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The effect of caffeine dose on caffeine and paraxanthine changes in serum and saliva and CYP1A2 enzyme activity in athletes: a randomized placebo-controlled crossover trial

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Abstract

Background Although caffeine (CAF) supplementation has been shown to improve exercise performance, its dose-dependent effect on CAF metabolism has not been sufficiently investigated. The aim of this study was to evaluate the effects of 3, 6 and 9 mg of CAF/kg_{BM} on changes of CAF and paraxanthine (PRX) in the serum and saliva at four time-points.

Methods In a randomized, double-blind, placebo-controlled crossover design, acute pre-exercise supplementation in 26 moderately-trained athletes, participating in high-intensity functional training (HIFT), was examined. The study protocol involved CAF/PRX biochemical analyses of serum and saliva with respect to *CYP1A2* polymorphism and CYP1A2 enzyme activity.

Results Despite significant differences between the serum and saliva levels of CAF and PRX, there was no difference in the PRX/CAF ratio. The interaction effect of dose and time-points for PRX concentration was revealed. The main effects of dose were observed for CAF and the PRX/CAF ratio. The main effect of time-points was registered only for serum CAF.

Conclusions Dose- and time-dependent effect of CAF supplementation on CAF and PRX in the serum and saliva of athletes was confirmed, but there was no effect of the CAF dose on CYP1A2 enzyme activity, nor was there an interaction of *CYP1A2* with enzyme inducibility. The CAF/PRX correlation indicated the possibility of interchangeable use of serum and/or saliva analyses in exercise studies.

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Clinical trial registration This trial was registered prospectively at ClinicalTrials.gov (NCT03822663, registration date: 30/01/2019).

Keywords Enzyme activity, Ergogenic support, High-intensity functional training, Metabolism, Sports dietetics, Supplementation

Introduction

Caffeine [(1,3,7-trimethylxanthine), CAF] is a purine alkaloid that exerts a wide range of psychophysiological effects, which might be partly influenced by its intake variation between individuals. Its consumption in humans commonly occurs via the ingestion of coffee and several other foods and beverages (e.g., cocoa, green tea, chocolate, guarana berries, yerba mate, kola nuts) [1, 2].

CAF is rapidly absorbed in the gastrointestinal tract [approximately 99% within 45 min, mainly in the small intestine or in the stomach], which is independent of sex, age, genetic factors, diseases or exposure to nicotine, alcohol or drugs [1]. However, the time of absorption depends on the CAF-based product; for example, CAF from the capsule is absorbed in 30 min, whereas that from the cola or chocolate is absorbed in 1.5–2 h. Its maximum concentration is reduced by approximately 25% for drinks in comparison to ~2.05 µg/mL for a capsule [1]. Although CAF is mainly absorbed via the gastrointestinal tract (80% via the small intestine and 20% via the stomach) [1, 3], recently it has been hypothesized that CAF absorption via the oral mucosa can play a role in enhancing exercise performance. Although it is not clear how much of CAF may be absorbed through oral mucosa, research demonstrated that delivering CAF in alternate forms, like in a chewing gum, may speed the rate of its delivery to the blood by absorption through the buccal mucosa [4, 5]. It may occur due to the extensive vascularization in this region. This rapid absorption is particularly relevant for athletes, as it can lead to quicker improvements in reaction time and cognitive alertness during exercise, which are crucial for performance [6]. After CAF enters the tissue water compartment, it is evenly distributed in body fluids, but it does not accumulate in the body. Because of its lipophilic character, CAF is able to cross all cellular membranes, in contrast to its metabolites. A blood/plasma ratio close to 1 reflects free movement into/out blood cells and limited plasma protein binding [1]. CAF is excreted through the renal system. Approximately 70% of its dosage is recovered in urine, with 0.5-2% being excreted unmetabolized in urine [1]. Notably, approximately 98% of CAF is reabsorbed from the renal tube, which explains the low level of CAF urine excretion. Additionally, 2–7% of the CAF dose is excreted in feces over 48 h, which consists mainly of CAF metabolites from the liver–intestine cycle and metabolism [1, 2].

CAF is eliminated via first-order kinetics at dosages of 2–10 mg_{CAF}/kg_{BM}; its first-pass metabolism is minimal, and its pharmacokinetics does not depend on the route of administration [1]. It is metabolized primarily in the liver, where it undergoes demethylation and oxidation processes. The majority of CAF is converted in the liver into paraxanthine (PRX, ~81.5%), which is exclusively mediated via the CYP1A2 enzyme and is suggested to be as potent as CAF for the adenosine receptors blockade. Other CAF metabolites, such as theobromine and theophylline, are metabolized by other enzymes. Different factors, such as pregnancy, lifestyle (e.g. smoking), diseases or the environment, may affect CAF metabolism [1, 2]. Interestingly, dose-dependent CAF metabolism has been suggested in several in vivo studies, showing its nonlinear clearance and saturation of 70–300 mg of CAF. The plasma kinetics of CAF may be influenced by the dose, the presence of dietary constituents in the stomach, gastric pathologies or fluid intake, which affect renal clearance and CAF pharmacokinetics [1, 2]. Nevertheless, most of the variations are accounted for by genetics. The peak plasma concentration of CAF is reached at 30–120 min, and the half-time ranges from 1.5 to 10 h [7]. The total plasma clearance of CAF and its main metabolite is identical (2.07–2.20 $mL/min/kg_{BM}$). The PRX concentration decreases more slowly than the CAF concentration does; in fact, it becomes even higher than the CAF concentration at approximately 8–10 h after CAF ingestion [1, 2]. Moreover, the concentration of CAF in saliva reaches 65–85% of its plasma concentration [1].

Considering that the CYP1A2 enzyme influences the disposition of CAF, one of the important parts of treatment personalization is assessing its enzymatic activity, which depends on several factors, such as sex and race [1]. Moreover, a functional polymorphism in the *CYP1A2* gene may contribute to inter-individual differences in CYP1A2 enzyme activity [8]. The A allele at position −163 (rs762551) leads to lower enzyme inducibility than the C allele does, which is reflected by the urinary or plasma ratio of CAF/metabolites after CAF ingestion. C-allele carriers (AC, CC), who constitute 54% of the population, are referred to as 'slow metabolizers'. This C variant is associated with slow CAF clearance. In turn, AA homozygotes are considered 'fast metabolizers' [1, 2, 9].

The enzyme CYP1A2 is found primarily in the liver, and CAF is the most widely used probe to measure its

activity. The 'gold standard' measurement is the apparent clearance of CAF by extensive blood sampling. However, the general enzymatic activity of CYP1A2 can also be measured by the PRX to CAF ratio (PRX/CAF RATIO) after time- and dosage-controlled administration of CAF [10–13] in a number of biological human matrices (such as blood or saliva) and metrics at a single time-point (concentration at a single time-point, C_t), which apparently correlates with clearance. Extensive pharmacokinetics measurements recommend the use of PRX/CAF C_t in serum/plasma at 2, 4, 3–12 or 5–7 h after administration or at 1–2, 2, 5 and 6–10 h in saliva [12]. Nevertheless, although most studies actually use a 6 h time period and PRX/CAF RATIO or CAF-based metabolic phenotyping, there is no clear consensus on such a scientific approach [14]. Moreover, the time- and dose-dependent effects of PRX/CAF RATIO have not been reported thus far and are especially relevant for phenotyping at very low doses [14]. The results from a recent systematic analysis [14] revealed that even though multiple studies and clinical investigations used different dosing protocols, the results of metabolic phenotyping with CAF were very consistent. The correlation coefficients between the CAF and PRX concentrations in saliva and blood/serum were 0.84 and 0.76, respectively. An even stronger correlation (0.88) was found between saliva-based and blood-based CAF clearance. The data indicated that clearance may be calculated from both blood/serum- and saliva-based measurements [14]. In conclusion, the saliva-based metabolic phenotyping approach has a very good correlation with blood-based approaches (as was previously confirmed in non-exercise conditions) [14].

Although both the pharmacokinetics and pharmacodynamics of CAF are well-studied and this ergogenic supplement is widely used in various doses by athletes, data on CAF metabolism under exercise conditions are scarce. Therefore, the aim of the current study was to evaluate the effects of three different doses of acute CAF supplementation on CAF, PRX, and PRX/CAF RATIO changes in serum and saliva at different time-points after supplementation in a group of young, healthy, and moderately trained people participating in high-intensity functional training (HIFT). We also investigated the effects of the CAF dose and single nucleotide polymorphism (SNP) of the *CYP1A2* gene (rs762551) on CYP1A2 enzyme activity, as measured by the mean time-corrected PRX/CAF RATIO in saliva. We hypothesized that a higher CAF dose (9 mg_{CAF}/kg_{BM}) will induce greater CAF and PRX changes in both biological human matrices and that the peak change in CAF concentration from pre-supplementation will be elicited 60 min after intake. Moreover, we hypothesized that a higher CAF dose will affect CYP1A2 enzyme activity more profoundly and that C-allele carriers will have different CYP1A2 enzyme activity responses than AA homozygotes.

