

Preanalytical Aspects and Sample Quality Assessment in Metabolomics Studies of Human Blood

Peiyuan Yin,^{1†} Andreas Peter,^{2,3†} Holger Franken,^{4†} Xinjie Zhao,¹ Sabine S. Neukamm,^{2,3} Lars Rosenbaum,⁴ Marianna Lucio,⁵ Andreas Zell,⁴ Hans-Ulrich Häring,^{3,6} Guowang Xu,^{1*} and Rainer Lehmann^{2,3*}

BACKGROUND: Metabolomics is a powerful tool that is increasingly used in clinical research. Although excellent sample quality is essential, it can easily be compromised by undetected preanalytical errors. We set out to identify critical preanalytical steps and biomarkers that reflect preanalytical inaccuracies.

METHODS: We systematically investigated the effects of preanalytical variables (blood collection tubes, hemolysis, temperature and time before further processing, and number of freeze–thaw cycles) on metabolomics studies of clinical blood and plasma samples using a nontargeted LC-MS approach.

RESULTS: Serum and heparinate blood collection tubes led to chemical noise in the mass spectra. Distinct, significant changes of 64 features in the EDTA-plasma metabolome were detected when blood was exposed to room temperature for 2, 4, 8, and 24 h. The resulting pattern was characterized by increases in hypoxanthine and sphingosine 1-phosphate (800% and 380%, respectively, at 2 h). In contrast, the plasma metabolome was stable for up to 4 h when EDTA blood samples were immediately placed in iced water. Hemolysis also caused numerous changes in the metabolic profile. Unexpectedly, up to 4 freeze–thaw cycles only slightly changed the EDTA-plasma metabolome, but increased the individual variability.

CONCLUSIONS: Nontargeted metabolomics investigations led to the following recommendations for the preanalytical phase: test the blood collection tubes, avoid hemolysis, place whole blood immediately in ice

water, use EDTA plasma, and preferably use nonre-frozen biobank samples. To exclude outliers due to preanalytical errors, inspect the biomarker signal intensities reflecting systematic as well as accidental and preanalytical inaccuracies before processing the bioinformatics data.

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Metabolic profiling approaches are increasingly applied in clinical research (1). Sophisticated study designs, high-quality analytical instrumentation, and bioinformatic data evaluation by experienced personnel are prerequisites for successful metabolomics projects. However, even the best study designs, the most expert operators, and the most sophisticated instrumentation are of little use if the sample collection and handling procedures are poorly standardized. For this reason, the preanalytical process is a critical factor for the success of metabolomics research studies. There are 2 major preanalytical phases: first, the period comprising patient preparation, blood sample collection, and storage in a freezer; and second, the pretreatment of stored samples for subsequent metabolomics analysis, e.g., by LC-MS (2–6). Typically, the sample pretreatment process follows a standard operating procedure (SOP)⁷ based on evaluated protocols (7). The initial preanalytical phase of blood collection is frequently embedded in the clinical work flow and is less standardized. Blood processing involves multiple steps, including transportation, centrifugation, and sample handling, that may cause accidental preanalytical errors

¹ CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China; ² Division of Clinical Chemistry and Pathobiochemistry (Central Laboratory), University Hospital Tuebingen, Tuebingen, Germany; ³ Paul Langerhans Institute Tuebingen (Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Centre Munich at the University of Tuebingen), Tuebingen, Germany; ⁴ Centre for Bioinformatics (ZBIT), University of Tuebingen, Tuebingen, Germany; ⁵ Helmholtz-Center Munich—German Research Center for Environmental Health, Institute for Ecological Chemistry, Ingoldstaedter Landstraße, Neuherberg, Germany; ⁶ Department of Internal Medicine, Division of Endocrinology, Diabetology, Vascular Medicine, Nephrology and Clinical Chemistry, University Hospital Tuebingen, Tuebingen, Germany.

† Peiyuan Yin, Andreas Peter, and Holger Franken contributed equally to the work, and should be considered as first authors.

* Address correspondence to: G.X. at CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Rd., Dalian 116023, China. Fax +86-411-84379559; e-mail xugw@dicp.ac.cn. R.L. at Division of Clinical Chemistry and Pathobiochemistry, University Hospital Tuebingen, Otfried-Mueller-Str. 10, D-72076 Tuebingen, Germany. Fax +49-(0)7071-29-5348; e-mail Rainer.Lehmann@med.uni-tuebingen.de.

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⁷ Nonstandard abbreviations: SOP, standard operating procedure; UPLC, ultra-performance liquid chromatography; qTOF-MS, quadrupole-time of flight mass spectrometry; MS/MS, tandem MS; PCA, principal component analysis; FDR, false discovery rate; AST, aspartate aminotransferase; CRP, C-reactive protein; lyso-PC, lysophosphatidylcholine; S-1-P, sphingosine 1-phosphate.

that result in sample outliers. In routine clinical diagnostics, the preanalytical phase may account for 60%–80% of laboratory testing errors (8–10). Notably, metabolomics study analysts who receive only frozen or deproteinized sample aliquots cannot check the initial sample quality to detect these errors.

Selected preanalytical aspects of targeted metabolomics investigations of human blood and urine by GC-MS and LC-MS (11–17), as well as the effects of delayed storage on human cerebrospinal fluid metabolites (18), have been reported. Recently, protocols for metabolic profiling of serum and plasma have been published (3, 7, 19). However, none of these protocols or even mandatory preanalytical SOPs can absolutely prevent random errors that produce poor individual sample quality.

