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SHORT COMMUNICATION

Preanalytical standardization of amino acid and acylcarnitine metabolite profiling in human blood using tandem mass spectrometry

Romy Brauer · Alexander Benedikt Leichtle · Georg Martin Fiedler · Joachim Thiery · Uta Ceglarek

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Abstract Quantitative metabolite profiling in biological samples has the potential to reflect physiological status and to identify disease associated disturbances in metabolic networks. However, this approach is hampered by a wide range of preanalytical variables. Hence, the aim of our study was to develop a standardized preanalytical protocol for metabolite profiling of amino acids and acylcarnitines in human blood. Amino acids and acylcarnitines were simultaneous analyzed after butylation of 3 μL dried blood or 10 μL whole blood, serum and anticoagulated plasma using electrospray tandem-mass spectrometry. The influence of exogenous and endogenous preanalytical variables was investigated in healthy volunteers. Different sampling materials and anticoagulants for blood taking were investigated. Concentrations of long-chain acylcarnitines were 5-fold higher in EDTA-whole blood or dried whole blood compared to serum and anticoagulated plasma. Significant differences in amino acid concentrations were found for capillary versus venous blood taking. Fasting for 8 h before specimen collection minimized the nutritional influence. Physical activity significantly alters amino acid and short chain acylcarnitine concentrations. As a result of our preanalytical investigation we developed a pre-treatment protocol based on EDTA whole blood dried on filter paper to reduce the preanalytical variability and facilitate reproducible quantitative metabolite profiling in clinical trials.

Keywords Metabolite profiling · Preanalytical standardization · Amino acids · Acylcarnitines · Dried blood

1 Introduction

The quantification of small molecular weight components (metabolites) of biological systems becomes more and more important to get deep insight into molecular networks and the influence of genetics, epigenetic environmental exposures, diet and behaviour (Dunn 2008; Hall 2006; Mashego et al. 2007). Clinical metabolomics is a rapidly growing area of contemporary science focussing on various disease-related metabolic effects. In large scale clinical studies standardized preanalytical protocols are necessary to minimize in vivo and in vitro variability (Bruce et al. 2008; Gika et al. 2008). Careful validation of the experimental setup and consideration of the biological variations are essential for metabolome analysis in human body fluids because actual dietary and environmental factors may significantly affect metabolite concentrations. A wide range of exogenous (e.g. sample specimen, storage conditions) and endogenous factors (e.g. physical activity,

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R. Brauer · A. B. Leichtle · G. M. Fiedler · J. Thiery · U. Ceglarek (✉)
Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University Hospital Leipzig, Liebigstrasse 27, 04103 Leipzig, Germany
e-mail: uta.ceglarek@medizin.uni-leipzig.de

R. Brauer
e-mail: romy.brauer@medizin.uni-leipzig.de

A. B. Leichtle
e-mail: alexander.leichtle@medizin.uni-leipzig.de

G. M. Fiedler
e-mail: martin.fiedler@medizin.uni-leipzig.de

J. Thiery
e-mail: thiery@medizin.uni-leipzig.de

nutrition) have to be considered (Ceglarek et al. 2009; Narayanan 2000; Zivkovic et al. 2009a).

In our study we developed a standardized protocol for reproducible profiling of amino acid (AA) and acylcarnitine (AC) analysis in various human blood specimens. AAs and ACs are actually determined in worldwide screening programs for the diagnosis of inborn metabolic disorders of the amino acid and fatty acid metabolism (Arn 2007; Chace et al. 1997; Chace and Kalas 2005; Wilcken 2007; Wilcken and Wiley 2008). Sample pretreatment protocols were established with the focus on an accurate diagnosis of inborn errors, associated with a dramatic increase or decrease of specific amino acids or acylcarnitines (Ceglarek et al. 2002; De Jesus et al. 2010; Rashed et al. 1995). Beyond that, the different metabolic pathways, which could be mapped by the metabolic screening of amino acids and acylcarnitines, are modulated by a wide variety of enzyme systems. As example, the pathways of branched chain amino acids, the fatty acid beta oxidation cycle, or the carnitine shuttle system are involved in the production of energy or in energy balance (Foster 2004) and seem to be equally exciting for metabolome studies in the field of life-style associated disease as metabolic syndrome or diabetes mellitus (Jogl et al. 2004; Ronnett et al. 2005). However, for the prospective detection of commonly slighter effects of disease-related metabolic alterations, additional emphasis should be placed on the preanalytical variability of amino acid and acylcarnitine metabolite profiling. Hence, the aim of this study was to establish a well-defined protocol for blood sample collection, storage, and processing procedures for precise metabolite analyses and minimization of variability of preanalytical origin. We optimized sample storage and pretreatment procedures to improve accuracy and data reproducibility. Applying the standardized protocol, we additionally investigated the influences of diet and physical activity as examples for in vivo variability on the amino acid and acylcarnitine metabolite profile.

