less potent metabolite AA does not reach the IC50 values even after the administration of 1000 mg [3]. For that reason, it is likely that impairment in the oxidative metabolism of metamizole would be related to adverse drug reactions. Hence it is surprising that the only metabolic polymorphism that has been studied with regard to metamizole adverse drug reactions is the acetylation polymorphism [12].

In this study, we have identified phenotypic (gender, age or drinking habits) and genotype (*CYP2C9*, *CYP2C19* and *NAT2*) factors that modify metamizole metabolic profiles. This is the first study that identified genetic factors related to oxidative metamizole metabolism *in vivo*. Our findings support a relevant role

for the CYP2C19 and CYP2C9 enzymes and confirm that functional polymorphisms affecting the *CYP2C19* gene modify the N-demethylation and formylation pathways in agreement with *in vitro* evidences [37]. Our findings confirm that the acetylation of AA is more efficient in women, in non-drinkers and in carriers of non-mutated *NAT2* alleles, with a differential effect of *NAT2*5* and *NAT2*6* alleles consistent with that described with other NAT2 substrate [33]. A gene-dose effect is present for all genetic associations identified in this study.

We did not identify oxalic acid derivatives previously described [2]. By turn, we identified for the first time in man the occurrence of arachidonoyl amides. The recoveries of these metabolites are higher in women and are related to *CYP2C9*, *CYP2C19* and *NAT2* polymorphisms. The presence of the rapid *CYP2C19*17* allele is related to a low recovery of arachidonoyl amides. In addition, our findings suggest that the *NAT2* slow acetylator status drives the metabolism towards arachidonoyl amides, which is an unexpected finding. Because the concentrations of these amides strongly correlate with MAA (supplemental Table S10) and because the NAT2 status strongly correlated with the recovery of MAA (Table 3), it is likely that the observed association of slow *NAT2* genotypes and higher production of arachidonoyl amides, as well as a lower N-demethylation or formylation ratios, would be related to an increased availability of the precursor metabolite MAA. Both arachidonoyl amides inhibit COX enzymes and both binds to cannabinoid CB1 and CB2 receptors with Ki values in the low micromolar concentration (between 3 and 8 µM) [34]. It is particularly interesting that the arachidonoyl amide designated as ARA-NMA is nearly 2.7 fold more potent inhibitor of the enzyme COX1 and 4.4 fold more potent inhibitor of COX2, as compared with MAA, which is considered the active metabolite of metamizole [34]. Taking into consideration the recovered concentration of MAA and ARA-NMA and their relative potencies for inhibiting the COX enzymes, in some individuals analyzed, particularly in women with the slow acetylation genotype, the contribution of these metabolites to COX inhibition is expected to have a similar extent.

In summary, we identified genetic and non-genetic factors that cosegregate with metamizole metabolic profiles, most of these factors being related to increased

recovery of the active metabolite MAA. These factors (summarized on supplemental Figure S12) could be tested as putative biomarkers for drug response and for the risk of developing adverse reactions after metamizole use (for instance for the assessment of hypersensibility reactions when no challenge is recommended due to the risk of anaphylaxis). Morevover, toxic photoproducts of MAA have been described, whereas FAA and AAA showed slower photodegradation kinetics [38]. This raises the hypothesis that individuals with genotypes or phenotype factors leading to increased MAA concentrations may be at higher risk of developing toxic reactions related to metamizole and, once relevant factors are disclosed (this study) further studies are required to elucidate the role of these factors in adverse reactions. In addition, we identified two pharmacologically active arachidonoyl metabolites, and we assessed genetic and non-genetic factors that are related with the production of these metabolites. Due to their pharmacological potency, further studies on the role of these arachidonoyl metabolites in drug response and in the risk of developing adverse effects are warranted.

ACKNOWLEDGEMENTS

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Table 1. Characteristics of the participants (n=362).

Men (n; %)	131 (36.2%)		
Women (n, %)	231 (63.8%)		
Age, years (mean ± SD; range)	21.09 ± 2.06 (19-36)		
Weight, kg. (mean ± SD; range)	63.7 ± 12.36 (42.6-115.0)		
Height, cm. (mean ± SD; range)	168.59 ± 8.59 (152-191)		
Body mass index (mean ± SD; range)	22.28 ± 3.11 (16.64-35.16)		
Urine volume, mL. (mean ± SD; range)	1289.72 ± 538.34 (450-4200)		
Non-drinkers (n, %)	216 (59.7%)		
Drinkers (n, %)	146 (40.3%)	Drinks per week (mean ± SD; range)	5.46 ± 3.13 (1-14)
Non-smokers. (n, %)	275 (76.2%)		
Smokers (n, %)	86 (23.8%)	Cigarettes per day (mean ± SD; range)	8.61 ± 6.47 (1-25)

Table 2. Effect of non-genetic factors on major metamizole metabolite recoveries and metabolic ratios.