Therefore, we sought to identify metabolite biomarkers to detect outliers with poor sample quality due to preanalytical errors in nontargeted metabolomics studies and to eliminate those samples that could result in misleading interpretations before time-consuming bioinformatics data evaluations. We focused on the most error-prone bedside-to-bench preanalytical steps; i.e., from blood drawing to blood cell separation by centrifugation and additional repetitive thawing of biobank samples.

Materials and Methods

SAMPLES AND STUDY DESIGN

To study the impact of blood collection tubes on the results of metabolomics analyses, we investigated the suitability of 5 different S-monovettes (Sarstedt). The tubes (4 plasma tubes and 1 serum tube) were filled with either blank solution (50 g/L human albumin in normal saline) or blood from 3 healthy volunteers. Serum was allowed to clot in an upright position for 25 min at room temperature. The 4 different blood plasma collection tubes (lithium-heparinate, sodium fluoride, sodium citrate, and potassium EDTA) were gently mixed and kept in ice water for 25 min. Metabolomic sample pretreatment was immediately performed after centrifugation at 2200g for 7 min at 4 °C. For all subsequent experiments performed to study the effects of sample handling, blood was collected into K⁺-EDTA sample tubes (Sarstedt). Informed written consent was obtained from all study participants, and the local medical ethics committee approved the protocol.

To study the impact of moderate and strong hemolysis, EDTA blood from 10 healthy volunteers (5 females/5 males) who had fasted overnight was used. Hemolysis was generated by aspirating blood either once or twice through a 26-gauge cannula. Control sample aliquots were centrifuged at 4 °C directly after

the blood sample was drawn (2200g, 7 min) and the metabolomics sample pretreatment was performed immediately as described below.

The effects of the exposure of EDTA blood to room temperature (n = 10; 22 °C in an air-conditioned room) were studied at 4 time points (2, 4, 8, and 24 h), and EDTA blood chilled in ice water (n = 10) was analyzed after 2 and 4 h of incubation. Control samples (n = 10) were again generated immediately after the blood sample was drawn. To confirm the kinetics of the increase in hypoxanthine and S-1-P peak areas in independent sample sets, exposure of EDTA blood to room temperature for 4 and 24 h was repeated (n = 5). In addition, a set of 10 EDTA plasma samples was generated under routine clinical conditions. Eight EDTA blood samples were placed in ice water for 1 h before further processing, and the other 2 were exposed to room temperature for 4 h. The hypoxanthine and S-1-P peak areas were again assessed.

To investigate the effects of repetitive freeze-and-thaw cycles, EDTA plasma (n = 10) was generated without delay after blood collection and directly frozen in cryotubes (Nunc, Thermo Scientific) at –80 °C. Thawing of the samples 1, 2, or 4 times was performed in ice water, followed by vortex mixing and either refreezing at –80 °C or metabolomics sample pretreatment, as described below. EDTA plasma control samples from the same study participants were not frozen after blood collection, but the metabolomics sample pretreatment was performed immediately.

ULTRAPERFORMANCE LIQUID CHROMATOGRAPHY QUADRUPOLE TOF-MASS SPECTROMETRY ANALYSIS

Metabolomics sample pretreatment. Following the different preanalytical investigations described above, 400- μ L aliquots of plasma or serum were deproteinized with 2 volumes of acetonitrile (final concentration 66%), mixed by vortex mixing (2 min), and centrifuged (15 700g, 20 min, 4 °C). The supernatant was dried in a vacuum centrifuge and stored at –80 °C. For analysis, samples were reconstituted in 200 μ L acetonitrile/water (160 μ L/40 μ L, respectively).

Chromatographic analysis. We performed the hemolysis study using an ACQUITY UPLC (ultraperformance liquid chromatography) system equipped with a 100 \times 2.1-mm ACQUITY 1.7 μ m/C18 column and coupled to a Micromass quadrupole TOF-mass spectrometer (qTOF-MS) (Waters). For all other studies the same column and a 1220 ultra-HPLC system coupled to a 6510 qTOF MS (Agilent) was used, except for the targeted analyses of hypoxanthine and S-1-P, for which an LTQ Orbitrap XL (Thermo Fisher) was used. The column temperature was 50 °C with a flow rate of 0.35

mL/min. For nontargeted metabolomics, a linear gradient was applied for 26 min, which changed from 98% A (0.1% formic acid in water) to 100% B (acetonitrile). For the targeted analysis of hypoxanthine and S-1-P, a linear gradient was applied for 11 min, which changed from 99% A (0.1% formic acid in water) to 100% B (acetonitrile).

The mass accuracy was <10 ppm on the Waters qTOF-MS, < 5 ppm on the Agilent qTOF-MS, and <5 ppm on the Orbitrap. A signal-to-noise ratio of 5 was set as the limit of detection. Pooled QC samples were prepared by mixing 10 μ L from each sample. The QC samples were analyzed after every fifth injection.

MASS SPECTROMETRIC PROCEDURES AND DATA COLLECTION

The qTOF-MS and Orbitrap settings are given in Table 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol59/issue5>. Potential biomarkers were identified following our recently published strategy, including tandem MS (MS/MS) and the final confirmation of metabolite identity by comparison with the reference compound (20). For statistical calculations, the peak areas of the features were used. "Features" are ions at a certain retention time extracted from raw spectral data. The data were combined into a single matrix by aligning peaks with the same mass-retention time from each data file in the data set. The peak area was normalized to the sum of the peak areas for each data set to enable comparison of relative peak areas between different data files. Detailed settings were recently described by our group (21).