2 Materials and methods

2.1 Chemical and reagents

Methanol and isopropanol were purchased from Merck (Darmstadt, Germany). Water (HPLC grade) was obtained from J. T. Baker (Deventer, Netherlands). AA and AC reference isotope labelled standard kits (NSK-A, NSK-B, Cambridge Isotope Laboratories, Andover, USA) were used as internal standard. 3N butanolic HCl was made in-house using 1-butanol (for spectroscopy) from Merck (Darmstadt, Germany) and acetyl chloride (p.a.) from Sigma-Aldrich (Steinheim, Germany). Dried blood controls for amino acids and acylcarnitines (Levels 1 and 2) were obtained from Chromsystems (Munich, Germany). Amino acid standards (A6407 acidic and neutral and A6282 alkaline) were purchased from Sigma-Aldrich (Steinheim, Germany). Lactate was measured on the Cobas 6000 system from Roche (Grenzach-Wyhlen, Germany) using capillary plasma taken with heparin-fluorid microvettes of Sarstedt (Nümbrecht, Germany).

Filter paper grade 903 was obtained from Whatman GmbH (Dassel, Germany). Multifly needle sets and polypropylene monovettes with and without anticoagulants (EDTA, citrate, and lithium-heparinate) were obtained from Sarstedt (Nümbrecht, Germany). Tubes for capillary blood taking (200 µL) were purchased by Kabe (Nümbrecht-Elsenroth, Germany). Foil-barrier ziploc bags and desiccant packets were obtained from Whatman GmbH (Dassel, Germany).

2.2 Study subjects

Blood samples from 5 healthy volunteers (3 female and 2 male; age range 24–34 years) were obtained by peripherally collected native and EDTA whole blood dropped on filter paper, serum, EDTA plasma, lithium-heparinate plasma, and citrate plasma.

To examine the influence of nutrition we analyzed 10 healthy volunteers (5/5 m/f, age 22–29 years, BMI 18–25) fasting >5 h, 3 h postprandial and 5 h postprandial after a standardized meal. For the investigation of physical activity, residual whole blood from lactate determinations of 15 healthy volunteers (15/0 m/f, age 17-36 years, BMI 22-29) before, directly after intense running exercise (running speed 4.5 m/s maximum for 1600 m, total 8000 m), and after a recovery phase (running speed 2.5 m/s for 1600 m) was used. All volunteers gave informed consent. The study was conducted according to the Helsinki declaration and approved by the local Ethics Committee.

2.3 Sample collection

Venous native and EDTA whole blood was spotted on filter paper in 40 µL portions. Samples were dried at least 3 h before processing. Dried blood spots were punched using a Multipuncher (PerkinElmer Wallac GmbH; Freiburg, Germany). Samples were analyzed in three replicates for each specimen and volunteer. For investigation of blood taking conditions (venous versus capillary blood taking) venous blood was collected in EDTA monovettes and capillary blood in 200 µL EDTA tubes with capillaries ($n = 12$ for each material and volunteer). The influence of the hematocrit was examined by obtaining samples (hematocrit range 39.5-46.3%) of peripherally collected EDTA whole blood. We prepared 5 dilutions from 100%

EDTA whole blood to 33% EDTA whole blood ($n = 12$ for each dilution and volunteer). Central and peripheral spots from the dropped whole blood were punched out. To evaluate the influence of the drying time, pooled EDTA whole blood was dropped on filter paper for a drying time of 3 and 24 h at room temperature and 4°C ($n = 12$). To examine batch versus consecutive processing and analysis, dried EDTA whole blood was stored at −80°C in foil-barrier ziploc bags containing MiniPax sorbent packets until pretreatment ($n = 10$).