	MAA (mg)	Intergroup comparison values	FAA (mg)	Intergroup comparison values	AA (mg)	Intergroup comparison values	AAA (mg)	Intergroup comparison values	Formylation ratio (FAA/MAA)	Intergroup comparison values	Demethylation ratio ((AA+AAA)/MAA))	Intergroup comparison values	Acetylation ratio (AAA/AA)	Intergroup comparison values
Men (n= 131)	10.18 ± 4.98	$p = 0.532$	45.03 ± 18.09	$p = 0.248$	10.90 ± 5.58	$p = 0.086$	63.73 ± 45.25	$p < 0.001$	5.51 ± 3.29	$p = 0.065$	10.25 ± 9.24	$p = 0.162$	7.07 ± 5.36	$p < 0.0$
Women (n = 231)	11.01 ± 6.26		42.71 ± 18.02		10.60 ± 8.39		76.02 ± 39.49		4.91 ± 2.98		10.91 ± 9.05		10.14 ± 8.21	
Age		$p = 0.016$		$p = 0.005$		$p = 0.090$		$p = 0.178$		$p < 0.001$		$p = 0.103$		$p = 0.0$
Weight		$p = 0.641$		$p = 0.762$		$p = 0.639$		$p = 0.041$		$p = 0.414$		$p = 0.437$		$p = 0.1$
Height		$p = 0.657$		$p = 0.308$		$p = 0.671$		$p = 0.003$		$p = 0.076$		$p = 0.234$		$p = 0.0$
Body mass index		$p = 0.884$		$p = 0.440$		$p = 0.935$		$p = 0.059$		$p = 0.458$		$p = 0.205$		$p = 0.3$
Urine volume (ml)		$p = 0.767$		$p = 0.393$		$p = 0.001$		$p = 0.065$		$p = 0.943$		$p = 0.665$		$p = 0.0$
Drinkers	10.20 ± 5.22	$p = 0.999$	42.58 ± 19.13	$p = 0.466$	11.91 ± 9.22	$p = 0.037$	67.34 ± 38.83	$p = 0.111$	5.12 ± 2.82	$p = 0.888$	10.62 ± 8.74	$p = 0.671$	7.18 ± 4.89	$p = 0.0$
Non-drinkers	10.57 ± 6.08		44.42 ± 17.22		9.89 ± 6.01		73.75 ± 42.33		5.33 ± 3.36		10.97 ± 9.73		10.42 ± 8.90	
Drinks per week		$p = 0.516$		$p = 0.037$		$p = 0.043$		$p < 0.001$		$p = 0.744$		$p = 0.298$		$p < 0.0$
Smokers	8.85 ± 5.48	$p = 0.013$	36.46± 18.83	$p = 0.007$	12.70 ± 7.67	$p = 0.056$	77.77 ± 40.39	$p = 0.134$	5.37 ± 2.90	$p = 0.616$	14.72 ± 10.04	$p = 0.001$	8.51 ± 6.58	$p = 0.5$
Non-smokers	10.68 ± 5.75		44.78± 17.66		10.46 ± 7.55		70.02 ± 40.93		5.21 ± 3.18		10.19 ± 9.04		9.11 ± 7.76	
Cigarettes per day		$p = 0.077$		$p = 0.022$		$p = 0.199$		$p = 0.260$		$p = 0.750$		$p = 0.017$		$p = 0.8$

Table 3. Effect of genetic factors on major metamizole metabolite recoveries and metabolic ratios.

	MAA (mg)	Intergroup comparison values	FAA (mg)	Intergroup comparison values	AA (mg)	Intergroup comparison values	AAA (mg)	Intergroup comparison values	Formylation ratio (FAA/MAA)	Intergroup comparison values	Demethylation ratio ((AA+AAA)/MAA)	Intergroup comparison values	Acetylation ratio (AAA/AA)	Intergroup comparison values
CYP2C8*1/*1	10.92 ± 6.22		43.31 ± 19.43		10.81 ± 8.06		70.29 ± 45.66		5.09 ± 3.04		10.68 ± 9.15		8.70 ± 7.32	
CYP2C8*1/slow	10.63 ± 5.37	p = 0.927	43.71 ± 15.33	p = 0.828	10.43 ± 6.67	p = 0.551	69.80 ± 34.39	p = 0.547	5.18 ± 3.30	p = 0.877	10.49 ± 9.31	p = 0.710	9.56 ± 8.06	p = 0.174
CYP2C8 slow/slow	9.77 ± 3.53		41.99 ± 22.00		9.52 ± 6.87		83.99 ± 45.57		4.86 ± 3.06		11.08 ± 7.68		10.48 ± 5.87	
CYP2C9*1/*1	10.82 ± 6.34		44.86 ± 19.17		10.46 ± 5.79		69.59 ± 44.42		5.39 ± 3.36		10.70 ± 9.50		8.50 ± 7.19	
CYP2C9*1/slow	10.67 ± 5.10	p = 0.715	41.87 ± 16.43	p = 0.055	10.58 ± 8.60	p = 0.568	73.61± 38.49	p = 0.123	4.84 ± 2.68	p = 0.011	10.84 ± 8.69	p = 0.264	10.20 ± 8.10	p = 0.101
CYP2C9 slow/slow	11.42 ± 5.02		35.48± 17.12		13.655 ± 16.22		63.33± 43.33		3.34 ± 1.66		7.94 ± 6.75		7.38 ± 6.12	
CYP2C19*1/*1	10.32 ± 5.67		43.06 ± 18.74		10.89 ± 6.69		74.21 ±46.80		5.35 ± 3.33		11.96 ± 8.06		8.78 ± 6.50	
CYP2C19*1/slow	11.55 ± 6.21	p = 0.056	46.28 ± 18.61	p = 0.317	10.46 ± 5.87	p = 0.474	65.40 ± 35.57	p = 0.132	4.80 ± 2.70	p = 0.048	8.05 ± 5.81	p = 0.001	8.50 ± 7.80	p = 0.785
CYP2C19 slow/slow	10.01 ± 0.15		37.10 ± 15.11		6.97 ± 5.76		39.72 ± 9.53		2.64 ± 1.07		3.33 ± 0.81		9.82 ± 7.28	
CYP2C19*1/rapid	10.81 ± 6.39		42.89 ± 17.83		10.54 ± 10.32		66.75 ± 39.58		5.20 ± 3.10		10.54 ± 9.32		9.80 ± 9.31	
CYP2C19 rapid/rapid	11.90 ± 3.88	p = 0.233	36.18 ± 12.73	p = 0.332	9.71 ± 5.62	p = 0.261	69.46 ± 37.98	p = 0.456	3.34 ± 1.76	p = 0.038	7.65 ± 4.72	p = 0.164	8.99 ± 6.45	p = 0.938
NAT2*4/*4	6.71 ± 3.02		48.80 ± 20.38		9.91 ± 4.37		123.90 ± 30.52		8.18 ± 3.30		22.13 ± 7.87		16.02 ± 10.12	
NAT2*4/slow	8.87 ± 4.78	p = 0.001	42.14 ± 18.44	p = 0.335	11.36 ± 7.32	p = 0.558	91.66 ± 46.26	p = 0.001	6.28 ± 3.74	p = 0.001	15.81 ± 9.92	p = 0.001	10.85 ± 7.87	p = 0.001
NAT2 slow/slow	12.82 ± 6.15		43.56 ± 17.81		10.22 ± 8.12		47.050 ± 19.29		3.77 ± 1.45		5.02 ± 2.02		6.64 ± 5.54	