Methods

Study design, protocol and visits

In this randomized, double-blind, placebo-controlled crossover design, three doses of CAF (3, 6, 9 mg_{CAF}/kg_{BM}; *LOW*, *MEDIUM*, and *HIGH*, respectively) or placebo (*PLA*) were acutely supplemented to the participants during the study protocol, which consisted of five visits (T_0–T_4) to the laboratory (Fig. 1). At the beginning, the volunteers were familiarized with the entire protocol and subsequently subjected to the first visit (T_0; baseline (BASE), without supplementation treatment), after which they were randomly assigned (stratified randomization on the basis of exercise performance results) to the treatment order with specific codes by an impartial biostatistician. The main study protocol involved four separate visits (T_1–T_4) performed at the same time of day for the participant, three hours after a standardized meal (carbohydrates: 2 g/kg_{BM}, proteins: 25 g, water: 7 mL/kg_{BM}) [15, 16]. The participants were aware to avoid any CAF-containing products for the 24 h before the study meeting. Each visit consisted of body composition measurements, the HIFT exercise protocol and blood/saliva sampling. Given the kinetics of CAF excretion from the body, a 7-day washout period was introduced between treatments [17]. Moreover, participants were divided post hoc into groups according to the *CYP1A2* genotype (AA homozygotes and C allele carriers).

The primary outcome in our study was a change from the first time-point (prior to supplement administration) to the next three time-points in terms of the CAF and PRX concentrations and the PRX/CAF RATIO in the serum and saliva. In turn, CYP1A2 enzyme activity and SNP in the *CYP1A2* genotype were defined as secondary outcomes.

Participants

Twenty-six moderately HIFT-trained participants (10 females, 16 males) completed the entire study protocol and were included in the analyses (Fig. 1; Table 1). All the athletes were enrolled from HIFT clubs in Poznań, Poland. The inclusion criteria for participation in the study were good health conditions, a valid and up-to-date medical certificate confirming the athlete's ability to practice sports, at least 2 years of HIFT training experience, a minimum of 4 workout sessions a week, and a habitual moderate intake of CAF-containing products (which refers to more than two 240 mL cups of coffee, where the typical average CAF content is ~ 170 mg (range: from 120 to 240 mg)) [18]. The exclusion criteria were current injury, health-related contraindications, a general feeling of being unwell, an unwillingness to follow the study

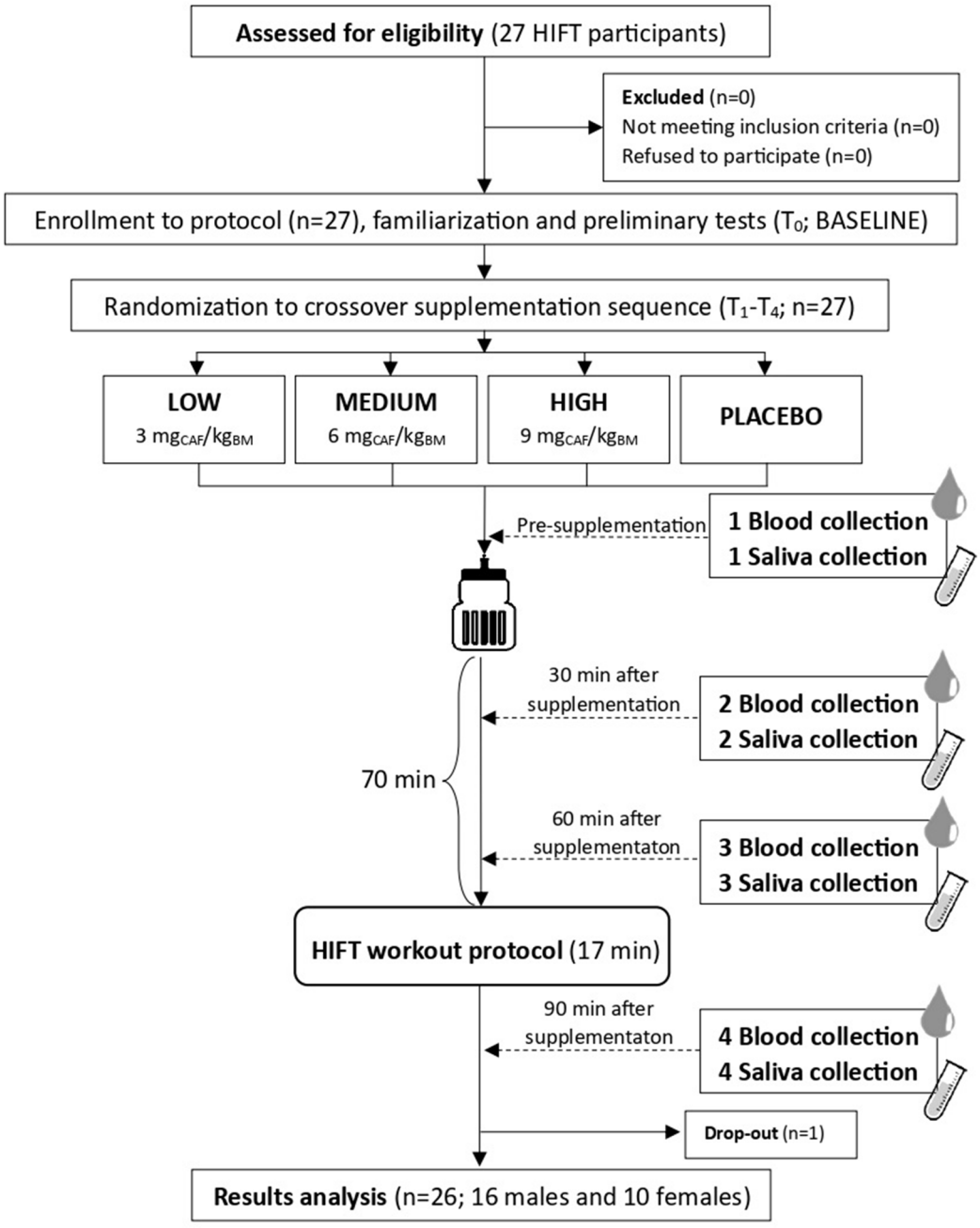

Fig. 1 Flow chart of the study design. HIFT – high-intensity-functional training

protocol, smoking, and failure to adhere to CAF abstinence. The study protocol was conducted from July 2021 to December 2022 at the Department of Sports Dietetics (Poznan University of Physical Education, Poland). This trial was reviewed and approved by the Bioethics Committee at Poznan University of Medical Sciences (reference number 293/17 of May 11, 2017) and was registered prospectively at ClinicalTrials.gov (NCT03822663, registration date: 30/01/2019). The study complies with the CONSORT Statement for randomized trials, as shown in Fig. 1. All study participants provided written informed consent. All procedures were carried out in accordance with the ethical standards of the Helsinki Declaration of 2013.

Procedures

Supplementation

In the experimental procedure, each participant ingested an acute dose of CAF [3- (*LOW*), 6- (*MEDIUM*) or

Table 1 Baseline characteristics of the studied group ($n = 26$)

Body composition variable	Mean ± SD
Age [years]	35.4 ± 6.5
Body mass [kg]	77.4 ± 16.6
Height [cm]	175 ± 9
Total Body Water [%, *L*]	57.2 ± 5.1, *44.1 ± 9.7*
Fat Mass [%, *kg*]	19.9 ± 5.6, *15.5 ± 6.1*
Fat-Free Mass [%, *kg*]	80.1 ± 5.7, *61.9 ± 13.6*
Habitual CAF intake [mg/day]*	231 ± 121
CYP1A2 genotypes	*n*
'fast metabolizers'	6 AA
'slow metabolizers'	20 (3 CC, 17 AC)

SD – standard deviation, *n* – number of participants, * habitual daily intake, not including the day of the study and the 24 h preceding the visit, when participants were required not to consume any CAF sources

9- (*HIGH*) mg_{CAF}/kg_{BM}] and *PLA* treatment in a cross-over regimen. CAF doses (pure pharmaceutical CAF, Ostrovit, Poland) and *PLA* (bitter aroma) were administered dissolved in 20 mL of orange juice and 80 mL of plain water. The supplements were administered after the first blood sampling, 70 min before the HIFT exercise (Fig. 1), in containers marked with a unique code, made in advance by the researcher who did not directly participate in the investigations. With respect to double blinding, neither the researchers nor the participants knew whether CAF or *PLA* was administered. The randomization details were anonymized and revealed after the cessation of the protocol. The preparations were administered at a strictly specified time before the exercise tests, and consumption compliance was fully controlled by the investigators.