To investigate the influence of physical activity, dried whole blood, lactate, AS and AC concentrations were measured before, at the point of maximum exercise and after the recovery phase.

2.4 Sample pretreatment and analysis

A sample pretreatment protocol was used according to our formerly described procedures (Ceglarek et al. 2002). 3.0 mm diameter punches (3 µL whole blood) of the dropped filter paper were placed into 96-well polypropylene microtiter plates. Dried blood spots were extracted with 100 µL of a methanol solution containing isotope labelled amino acid and acylcarnitine standards for 30 min. Serum and plasma samples were diluted 1:10 with methanol. After centrifugation, 10 µL of the supernatant was diluted with 100 µL of the internal standard solution and placed in 96-well polypropylene microtiter plates. Samples were evaporated at 70°C for 40 min and derivatized using 60 µL of 3N butanolic-HCL at 65°C. After evaporation the samples were reconstituted with 150 µL of the mobile phase (1/1 v/v isopropanol/water).

An API 2000 tandem mass spectrometer (Applied Biosystems, Germany) using a Turbo Ion Spray Source (TIS) in combination with a HTC Pal autosampler and a PE 200 micro gradient pump was used for flow injection analysis (FIA). 25 µL of the sample were directly injected at a flow rate of 100 µL/min in an analysis time of 1.5 min. We detected AA by a neutral loss scan of 102 in the mass range of 130–280 or multiple reaction monitoring (MRM). For AC a precursor ion scan of m/z 85^+ was used scanning from m/z 200–510 (Mueller et al. 2003). Quantitative analysis using the internal standard concentrations for 26 AA, free carnitine, and 34 AC was performed using ChemoView™ 1.4.2 (AB SCIEX, Darmstadt, Germany). In Tables 1–3 of the supplement the analytes, the detection mode, and the corresponding internal standards used for quantification are summarized (Fahy et al. 2007).

2.5 Precision, accuracy and linearity

Accuracy and linearity were proven for amino acids using a methanolic amino acid standard solution at 2.5, 25 and 250 µmol/L. Within-day variability was determined by analyzing 20 dried blood quality control samples in two different concentration ranges. Between-day variability was determined by analyzing two commercial dried blood quality control samples (L1 and L2) on 20 consecutive days.

2.6 Statistical analysis

SPSS17 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Biometrical data are reported as means ±2 standard deviations (SD), or medians and interquartile ranges, where appropriate. The differences of the means were compared by the F-test or Students t-test. Statistical significance was defined as $P < 0.05$.

3 Results and discussion

3.1 Method validation

The application of our mass spectrometric method enables the simultaneous detection of about 26 amino acids and 35 acylcarnitines in only 1.5 min. As shown in Table 4 of the supplement, the direct quantification via a corresponding internal standard resulted in recovery rates between 80 and 120% for alanine, citrulline, glutamic acid, glycine, methionine, phenylalanine, proline, tyrosine, lysine, arginine and ornithine. For AA without corresponding isotope labelled internal standard overestimation (e.g. histidine 338–360%), or underestimation (e.g. threonine 8–18%) were observed. The lack in accuracy reflects the different ion suppression effects of the sample matrix on the analyte and the internal standard.

However, sufficient linearity from 2.5 to 250 µmol/L and regression coefficients >0.96 was achieved for all amino acids. Additionally, variation coefficients were found in the range between 4 and 30%. Hence, the semi-quantitative analysis of AA using our mass spectrometric method is appropriate for the identification of relative concentration differences between defined study groups. Similar results were shown for free carnitine, octanoylcarnitine, and hexadecanoylcarnitine as previously published (Mueller et al. 2003). For 33 amino acids and acylcarnitines between-day variability <25% (AA) and <30% (AC) for 2 sets of commercial dried blood controls were determined as shown in Table 5 of the supplement.

