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Paraxanthine/caffeine concentration ratios in hair: an alternative for plasma-based phenotyping of cytochrome P450 1A2?

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Abstract

Background and Objective Although metabolite-to-parent drug concentration ratios in hair have been suggested as a possible tool to study the metabolism of drugs in a non-invasive way, no studies are available that evaluate this in a systematic way. Cytochrome P450 (CYP) 1A2 is a drug metabolizing enzyme characterized by large inter-individual differences in its activity. The standard approach for CYP1A2 phenotyping is to determine the paraxanthine/caffeine ratio in plasma, at a fixed time point after intake of a dose of the CYP1A2 substrate caffeine. The aim of this study was to evaluate whether paraxanthine/caffeine ratios measured in hair samples reflect the plasma-based CYP1A2 phenotype.

Methods Caffeine and paraxanthine concentrations were measured in proximal 3-cm segments of hair samples from 60 healthy volunteers and resulting paraxanthine/caffeine ratios were correlated with CYP1A2 phenotyping indices in plasma.

Results Paraxanthine/caffeine ratios in hair ranged from 0.12 to 0.93 (median 0.41), corresponding ratios in plasma ranged from 0.09 to 0.95 (median 0.40). A statistically significant correlation was found between ratios in hair and plasma ($r = 0.41$, $p = 0.0011$). However, large deviations between ratios in both matrices were found in individual cases. Although the influence of several factors on paraxanthine/caffeine ratios and hair-plasma deviations was investigated, no evident factors explaining the observed variability could be identified.

Conclusion The variability between hair and plasma ratios complicates the interpretation of hair ratios on an individual basis and, therefore, compromises their practical usefulness as alternative CYP1A2 phenotyping metrics.

Key Points

- For the first time, the usefulness of paraxanthine/caffeine molar concentrations ratios in hair for CYP1A2 phenotyping was evaluated by comparing hair ratios from 60 healthy volunteers with reference, plasma-based CYP1A2 phenotyping indices .
- Although ratios in hair and plasma showed a statistically significant correlation, large deviations were found in individual cases. Investigating the influence of several factors did not provide a clear explanation for the observed deviations, complicating the interpretation of hair ratios on an individual basis.

1 Introduction

Sampling and analysis of hair has earned an established place in bioanalysis, with applications in diverse fields, including, amongst others, postmortem toxicology, workplace drug testing, doping control, driving ability examination and detection of pre- and postnatal drug exposure. Owing to the ability to retrospectively determine drug use over a time window of several months (or even years), together with the long-term stability of many drugs in hair and the non-invasive sample collection, hair strands are collected as an alternative for, or in combination with, traditional blood or urine samples. On the other hand, important limitations associated with hair analysis are the lack of a clear-cut correlation between hair and plasma concentrations -the latter reflecting drug dose- for many substances and the risk of misinterpreting hair results due to external contamination [1,2].

In addition to the determination of parent drugs, highly sensitive analytical techniques, such as liquid chromatography (LC)- or gas chromatography (GC)-tandem mass spectrometry (MS/MS), also allow to quantity specific drug metabolites in hair, which are often present at low concentration levels. Resulting metabolite-to-parent drug concentration ratios have been applied to distinguish active ingestion of a drug from external contamination or passive exposure. For this purpose, cut-off values have been proposed for several drugs, such as cocaine, heroin and tramadol [3,4]. Apart from the latter application, the use of metabolite-to-parent drug concentration ratios in hair has recently been proposed as a possible means to assess the metabolic phenotype of drug metabolizing enzymes, as reviewed by De Kesel *et al*. [5]. Thieme *et al.* found that nortriptyline/amitriptyline concentration ratios measured in hair samples of children (n = 23) correlated significantly with the number of functional alleles of CYP2C19, the enzyme that is predominantly responsible for the demethylation of amitriptyline. Poor metabolizers showed significantly lower nortriptyline/amitriptyline ratios in hair compared to intermediate or rapid extensive metabolizers [6]. Eisenhut *et al.* determined concentrations of the anti-tuberculosis drug isoniazide and its metabolite acetylisoniazide in hair samples (n = 24) and correlated the corresponding metabolite-to-parent drug concentration ratios to the arylamine *N*-acetyltransferase-2 (*NAT-2*) genotype. Acetylisoniazide/isoniazide ratios in hair samples of slow acetylators were significantly lower than ratios in subjects possessing rapid acetylator genotypes [7]. In a recent study, LeMasters *et al.* found higher cotinine levels in hair of secondhand smoke exposed children carrying the *NAT-1* minor allele compared to children with the major allele [8]. Several groups also reported on the use of metabolite-to-parent drug ratios to (retrospectively) detect changes in drug metabolism within individuals during pregnancy. Koren *et al.* found higher cotinine/nicotine ratios in hair segments corresponding to the third trimester of

pregnancy than in segments corresponding to the first trimester, as determined in hair samples of 74 pregnant women. According to the authors, these results suggest an increased CYP2A6-mediated nicotine metabolism during pregnancy [9]. Similar findings for the antidepressant citalopram and its metabolite norcitalopram, determined in hair samples of 4 subjects, were described by O'Brien *et al.* [10]. Finally, by analyzing proximal hair segments from 29 women involved in a methadone-assisted therapy at different time points during pregnancy, Himes *et al.* found a positive correlation between 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)/methadone ratios and cumulative methadone dose. Also here, the authors suggested that these findings could indicate an altered metabolism during gestation [11]. Although the above mentioned results seem very promising, it should be noted that in the latter 3 studies no control samples from non-pregnant women or reference phenotyping indices determined in non-hair matrices were included to support the validity of hair ratios in reflecting changes in drug metabolism.