CYP2C8 slow include *CYP2C8**3 and *4; *CYP2C9* slow include *CYP2C9**2 and *3; *CYP2C19* slow include *CYP2C19**2 and *3; *CYP2C19* rapid include *CYP2C19**17; *NAT2* slow include *NAT2**5, *6, *7 and *14.

Table 4. Effect of genetic factors on araquidonate metabolites recoveries.

	ARA-NMA (mg)	Intergroup comparison values	ARA-MA (mg)	Intergroup comparison values
CYP2C8*1/*1 n = 36	0.75 ± 1.34	reference	1.06 ± 2.26	reference
CYP2C8*1/ slow n = 10	0.79 ± 1.01	p = 0.938	1.66 ± 2.82	p = 0.271
CYP2C8 slow/ slow n = 8	0.15 ± 0.09	p = 0.086	0.75 ± 0.06	p = 0.108
CYP2C9*1/*1 n = 37	0.81 ± 1.34	reference	1.29 ± 2.57	reference
CYP2C9*1/ slow n = 10	0.24 ± 0.13	p = 0.040	0.33 ± 0.41	p = 0.091
CYP2C9 slow/ slow n = 8	0.59 ± 1.03	p = 0.706	0.66 ± 1.39	p = 0.434
CYP2C19*1/*1 n = 21	0.94 ± 1.65	reference	1.69 ± 3.34	reference
CYP2C19*1/ slow n = 6	1.16 ± 0.94	p = 0.533	1.32 ± 0.94	p = 0.164
CYP2C19 slow/slow n = 3	0.26 ± 0.10	p = 0.208	0.27 ± 0.07	p = 0.154
CYP2C19*1/ rapid n = 14	0.50 ± 0.86	p = 0.123	0.67 ± 1.04	p = 0.028
CYP2C19 slow/rapid n = 2	0.32 ± 0.21	p = 0.340	0.30 ± 0.02	p = 0.234
CYP2C19 rapid/rapid n = 9	0.24 ± 0.14	p = 0.037	0.25 ± 0.18	p = 0.020
NAT2*4/*4 n=8	0.12 ± 0.08	reference	0.10 ± 0.06	reference
NAT2*4/slow n = 17	0.46 ± 0.69	p = 0.075	0.55 ± 0.77	p = 0.022
NAT2 slow/slow n = 30	0.94 ± 1.45	p= 0.045	1.54 ± 2.83	p = 0.039

CYP2C8 slow include *CYP2C8**3 and *4; *CYP2C9* slow include *CYP2C9**2 and *3; *CYP2C19* slow include *CYP2C19**2 and *3; *CYP2C19* rapid include *CYP2C19**17; *NAT2* slow include *NAT2**5, *6, *7 and *14.

Supplemental table S1. Genotyping assays used in this study.

Gene	Allele signature	rs number	chromosomal location	Functional effect	Assay identification
CYP2C8	*CYP2C8*3*	rs11572080	10:96827030	Decreased metabolism	C__25625794_10
	*CYP2C8*4*	rs1058930	10:96818119	Decreased metabolism	C__25761568_20
CYP2C9	*CYP2C9*2*	rs1799853	10:96702047	Decreased metabolism	C__25625805_10
	*CYP2C9*3*	rs1057910	10:96741053	Decreased metabolism	C__27104892_10
CYP2C19	*CYP2C19*2*	rs4244285	10:96541616	Decreased metabolism	C__25986767_70
	*CYP2C19*3*	rs4986893	10:96540410	Decreased metabolism	C__27861809_10
	*CYP2C19*17*	rs12248560	10:96521657	Increased expression	C____469857_10
NAT2	*NAT2*5*	rs1801280	8:18257854	Decreased metabolism	C___1204093_20
	*NAT2*6*	rs1799930	8:18258103	Decreased metabolism	C___1204091_10
	*NAT2*7*	rs1799931	8:18258370	Decreased metabolism	C____572770_20
	*NAT2*14*	rs1801279	8:18257704	Decreased metabolism	C____572771_10