3.2 Exogenous influencing factors

3.2.1 Specimen collection conditions

Main criteria of specimen selection for clinical studies are the availability and amount of the body fluid for sampling,

the concentration of the required analytes in the respective material, and the possibility of standardized sampling and storage. Metabolome studies were already performed using serum, plasma or urine samples (Illig et al. 2010; Lv et al. 2010; Seeger 2009; Zhao et al. 2010). However, the use of dried blood has been shown to be advantageous for large scale studies due to the easy sample management, storage and shipment (Leichtle et al. 2010; Michopoulos et al. 2010).

The comparison of dried whole blood and dried EDTA-whole blood resulted in comparable concentrations of AA and AC. In serum and anticoagulated plasma the concentrations of AA and AC (C0–C10) were 1.5-2-fold higher compared to dried whole blood, which can be explained by a lower intracellular concentration of AA in erythrocytes (Johnson and Bergeim 1951). Additionally, we obtained 10% lower concentration in plasma compared to serum. This is caused by dilution effects of the anticoagulants and its osmotic effects in the plasma samples (Fiedler and Thiery 2004). Interestingly, we detected 6–8-fold higher arginine concentrations in serum and plasma compared to dried blood (Fig. 1). It is known from literature that in erythrocytes and monocytes arginase can be released by hemolysis and could convert arginine to ornithine (Morris 2009; Omodeo-Sale et al. 2010). This may explain the low arginine concentration in dried blood samples. However, the expected increase of ornithine concentrations could not be observed. For long chain AC (C14–C18:2), which are mainly involved in the intracellular beta-oxidation, in dried blood up to 5-fold higher concentrations were found compared to serum (Fig. 1). Hence, we decided to apply native or EDTA dried whole blood in our preanalytical protocol (Fig. 2).

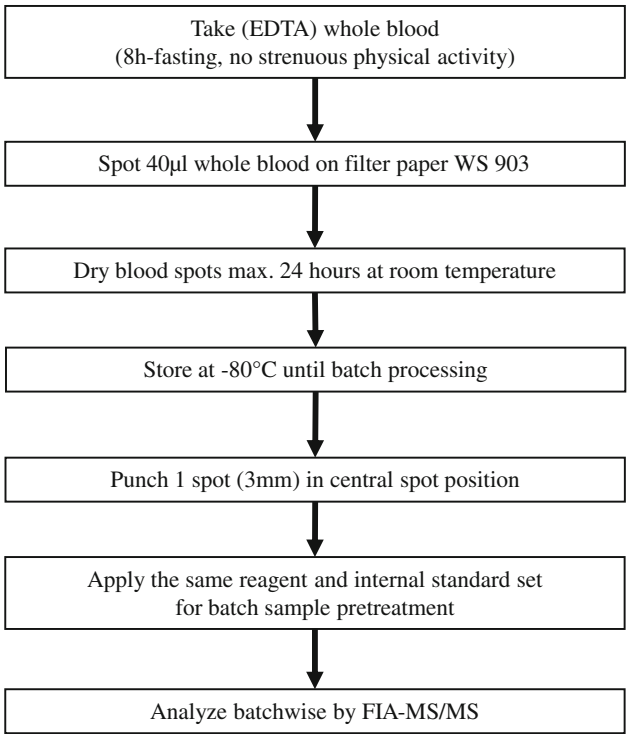

Fig. 2 Standardized preanalytical protocol for blood taking and processing for the determination of amino acids and acylcarnitines in dried blood

3.2.2 *Dried blood processing and storage*

With declining hematocrit we observed a decreased concentration of AAs and ACs as described by Holub et al. (Holub et al. 2006). The hematocrit dependency of the sample volume/spot was previously shown (Leichtle et al.