Body composition and nutritional assessment

The anthropometric and body composition measurements and nutritional assessment methods used in this study are thoroughly described elsewhere [19]. At the beginning of each study visit, anthropometric indices were measured in duplicate via a calibrated scale with a stadiometer (WPT 60/150 OW, Radwag®, Radom, Poland), and bioelectric impedance was measured with a Bodystat 1500 (Bodystat Inc., Douglas, UK). With respect to nutritional assessment, participants completed the special questionnaire prepared for this study for the quantification of daily CAF intake and additionally recorded their diet for the period of two consecutive days before each study visit to ensure 24 h CAF abstinence. The basic results of body composition analysis and habitual CAF intake are presented in Table 1. More detailed information was previously presented [19].

Exercise

HIFT workout protocol, with well-documented high repeatability (ICC 0.9, SEM 6%), was performed for 17 min in accordance with the protocol from our previous studies [19–22]. Additionally, before its commencement, the participants followed a 20 min self-prepared warm-up, which was the same for a particular participant during every study meeting. The results of the HIFT workout protocol, along with the heart rate and rate of perceived exertion, are published elsewhere [19].

Blood and saliva collection and samples preparation

Capillary blood and saliva samples were obtained at four time-points (1st – pre-supplementation (0'); 2nd – 30 min after supplementation (30'); 3rd – 60 min after supplementation (60'), pre-exercise; 4th – 3 min after exercise (90 min after supplementation, 90')) during every study meeting. All the samples were taken by a medical professional and were obtained with the participants in a seated upright position. Blood samples (600 µL) were collected from a fingertip of the nondominant hand via a disposable lancet-spike Medlance® Red (HTL-STREFA, Łódź, Poland) with a 1.5 mm blade and a 2.0 mm penetration depth. The capillary blood was collected into two Microvette® CB 300 Serum CAT tubes (Sarstedt, Nümbrect, Germany) with a clotting activator, and then the separated serum was used to measure the CAF and PRX concentrations. Saliva samples (1 mL) were collected at the same time-points as blood samples, via saliva receptacles [Salivette® swab (Sarstedt, Nümbrecht, Germany)] according to the manufacturer's instructions (the swab was removed from the Salivette®, then it was placed in the mouth and chewed for approximately 60 s to stimulate salivation, and then the swab with the absorbed saliva was returned to the Salivette® and the stopper was replaced). Saliva was extracted by centrifugation for 2 min at 1000 × g. Collected serum and saliva samples were frozen and stored at -80 °C until further analyses were performed.

CAF and PRX concentrations' determination

Serum and salivary CAF and PRX concentrations (µg/mL) were quantified following liquid–liquid extraction and high-pressure liquid chromatography as described previously [11, 23]. The concentrations were measured via a Hitachi Lachom 7000 high-performance liquid chromatography (HPLC) analyzer (Merck, Darmstadt, Germany), in which a LiChroCART®250-4 (LiChrospher®100, RP-18, 5 µm, Merck, Darmstadt, Germany) column was used. The mobile phase consisted of acetonitrile–acetic acid–H_2O (100:1:899). CAF, PRX and the internal standard benzotriazole were quantified via UV detection at 280 nm. With an eluent flow rate of 1 mL/min, the retention times were 14 min (CAF) and 7 min (PRX). Additionally, the PRX/CAF RATIO was calculated by simply dividing the concentration of PRX by the concentration of CAF.

CYP1A2 enzyme activity assessment by the determination of the mean time-corrected PRX/CAF RATIO in saliva

Previous analyses concluded that saliva PRX/CAF RATIO 6 h after CAF ingestion has the best correlation to CAF/PRX clearance, providing the most valid CYP1A2 enzyme activity validation [24, 25]. In view of the 4 different time-points of sampling in our current study and the relatively short duration of the study meeting, which prevented sampling 6 h after CAF ingestion, we decided to use a previously described [8] developed method, which enabled adjusting the CYP1A2 enzyme activity via the PRX/CAF RATIO values to the optimal 6 h post-dose sampling time-point. In this method, the equation from the study by Spigset et al. [26] was adapted to estimate PRX/CAF (y) if the time span between CAF intake and sampling (x) was known. It was concluded that a time span of 6 h equates to a mean PRX/CAF of 0.725 [8].

$$y = 0.016 + (0.141 * x) + \left(-0.004 * x^2\right)$$

Then, following the instructions, (1) the actual PRX/CAF RATIO was calculated on the basis of the molar concentrations of CAF and PRX. Next, (2) the correct mean PRX/CAF RATIO was estimated as mentioned before. (3) The difference between the actual and correct PRX/CAF RATIO values was subsequently calculated. Finally, (4), the mean time-corrected PRX/CAF RATIO, assuming a 6 h ideal time span, was determined by adding 0.725 to the difference calculated in step 3. In step 2, as x in the equation, we assumed a time span from the 3rd time-point (60 min after supplementation) because the consensus statement on CAF supplementation in sports recommends its intake 60 min before exercise because of its likely highest concentration [7].

CYP1A2 Genotyping

Samples for *CYP1A2* (rs762551) genotyping were collected at the first study visit. The swab was inserted into the mouth and rubbed firmly against the inside of the cheek and underneath the lower and upper lips for 1 min. DNA was isolated from exfoliated buccal epithelial cells via a standard kit (EXTRACTME® Genomic DNA KIT EM13, Blirt S.A., Poland). Genotyping was performed via commercially available TaqMan®SNP genotyping assays (ThermoFisher Scientific, Waltham, MA, USA; ID C___8881221_40) on a LightCycler 480 instrument (Roche Diagnostics, Switzerland), as described previously [23]. The results of genotyping are presented in Table 1.

Statistical analysis

All analyses were performed in Statistica 13.3 (TIBCO Software Inc. 2017) and RStudio via DescTools (Posit Software, PBC 2009–2023). The Shapiro-Wilk test was used to check the assumption of normality. Data analysis included both raw data (0', 30', 60', 90') and concentration changes calculated from the difference between the first concentration measurement, prior to supplement ingestion (0'), and subsequent measurements (30', 60', 90'). Correlation analysis, polynomial regression and difference-of-dependent variables analysis (t-test or Wilcoxon test) were used to analyze the raw data. General Linear Model (GLM) was used to analyze differences between change variables. Pearson and Spearman correlations for the assumption of a relationship between concentrations (CAF, PRX or PRX/CAF RATIO) in serum and saliva were used. Polynomial regression was performed for the differences in the CAF and PRX concentrations between the serum and saliva samples but not for the PRX/CAF RATIO, because of the level of model fit. For raw data correlation, difference and polynomial regression analysis, 520 variables were compared (without splitting the data into groups by dose). The GLM considered two within-group factors [dose: D (*LOW*, *MEDIUM*, *HIGH*, *PLA*); time-point: TP (30', 60', 90')] and one between-group factor [*CYP1A2*: C (SLOW, FAST)]. D*TP and D*TP*C interactions were also analyzed. If the sphericity condition was not met, the Huynh-Feldt correction was applied. Significant GLM results were further subjected to Bonferroni post hoc pairwise comparison analysis. An alpha level of 0.05 was considered the cutoff point for statistical significance. The means with standard deviations (SD) were presented in the tables and figures. Additional GLM analysis was performed on the transformed data, taking into account the predicted concentrations at 6 h after supplementation ingestion. For this purpose, the data were transformed from µg/mL to µmol/L, assuming a molar mass PRX of 180 and a CAF of 194 g/mol. Sample size estimation was performed via G*Power software (version 3.1.9.6, Germany). On the basis of the umbrella review [27], a medium effect size (ES): f(U)=0.38 was used. A sample size of 26 participants was adequate for this study.

Results

Correlation analysis between the raw result of 0' and changes in 30', 60' and 90' showed significance only in *BASE* for CAF ($p<0.001$) and PRX ($p=0.015$) and in *PLA* for CAF ($p=0.006$) but not in *LOW*, *MEDIUM* or *HIGH* dose in both CAF and PRX concentrations, which enabled the utilization of concentration changes calculated from the difference between the first concentration measurement, prior to supplement ingestion (0'), and subsequent measurements (30', 60', 90'). The raw data of the CAF and PRX concentrations and PRX/CAF RATIO, which was not the object of this study, are shown in Supplemental Table S1. Additionally, we did not intend to assess differences between sexes; therefore, neither males nor females were recruited for the study participation in

an intended even distribution. Hence, no sub-analyses of males/females differences were performed. Nevertheless, to clarify we added CAF, PRX and PRX/CAF RATIO and CYP1A2 enzyme activity raw results in all time-points in serum and saliva for all participants together, as well as separately for males and females into Supplemental Table S1.