Supplemental table S2. Normality test for the phenotypic characteristics analyzed in this study:

Characteristic	Z (Kolmogorov-Smirnov)	P value
Age	5.72	<0.001
Weight	2.50	<0.001
Height	1.81	=0.003
Body mass index	1.41	=0.038
Urine volume (24 hours)	2.34	<0.001
Drinks per week	5.98	<0.001
Smoker/Non smoker	9.56	<0.001
FAA (mg)	0.76	=0.618
AAA (mg)	2.71	<0.001
AA (mg)	2.24	<0.001
MAA (mg)	1.77	=0.004
ARA-NMA (mg)	2.32	<0.001
ARA-MA (mg)	2.40	<0.001
Recovery (percent of dose)	1.17	=0.127
Formylation ratio 1 (FAA/MAA)	2.73	<0.001
Formylation ratio 2 (FAA/(MAA+AA+AAA))	1.55	=0.017
Demethylation ratio 1 ((AA+AAA)/MAA))	3.65	<0.001
Demethylation ratio 2 ((AA+AAA)/(MAA+FAA))	2.48	<0.001
Acetylation ratio 1 (AAA/AA)	2.87	<0.001
Acetylation ratio 2 ((AAA/(AA+MAA+FAA)	2.60	<0.001

Supplemental table S3: Association between non-genetic factors.

	Age	Weight	Height	Body Mass Index	Urine volume	Drinker (yes/no)	Drinks per week	Smoker (yes/no)	Cigarettes per day
Gender (men/women)	p = 0.005	p < 0.001	p < 0.001	p <0.001	p = 0.001	p < 0.001	p < 0.001	p= 0.470	p = 0.720
Age		p = 0.001	p = 0.001	p = 0.041	p = 0.519	p = 0.467	p = 0.194	p = 0.235	p = 0.588
Weight			p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p = 0.401	p = 0.958
Height				p < 0.001	p < 0.001	p < 0.001	p < 0.001	p = 0.252	p = 0.731
Body Mass Index					p < 0.001	p = 0.013	p = 0.004	p = 0.774	p = 0.912
Urine volume						p = 0.053	p = 0.032	p = 0.011	p = 0.064
Drinker (yes/no)							p < 0.001	p < 0.001	p < 0.001
Drinks per week								p < 0.001	p < 0.001
Smoker (yes/no)									p < 0.001

The significance values for bilateral Spearman correlations are shown.

Supplemental table S4: *NAT2* SNPs and inferred acetylation phenotype by gender.

SNP identifier	Men, No. (%; 95 % CI)	Women, No. (%; 95 % CI)	Intergroup comparison values
rs1801280 (*NAT2*5*)			
T/T	45 (34.4; 26.2-42.5)	85 (36.8; 30.6-43.0)	Chi-square = 2.88; p = 0.238
T/C	70 (53.4; 44.9-62.0)	105 (45.5; 39.0-51.9)	
C/C	16 (12.2; 6.6-17.8)	41 (17.7; 12.8-22.7)	
rs1799930 (*NAT2*6*)			
G/G	55 (42.0; 33.5-50.4)	103 (44.6; 38.2-51.0)	Chi-square = 0.24; p = 0.888
G/A	62 (47.3; 38.8-55.9)	105 (45.5; 39.0-51.9)	
A/A	14 (10.7; 5.4-16.0)	23 (10.0; 6.1-13.8)	
rs1799931 (*NAT2*7*)			
G/G	126 (96.2; 92.9-99.5)	220 (95.2; 92.5-98.0)	Chi-square = 0.18; p = 0.674
G/A	5 (3.8; 0.5-7.1)	11 (4.8; 2.0-7.5)	
A/A	0 (0.0; 0.0-0.0)	0 (0.0; 0.0-0.0)	
Inferred acetylation phenotye			
Rapid	9 (6.9; 2.5-11.2)	18 (7.8; 4.3-11.2)	Chi-square = 0.14; p = 0.932
Intermediate	54 (41.2; 32.8-49.7)	92 (39.8; 33.5-46.1)	
Slow	68 (51.9; 43.4-60.5)	121 (52.4; 45.9-58.8)	

Supplemental Table S5. SNP frequencies observed in the present study.

SNP identifier	No. (%; 95 % CI)	Hardy-Weinberg's P (Pearson)
rs11572080 (*CYP2C8*3*)		
C/C	269 (74.3; 69.8-78.8)	
C/T	84 (23.2; 18.9-27.6)	p = 0.431
T/T	9 (2.5; 0.9-4.1)	
rs1058930 (*CYP2C8*4*)		
G/G	324 (89.5; 86.3-92.7)	
G/C	35 (9.7; 6.6-12.7)	p = 0.070
C/C	3 (0.8; 0.-1-1.8)	
rs1799853 (*CYP2C9*2*)		
C/C	237 (65.5; 60.6-70.4)	
C/T	111 (30.7; 25.9-35.4)	p = 0.824
T/T	14 (3.9; 1.9-5.9)	
rs1057910 (*CYP2C9*3*)		
A/A	302 (83.4; 79.6-87.3)	
A/C	58 (16.0; 12.2-19.8)	p = 0.660
C/C	2 (0.6; 0.-2-1.3)	
rs4244285 (*CYP2C19*2*)		
G/G	274 (75.7; 71.3-80.1)	
G/A	81 (22.4; 18.1-26.7)	p = 0.724
A/A	7 (1.9; 0.5-3.4)	
rs4986893 (*CYP2C19*3*)		
G/G	362 (100.0; 100.0-100.0)	
G/A	0 (0.0; 0.0-0.0)	--
A/A	0 (0.0; 0.0-0.0)	
rs12248560 (*CYP2C19*17*)		