The aim of this study was to evaluate the usefulness of determining paraxanthine/caffeine concentration ratios in hair for CYP1A2 phenotyping by comparing hair ratios of healthy volunteers with their reference, plasma-based CYP1A2 phenotyping indices. CYP1A2, a member of the CYP family of drug metabolizing enzymes, plays a role in approximately 9 % of drug metabolism pathways in which CYP isoforms are involved [12]. Several therapeutically used drugs are metabolized by CYP1A2, including olanzapine [13], clozapine [14], zolmitriptan [15] and warfarin [16]. CYP1A2 activity is affected by a combination of genetic, nongenetic and environmental factors [12] and, consequently, its activity displays a high inter-individual variability [17]. Therefore, a phenotyping approach involving controlled administration of a selective probe drug is preferred to assess CYP1A2 activity. As caffeine is predominantly metabolized by CYP1A2, it is the probe drug of choice [17,18]. In typical phenotyping approaches the paraxanthine/caffeine concentration ratio is determined 4-6 h post-administration in serum or plasma samples [19], although dried blood spots [20] and saliva [21] have proven to be appropriate matrices for this purpose as well. A CYP1A2 phenotyping procedure based on hair analysis would eliminate the need to administer a caffeine test dose, since it is expected that caffeine and paraxanthine will readily be detected in hair samples of a large part of the population given the widespread consumption of caffeine-containing beverages (e.g. coffee, tea, soft drinks) and food products (e.g. chocolate). Our study is the first to compare hair ratios with plasma ratios for phenotyping purposes. The comparison between paraxanthine/caffeine ratios in hair and those obtained using a classical plasma-based approach may provide valuable insights into the usefulness and potential of hair analysis for this purpose. This could e.g. be relevant in fatal cases where it might be possible to deduce a phenotype postmortem by analyzing hair.

2 Methods

2.1 CYP1A2 phenotyping study

A CYP1A2 phenotyping study involving healthy volunteers was approved by the Ethics Committee of Ghent University Hospital (B670201111655). Scalp hair samples were collected at the posterior vertex region after obtaining written informed consent from each participant. Caffeine and paraxanthine concentrations were determined in proximal 3-cm segments and the corresponding paraxanthine/caffeine molar concentration ratios were calculated. For 4 volunteers, a segmental analysis of 6 consecutive 3-cm hair segments was performed as well. At the day of hair sampling, a standard CYP1A2 phenotyping procedure was conducted in the same study population, as described elsewhere [20]. Briefly, caffeine and paraxanthine concentrations were determined with a validated method in plasma samples from volunteers that had taken a 150 mg caffeine capsule 6 hours (± 5 minutes) before sample collection [20,22]. By means of a written questionnaire, data on sex, age, general health status, smoking habits, caffeine intake, alcohol consumption, intake of drugs (including hormonal contraception) and cosmetic hair colouring were obtained. Caffeine intake was defined as the number of caffeine-containing drinks (including coffee, tea, soft and energy drinks) per day, while alcohol use was defined as the number of alcohol-containing drinks per week. Volunteers were asked to report chronic and occasional medication use at the time of inclusion and in the past without defining a time frame. A list of substrates, inhibitors or inducers of CYP1A2 was not available. Hair color was assessed by visual inspection of the collected samples in the laboratory.

2.2 Hair sample collection, pretreatment and analysis

Hair locks were cut as close as possible to the scalp at the posterior vertex region of the head and stored in aluminum foil envelopes at room temperature until analysis. Using adhesive tape, hair samples were either segmented into 3-cm sections or only the proximal 3-cm segment was used. The resulting segments were decontaminated in methylene chloride (20 mL, 2 min), followed by two washes in water (20 mL, 2 min). Following each wash step, the wash solvents were removed and the hair was air-dried. Dried samples were cut into small snippets (< 5 mm) using scissors before being manually ground in a mortar with liquid N_2. One mL of a solution of protease type VIII (1.2 IU/mL) in Tris buffer (pH 7.5, 50 mmol/L), containing the internal standards caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$, was then added to 20 mg of ground hair. The samples were shaken for 1 hour at 37 °C and 750 rpm and centrifuged for 10 minutes at 10000 x *g.* Subsequently, the supernatants were cleaned-up by solid phase extraction (SPE) using Phenomenex Strata-X™ cartridges (200 mg, 3 mL). These were conditioned and equilibrated with 3 mL of methanol and 2 mL of water, respectively, prior to application of the hair extracts. The cartridges were washed with 2 mL of a water/methanol mixture (65/35, v/v) and dried for 2 minutes. The analytes were eluted from the columns with 4 × 500 µL of a

methanol/acetonitrile mixture (50/50, v/v). The obtained solutions were evaporated under a stream of N_2 and redissolved in 150 µL of the mobile phase. Data on the optimization of the hair extraction procedure are described elsewhere [De Kesel *et al.*, submitted].