DATA ANALYSIS

Raw data were processed by Masslynx 4.1 (Waters), Mass Hunter B.02.00 (Agilent), and Xcalibur (Thermo Fisher) for peak detection and alignment. The preprocessed data were exported into SIMCA-P (Umetrics) for multivariate statistical analysis. The 80% rule was used to remove missing values (22, 23). If, in at least 1 group, more than 80% nonzero measurement values were detected, these features were retained in the peak list. These preprocessing steps define the total number of remaining ions. Consequently, experimental conditions led to different numbers of nonzero measurements that resulted in differences in the total number of features in the data sets. Principal component analysis (PCA) was applied to elucidate and visualize internal structures in the data that reflect the different preanalytical conditions under investigation. To assess the individual features that account for the observed structures, we applied univariate techniques with the use of the Kruskal–Wallis test ($P < 0.05$) to make a preliminary selection of significantly altered features over all time points for 1 preanalytical condition. To account

for the higher rate of false positives in multiple parallel univariate hypothesis testing, the false discovery rate (FDR) was adjusted to 0.1 using the Benjamini–Hochberg method. Features that changed significantly according to the Kruskal–Wallis test and FDR were selected and plotted in a heat map. The Wilcoxon signed-rank test was then applied only to these preselected features to find pairwise significant changes between the individual time points ($P < 0.05$). The statistical analysis and heat map generation were performed using Mev 4.8.1 (<http://www.tm4.org/mev/>). We computed data concerning routine clinical parameters of the individuals using JMP (SAS Institute).

ANALYSIS OF ROUTINE CLINICAL PARAMETERS IN PLASMA

Free plasma hemoglobin concentrations were measured in EDTA plasma using the Dimension XL chemistry system, and in lithium heparinate plasma potassium, aspartate aminotransferase (AST), and C-reactive protein (CRP) were analyzed with the ADVIA 1650 clinical chemical analyzer (both Siemens Healthcare Diagnostics).

Results

IMPACT OF BLOOD COLLECTION TUBES

Contaminants present in the blood collection tubes may affect the ionization process during an LC-MS run, thereby suppressing metabolite ionization and/or introducing interfering peaks. Consequently, the first important step before initiating a metabolomics study is to examine every specific blood collection tube used. The total ion chromatogram of K^+ -EDTA- and Na^+ -fluoride-blanks and -plasma were inconspicuous (Fig. 1, A and C). Citrate showed few additional signals (Fig. 1B). However, clear patterns of noise signals were detected for lithium heparinate and serum blood collection tubes (Fig. 1, D and E) that were identified as polyethylene glycol with a typical ion cluster (Fig. 1F). Notably, both tubes contain plastic beads, which are the potential origin of the chemical noise. Therefore, we continued our investigations using EDTA blood collection tubes.

IMPACT OF HEMOLYSIS

First, routine parameters reflecting hemolysis were analyzed. Fig. 2A shows significant increases in free hemoglobin, AST, and potassium in moderate and strong hemolytic plasma samples. As a control, CRP concentrations showed no significant changes. Next, a UPLC-qTOF-MS–driven nontargeted metabolomics analysis was performed that revealed pronounced effects of hemolysis on the plasma metabolome. Although control samples clustered in the PCA score plot (Fig. 2B), hemolytic samples showed a high variability. The heat