C/C	232 (64.1; 59.1-69.0)	
C/T	116 (32.0; 27.2-36.9)	p = 0.916
T/T	14 (3.9; 1.9-5.9)	
rs1801280 (*NAT2*5*)		
T/T	130 (35.9; 31.0-40.9)	
T/C	175 (48.3; 43.2-53.5)	p = 0.881
C/C	57 (15.7; 12.0-19.5)	
rs1799930 (*NAT2*6*)		
G/G	158 (43.6; 38.5-48.8)	
G/A	167 (46.1; 41.0-51.3)	p = 0.462
A/A	37 (10.2; 7.1-13.3)	
rs1799931 (*NAT2*7*)		
G/G	346 (95.6; 93.5-97.7)	
G/A	16 (4.4; 2.3-6.5)	p = 0.667
A/A	0 (0.0; 0.0-0.0)	
rs1801279 (*NAT2*14*)		
G/G	362 (100.0; 100.0-100.0)	
G/A	0 (0.0; 0.0-0.0)	--
A/A	0 (0.0; 0.0-0.0)	

Supplemental table S6: Effect of SNPs on major metamizole metabolite recoveries and metabolic ratios.

	MAA (mg)	Intergroup comparison values	FAA (mg)	Intergroup comparison values	AA (mg)	Intergroup comparison values	AAA (mg)	Intergroup comparison values	Formylation ratio (FAA/MAA)	Intergroup comparison values	Demethylation ratio ((AA+AAA)/MAA))	Intergroup comparison values	Acetylation ratio (AAA/AA)	Intergroup comparison values
CYP2C8*3 rs11572080 C/C	11.11 ± 6.31		44.02 ± 19.00		10.90 ± 7.72		69.87 ± 43.92		5.12 ± 3.07		10.49 ± 8.99		8.48 ± 7.10	
CYP2C8*3 rs1157208 C/T	10.07 ± 4.48	p = 0.576	42.51 ± 15.61	p = 0.163	9.95 ± 7.13	p = 0.104	71.45 ± 36.37	p = 0.618	5.15 ± 3.29	p = 0.618	10.89 ± 9.70	p = 0.453	10.50 ± 8.56	p = 0.049
CYP2C8*3 rs1157208 T/T	8.96 ± 3.56		33.74 ± 19.52		9.71 ± 8.43		86.68 ± 50.11		4.32 ± 2.90		11.91 ± 8.6		11.57 ± 6.99	
CYP2C8*4 rs1058930 G/G	10.62 ± 5.77		42.88 ± 18.58		10.59 ± 7.85		71.22 ± 43.62		5.10 ± 3.10		10.81 ± 9.25		9.25 ± 7.70	
CYP2C8*4 rs1058930 G/C	12.52 ± 6.85	p = 0.440	45.88 ± 13.72	p = 0.016	11.14 ± 4.95	p = 0.270	61.71 ± 26.58	p = 0.203	4.93 ± 3.25	p = 0.163	8.50 ± 7.71	p = 0.062	6.76 ± 5.05	p = 0.253
CYP2C8*4 rs1058930 C/C	9.66 ± 12.52		71.09 ± 1.33		11.56 ± 0.91		104.00 ± 32.37		7.98 ± 2.52		13.62 ± 6.80		9.17 ± 3.37	
CYP2C9*2 rs1799853 C/C	10.85 ± 6.25		43.70 ± 19.01		10.68 ± 7.72		69.31 ± 43.79		5.22 ± 3.22		10.66 ± 9.23		8.64 ± 7.08	
CYP2C9*2 rs1799853 C/T	10.81 ± 4.61	p = 0.439	43.48± 15.65	p = 0.131	10.60 ± 7.06	p = 0.619	74.17 ± 36.14	p = 0.436	4.81 ± 2.77	p = 0.391	10.36 ± 8.83	p = 0.699	10.16 ± 8.71	p = 0.347
CYP2C9*2 rs1799853 T/T	8.84 ± 3.96		30.92 ± 15.35		10.24 ± 9.66		82.82 ± 55.24		4.02 ± 2.41		11.74 ± 8.93		11.25 ± 7.84	
CYP2C9*3 rs1057910 A/A	10.68 ± 5.88		44.17 ± 18.39		10.51 ± 6.28		71.57 ± 42.85		5.26 ± 3.22		10.79 ± 9.36		9.09 ± 7.72	
CYP2C9*3 rs1057910 A/C	11.54 ± 5.94	p = 0.550	40.26 ± 17.07	p = 0.040	11.34 ± 9.56	p = 0.336	67.05 ± 40.33	p = 0.529	4.41 ± 2.31	p = 0.027	9.80 ± 7.93	p = 0.899	8.96 ± 6.29	p = 0.055
CYP2C9*3 rs1057910 C/C	8.96		16.30		40.88		38.09		1.83		7.87		1.28	
CYP2C19*2 rs4244285 G/G	10.51 ± 5.83		42.70 ± 18.20		10.72 ± 8.12		71.29± 44.07		5.22 ± 3.20		11.28 ± 9.80		9.13 ± 7.62	
CYP2C19*2	11.14 ±	p = 0.047	46.13 ±	p = 0.270	10.72 ±	p = 0.314	70.65 ±	p = 0.167	4.90 ± 2.77	p = 0.060	8.93 ± 6.15	p = 0.006	8.61 ± 7.23	p = 0.936