To evaluate the effect of the analytical wash procedure, the second (i.e. 3-6 cm) segments of 10 samples from the data set were decontaminated and all wash solvents were subsequently analyzed. The samples originated from 5 individuals with the largest positive differences between hair and plasma ratios and 5 individuals with the largest negative differences (see further).

All analyses were performed on an LC-MS/MS configuration consisting of a Waters Acquity UPLC® system (Waters, Milford, MA, USA) and an AB SCIEX API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA), controlled by AB SCIEX Analyst 1.5.2 and Waters Acquity console software. Chromatographic conditions for the determination of caffeine and paraxanthine in hair were identical to those previously described for the analysis of dried blood spots, whole blood and plasma [22]. The same holds for the API 4000™ mass spectrometer parameters, except for some minor modifications [De Kesel *et al.*, submitted]. All compounds were detected in scheduled multiple reaction monitoring (sMRM) mode. The monitored precursor to product ion transitions were identical as previously described [22].

Validation of the hair analysis procedure was based on U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [23,24]. For both compounds, no carry-over or interferences were seen in the chromatogram. Linear calibration curves for caffeine and paraxanthine ranged from 20 to 500 pg/mg, with 20 pg/mg being the lower limit of quantification (LLOQ) for both compounds. Intra- and interbatch precision was below 12% (% relative standard deviation, %RSD) and accuracy below 7% (%bias) for all QCs (20, 60, 200 and 400 pg/mg). Matrix effects were determined at two concentrations levels using hair samples from five different individuals. Ion suppression was observed for caffeine (56.58 – 66.24 % absolute matrix effect) and paraxanthine (79.70 – 81.16 % absolute matrix effect). When compensation by the IS was taken into account, absolute matrix effects approximated 100 % and relative matrix effects were within 15 % limits. The same five hair samples were used to determine the recovery of the sample preparation procedure by spiking samples before and after extraction and clean-up. Recovery was high (> 87 %) and reproducible (%RSD < 15 %). Incurred and processed samples were stable under the evaluated conditions. Finally, it was shown that extracts of hair samples could be safely diluted 5-fold. Full details on the validation of the hair method are described elsewhere [De Kesel *et al.*, submitted].

2.3 Chemicals and stock solutions

Ultrapure water was obtained from a Synergy® Water Purification System (Merck Millipore, Overijse, Belgium). Methanol, acetonitrile and methylene chloride were purchased from Biosolve (Valkenswaard, The Netherlands). Caffeine, caffeine-$^{13}C_3$, paraxanthine, paraxanthine-$^{13}C_4$-$^{15}N_3$, theophylline, theobromine, formic acid, proteinase K, protease type VIII, protease type XIV, dithiothreitol and trishydroxymethylaminomethane (Tris) were all purchased from Sigma-Aldrich (Diegem, Belgium). HCl (1N) was obtained from Merck KGaA (Darmstadt, Germany) and liquid N_2 from Air Liquide (Brussels, Belgium). Stock solutions were prepared as described elsewhere [22].

2.3.1 Statistical analysis

Statistical evaluation and graphical representation of the data was performed using Microsoft Excel® 2010 (Microsoft, Redmond, WA, USA), IBM SPSS Statistics 20 (IBM Corp., Armonk, NY) and Medcalc 12.7.5 (MedCalc Software bvba, Ostend, Belgium). Normality of paraxanthine/caffeine ratios in plasma and hair was assessed by Kolmogorov-Smirnov test. Correlation was examined by Pearson coefficient of correlation. Differences between 2 groups were determined by independent samples t-tests, while one-way analysis of variances (ANOVA) and Tukey post-hoc tests were used when more than 2 groups were compared. Significance was defined as $p < 0.05$.

3 Results

Caffeine and paraxanthine concentrations in 6 consecutive 3-cm hair segments from 4 female, healthy volunteers overall decreased with increasing distance from the hair root (Figure 1 a-d). Corresponding paraxanthine/caffeine molar concentration ratios showed a considerable variability between the consecutive hair segments (Figure 1 e). Therefore, we decided to analyze proximal 3-cm hair segments in the actual comparative phenotyping study.

Paraxanthine was not detected in any of the analyzed wash solvents. For caffeine, responses below the LLOQ were seen in all wash solutions from all 10 participants of the phenotyping study. Although no real quantitative conclusions can be drawn from these data, peak areas were compared with the peak area of the LLOQ. The average peak area of caffeine was 54.08 ± 10.16 % of the LLOQ for the methylene chloride wash, 39.80 ± 11.44 % for the first water wash and 21.46 ± 6.10 % for the second water wash.

Sixty healthy volunteers were involved in the CYP1A2 phenotyping study, including 43 women and 4 smokers. The age of the participants ranged from 24 to 48 years (mean ± standard deviation (SD); 28.8 ± 5.7). Paraxanthine/caffeine molar concentration ratios in plasma and hair samples were normally distributed (Kolmogorov-Smirnov test; $p = 0.200$ and $p = 0.093$, respectively). The median

paraxanthine/caffeine ratios in both matrices, along with the measured ranges, are listed in Table 1. Pearson correlation analysis revealed a correlation coefficient of 0.410 for ratios in hair and plasma ($p = 0.001$). Although the correlation was found to be statistically significant, a wide scatter of the data was observed, with large deviations between corresponding ratios in hair and plasma in individual cases (Figure 2). To evaluate further into depth to what extent ratios in hair correlate with corresponding plasma-based ratios, the datasets from both matrices ($n = 60$) were sorted from low to high and arbitrarily divided into 3 equally-sized groups ($n = 20$ for each group). From the 20 individuals showing the lowest ratios in plasma, 12 also showed the lowest ratios in hair, 6 were in the intermediate subgroup and 2 were in the subgroup with the highest ratios in hair (Figure 3 a). A similar pattern was observed for the 20 individuals showing the highest ratios in plasma: 12 subjects were in the subgroup with the highest ratios in hair, while 5 were in the intermediate subgroup and 3 were in the subgroup with the lowest ratios in hair (Figure 3 b).