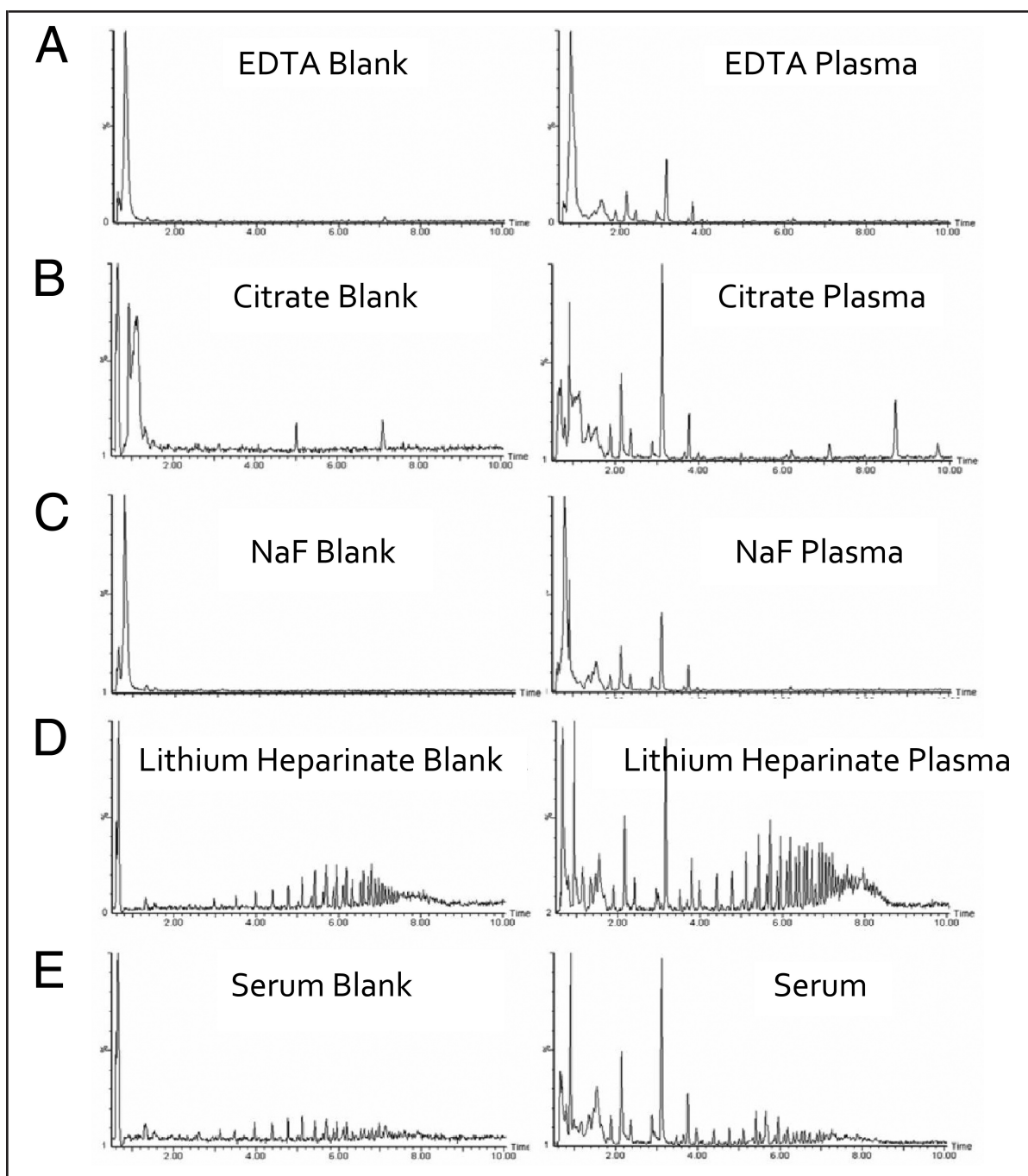
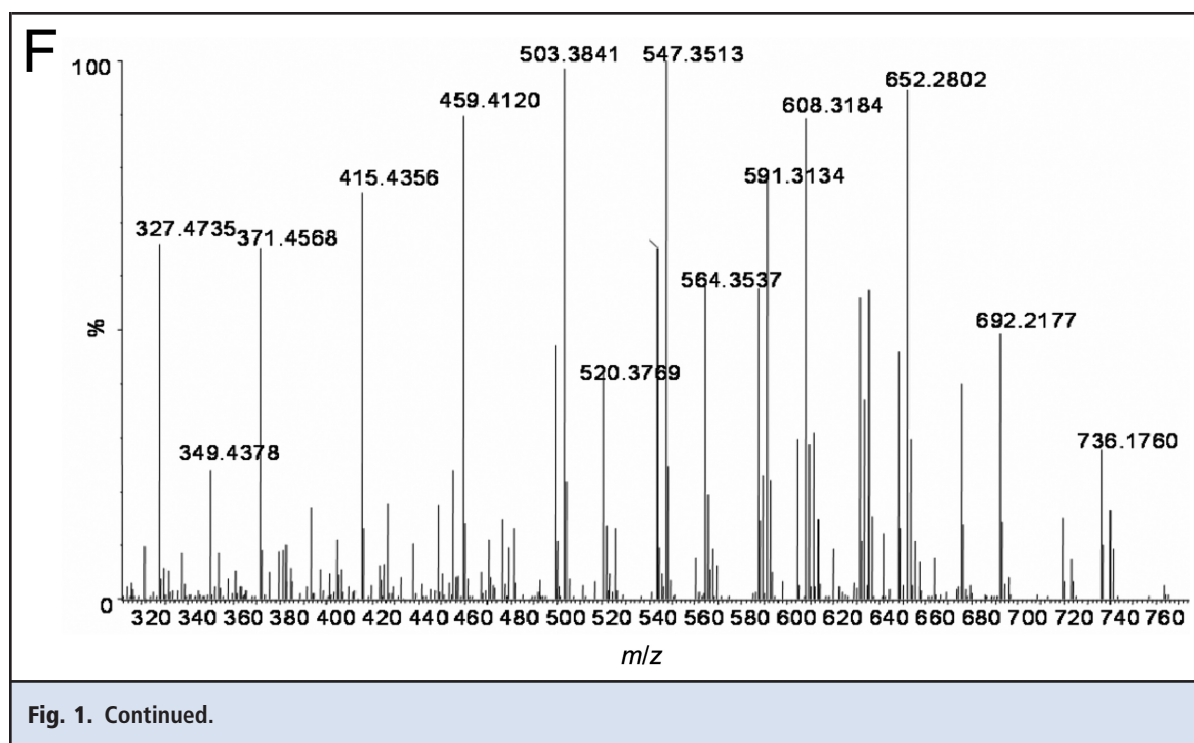


Fig. 1. Investigation of chemical noise arising from different blood collection tubes.

The blanks (50 g/L human albumin in normal saline) and blood samples were collected in commercial blood collection tubes with the following additives: (A), K^+ -EDTA; (B), Na^+ -citrate; (C), Na^+ -fluoride (NaF); (D), lithium-heparinate and (E), kaolin to enhance the generation of serum. (F), Mass spectra of chemical noise $[(-CH_2CH_2O)_n]$ detected in lithium-heparinate plasma, which exhibits typical polyethylene glycol clusters (38). Typical total ion chromatograms of blanks, plasma, and serum are given.