Polynomial regression (saliva to serum; serum to saliva)

The polynomial regression analysis revealed a statistically significant model for estimation of the CAF concentration from saliva to serum samples ($F(1,517)=619.58$, $p<0.001$, $y=0.1281+1.5082*x-0.0375*x^2$, $R^2=0.79$) and from serum to saliva samples ($F(1,517)=506.32$, $p<0.001$, $y=-0.2465+1.0698*x-0.017*x^2$, $R^2=0.77$) (Fig. 2). The Pearson correlation coefficient between CAF variables in serum and saliva samples was 0.87 (Spearman rho 0.91). Additionally, with respect to the PRX concentration, a significant model of saliva to serum samples ($F(1,517)=210.99$, $p<0.001$, $y=0.0247+1.5501*x-0.1803*x^2$, $R^2=0.61$) and of serum to saliva samples ($F(1,517)=359.81$, $p<0.001$, $y=0.069+0.8603*x-0.0738*x^2$, $R^2=0.63$) was observed. The Pearson correlation coefficient between the PRX variables in the serum and saliva samples was 0.77 (Spearman rho 0.83). The regression models are shown in Fig. 2.

For the PRX/CAF RATIO variables in both the serum to saliva and of saliva to serum, the distribution obtained did not allow regression analysis to be performed. In this case, the Pearson correlation coefficient was 0.29 (Spearman rho 0.83). Both the CAF and PRX concentrations between the serum and saliva showed a significant difference ($Z=12.37$, $p<0.001$ and $Z=9.52$, $p<0.001$, respectively) in the pairwise comparisons, but for the PRX/CAF RATIO the differences between the serum and saliva were not significant ($Z=1.36$, $p=0.174$).

CAF and PRX concentrations' changes in serum and saliva

GLM analysis of changes under the influence of CAF supplementation for CAF showed significant differences in terms of the D main effect in both the serum ($p<0.001$) and saliva ($p<0.001$) samples and in terms of the time-point main effect but only in the saliva ($p=0.014$) sample.

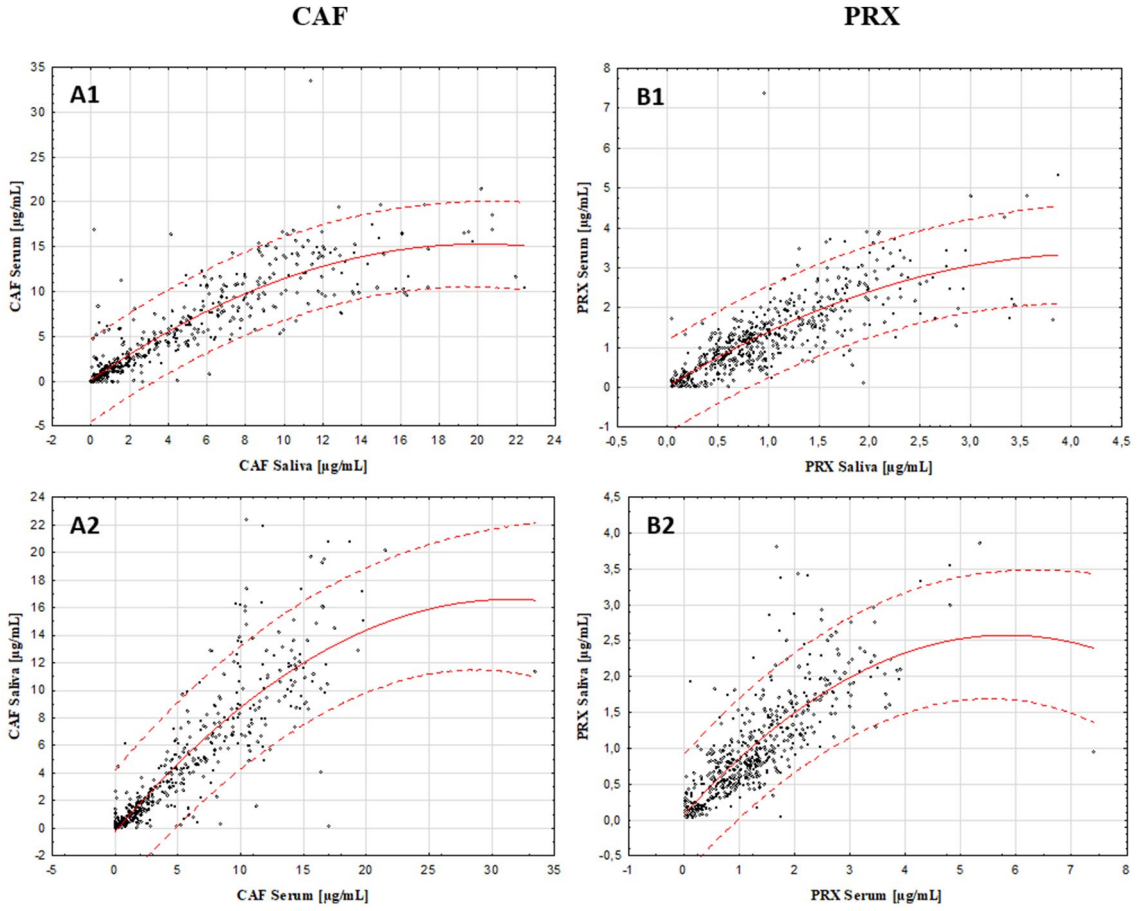

Fig. 2 Regression model with predictions. A1 – CAF concentration of saliva to serum; A2 – CAF concentration of serum to saliva; B1 – PRX concentration of saliva to serum; B2 – PRX concentration of serum to saliva

Table 2 CAF, PRX and PRX/CAF RATIO changes in serum and saliva (µg/mL)

	SERUM				SALIVA		
CAF	**1**[3]	**2**[3]	**3**[1,2]		**1**[2,3]	**2**[1]	**3**[1]
LOW[M,H,P]	4.13 ± 1.94	3.40 ± 1.70	3.32 ± 1.06	*LOW*[M,H,P]	3.77 ± 2.11	2.90 ± 1.41	2.80 ± 1.50
MEDIUM[L,H,P]	10.81 ± 5.60	11.12 ± 4.29	9.96 ± 5.05	*MEDIUM*[L,H,P]	9.59 ± 4.84	7.36 ± 3.16	7.09 ± 2.84
HIGH[L,M,P]	12.94 ± 2.04	12.79 ± 0.63	12.27 ± 1.73	*HIGH*[L,M,P]	11.92 ± 4.92	9.94 ± 2.76	10.18 ± 2.66
PLA[L,M,H]	-0.09 ± 0.55	-0.27 ± 0.31	-0.40 ± 0.29	*PLA*[L,M,H]	-0.34 ± 0.31	-0.36 ± 0.35	-0.35 ± 0.36
PRX	**1**	**2**	**3**		**1**	**2**	**3**
LOW	0.40 ± 0.26[H,P]	0.37 ± 0.29[3,M,H,P]	0.22 ± 0.32[2,M,H,P]	*LOW*	0.13 ± 0.43[3]	0.23 ± 0.44[M,H]	0.44 ± 0.65[1,M,H,P]
MEDIUM	0.62 ± 0.31[2,P]	1.09 ± 0.52[1,3,L,P]	0.83 ± 0.45[2,L,P]	*MEDIUM*	0.38 ± 0.16[3,P]	0.58 ± 0.34[L,P]	0.86 ± 0.53[1,L,P]
HIGH	0.76 ± 0.38[2,L,P]	1.18 ± 0.52[1,3,L,P]	0.78 ± 0.41[2,L,P]	*HIGH*	0.34 ± 0.48[2,3,P]	0.65 ± 0.67[1,L,P]	0.87 ± 0.51[1,L,P]
PLA	-0.00 ± 0.33[L,M,H]	-0.30 ± 0.50[L,M,H]	-0.42 ± 0.47[L,M,H]	*PLA*	-0.04 ± 0.13[M,H]	-0.02 ± 0.24[M,H]	0.08 ± 0.17[L,M,H]
PRX/CAF RATIO	**1**	**2**	**3**		**1**	**2**	**3**
LOW	-0.62 ± 0.71	-0.59 ± 0.72	-0.64 ± 0.71	*LOW*[P]	-0.90 ± 0.98	-0.87 ± 0.97	-0.82 ± 0.96
MEDIUM[P]	-2.94 ± 4.40	-2.92 ± 5.40	-2.92 ± 5.40	*MEDIUM*[P]	-1.28 ± 2.21	-0.25 ± 2.20	-1.21 ± 2.20
HIGH[P]	-3.66 ± 6.62	-3.63 ± 6.63	-3.65 ± 6.62	*HIGH*[P]	-1.46 ± 2.05	-1.43 ± 2.06	-1.41 ± 2.05
PLA[M,H]	0.88 ± 2.08	0.44 ± 1.32	0.43 ± 0.82	*PLA*[L,M,H]	0.39 ± 0.81	0.58 ± 0.87	0.43 ± 1.27