The influence of various factors, both related to the use of hair as a bioanalytical matrix and to a possible effect on CYP1A2 activity, was evaluated. The median values and corresponding ranges for paraxanthine/caffeine ratios in plasma and hair are listed in Table 1. Hair color had no apparent effect on the paraxanthine/caffeine ratios. Ratios in plasma as well as in hair did not differ significantly between individuals with non-cosmetically-treated blond, brown, dark or black hair ($p = 0.836$ and 0.708, respectively). In addition, paraxanthine/caffeine ratios in subjects with cosmetically coloured hair were compared with ratios from subjects with non-cosmetically treated hair. Again, no significant differences were found for the ratios in both plasma and hair ($p = 0.941$ and 0.959, respectively). Concerning the effect of factors affecting CYP1A2 activity, we found that hormonal contraception users had 1.7-fold lower paraxanthine/caffeine ratios in plasma compared with women not taking hormonal contraception ($p < 0.0001$). Although also in hair the median ratio was lower in hormonal contraception users, this difference did not reach statistical significance when comparing hormonal contraception users and non-users ($p = 0.143$). Apart from hormonal contraceptives, none of the participants reported the use of any drugs that interfere with CYP1A2 activity. Both in plasma and hair, women showed lower ratios compared to men ($p = 0.00136$ and 0.0465, respectively); however, this sex effect disappeared when hormonal contraception users were excluded ($p = 0.317$ and 0.412 for plasma and hair, respectively). Paraxanthine/caffeine ratios in the small subgroup of smokers ($n = 4$) were not significantly different from ratios in non-smokers ($p = 0.119$ and 0.274 for plasma and hair, respectively). Also average caffeine intake had no apparent effect on measured ratios ($p = 0.154$ and 0.915 for different groups of caffeine intake in plasma and hair, respectively). Yet it is interesting to note that hair caffeine concentrations actually showed an increasing trend in subjects consuming higher amounts of caffeine, with significantly higher

concentrations in hair from subjects reporting intake of > 7 caffeine consumptions/day ($p < 0.0001$) (Supplementary Figure S1). Concerning the influence of alcohol intake, individuals not consuming alcohol showed higher paraxanthine/caffeine ratios in plasma compared to individuals consuming 1-3 consumptions/week ($p = 0.031$). However, again, the difference was no longer significant when excluding hormonal contraception users from the comparison ($p = 0.629$). Finally, we found no effect of age on paraxanthine/caffeine ratios ($p = 0.343$ and 0.880 for plasma and hair, respectively).

To further investigate the effect of these factors on the variability between ratios in hair and plasma, 2 subgroups ($n = 15$) were selected from the dataset, with either a good or a poor correlation between ratios in hair and plasma (Figure 2), with respective differences between corresponding ratios below 15 % and above 60 %. The distribution of all groups of participants in the poor and good correlation subgroup, as well as in the entire dataset, is displayed in Figure 4. For none of the evaluated factors, a clear effect on the variability between hair and plasma ratios was found as the distribution in both subgroups was similar in all cases.

4 Discussion

Determining metabolite-to-parent drug concentration ratios in traditional matrices, such as plasma or urine [19], or alternative samples, such as dried blood spots [20] or saliva [21] is an accepted approach to assess the metabolic activity of enzymes. In the last few years, the use of hair analysis has been introduced in this field [5]. Already in 1996, Mizuno *et al.* suggested the use of the caffeine content in hair as an indicator of liver function, as they found higher caffeine concentrations in hair of liver cirrhosis patients compared to healthy volunteers [25]. In this study, using an optimized and validated LC-MS/MS method, paraxanthine/caffeine concentration ratios were determined in hair for the first time as a potential measure of CYP1A2 activity. This is, to the best of our knowledge, the first study in which metabolite-to-parent drug ratios in hair were compared with reference, plasma-based phenotyping metrics.

Segmental hair analysis showed decreasing caffeine and paraxanthine concentrations from the hair root to the distal end. As all 4 volunteers reported to generally have a constant caffeine intake over time and none of the hair samples had been cosmetically treated (i.e. coloured, bleached or permed), the observed decrease in analyte concentrations along the hair shaft is probably due to a wash-out effect caused by regular hair washing, mechanical stress and influence of UV-light over time [1]. Although the underlying reason for the observed variability between paraxanthine/caffeine ratios is not known, we cannot exclude the theoretical possibility that caffeine and paraxanthine might be unequally removed from the hair as a result of regular hair washing or other environmental