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map showed 69 features that were significantly changed ($P < 0.05$, FDR < 0.1), which demonstrates the pronounced effects of hemolysis (Fig. 2C). Significant changes in the lysophosphatidylcholines (lyso-PCs) C16:0 and C18:0 were detected with Wilcoxon signed-rank tests, ($P < 0.05$, Table 1). Free hemoglobin and lyso-PC C16:0 showed a strong correlation ($P = 0.0003$; $r = 0.61$).

EFFECTS OF SAMPLE HANDLING ON FRESHLY DRAWN BLOOD

Effects of exposure to room temperature. The blood samples were prepared immediately after drawing (controls) and after exposure to room temperature for 2, 4, 8, and 24 h. The most pronounced effect of room temperature exposure was a decrease in signal intensities. The PCA score plot demonstrated a high variability of samples exposed to room temperature (see online Supplemental Fig. 1). The heat map presented in Fig. 3A shows 64 features that were significantly altered during longer room-temperature-exposure times ($P < 0.05$, FDR < 0.1). Three of the most altered features were unequivocally identified by further MS investigations as the metabolites hypoxanthine, sphingosine 1-phosphate (S-1-P), and linolenyl carnitine ($P < 0.05$ by Wilcoxon signed-rank tests for all 3 metabolites). The time courses of changes in hypoxanthine and S-1-P levels are illustrated in Fig. 3B. The detected kinetics of these metabolites were reproduced in an inde-

pendent set of 5 plasma samples (data not shown). Of note, linolenyl carnitine concentrations did not significantly differ between controls and samples stored for up to 4 h at room temperature, but increased by 1780% after 8 h at room temperature (Table 1).

Effects of blood storage in ice water. Immediately after collection, control samples were prepared and the remaining EDTA-blood samples were directly placed in ice water for 2 or 4 h before sample preparation. These time points were chosen because the separation of plasma from the blood can be realistically done in 2 h in most studies and in 4 h in almost all studies. The PCA score plot showed no clustering of all study participant metabolomes at 3 different time points, whereas the metabolomes of each individual clustered for all 3 time points (marked by circles in Fig. 4A). This result indicates that no obvious changes of the plasma metabolome occurred during storage of any individual sample for up to 4 h in ice water. Furthermore, the plots of PCA scores after 2 and 4 h in ice water differed significantly from those for samples stored at room temperature (see online Supplemental Fig. 1). Of note, all 64 features that were seriously affected by room temperature showed very few, if any, changes in ice water (data not shown). In particular, the dominating markers hypoxanthine and S-1-P, which showed 1810% and 710% increases after storage of blood at room temperature for 4 h, were absolutely unremarkable with storage in