Genotype														
rs4244285 G/A	6.03		18.76		5.65		37.36							
CYP2C19*2 rs4244285 A/A	14.08 ± 0.15		37.10 ± 15.11		6.97 ± 10.72		39.72 ± 9.53		2.64 ± 1.07		3.32 ± 0.81		9.82 ± 7.28	
CYP2C19*17 rs12248560C/C	10.67 ± 5.77		43.86 ± 18.72		10.70 ± 6.47		71.19 ± 44.01		5.17 ± 3.16		10.77 ± 9.41		8.72 ± 6.87	
CYP2C19*17 rs12248560C/T	10.89 ± 6.31	p = 0.231	43.57 ± 17.87	p = 0.148	10.69 ± 9.69	p = 0.485	69.93 ± 40.10	p = 0.930	5.22 ± 3.08	p = 0.018	10.74 ± 8.95	p = 0.510	9.68 ± 8.76	p = 0.745
CYP2C19*17 rs12248560T/T	12.13 ± 3.83		34.54 ± 13.70		9.64 ± 5.40		70.60 ± 36.73		3.17 ± 1.81		7.55 ± 4.55		9.05 ± 6.21	
NAT2*5 rs1801280 T/T	9.49 ± 5.37		45.45 ± 18.31		11.01 ± 6.51		85.82 ± 50.09		6.17 ± 3.62		14.79 ± 10.73		10.29 ± 8.05	
NAT2*5 rs1801280 T/C	11.23 ± 5.76	p = 0.003	42.89 ± 17.45	p = 0.306	10.40 ± 5.94	p = 0.181	66.83 ± 36.50	p < 0.001	4.74 ± 2.87	p < 0.001	9.21 ± 7.91	p < 0.001	8.84 ± 7.82	p = 0.019
NAT2*5 rs1801280 C/C	12.41 ± 6.77		40.50 ± 20.41		10.60 ± 12.69		48.36 ± 24.50		3.81 ± 1.43		5.50 ± 0.15		6.77 ± 3.90	
NAT2*6 rs1799930 G/G	10.00 ± 5.93		42.43 ± 20.06		10.70 ± 9.36		80.76 ± 43.48		5.58 ± 3.34		12.63 ± 9.53		10.39 ± 8.22	
NAT2*6 rs1799930 G//A	10.88 ± 5.75	p < 0.001	43.13 ± 16.21	p = 0.114	10.51 ± 6.11	p = 0.369	67.09 ± 42.06	p < 0.001	4.97 ± 3.03	p = 0.010	10.18 ± 9.05	p < 0.001	8.65 ± 7.20	p < 0.001
NAT2*6 rs1799930 A/A	13.48 ± 5.67		48.42 ± 19.08		10.88 ± 5.55		47.52 ± 26.65		3.92 ± 2.04		4.93 ± 3.87		5.60 ± 3.83	
NAT2*7 rs1799931 G/G	10.79 ± 6.17		43.27 ± 18.75		10.26 ± 7.61		70.42 ± 42.85		5.19 ± 3.20		10.79 ± 9.26		9.37 ± 7.70	
NAT2*7 rs1799931 G/A	12.15 ± 2.54	p = 0.049	50.91 ± 10.72	p = 0.062	11.44 ± 2.84	p = 0.093	59.62 ± 31.62	p = 0.421	4.38 ± 1.40	p = 0.761	6.03 ± 0.50	p = 0.087	5.76 ± 3.60	p = 0.026

Supplemental Figure S7: Scheme and linkage analysis of the *CYP2C* SNPs analyzed in this study.
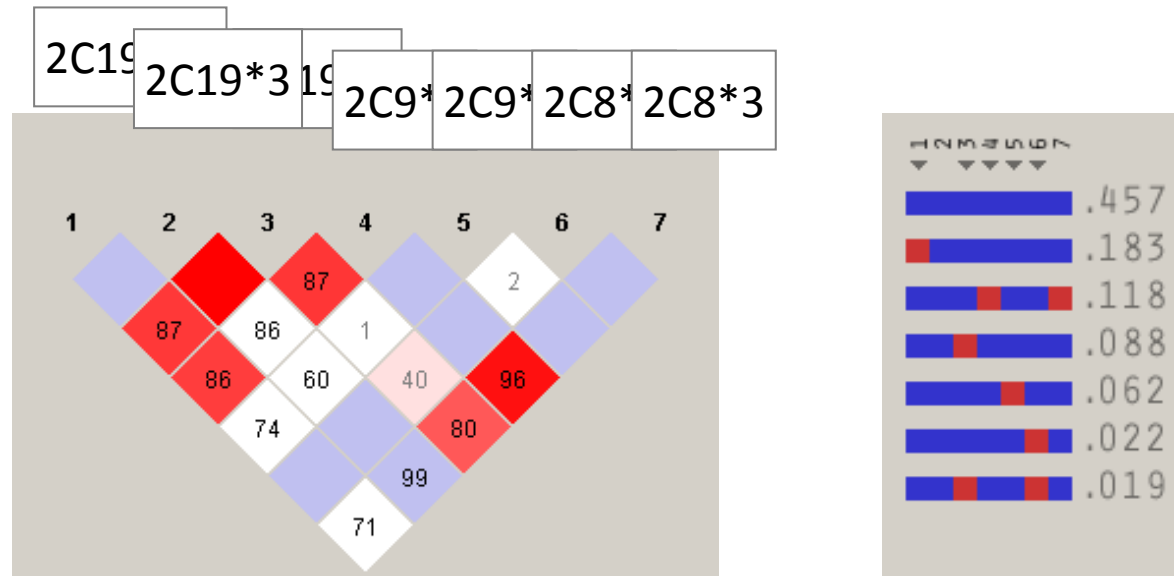
The linkage figure (left) and the haplotype figure (right) were composed with Haploview Ver. 4.1. The linkage figure was performed according to the standard colour scheme (D'/LOD), and the D' values (x 100) are shown when relevant. The haplotype frequencies (right) indicate that the commonest *CYP2C* haplotype was the haplotype containing no variant alleles.