factors to which hair is exposed over time, which could affect paraxanthine/caffeine ratios. Data on hair washing habits were not collected, as it would be very difficult to adequately assess the effect of these factors, given the large variability in regular hair washing procedures that can be expected. As a result, proximal 3-cm hair segments were used in this study, as is also commonly applied when determining markers of alcohol use in hair [26]. Furthermore, from a practical point of view, analyzing the first 3-cm segment was more convenient as it allowed to apply the procedure to individuals with short hair as well, thereby preventing that hair length would be a limiting factor for inclusion in the study. Although 3-cm segments could be obtained from all participants, hair length would be even less of a concern when shorter segments were used. However, this was not an option in this study for two reasons. First, we wished to avoid that samples needed to be excluded from the study due to limited sample amount (i.e. less than 20 mg of ground hair) or measured concentrations below the LLOQ, especially for the metabolite paraxanthine which was expected to be present in hair at lower concentrations. Second, while the aforementioned sensitivity issue might be coped with by sampling a shorter segment from a larger head area, this would undoubtedly have had a negative cosmetic impact, rendering it difficult to recruit sufficient volunteers.

Using data from 60 healthy volunteers, a statistically significant correlation between ratios in hair and plasma was found, albeit that large deviations were observed for some individuals. Given the lack of a clear link between CYP1A2 activity and known polymorphisms of the *CYP1A2* gene, a distinction between poor or extensive metabolizer status, as is the case for other CYP450 isoforms such as CYP2D6, cannot be made for CYP1A2 [12,27]. In addition, there are also no established reference ranges or cut-off values for CYP1A2 phenotyping metrics available. In this light, Figure 3 illustrates to what extent ratios in hair correlate with corresponding plasma-based ratios. This graphical representation shows that, in some cases, ratios in hair do not provide a reliable reflection of the CYP1A2 activity. For example, 3 individuals (2 women, 1 men) belonging to the subgroup of participants with the highest ratios in plasma, showed hair ratios that were among the 5 lowest measured in the entire study population (Figure 3 b). As described below, the effect of several variables was evaluated for these individuals, as was done for the entire data set. They were non-smokers, had different hair color and did not report cosmetic hair colouring, use of CYP1A2-affecting medication, excessive caffeine or alcohol intake. Therefore, no clear explanation for their deviating results was available.

In general, many factors may contribute to the observed deviations between ratios in hair and plasma. We consider it most unlikely that analytical issues contributed to the observed variability. Validated LC-MS/MS methods were used for both matrices, with highly comparable data for precision (%RSD) and accuracy (%bias) in hair [De Kesel *et al.*, submitted] and plasma [22]. In

addition, the differences between hair and plasma ratios cannot be explained by breakdown of the analytes during storage, as caffeine and paraxanthine were stable in hair stored at room temperature for at least 644 days [De Kesel *et al.*, submitted] as well as in plasma stored at -20 °C for at least 172 [22]. Our evaluation of the analytical wash procedure only demonstrated the presence of caffeine in the wash solvents, with peak areas corresponding to less than 30 % of the LLOQ in the last wash step, consistent with our observations in the validation of this procedure [De Kesel *et al*., submitted]. In general, these amounts will have a limited impact on measured paraxanthine/caffeine ratios.

Another aspect that may have an effect on the observed variability is the methodological nature of the conducted comparative phenotyping study. Paraxanthine/caffeine ratio determined in plasma samples represent a measure of the CYP1A2 activity at the actual time of sampling. Ratios measured in 3-cm hair segments, on the other hand, rather may provide an image of the enzyme activity over a period of approximately 3-4 months prior to sampling, assuming that it takes approximately 2 weeks until a hair reaches the scalp and that hair grows with an average rate of 1.06 cm/month [28]. As a consequence, high intra-individual variability in CYP1A2 activity over time might be raised as an explanation for the differences between ratios in hair and plasma. However, using a similar CYP1A2 phenotyping approach as applied here, Simon *et al.* found that paraxanthine/caffeine ratios, determined in plasma 5 hours after administration of a 140 mg caffeine dose, did not differ significantly over a period of 12 weeks. The authors reported an intra-individual coefficient of variation (CV) of 17.6 ± 6 % and 16.2 ± 5.9 %, respectively in young and elderly subjects [29]. Based on the findings from this study, combined with the fact that none of the volunteers in our study reported to take (or have taken) medication of which an inhibiting or inducing effect on CYP1A2 has been described (apart from hormonal contraception), we consider it unlikely that intra-individual variability can explain the observed deviations.

In addition to the factors described above, the incorporation of caffeine and paraxanthine in hair may be a matter of concern. Incorporation of analytes into hair is a complex phenomenon in which multiple mechanisms are involved. Compounds can be incorporated via passive diffusion from blood capillaries or other tissues surrounding the hair follicle, from sweat or sebum or through external contamination [30]. Many factors affect these processes, such as the physicochemical properties of the analyte and its affinity for binding to melanin in hair. As melanin determines hair pigmentation, a color-dependent incorporation has been described for several compounds [31,32]. Here, no influence of hair color on paraxanthine/caffeine ratios was found. Furthermore, it is known that cosmetic treatment of hair may decrease drug concentrations [33]. However, in our population, cosmetic hair colouring did not affect measured ratios in hair. In addition, both factors had no apparent effect on the observed hair-plasma deviations. Another aspect that may play a role is the

site of hair sampling. Dussy *et al.* determined caffeine concentrations in hair locks from 3 individuals collected at 10 different areas of the scalp. Coefficients of variation of measured caffeine concentrations ranged from 12.6 to 61.9 %, while a CV of 4.5 % was found for a homogenized control sample analyzed in six-fold [34]. However, it should be noted that the areas of sampling were widely distributed over the scalp, which could have contributed to the observed variability in measured concentrations as the proportions of hair in the anagen or telogen phase may vary in function of the location. In our study, all hair samples were cut from the posterior vertex region of the head. It is generally recognized that the proportion of hair in the telogen phase is the lowest and that hair growth rate shows less variability in this region [1,2]. Furthermore, all samples were collected by the same operator to minimize variation in the actual sampling site.