Data are means with standard deviations; 1,2,3 – change from 0' to 30', from 0' to 60', from 0' to 90' respectively; [L,M,H,P] – statistically significant differences of post hoc Bonferroni analysis between doses (L – low, M – medium, H – high dose of caffeine, or P – placebo); [1,2,3] – statistically significant differences of post hoc Bonferroni analysis between time-points; CAF – caffeine; PRX – paraxanthine

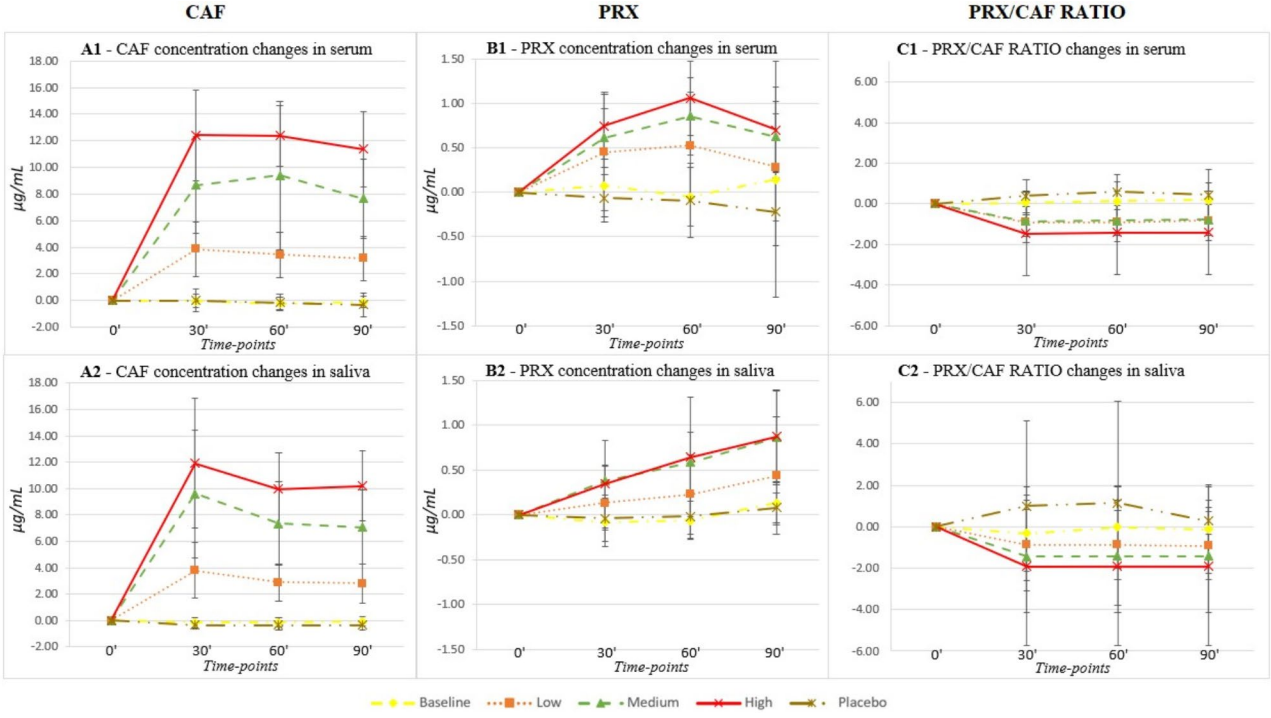

Fig. 3 Changes in CAF and PRX concentrations in serum and saliva. Data are means with standard deviations. A1 – CAF concentration changes in serum; A2 – CAF concentration changes in saliva; B1 – PRX concentration changes in serum; B2 – PRX concentration changes in saliva; C1 – PRX/CAF RATIO changes in serum; C2 – PRX/CAF RATIO changes in saliva

In both the serum and saliva samples, post hoc Bonferroni analysis showed statistical differences ($p<0.001$) between all the doses and *PLA* in terms of the D main effect, with the lowest CAF concentration change after *PLA*, and successively higher after *LOW*, *MED*, *HIGH* doses. A post hoc Bonferroni of the time-point effect on the serum samples revealed significantly higher CAF concentration changes at the 30' vs 90' ($p=0.040$) and at the 60' vs 90' time-points ($p=0.035$); however, the CAF concentrations at the 30' and 60' time-points have been not statistically different ($p>0.05$). For the time-point main effect of saliva samples, higher CAF concentration changes were detected at the 30' vs 60' ($p<0.001$) and 90' ($p<0.001$) time-points; 60' and 90' time-points have been not statistically different ($p>0.05$) (Table 2; Fig. 3, Supplemental Table S2).

For the PRX variables, GLM analysis revealed a significant effect of the D*TP interaction in both the serum ($p<0.001$) and saliva ($p=0.017$) samples. Pairwise post hoc comparison of the serum D*TP interaction with the Bonferroni test showed higher PRX concentration change at the 60' than at the 90' time-point for the *LOW* ($p=0.015$) dose. At the *MEDIUM* dose, a lower PRX concentration change was observed at the 30' vs 60' ($p=0.018$); however higher change was observed at the 60' vs 90' ($p=0.037$) time-points. With respect to the *HIGH* dose, higher PRX concentration change occurred at 60' than at 30' ($p<0.001$) and 90' ($p<0.001$). All of the doses (*LOW*, *MEDIUM*, and *HIGH*) were characterized by significantly higher PRX concentration changes than in the *PLA* ($p<0.001$) at each time-point. Additionally, at the *LOW* dose in 30', 60' and 90' time-points, the change in the PRX concentration was significantly lower vs *HIGH* ($p<0.001$) dose, and at the 60' and 90' time-points of *MEDIUM* ($p<0.001$). Moreover, pairwise post hoc comparison of the saliva D*TP interaction with the Bonferroni test revealed significantly lower PRX concentration changes at 30' vs 90' for the *LOW* ($p=0.016$) and *MEDIUM* ($p<0.001$) doses and at 30' vs 60' ($p=0.019$) and 90' ($p<0.001$) for the *HIGH* dose. Additionally, at the *LOW* dose at the 60' and 90' time-points, the change in the PRX concentration was lower than that at the *MEDIUM* ($p=0.002$; $p<0.001$) and *HIGH* ($p<0.001$; $p<0.001$) doses. All time-points of *MEDIUM* and *HIGH* doses showed higher PRX concentration change ($p<0.001$) in comparison with all *PLA* time-points. (Table 2; Fig. 3, Supplemental Table S2).

The GLM analysis also revealed significant differences in terms of the D main effect in both the serum ($p=0.009$) and saliva ($p=0.018$) samples in terms of the PRX/CAF RATIO. In the serum, post hoc Bonferroni analysis revealed differences between the *PLA* vs *MEDIUM* ($p=0.018$) and *HIGH* ($p=0.002$) doses in terms of the D main effect, with a decrease in the PRX/CAF RATIO after *MEDIUM* and *HIGH* dose, and an increase after *PLA*. The difference between the *LOW* dose and *PLA* was not significant; however, a decrease in the PRX/CAF RATIO was also observed after the *LOW* dose. Saliva PRX/CAF RATIO variables post hoc Bonferroni analysis revealed differences between the *PLA* vs *LOW* ($p=0.025$), *MEDIUM* ($p=0.002$) and *HIGH* ($p<0.001$) doses in terms of the D main effect, with a decrease in the PRX/CAF RATIO at all doses successively and an increase after *PLA*. In the serum and saliva samples, post hoc Bonferroni analysis of the time-point effect revealed no statistically significant differences (Table 2; Fig. 3, Supplemental Table S2).

CYP1A2 enzyme activity

For the prediction of the PRX/CAF RATIO variables at 6 h after CAF ingestion (CYP1A2 enzyme activity), a GLM showed a statistically significant main effect of D. A post hoc Bonferroni showed differences between the *BASE* vs *LOW* ($p=0.004$), *MEDIUM* ($p<0.001$) and *HIGH* ($p<0.001$) doses and between the *PLA* vs *LOW* ($p<0.001$), *MEDIUM* ($p<0.001$), and *HIGH* ($p<0.001$) doses, with lower variables occurring after CAF supplementation. There were no significant differences between the *LOW*, *MEDIUM* and *HIGH* doses (Table 3, Supplemental Table S1).

All the GLM results are presented in Tables 2 and 3, with additional graphic representation of these results in Fig. 3.

SNP of *CYP1A2*

There were no significant interactions of the *CYP1A2* genotype with CAF, PRX, or PRX/CAF RATIO in serum or saliva or with CYP1A2 enzyme activity (effects presented in Table 3 and Supplemental Tables S1 and S2).