Supplemental Table S8: Effect of *CYP2C* haplotypes on metamizole metabolite recoveries and metabolic ratios.

	MAA (mg)	Intergroup comparison values	FAA (mg)	Intergroup comparison values	AA (mg)	Intergroup comparison values	AAA (mg)	Intergroup comparison values	Formylation ratio (FAA/MAA)	Intergroup comparison values	Demethylation ratio ((AA+AAA)/MAA))	Intergroup comparison values	Acetylation ratio (AAA/AA)	Intergroup comparison values
Non mutated homozygous n= 65	10.41 ± 6.33		45.59 ± 20.52		11.81 ± 6.39		74.71 ± 52.48		5.65 ± 3.33		12.57 ± 11.01		7.80 ± 6.59	
Non mutated / mutated n = 173	10.67 ± 6.30	p = 0.146	43.45 ± 18.71	p = 0.538	9.99 ± 6.34	p = 0.089	69.34 ± 42.87	p = 0.677	5.32 ± 3.27	p = 0.016	10.76 ± 9.55	p = 0.174	9.29 ± 7.74	p = 0.260
Mutated Homozygous n = 124	11.19 ± 5.00		42.20 ± 16.44		10.97 ± 9.54		70.37 ± 35.43		4.51 ± 2.66		9.39 ± 7.01		9.31 ± 7.60	

Supplemental table S9: Analysis of the differential effect of *NAT2*5* and *NAT2*6* alleles in the recoveries of MAA, AAA and the acetylation ratio.

Genotype	Number of individuals	MAA (mg)	Comparison (Mann-Whitney)	AAA (mg)	Comparison (Mann-Whitney)	Acetylation ratio AAA/AA	Comparison (Mann-Whitney)
NAT2*4/*4	27	6.71 ± 3.02	reference value	123.90 ± 30.52	reference value	16.02 ± 10.12	reference value
NAT2*4/*5	78	9.32 ± 5.04	p = 0.311	91.22 ± 38.91	p = 0.173	10.66 ± 8.55	p = 0.238
NAT2*4/*6	69	8.34 ± 4.43		101.09 ± 46.82		11.07 ± 7.05	
NAT2*5/*5	57	12.49 ± 6.81	p = 0.155	49.70 ± 23.13	p = 0.034	6.83 ± 3.91	p = 0.004
NAT2*6/*6	37	13.91 ± 5.66		40.88 ± 12.61		5.19 ± 3.68	

The differential effect of the slow alleles *NAT2*5* and *NAT2*6* on the recoveries of MAA and AAA, as well as the effect in the acetylation ratio was analyzed. The basal values for carriers of *NAT2*4* (rapid alleles) in homozygosity are shown for reference. The comparison of the metabolite recoveries and the acetylation ratio between carriers of *NAT2*5* and *NAT2*6* in heterozygosity with *NAT2*4* revealed non-significant differences in the effect of *NAT2*5* and *NAT2*6*. However, when no *NAT2*4* alleles were present, significant differences between homozygous carriers of *NAT2*5* and homozygous carriers of *NAT2*6* are observed for the recovery of AAA and for the acetylation ratio. *NAT2*6* is associated to a higher impairment in the acetylation capacity as compared to *NAT2*5*, which is in agreement with previous findings obtained with a different NAT2 substrate [33].