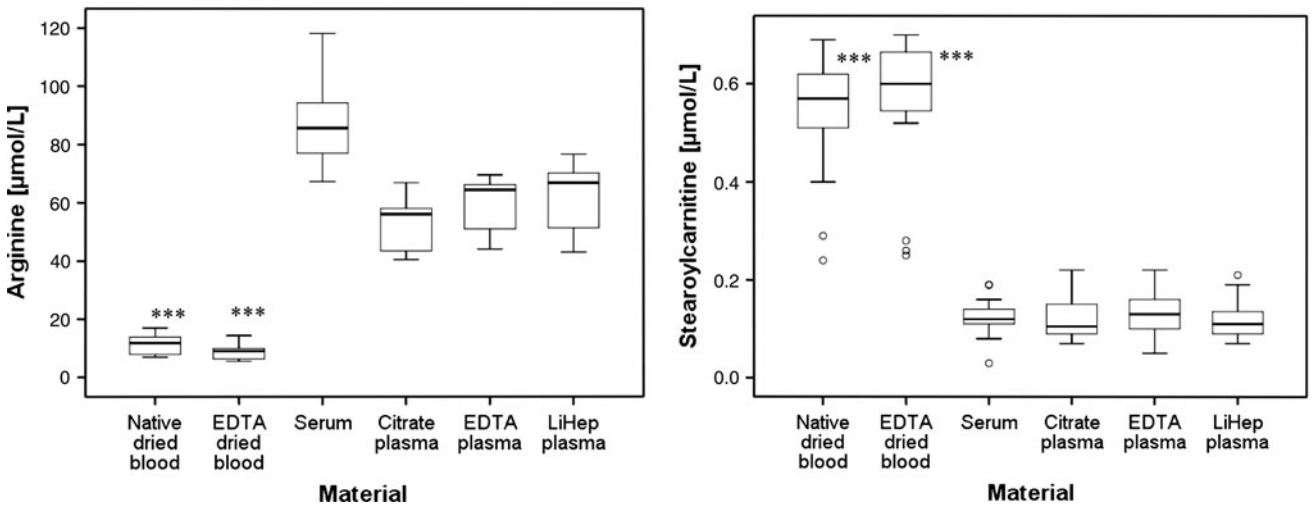

Fig. 1 Differences in arginine and C18-carnitine concentrations in dried whole blood, dried EDTA-whole blood, serum, EDTA-, citrate-, and heparinate plasma ($n = 5$). *** $P < 0.001$

2010). Furthermore, we observed an increase in the blood spot diameter with declining hematocrit. In samples with low hematocrit, levels of most AA were higher in the peripheral than in the central spot ($P < 0.05$). Therefore, spots should be punched always from the centre (see protocol in Fig. 2). The influence of the hematocrit is a limitation of dried blood compared to plasma or serum processing, especially if a varying hematocrit is prevalent in the study population.

The comparison of dried EDTA-whole blood derived from venous versus capillary blood taking resulted in significant differences for ornithine (42 µmol/L capillary versus 78 µmol/L venous), serine (81 µmol/L capillary versus 109 µmol/L venous) and taurine (0.59 µmol/L capillary versus 1.20 µmol/L venous). These differences may reflect different metabolic conditions in the central venous and arterial capillary blood vessels. In Fig. 3 the influence of venous versus capillary blood taking on the concentration of ornithine with an individual difference between 50 and 65% is presented. As shown for free carnitine, these effects were not observed for acylcarnitines. Most AAs and ACs showed no significant concentration changes up to 24 h storage at room temperature or 5°C, respectively. Only arginine increased as shown in Fig. 4 and the concentration of ornithine, sarcosine and serine decreased significantly for both storage temperatures ($P < 0.05$). Storage at −80°C for 3 months revealed no significant changes of AA and AC concentration. Similar long-term storage conditions were recommended for lipids (fatty acids, phospholipids, cholesterol ester), too (Zivkovic et al. 2009b).

Daily variation in sample preparation and analysis conditions may significantly influence the variability of the results in large clinical studies. The differences of quantitative amino acid and acylcarnitine profiling results between consecutive analysis of each dried blood sample (at least 24 h after each blood taking) and batch metabolite analysis of all study samples were previously shown (Ceglarek et al. 2009). We could verify this observation in our actual study. Therefore, batch wise sample processing is recommended in our protocol (Fig. 2).