Discussion

To our knowledge, this is the first study evaluating the effect and differences between three different doses of CAF or *PLA* on the changes in the concentrations of CAF, PRX and PRX/CAF RATIO at different time-points in both the serum and saliva during a study protocol involving moderately trained people, additionally taking into account CYP1A2 enzyme activity and the *CYP1A2* gene. We demonstrated that, in line with our hypothesis, the higher the CAF dose was, the greater the changes in the CAF and PRX concentrations in both the serum and saliva. Nevertheless, contrary to our hypothesis, the peak change in CAF concentration from pre-supplementation was elicited not 60 but 30 min after intake; CYP1A2 enzyme activity was not dose-dependent, and the *CYP1A2* genotype had no interaction with enzyme inducibility.

As mentioned above, in our study, CAF and PRX concentrations were measured in two biological human matrices. We revealed moderate to strong correlations between serum and saliva CAF and PRX concentrations, indicating the possibility of the common use of less invasive saliva sampling under exercise conditions. However, with respect to CAF, the dependent variables (in saliva to serum) were explained in 79% and PRX in 61%. The good correlation observed in the literature between CAF concentrations in serum and saliva indicates that saliva analysis can be efficiently used to reliably reflect CAF concentrations in serum [28]. In a systematic analysis, Grzegorzewski et al. [14] performed linear regressions to quantify the relationships between saliva and blood-based CAF and PRX measurements. The resulting scaling

Table 3 Main, simple and interaction effects on the CAF, PRX and PRX/CAF RATIO variables

	df	*F* value	*p*-value	η_p^2	df	*F* value	*p*-value	η_p^2	df	*F* value	*p*-value	η_p^2
SERUM	**CAF**				**PRX**				**PRX/CAF RATIO**			
CYP1A2 [C]	1, 24	2.27	0.145	0.09	1, 24	0.00	0.963	0.00	1, 24	1.45	0.241	0.06
Dose [D]	2.23, 53.46	145.00	0.000*	0.86	2.23, 53.46	24.56	0.000*	0.51	1.81, 43.34	5.49	0.009*	0.19
Time-point [TP]	2, 48	2.62	0.084	0.10	2, 48	11.77	0.000*	0.33	1.08, 25.83	0.40	0.550	0.02
Interaction of dose and time-point [D*TP]	3.19, 76.61	0.55	0.662	0.02	5.31, 127.37	4.70	0.000*	0.16	1.09, 26.18	0.35	0.577	0.01
Interaction of dose and *CYP1A2* [D*C]	2.23, 53.46	1.82	0.168	0.07	2.23, 53.46	0.79	0.471	0.03	31.81, 43.34	1.06	0.348	0.04
Interaction of time-point and *CYP1A2* [TP*C]	2, 48	0.17	0.841	0.01	2, 48	0.02	0.985	0.00	1.08, 25.38	0.23	0.656	0.01
Interaction of dose, time-point and *CYP1A2* [D*TP*C]	3.19, 76.61	0.04	0.991	0.00	5.31, 127.37	2.18	0.056	0.08	1.09, 26.18	0.24	0.649	0.01
SALIVA	**CAF**				**PRX**				**PRX/CAF RATIO**			
CYP1A2[C]	1, 24	0.32	0.579	0.01	1, 24	0.15	0.703	0.01	1, 24	0.00	0.947	0.00
Dose [D]	2.72, 65.27	111.78	0.000*	0.82	3, 72	16.03	0.000*	0.40	2.43, 58.36	3.96	0.018*	0.14
Time-point [TP]	1.43, 34.23	5.63	0.014*	0.19	2, 48	15.10	0.000*	0.39	1.70, 40.75	0.80	0.437	0.03
Interaction of dose and time-point [D*TP]	2.36, 56.64	1.26	0.294	0.05	4.61, 110.57	2.97	0.017*	0.11	1.62, 38.89	1.05	0.346	0.04
Interaction of dose and *CYP1A2* [D*C]	2.72, 65.27	0.41	0.727	0.02	3, 72	2.32	0.082	0.09	2.43, 58.36	0.66	0.547	0.03
Interaction of time-point and *CYP1A2* [TP*C]	1.43, 34.23	1.30	0.277	0.05	2, 48	1.63	0.207	0.06	1.70, 40.75	1.12	0.329	0.04
Interaction of dose, time-point and *CYP1A2* [D*TP*C]	2.36, 56.64	0.80	0.470	0.03	4.61, 110.57	2.08	0.079	0.08	1.62, 38.69	0.95	0.378	0.04
CYP1A2 enzyme activity	**CAF**											
CYP1A2 [C]	1, 25	1.75	0.198	0.07								
Dose [D]	1.67, 40.10	12.36	0.000	0.34								
Interaction of dose and *CYP1A2* [D*C]	1.67, 40.10	2.81	0.081	0.10								

* - statistical significance p<0.05; C – *CYP1A2* genotype; D – dose of CAF (LOW, MEDIUM, HIGH) and PLACEBO; TP – measurement time-points (30', 60', 90'); D*TP, D*C, TP*C, D*TP*C – interactions; CAF – caffeine; PRX – paraxanthine; *df* – degrees of freedom; *F* value – statistic value of general linear model; *p*-value – probability variable; η_p^2 – effect size (partial eta square)

factors and Pearson correlation coefficients between saliva to blood-based concentrations of CAF and PRX were 0.79 and 0.68 and 0.84 and 0.76, respectively.

Although current guidelines recommend CAF intake of 3–6 mg_{CAF}/kg_{BM} 30–60 min before the commencement of exercise [7], owing to its effect on the central nervous system, CAF is suspected to be the most ergogenic toward the end of exercise, when perceived effort is increased [29, 30]. Nevertheless, research has shown that after the plasma CAF concentration reaches its peak, it can remain at a steady elevated state even for several hours after intake [31].

Importantly, in contrast to our study, in the majority of research the assessment of CAF or CAF metabolites concentrations was performed solely in serum or plasma (as not the main but rather additional purpose), and blood was taken only at pre-supplementation time-point and one other time-point. Moreover, most exercise studies reported that the concentration of CAF at pre-supplementation time-point after 24 h of abstinence was usually 'zero'; nevertheless, the measured levels may be in fact above zero [32] (ranging from 0.1 to even 5.0 µg/mL [33]). Although the participants were subjected to 24 h of abstinence, similar concentrations were also observed in our study; for this reason, we adopted the method of adjusting the values and assessing the changes in CAF and PRX concentrations from standardized zero levels at the pre-supplementation time-point. Furthermore, it has been shown that major ergogenic effects are unlikely to be induced with doses lower than 3 mg_{CAF}/kg_{BM}, where plasma levels are 2.91–3.88 µg/mL [34]. On the basis of only one previous study [31] analyzing CAF concentrations in plasma at three CAF doses, it was concluded that the *MEDIUM* CAF dose (6 mg_{CAF}/kg_{BM}) may increase CAF plasma levels up to 7.76 µg/mL and that the *HIGH* CAF dose (9 mg_{CAF}/kg_{BM}) up to 11.64–13.58 µg/mL after 60 min of intake. Additionally, it was simultaneously shown that the PRX level did not differ between the 6 and 9 mg_{CAF}/kg_{BM}, suggesting that hepatic CAF metabolism has been already saturated earlier.

Generally, previous studies, including exercise studies, have shown similar values of mean peak CAF concentrations in serum/plasma after CAF intake. The results ranged from 3.90 to 8.19 µg/mL [35–41] for CAF and from 1.08 to 1.60 µg/mL [36, 38, 42] for PRX. Nevertheless, studies have demonstrated that the time to the CAF peak has large inter-individual variations [43, 44]. Moreover, it was speculated that achieving peak serum/plasma concentrations prior to endurance exercise may not be the most important factor in the maximization of CAF ergogenic potential. The other possible explanation may be the CAF concentration at the site of action (in the central nervous system or muscle cells), which is currently impossible to measure. Another possible factor might be the effect of CAF metabolites or the achievement of peak concentration later in endurance exercise, when fatigue and effort perception are heightened [41].