Supplemental table S10: Association between araquidonate metabolites and major metabolite recoveries and metabolic ratios in 55 healthy subjects.
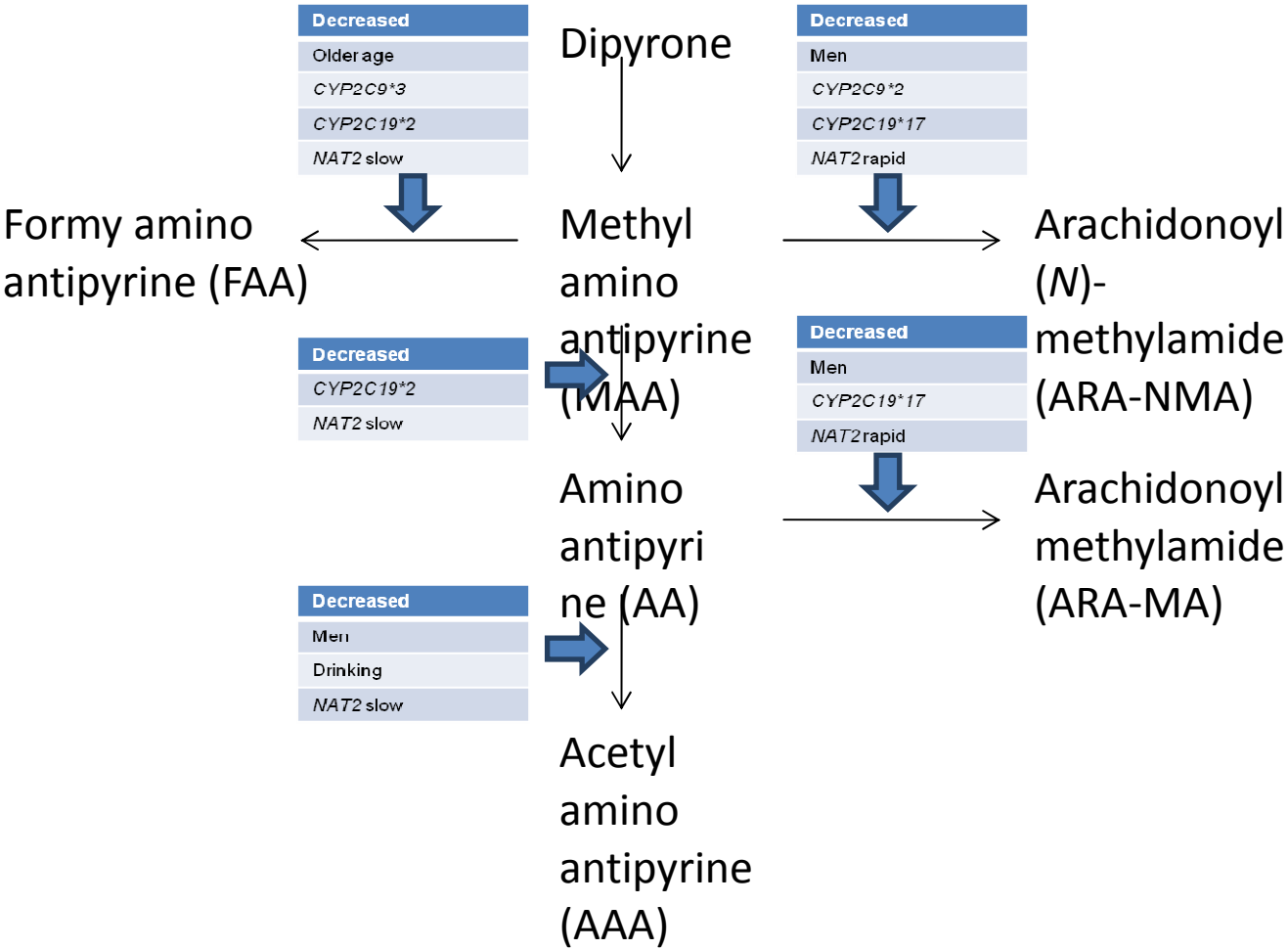
	ARA-MA (mg)	ARA-NMA+ARA-MA (mg)	MAA (mg)	FAA (mg)	AA (mg)	AAA (mg)	Formylation ratio (FAA/MAA)	Demethylation ratio ((AA+AAA)/MAA))	Acetylation ratio (AAA/AA)
ARA-NMA (mg)	p < 0.001	p < 0.001	p < 0.001	p = 0.075	p = 0.398	p = 0.212	p = 0.002	p < 0.001	p = 0.028
ARA-MA (mg)		p < 0.001	p < 0.001	p = 0.014	p = 0.464	p = 0.242	p = 0.001	p < 0.001	p = 0.053
ARA-NMA+ARA-MA (mg)			p < 0.001	p = 0.026	p = 0.451	p = 0.201	p = 0.001	p < 0.001	p = 0.038

The significance values for bilateral Spearman correlations are shown.

Supplemental Table S11. Effect of non-genetic factors on araquidonate metabolites recoveries

	ARA-NMA (mg)	Intergroup comparison values	ARA-MA (mg)	Intergroup comparison values
Men (n= 7)	0.49 ± 0.56	p= 0.382	0.74 ± 0.99	p = 0.515
Women (n = 48)	0.70 ± 1.24		1.07 ± 2.33	
Age		p= 0.134		p = 0.263
Weight		p = 0.805		p = 0.705
Height		p = 0.090		p = 0.452
Body mass index		p = 0.247		p = 0.450
Urine volume (ml)		p = 0.107		p = 0.971
Drinkers	0.69 ± 0.93	p = 0.039	0.93 ± 1.35	p = 0.001
Non-drinkers	0.40 ± 0.56		0.43 ± 0.51	
Drinks per week		p = 0.414		p = 0.325
Smokers	0.85 ± 1.15	p = 0.021	1.30 ± 1.73	p = 0.093
Non-smokers	0.51 ± 0.69		0.72 ± 1.48	
Cigarettes per day		p= 0.978		p = 0.564

Supplemental Figure S12: Summary of the effects observed on the metabolic profiles of metamizole.

Decreased
Older age
CYP2C9*3
CYP2C19*2
NAT2 slow

Dipyrone

Decreased
Men
CYP2C9*2
CYP2C19*17
NAT2 rapid

Formy amino antipyrine (FAA)

Methyl amino antipyrine (MAA)

Arachidonoyl (*N*)-methylamide (ARA-NMA)

Decreased
CYP2C19*2
NAT2 slow

Decreased
Men
CYP2C19*17
NAT2 rapid

Amino antipyrine (AA)

Arachidonoyl methylamide (ARA-MA)

Decreased
Men
Drinking
NAT2 slow

Acetyl amino antipyrine (AAA)

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