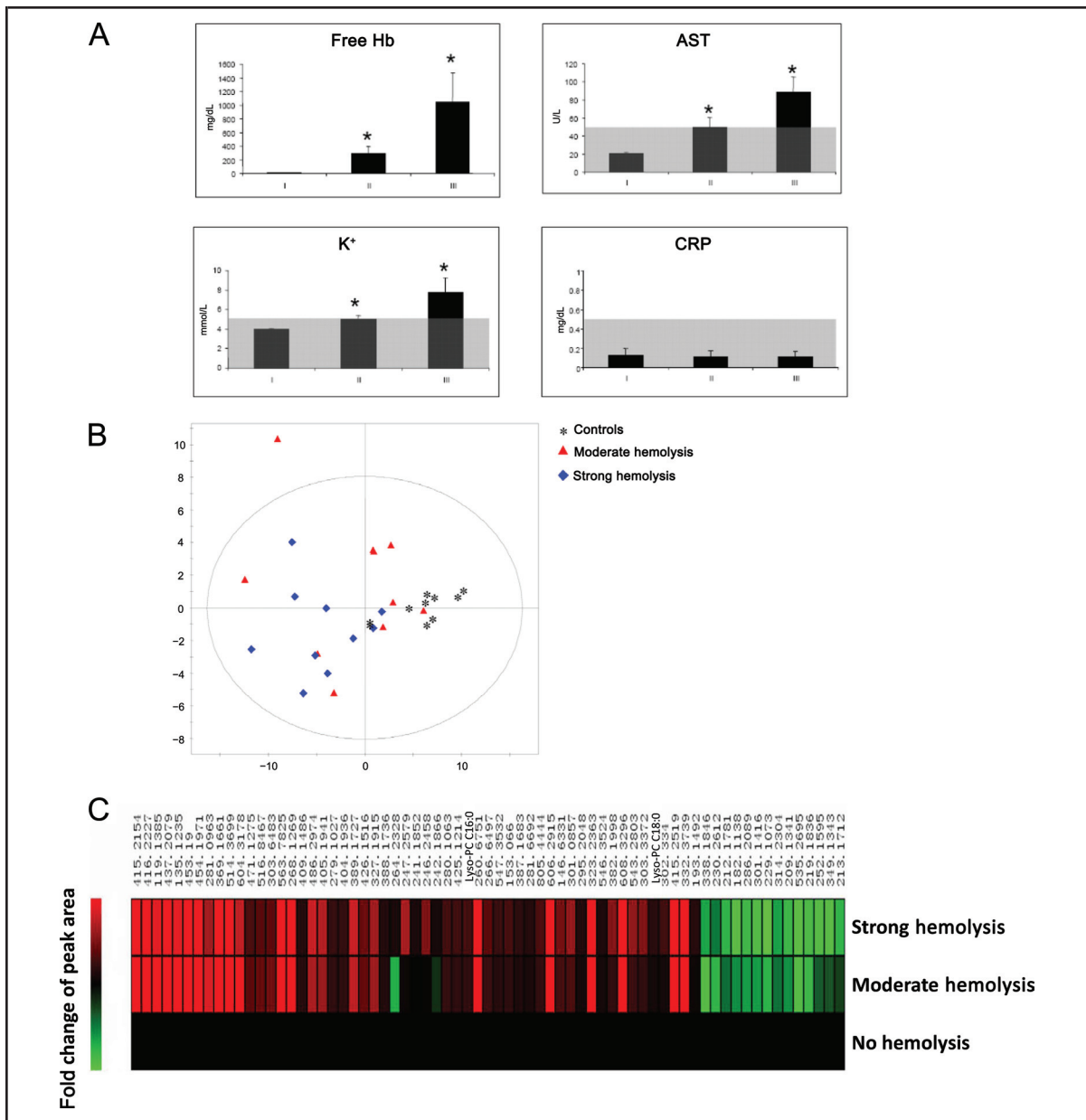


Fig. 2. Investigation of hemolytic samples (n = 10).

The plasma was analyzed either by routine clinical analyzers or by nontargeted metabolomics applying UPLC-qTOF electrospray ionization MS. (A), Routine clinical parameters that reflect hemolysis in plasma were measured [free plasma hemoglobin (free Hb), AST, potassium]. Additionally, CRP was measured as a control. I = controls; II = moderate hemolysis; III = strong hemolysis (* indicates $P < 0.05$ by a 2-tailed paired t test). The light grey background indicates the reference ranges (the reference range for free hemoglobin is below 10 mg/dL). (B), PCA score plot of the nontargeted metabolomics analysis of the same plasma samples ($R^2X_1 = 0.54$, $R^2X_2 = 0.09$; 383 features in the data set). The parameter R^2X is a quantitative measure for the goodness of fit, i.e., the fraction of variance in the data that is explained by the model (R^2X_1 represents the fraction of variance explained by the first principal component, R^2X_2 represents the fraction of variance explained by the second principal component). (*), Controls; (▲), moderate hemolysis; (◆), strong hemolysis. (C), Heat map of 69 significantly changed features in moderate and strong hemolytic samples compared to control plasma ($P < 0.05$, Kruskal–Wallis test; FDR < 0.1 , Benjamini–Hochberg method). The names of those metabolites unequivocally identified are given. Shades of red and green represent fold increase and fold decrease relative to baseline peak area of the features (see color scale).

Table 1. Identified plasma metabolites.^a

Metabolites	Hemolysis					Room temperature					MS identification parameter ^b		
	Moderate vs none	Strong vs none	2 h vs fresh	4 h vs fresh	8 h vs fresh	24 h vs fresh	Measured mass (positive mode)	Mass error (δ ppm)	MS/MS (30 V)				
S-1-P			0.02 (380% ↑)	0.002 (710% ↑)	0.002 (670% ↑)	0.002 (1250% ↑)	379.2481	1.58	264.27, 82.06				
Hypoxanthine			0.02 (800% ↑)	0.002 (1810% ↑)	0.002 (2740% ↑)	0.002 (4010% ↑)	136.0391	4.41	119.04, 110.04, 94.04, 82.04, 55.03				
Lyso-PC 16:0	0.017 (290% ↑)	0.013 (370% ↑)					495.3322	0.4	184.07, 104.1				
Lyso-PC 18:0	0.047 (270% ↑)	0.009 (320% ↑)					523.3633	0.76	184.07, 104.1				
L-carnitine							161.1051	0.62	85.03, 60.08				
Linolenyl carnitine							421.3185	1.67	85.03, 60.08				

^a Metabolites were preselected by having a *P* value below 0.05 by the Kruskal–Wallis test (FDR adjusted to 0.1 by the Benjamini–Hochberg method) and were finally tested for significant alterations in the peak area (*P* < 0.05) according to the Wilcoxon signed-rank test. Changes were induced by alterations in blood drawing during the preanalytical phase (hemolysis) and storage of whole blood before processing (at room temperature). Shown are the *P* values according to the Wilcoxon signed-rank test and the percentage change (↑, increase; ↓, decrease).
^b The identity of every metabolite was confirmed by a comparison with the corresponding reference compound.

ice water (data not shown). No significantly changed features were detected upon applying the criterion Kruskal–Wallis test *P* < 0.05 and FDR < 0.1. Next, we performed an independent experiment to test our biomarkers with blood samples from 10 study participants for which the analytical chemists were blinded. Two samples were exposed to room temperature for 4 h while the other samples were placed in ice water. The peak areas of hypoxanthine and S-1-P of the outlier samples clearly exceeded the mean + 3 SD (Fig. 4B) and were correctly identified as outliers by inspection of the signal intensities of these preanalytical biomarkers.