In our study, the CAF concentration was markedly dependent on the CAF dose (the highest at *HIGH* dose). Interestingly, the peak change occurred at each dose from 0' to 30', and then, the level was maintained until 90', suggesting serum CAF sustainability. This could be recognized as a valuable observation in the scientific and practical background. Simmonds et al. [38] demonstrated that 5 mg_{CAF}/kg_{BM} (in comparison with *PLA)* significantly elevated the CAF plasma concentration after 60 min of intake and improved supramaximal cycling in highly trained male cyclists. In a study by Sampaio-Jorge et al. [39], where recreationally trained male cyclists were supplemented with capsules containing 6 mg_{CAF}/kg_{BM} or *PLA*, significant time and treatment main effects on CAF serum concentrations were observed. However, again, blood samples were taken solely at pre-supplementation time-point and 60 min after the intake of CAF. In a study by Stadheim et al. [40] ten healthy highly trained male cross-country skiers were supplemented with 6 mg_{CAF}/kg_{BM} or *PLA* 75 min before the cross-country performance test (C-PT). Blood samples for CAF concentration analysis were taken more often - at rest, 35 min after ingestion, after the last workload on the incremental test and 1 and 14 min after the performance test. Ingestion of CAF increased plasma concentrations to approximately 7.8 µg/mL before 8-km C-PT, and, similarly to our results, it remained at a similar level throughout the exercise trial [40]. Furthermore, in a study by Mesquita et al. [45] blood samples were collected after the intake of 6 mg_{CAF}/kg_{BM}, immediately after the fatigue protocol and 6 h after intake, and the results demonstrated that the CAF serum concentration increased over time, with a peak level of ~8.51 µg/mL after 60 min of ingestion, which contradicts our results. Nevertheless, it is important to note that in this study, CAF was ingested in the form of capsules, not in powder form, as in our study. Additionally, the serum levels significantly decreased after the "fatigue protocol" (sets of 40 bilateral rebound jumps until task failure) and 6 h later. Bell et al. [46] examined the duration of the CAF ergogenic effect after the ingestion of 5 mg_{CAF}/kg_{BM} and registered significantly elevated CAF plasma concentrations throughout exercise. Nevertheless, this variable remained constant for trials conducted 3 and 6 h after ingestion, and its concentrations determined 1 and 3 h after ingestion were greater than those for the trial conducted 6 h after ingestion. In a more complex study, by Conway et al. [47], 3 or 6 mg_{CAF}/kg_{BM} (capsules) or *PLA* was administered 60 min before exercise, and additionally another 3 mg_{CAF}/kg_{BM} (where the previous dosage was also 3 mg_{CAF}/kg_{BM}) 45 min into exercise was given to the participants. Blood samples

were drawn at pre-supplementation time-point, 30 and 60 min after the first supplementation; and additionally 30, 45, 60, and 90 min after the start of exercise and at the end of exercise (120 min after its onset). Similarly to the current work, CAF plasma concentrations increased in a dose-related manner. Its level was significantly elevated after 30 min but peaked within 90 min in the high-dose group. The lower CAF group exhibited an initial peak after 30 min, which was then followed by a slow decline until the second supplementation. Additionally, the CAF concentration was significantly higher in the higher dose group until 60 min into exercise, when the concentrations were similar [47]. In a study by Skinner et al. [41] 14 cyclists and triathletes consumed 6 mg_{CAF}/kg_{BM} or *PLA* either 60 min prior to completing a 40 km time trial or when the start of exercise coincided with the individual peak serum CAF concentration, which was determined from a separate 'CAF profiling' session involving monitoring CAF concentrations in the serum every 30 min over a 4 h period. Surprisingly, the CAF peak concentration (~6.4 µg/mL) occurred as late as 120/150 min after intake, but significant performance improvements were noted when CAF was consumed 60 min prior to exercise. It was then concluded that the ergogenic effect of CAF may not be related to the peak CAF concentration in the serum at the onset of endurance exercise. The second study by Skinner et al. [48] showed that the peak CAF serum concentration (~6.79 µg/mL) was also achieved as late as 120–180 min after the ingestion of 6 mg_{CAF}/kg_{BM}. In the third study by Skinner et al. [49] the serum was analyzed for the CAF concentration at 6 time-points over 4 h following 6 or 9 mg_{CAF}/kg_{BM} (fasted vs fed conditions). It was shown that this CAF time to peak occurred 60 min after ingestions for fasted trials compared with 120 and 180 min after ingestions for fed trials (carbohydrate consumption with CAF). Moreover, a higher dose resulted in a greater CAF peak in the fed condition than in the moderate dose in the fasted condition. The authors suspected that if an athlete was to consume a meal within 60 min of exercise, then a higher CAF dose may be needed to achieve the same serum concentrations as a moderate dose taken when fasted. Furthermore, Davenport et al. [30] investigated the optimal timing of a 200 mg CAF-containing supplement on exercise performance [a) 35 min before the 30 min steady-state cycling (SS); b) at the onset of SS, or c) immediately before the 15 min time trial]. Blood samples were taken at five time-points. CAF plasma concentrations increased under all experimental conditions, and there was a significant interaction effect, with peak values observed at different time-points [30]. Although the intake of 1.5-3 mg_{CAF}/kg_{BM} at around 60 min before the cycling time trial may improve performance [50], the same intake at approximately 180 min before the trial does not explicit similar results [51]. This finding suggests that elevated plasma CAF may not be the only mechanism responsible for improved performance but rather the optimal timing of the ingestion of a low dose of CAF.

In our study, the CAF dose increased the PRX concentration in the serum, and the change in the PRX concentration was also the greatest with the *HIGH* dose and increased gradually until 60', when its peak was reached. There are only sparse studies measuring PRX concentration after CAF intake. Simmonds et al. [38] demonstrated elevated ($p=0.006$) PRX plasma concentrations after 60 min of 5 mg_{CAF}/kg_{BM} intake in comparison with *PLA*. Similarly to our results, Conway et al. [47] showed that the PRX plasma concentration was significantly greater in the higher CAF dose group. However, the authors reported that the increase in the PRX concentration occurred at a slower rate, and no peak was observed, as it continued to increase throughout exercise [47]. Additionally, the PRX concentrations were greater with higher CAF intake and under fasted conditions. The authors suspected that if PRX contributes to the ergogenic CAF potential, the reduced PRX concentration observed following a pre-exercise meal may even limit the ability of CAF to enhance performance [49]. Davenport et al. [30] reported that PRX plasma concentrations increased over the duration of the trial and that there was a main effect for condition, with PRX concentrations being higher at the CAF intake point of 35 min before exercise. Interestingly, although CAF was elevated before and during the exercise test with all strategies, PRX was elevated only prior to exercise when the supplement was ingested 35 min before exercise (the most currently postulated strategy with ergogenic effects) [30].

In terms of saliva measurements, our study revealed that the change in the CAF concentration in saliva was also the greatest in *HIGH* dose. The peak change occurred from 0' to 30'; nevertheless, in contrast to serum, we observed further significant differences between time-points, which suggested lower sustainability in saliva, which was probably influenced by other factors. Furthermore, the CAF dose increased the PRX concentration in the serum, and the change in the PRX concentration in the serum was also the greatest in HIGH dose. The saliva PRX peak was not achieved at the 60' time-point for each dose, but increased gradually at least until the 90' time-point. In another study, measuring saliva, Zawieja et al. [23] collected saliva samples at pre-supplementation time-point, 45 min post-ingestion of 5 mg_{CAF}/kg_{BM} and post-exercise (120 min post-ingestion) to quantify the CAF and PRX concentrations. The CAF concentration increased 30-fold and was approximately 20 times greater than that during the *PLA* trial at 45 min post-ingestion and decreased post-exercise. In

turn, the PRX concentration increased 2.5 times 45 min post-ingestion and then increased further to the next time-point.

Notably, in our study, serum CAF concentration changes did not differ significantly between the 30' and 60' time-point, suggesting the possibility of attaining an earlier plateau, causing a peak in CAF was achieved before exercise. Davenport et al. [30] observed this at approximately 50 min after CAF ingestion, which is in contrast with other studies showing peak CAF concentrations between 75 and 120 min post supplementation [4, 47, 52]. Interestingly, it has been suggested that performance improvements caused by a reduction in perceived exertion may be mediated not only by CAF but also by increased PRX concentrations [30]. It may be concluded that when the plasma CAF concentration decreases, the PRX concentration may still increase, with no indication of a plateau even after 180 min [47]. Its increase in plasma occurs at a slower rate than that of CAF during the period of 60 min [32].