As mentioned in the introduction section, CYP1A2 activity is characterized by a large inter-individual variability to which many factors have been reported to contribute. The influence of several factors on CYP1A2 activity was evaluated. The inhibiting effect of hormonal contraceptives found in our population is similar to that described in other studies [35,36]. When hormonal contraception users were excluded, no sex differences in paraxanthine/caffeine ratios were found. This is in agreement with other studies in which sex was found not to influence CYP1A2 activity [29,35]. Furthermore, an inducing effect of smoking [29,35,36] or caffeine consumption [35,36] was not observed in our study. However, as our study population contained only 4 smokers and 4 individuals reporting intake of > 7 caffeine consumptions/day, we might have been unable to detect an existing effect of these factors. In accordance with other studies [29,36], we also found no effect of age on paraxanthine/caffeine ratios. Finally, none of the above-mentioned factors could explain the variability between ratios in hair and plasma. However, it should be noted that subdividing the data set (n = 60) into different subgroups resulted in small (or, in some cases, very small) group sizes. Consequently, some statistical comparisons may be underpowered. Future studies with even more participants or with participants belonging to certain focus groups (e.g. more smokers) may be needed to make more conclusive statements.

5 Conclusion

In this study, the potential of using hair as an alternative matrix for CYP1A2 phenotyping was evaluated. Paraxanthine/caffeine molar concentration ratios were determined in proximal 3-cm hair segments from 60 healthy volunteers and compared with reference CYP1A2 phenotyping indices determined in plasma. Although paraxanthine/caffeine ratios in hair and plasma overall showed a statistically significant correlation, large deviations in individual cases impede the interpretation of hair results on an individual basis. The influence of several factors on the variability between ratios in

hair and plasma was evaluated, but, hitherto, none of these factors could explain the observed deviations. Therefore, hair sampling is not (yet) suitable for CYP1A2 phenotyping.

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Conflicts of Interest

The authors have no conflict of interest to declare. This study was financed by the Laboratory of Toxicology, Ghent University, Belgium. The authors received no additional funding directly related to the content of this study.

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Tables

Table 1. Paraxanthine/caffeine molar concentration ratios in plasma and hair samples. The median values together with the observed ranges are listed.

Variable		Paraxanthine/caffeine molar concentration ratio in plasma		Paraxanthine/caffeine molar concentration ratio in hair	
	n	median	range	median	range
All participants	60	0.40	0.09 – 0.95	0.41	0.12 – 0.93
Hair color					
Blond	13	0.41	0.14 – 0.95	0.37	0.12 – 0.81
Brown	16	0.38	0.14 – 0.73	0.41	0.21 – 0.82
Dark	15	0.42	0.12 – 0.86	0.40	0.21 – 0.93
Black	5	0.34	0.31 – 0.40	0.47	0.34 – 0.65
Cosmetic colouring					
Coloured	11	0.41	0.09 – 0.84	0.47	0.18 – 0.75
Non-treated	49	0.40	0.12 – 0.95	0.40	0.12 – 0.93
Sex					
Women	43	0.34 [a,b]	0.09 – 0.84	0.40 [a,b]	0.12 – 0.93
Men	17	0.51 [a,b]	0.31 – 0.95	0.59 [a,b]	0.13 – 0.82
Hormonal contraception					
Non-users	18	0.47 [a]	0.26 – 0.84	0.50 [a]	0.12 – 0.93
Users	25	0.28 [a]	0.09 – 0.66	0.37 [a]	0.18 – 0.68
Smoking					
Smoker	4	0.53	0.29 – 0.86	0.52	0.31 – 0.81
Non-smoker	56	0.40	0.09 – 0.95	0.41	0.12 – 0.93
Caffeine intake (consumptions/day)					
0	1	0.95		0.59	
1-3	23	0.45	0.09 – 0.84	0.48	0.12 – 0.75
4-6	32	0.37	0.12 – 0.70	0.37	0.13 – 0.93
> 7	4	0.43	0.28 – 0.86	0.43	0.19 – 0.82
Alcohol intake (consumptions/week)					
0	7	0.50 [a,b]	0.40 – 0.84	0.55	0.13 – 0.75
1-3	26	0.35 [a,b]	0.09 – 0.57	0.37	0.12 – 0.93
4-6	18	0.40	0.14 – 0.73	0.46	0.21 – 0.82
> 7	9	0.34	0.16 – 0.95	0.35	0.19 – 0.81
Age (years)					
20-29	43	0.39	0.09 – 0.95	0.40	0.12 – 0.82
30-39	13	0.41	0.19 – 0.84	0.41	0.13 – 0.93
40-49	4	0.41	0.26 – 0.66	0.50	0.34 – 0.57

a: significantly different , b: no longer significantly different when hormonal contraception users were excluded