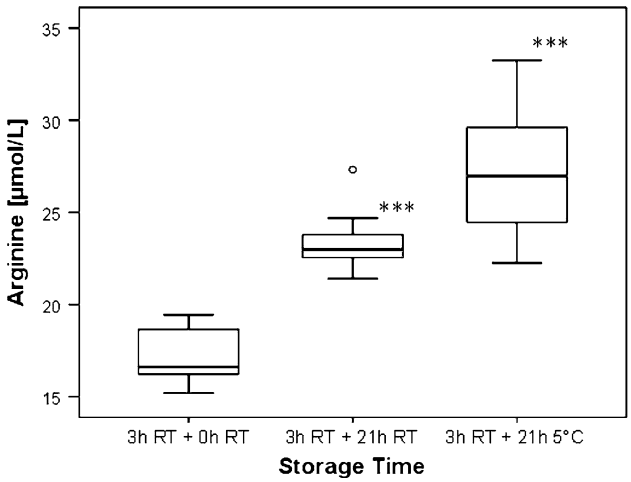

Fig. 4 Influence of the storage temperature and time on the arginine concentrations in dried EDTA-whole blood. RT-room temperature, *** $P < 0.001$

3.3 Endogenous influencing factors

3.3.1 Effect of fasting

It is well known that a fatty meal increases lipid concentrations up to 9 h. Ideally, probands should be instructed to fast overnight at least 12 h (Guder et al. 1996). Otherwise,

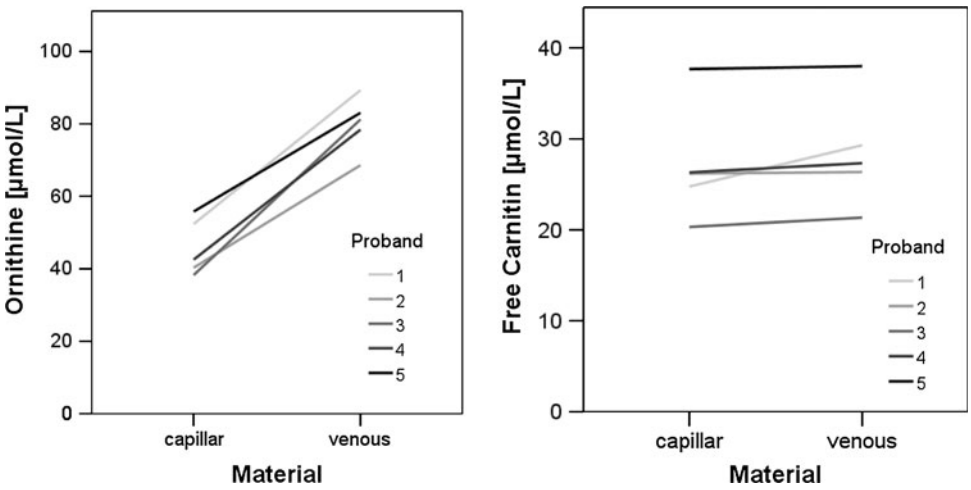

Fig. 3 Individual differences in ornithine and carnitine concentrations between venous and capillary blood taking ($n = 5$). *** $P < 0.001$

Table 1 Significantly differing amino acid and acylcarnitine concentrations in whole blood 3 and 5 h postprandial (10 healthy volunteers)

AS/AC [µmol/L]	Fasting (A)		3 h postprandial (B)		P_{A-B}	5 h postprandial (C)		P_{A-C}
	$\bar{x}$	95% CI	$\bar{x}$	95% CI		$\bar{x}$	95% CI	
Gln	10	7–11	11	9–16	*	12	9–15	*
OH-Prol	271	217–389	346	297–489	**	384	261–492	*
His	89	73–109	115	88–178	n.s.	162	101–255	*
Leu/Ile[a]	78	64–103	109	80–136	**	107	82–142	*
Met[a]	15	11–24	20	14–30	**	21	11–27	*
Orn	53	34–65	65	38–111	**	65	41–168	*
Phe[a]	40	29–61	58	51–67	**	55	53–68	*
Pro	118	67–166	185	118–240	**	194	118–256	**
Sarc	86	66–123	97	75–138	n.s.	103	83–147	*
Ser	66	53–77	70	54–85	n.s.	83	58–127	*
Tyr	37	30–60	57	45–68	**	58	48–84	*
Val[a]	160	136–210	193	162–248	*	188	166–220	n.s.
C2	13.4	9.5–17.0	11.4	4.1–13.0	**	11.4	6.2–14.3	n.s.
C6DC	0.01	0.00–0.01	0.01	0.00–0.05	n.s.	0.01	0.00–0.05	*
C8	0.15	0.09–0.73	0.04	0.00–0.29	**	0.11	0.04–0.20	n.s.
C10	0.25	0.14–0.71	0.12	0.04–0.42	**	0.13	0.06–0.24	*
C14:1	0.1	0.03–0.24	0.04	0.00–0.09	**	0.03	0.00–0.07	**