Additionally, we measured CYP1A2 enzyme activity via the mean time-corrected PRX/CAF RATIO in saliva and demonstrated that there was no CAF dose-dependency. Previously, a number of different biological human matrices and metrics, such as urine, plasma, serum, and saliva, have been employed, and CAF has been used as a probe for measuring CYP1A2 enzyme activity. The PRX/CAF concentration ratio at a single time-point correlates with apparent CAF clearance [12]. Metabolic phenotyping with PRX/CAF RATIO is time dependent (ratios increasing with time) and there is a clear CAF-dose dependency (smaller CAF doses increase the metabolic ratio) [14]. Although, it was demonstrated that to reliably assess CYP1A2 enzyme activity, no 24 h CAF abstinence is needed [12], in our study, this abstinence was followed by participants. The 'gold standard' for CYP1A2 enzyme activity estimation is considered to be the saliva molar PRX/CAF RATIO 6 h after CAF intake, owing to its best correlation with intrinsic CAF/PRX clearance [8, 24]. On the basis of these data, Urry et al. [8] developed a method enabling the adjustment of the CYP1A2 enzyme activity values to the optimal values and required 6 h post-dose sampling time-point. This novel technique accounts for the varied time intervals between CAF intake and saliva sampling. Perera et al. [12] quantified plasma and saliva CAF concentrations at different time-points after the ingestion of 100 mg of CAF. No significant difference was found between the PRX/CAF AUC_{0-24} ratio in plasma (0.79) and saliva (0.78) during the CAF abstinence period and during no abstinence (0.83 and 0.85, respectively). In particular, the PRX/CAF RATIO at 4 h demonstrated a strong correlation with all the reference metrics, such as CAF clearance or plasma CAF/PRX. Moreover, a study conducted in healthy males and females [53] assessing CAF as a probe for CYP1A2 enzyme activity reported no evidence of dose dependency. It was also concluded that even with high CAF consumption, steady-state concentrations are too low to saturate CYP1A2-mediated metabolism [13]. Considering the habitual intake of coffee, people who usually drink more than three cups/day usually have significantly higher normal CYP1A2 enzyme activity than individuals who usually consume less. This finding suggests that cessation of regular CAF intake before the measurement of CYP1A1 activity may have resulted in its decrease during the study; thus, it may not reflect an individual's usual CYP1A2 activity [13].

In our study, the functional rs762551 polymorphism of the *CYP1A2* gene was analyzed, but no interaction with CYP1A2 enzyme activity was found. It is a demonstrated determinant of inducible CYP1A2 activity, by which genotypes may be labeled 'highly inducible' (AA) and 'less inducible' (AC, CC) [8, 54]. On the basis of the literature, AA homozygotes may metabolize CAF more quickly and perceive a greater ergogenic effect [55]. In this case, it would appear that PRX may be a more potent adenosine-receptor antagonist, which is elevated sooner in the AA genotype [30]. Nevertheless, several authors [8, 56–59] have demonstrated that there is no significant effect of this SNP on CYP1A2 enzyme activity, as measured by the PRX/CAF RATIO or the mean time-corrected PRX/CAF RATIO, indicating that this *CYP1A2* genotype is not a robust predictor of the CYP1A2 phenotype. The −163 C>A polymorphism is correlated with RATIO only in smokers [58], since the more pronounced increase in CYP1A2 enzyme activity caused by this genetic variation is only observed in current smokers. Smokers with the −163 C/C genotype have been shown to have 40% lower plasma PRX/CAF RATIO than those with the −163 A/A genotype, while no influence of this polymorphism has been detected among nonsmokers [57]. Interestingly, according to Davenport et al. [30] although regular exercise increases *CYP1A2* expression, it seems that 'slow metabolizers' may be able to overcome any genetic disadvantage with training to increase CYP1A2 enzyme activity sufficiently.

Limitations

Notably, our research has several limitations. Firstly, we examined the CAF and PRX concentrations at different time-points over a relatively short period of time (0–90 min); the sampling was not constant over a long period of time, which prevented us from measuring the AUC, and we were not able to measure the possible peak of PRX, as it is believed to peak further than 90 min. Secondly, examining the PRX/CAF RATIO only at these time-points probably influenced the possibility of adequate CYP1A2 enzyme activity measurement. It is established that early and late time-sampling are least suitable

for phenotyping because at these time-points, concentrations may be low, resulting in relatively high random errors and thus a low single to noise ratio. Additionally, in this early stage, the distribution phase of the substance and its absorption and kinetics may further influence the outcome of metabolic ratios. Moreover, metabolic phenotyping with PRX/CAF RATIO is strongly time dependent (the ratio increases with time), and smaller CAF doses and concentrations increase the metabolic ratio (explaining why *BASE* and *PLA* values were profoundly higher) [8, 14, 32]. Nevertheless, as we were aware of this methodological issue, we used a previously developed method to adjust the CYP1A2 enzyme activity to the optimal 6 h after CAF administration sampling [8]. Moreover, the *CYP1A2* genotypes results should be accounted with caution due to relatively small sample of 'fast metabolizers' among the studied participants. Nevertheless, in accordance with the Hardy-Weinberg principle, the frequency of *CYP1A2* SNP in the population is approximately 45% for AAs, 45% for ACs and 10% for CCs [9]. Finally, although we included both males and females in the study protocol, because of the participation of both sexes in CrossFit/HIFT training, we were not able to perform sex-dependent analyses differentiating CAF effects between sexes (uneven groups).

Strengths

The unquestionable strengths of our study were its multiple crossover design protocol and the implementation of three different CAF doses, as well as the *PLA*, in HIFT-trained participants. Secondly, as the first study, we analyzed both serum and saliva CAF and PRX concentrations at different time-points in moderately trained people and measured their CYP1A2 enzyme activity. Thirdly, taking into account the possibility of higher than zero CAF pre-supplementation concentrations, we assessed the effects of CAF on CAF and PRX changes from the pre-supplementation time-point. Furthermore, we used a saliva to serum regression model, which to some extent accounted for changes in CAF or PRX concentrations with time in saliva (concentrations decreased over time) and serum (more constant). In addition, we calculated the required sample size, which was met by the number of participants who took part in the study. Furthermore, all study participants were habitual CAF users who maintained 24 h CAF abstinence and were not smokers. We also ensured full compliance with the intake of supplements and thorough standardization of the procedures.

Conclusions

In conclusion, the present study confirmed the dose- and time-point-dependent effects of CAF supplementation on CAF and PRX concentrations in the serum and saliva of HIFT-trained participants. Furthermore, the peak change in CAF concentration was elicited 30 min after intake, suggesting discrepancies from current CAF supplementation recommendations. Additionally, the earlier CAF plateau and possible late PRX plateau obtained in this study may be important scientific and practical indicators. Nevertheless, there was no evidence of a CAF dose effect on CYP1A2 enzyme activity, nor was there an interaction of the *CYP1A2* genotype with enzyme inducibility. Finally, a correlation between serum and saliva CAF and PRX concentrations was observed, indicating the possibility of common, interchangeable use of less invasive saliva sampling in exercise studies. Future studies should focus on common measurements of CAF and its metabolites in relation to performance and the possibility of achieving peak concentrations later in exercise, when fatigue occurs.

Practical implications

Our research on CAF supplementation in athletes highlights several key practical implications for sports nutrition. Firstly, our results demonstrating peak CAF levels occurring around 30 min post-ingestion may possibly shorten the recommended timing of CAF intake prior to exercise. Moreover, the revealed sustained elevated CAF levels for up to 90 min suggest prolonged ergogenic benefits during exercise. Additionally, while CAF's central nervous system effects are likely most beneficial toward the later stages of exercise when fatigue and perceived effort increase, our study suggests that the ergogenic benefits may not be strictly tied to peak CAF concentrations at the start of exercise. Secondly, our study also emphasizes the relevance of PRX, which continues to increase in concentration even after CAF begins to decline, peaking much later. This delayed increase in PRX might contribute to performance improvements, particularly in reducing perceived exertion toward the end of prolonged activities. Furthermore, the moderate to strong correlation between serum and saliva concentrations of both CAF and PRX indicates that saliva sampling could serve as a less invasive and practical alternative for monitoring these compounds in athletic settings. This nuanced understandings offers athletes and coaches insights into optimizing CAF intake for exercise performance.

Abbreviations

1^{st}	Pre-supplementation (0')
2^{nd}	30 min after supplementation (30')
3^{rd}	60 min after supplementation (60'), pre-exercise
4^{th}	3 min after exercise (90 min after supplementation, 90')
BASE, B	Baseline
BM	Body mass
CAF	Caffeine
HIFT	High-intensity functional training
HIGH, H	9 mg_{CAF}/kg_{BM}
LOW, L	3 mg_{CAF}/kg_{BM}
MEDIUM, M	6 mg_{CAF}/kg_{BM}
PLA, P	Placebo

PRX	Paraxanthine
PRX/CAF RATIO	Paraxanthine to caffeine ratio
SD	Standard deviation
SNP	Single nucleotide polymorphism
T_0–T_4	Order of study visits

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions
Study design/planning: N.G., K.D.-M.; data collection/entry: N.G., J.M., J.A., E.E.Z., A.C., K.D.-M.; data analysis/statistics: N.G., J.M.; data interpretation: N.G., J.M., K.D.-M.; preparation of the first draft version of the manuscript: N.G., J.M., K.D.-M.; literature analysis/search: N.G.; collection of funds: N.G., K.D.-M. All authors were involved in drafting versions and critically revising for important intellectual content. All authors have contributed to and approved the final version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was approved by the Bioethics Committee at Poznan University of Medical Sciences (reference number 293/17 of May 11, 2017. All participants gave written informed consent for participation in the study.

Consent for publication
All authors support the submission to this journal.

Competing interests
The authors declare no competing interests.

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