EFFECTS OF REPETITIVE FREEZE–THAW CYCLES

The metabolomic pattern of freshly prepared plasma compared with plasma thawed once in ice water was visualized by a PCA score plot (Fig. 5A). The plasma metabolomes of the majority of individuals lie close together, reflecting no obvious differences in individual metabolomes of freshly prepared plasma and plasma that was frozen 1 time. For 2 individuals we detected a difference in the metabolic fingerprint stability (see arrows in Fig. 5A). Since the pretreatment of all samples was strictly regulated by our protocol, we excluded differences in sample handling and preparation. Next, we investigated the effects of 2 freeze-and-thaw cycles in ice water compared to a single freeze-thaw cycle (Fig. 5B). No individual changes of the metabolite fingerprints were obvious, except again for individual no. 9. Following a Kruskal–Wallis test (*P* < 0.05) and FDR < 0.1, we detected only 4 significantly changed features (*P* < 0.05), 1 of which was identified as L-carnitine by MS/MS fragmentation and comparison with the reference compound. The L-carnitine peak area was significantly decreased by 70% after two and four freeze-thaw cycles in the Wilcoxon signed-rank test (Fig. 5C).

Discussion

Preanalytical questions are an essential and often underestimated part of clinical metabolomics study design. Precise instructions must be implemented regarding the handling of whole blood samples and the maximum allowable time interval before the separation of plasma or serum to achieve valid results. If reasonably practicable, immediate separation provides optimal analyte stability, as recently shown for 24 routine parameters (24). However, in clinical trials laboratory equipment and a centrifuge are often not immediately available where the blood is drawn (e.g., an outpatient clinic or a hospital ward). Therefore, the study protocol often includes a predefined delay before centrifugation of whole blood samples, which may vary

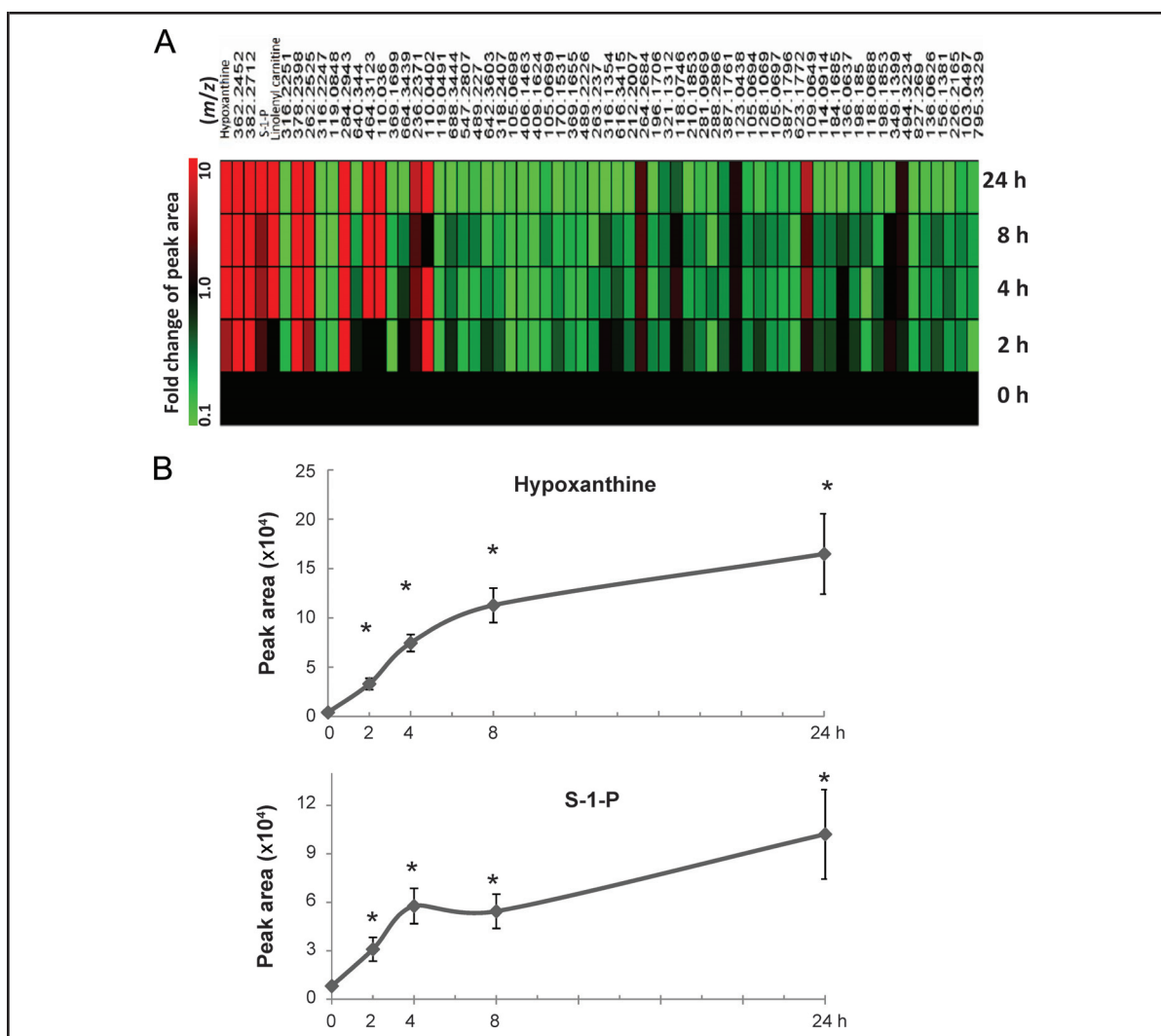


Fig. 3. Impact of exposing freshly drawn blood samples to room temperature (n = 10).