Figures

Fig. 1 Caffeine and paraxanthine concentrations (a-d) and paraxanthine/caffeine molar concentration ratios (e) in 6 consecutive 3-cm hair segments from 4 female, healthy volunteers

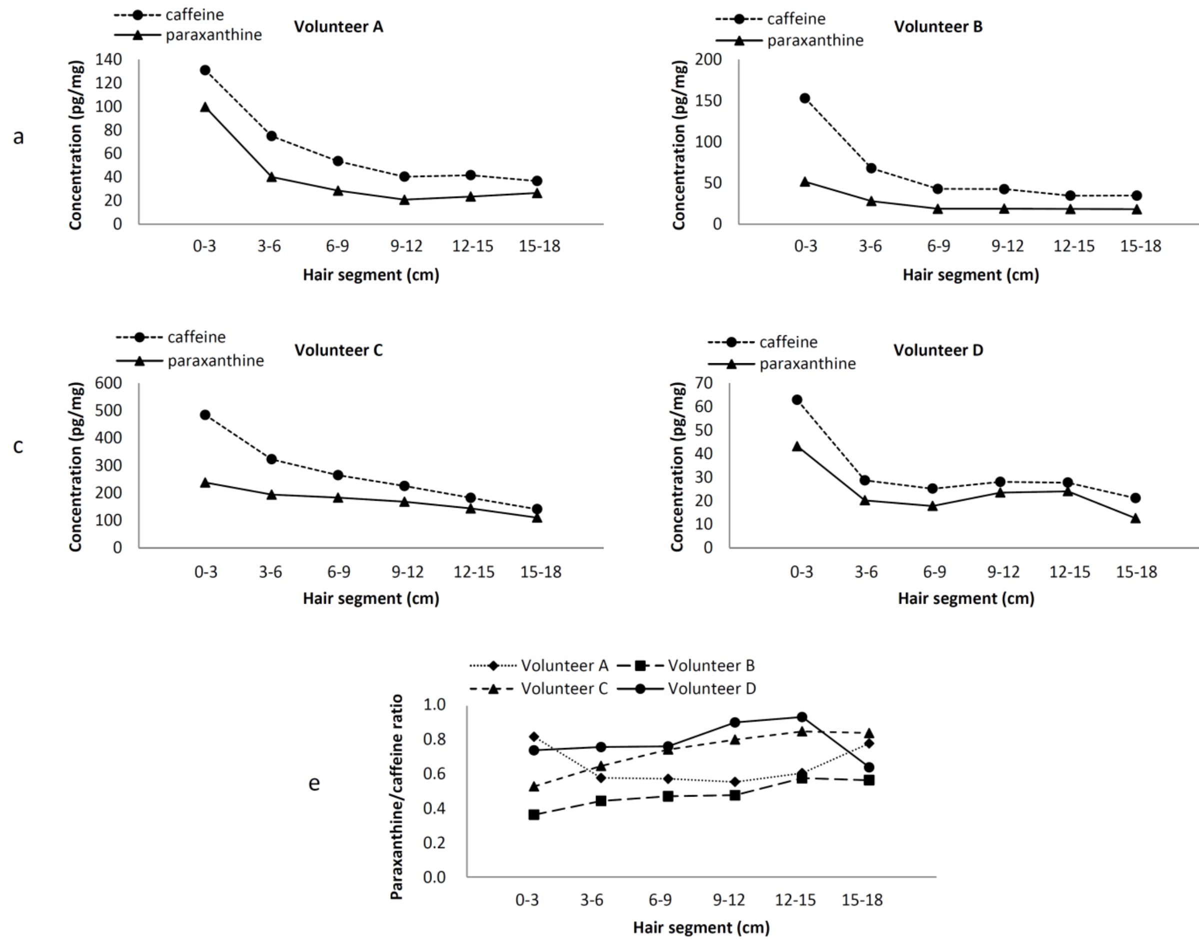

Fig. 2 Correlation between paraxanthine/caffeine molar concentration ratios determined in 3-cm proximal hair segments and in plasma samples from 60 healthy volunteers. Pearson correlation coefficient r is displayed, together with its p-value. Subgroups (n = 15) with a good and a poor correlation between ratios in hair and plasma are represented by green and red dots, respectively. The remaining subjects (n = 30) are represented by blue dots.