[a] Essential AA, $\bar{x}$ median, *P* significance level between group A and B (P_{A-B}) and group A–C (P_{A-C}) (Wilcoxon-test): * $P < 0.05$, ** $P < 0.01$, n.s. not significant

prolonged fasting influences the metabolism of valine, leucine or isoleucine (Bremer et al. 1981). Besides lysine, threonine and tryptophan all essential amino acids and glutamine, hydroxyproline, ornithine, proline, and tyrosine significantly increased 3 and 5 h postprandial. In contrast, the concentrations of acylcarnitines (C2, C6DC, C8, C10, C14:1) significantly decreased in concentration 3 and 5 h postprandial (Table 1). Other amino acids and acylcarnitines were not affected by nutritional influences. This observation indicates that a fasting period longer than 5 h has to be considered. As a compromise we decided to define an 8 h fasting period in our standard protocol.

3.3.2 Effect of strenuous exercise

Strenuous exercise affects laboratory results as known for creatinkinase, steroids, and catecholamines (Narayanan 2000). Muscular effort and the resulting changes in the energy balance should affect the amino acid and carnitine metabolism, too. We investigated metabolic changes of the amino acid and acylcarnitine levels in 15 healthy male athletes during a fitness test based on 5 increasing running levels and a following recovery phase under standardized preanalytical conditions. As shown in Table 2, lactate as marker for anaerobic metabolism increased with increasing physical strain and decreased till the end of the recovery phase in all subjects. This observation is confirmed by an increase of alanine and acetylcarnitine, too. Concentration of sarcosine, propionylcarnitine, and octanoylcarnitine changed in the same way. Elevations of sarcosine concentrations are known from patients with mitochondrial disorders due to the damage of muscle tissue (Blau et al. 2003). Other amino acids and acylcarnitines were not significantly influenced during the exercise experiment.

We divided study probands into three groups according to the maximum lactate level. As shown in Fig. 5, in the group of athletes with the lowest maximum lactate level the concentration of alanine, arginine and acetylcarnitine decline to the range of the starting conditions, whereas in the group with the highest lactate concentrations the metabolite concentrations still increased. These results indicate an individual varying adaption mechanism.

4 Conclusions

Our investigations underline the importance of various in vitro and in vivo preanalytical variables on the results of metabolite analysis in human blood. Therefore, all metabolic parameters need to be carefully evaluated before clinical studies regarding their preanalytical interferences. The application of our pretreatment protocol for

Table 2 Significant changes of amino acid and acylcarnitines concentrations after physical activity

Analyte	Before exercise (A)		Maximum exercise (B)			After recovery (C)			
	x̄	95% CI	x̄	95% CI	P_{A-B}	x̄	95% CI	P_{A-C}	P_{B-C}
Lactate [mmol/L]	2.0	0.9–3.9	12.5	6.3–18.2	***	3.24	1.3–7.0	***	***
Amino acids and acylcarnitines [μmol/L]									
Aba	19	13–27	15	10–18	**	15	10–27	*	n.s.
Ala	236	150–306	318	213–404	***	263	162–409	**	n.s.
Arg	21	13–36	19	13–30	n.s.	15	11–29	n.s.	*
Orn	52	45–88	39	25–56	**	46	33–65	*	n.s.
Sarc	145	114–199	207	155–251	***	182	134–253	**	n.s.
C2-Carn	11	8–16	17	13–21	***	16	9–23	**	n.s.
C3-Carn	1.2	0.9–2.6	2	0.9–2.6	*	1.4	0.8–2.5	n.s.	n.s.
C8-Carn	0.05	0.0–0.21	0.08	0.0–0.32	*	0.07	0.0–0.35	n.s.	n.s.