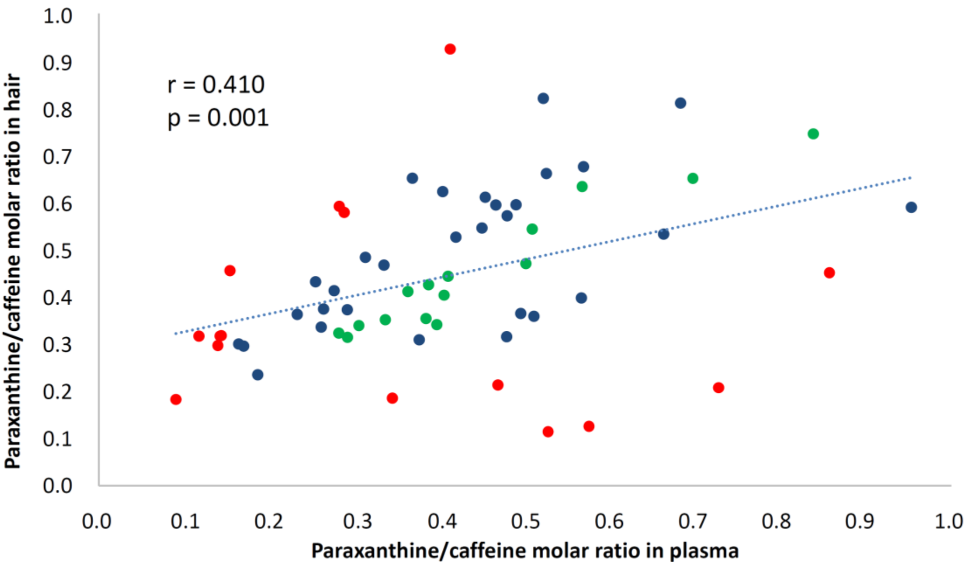

Fig. 3 Paraxanthine/caffeine molar concentration ratios in plasma and in hair from 60 healthy volunteers were arbitrarily divided into 3 subgroups containing the lowest, intermediate and highest ratios (n = 20 for each group). A: green lines between subjects showing the lowest ratios in plasma and in hair (n =12), orange lines between subjects showing the lowest ratios in plasma and intermediate ratios in hair (n = 6), red lines between subjects showing the lowest ratios in plasma and highest ratios in hair (n = 2). B: green lines between subjects showing the highest ratios in plasma and in hair (n = 12), orange lines between subjects showing the highest ratios in plasma and intermediate ratios in hair (n = 5), red lines between subjects showing the highest ratios in plasma and lowest ratios in hair (n = 3)

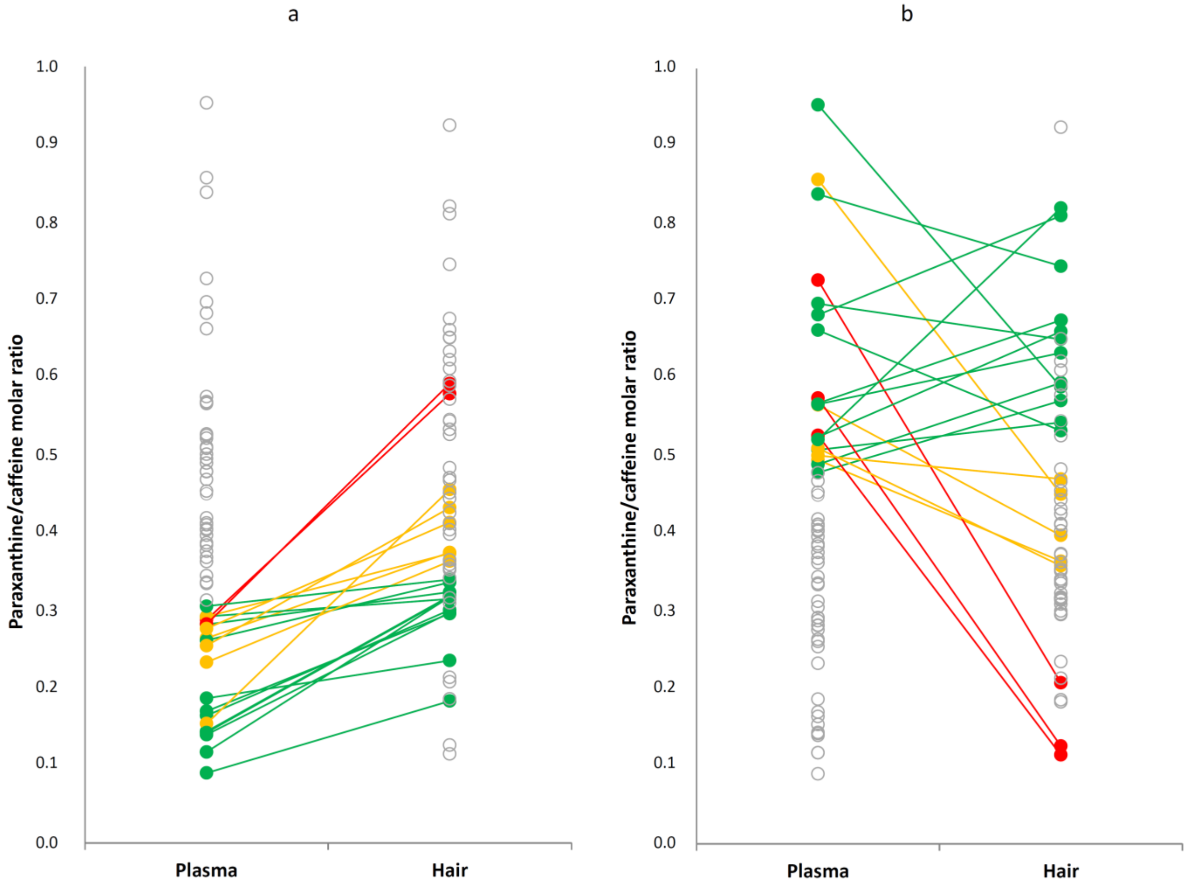

Fig. 4 Distribution of participants, sorted according to different factors (*), in the entire data set (n = 60) and in the subgroups of subjects showing a good (n= 15) or poor correlation (n = 15) between ratios in hair and plasma

(*) a: hair color (only non-cosmetically coloured samples), b: cosmetic hair colouring, c: hormonal contraception, d: sex, e: smoking, f: average number of caffeine consumptions per day, g: average number of alcohol consumptions per week, h: age