Before exercise (A), at maximum exercise (B) and after the recovery phase (C) ($n = 15$) (other AA and AC did not changed significantly)

[a] Essential AA; x̄, median; *P*-value (Mann–Whitney-U-test): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

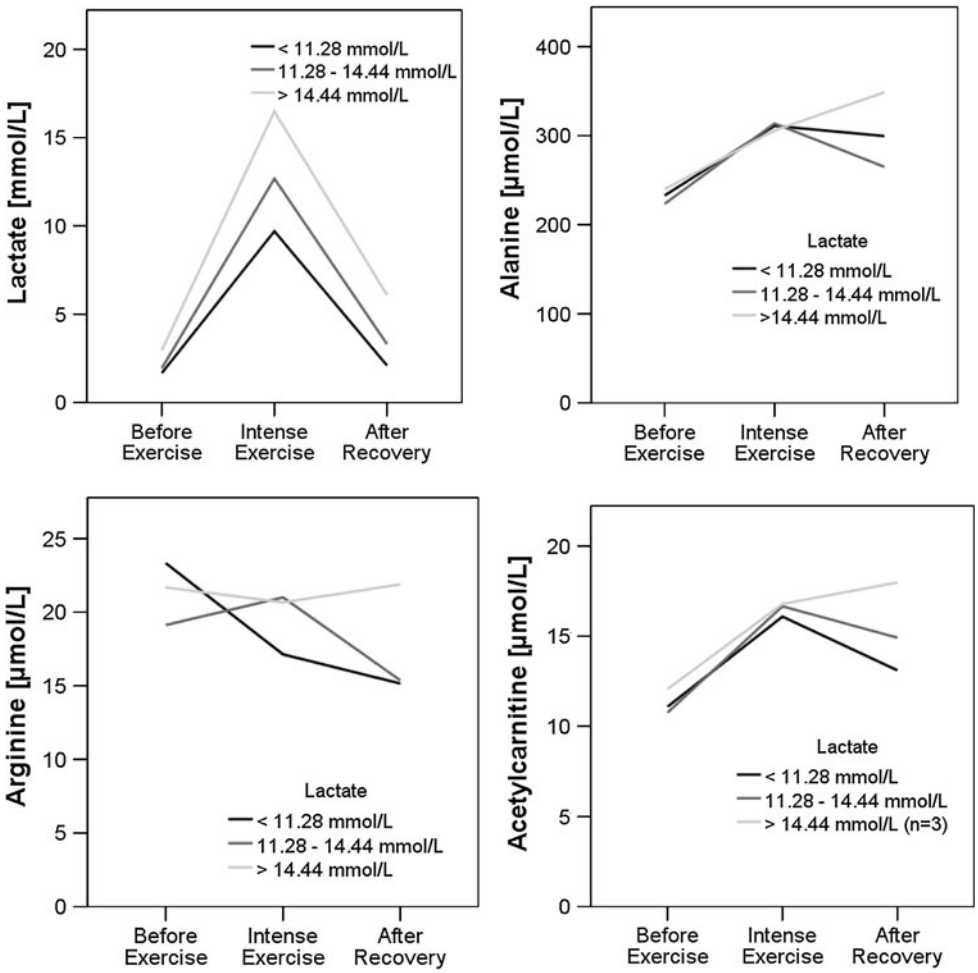

Fig. 5 Strenuous physical activity (endurance running test) and significant changes of lactate, alanine, arginine and acteylcarnitine concentrations in three groups divided according to the maximum lactate concentration <12.28 mmol/L (<25 percentile), 12.28–14.44 mmol/L (25–75 percentile), and >14.44 mmol/L (>75 percentile)

quantitative amino acids and acylcarnitine profiling in dried blood (Fig. 2) allows standardization of critical procedures in the preanalytical phase and facilitates the reproducible metabolite profiling.

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