Blood was exposed for 2, 4, 8, and 24 h to room temperature followed by nontargeted metabolomics analysis of plasma by UPLC-qTOF electrospray ionization MS (0 h indicates that metabolomics sample pretreatment was performed immediately after blood drawing). (A), Heat map of 64 features showing significant alterations in the peak area compared to 0 h ($P < 0.05$, Kruskal–Wallis test; FDR < 0.1 , Benjamini–Hochberg method). The name of those metabolites unequivocally identified is given. Shades of red and green represent fold increase and fold decrease relative to baseline peak area of the features (see color scale). (B), Time-dependent kinetics of the hypoxanthine and S-1-P peak areas showing significant alterations by the Wilcoxon signed-rank test ($P < 0.05$).

or be prolonged in occasional instances. Despite strict protocols or SOPs, random errors in the preanalytical phase may occur and could greatly affect individual sample quality and subsequent interpretation of results. One absolutely avoidable negative impact on sample quality is the choice of unsuitable blood collection tubes. Various, somewhat contradictory recommendations suggest that interference from the blood collection tubes may be manufacturer dependent (7, 25, 26). The exact tubes used for the study should

therefore be tested beforehand, since chemical noise may greatly interfere with metabolic profiling. In our study, effects were clearly shown for lithium heparinate and serum blood collection tubes, results that are in contrast to a recently published recommendation that these tubes be used with preference for metabolomic study sample collection (7).

Shurubor et al. recently reported sufficient stability of 47 of 61 tested plasma metabolites in whole blood exposed for 48 h to room temperature in a targeted

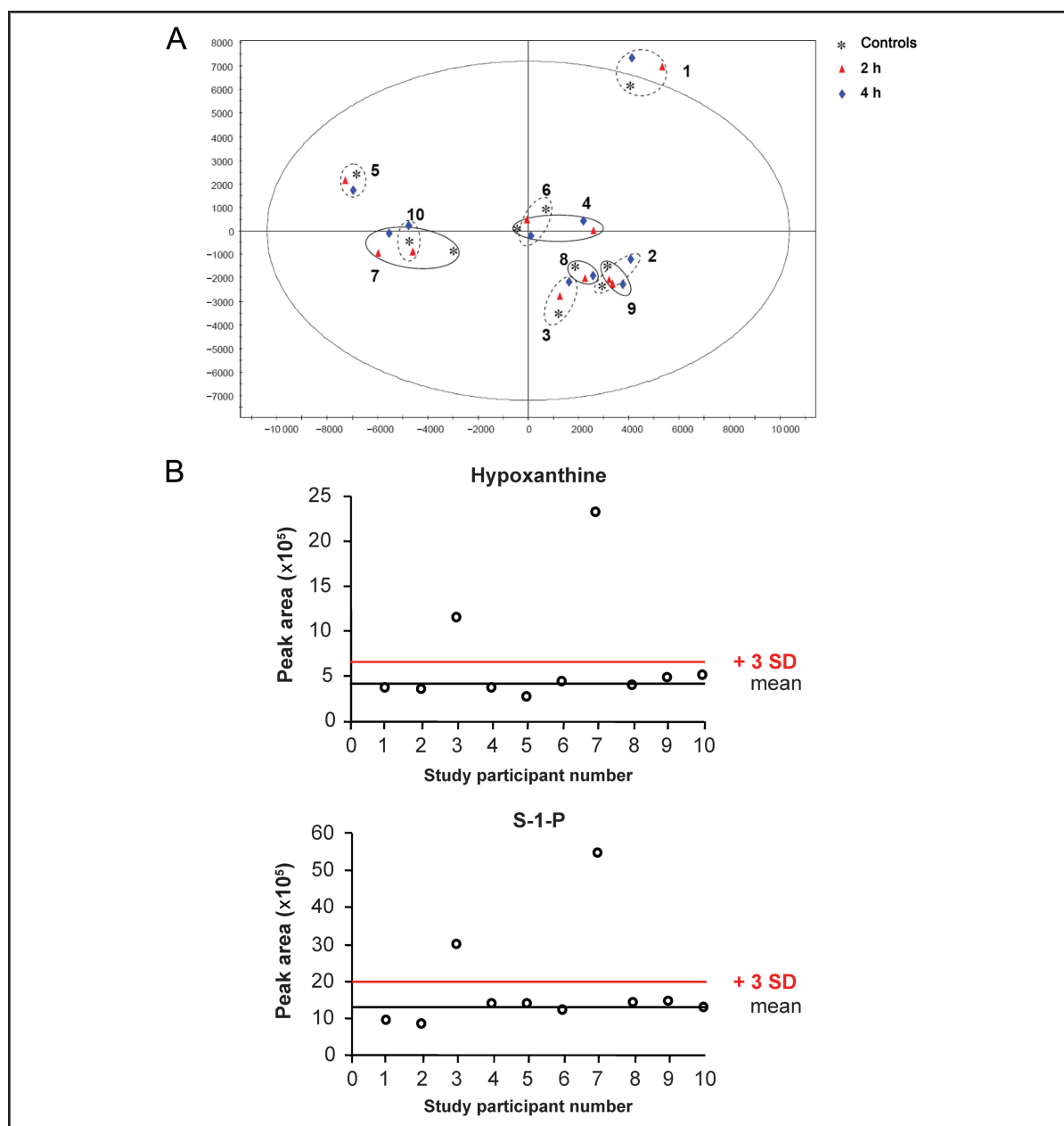
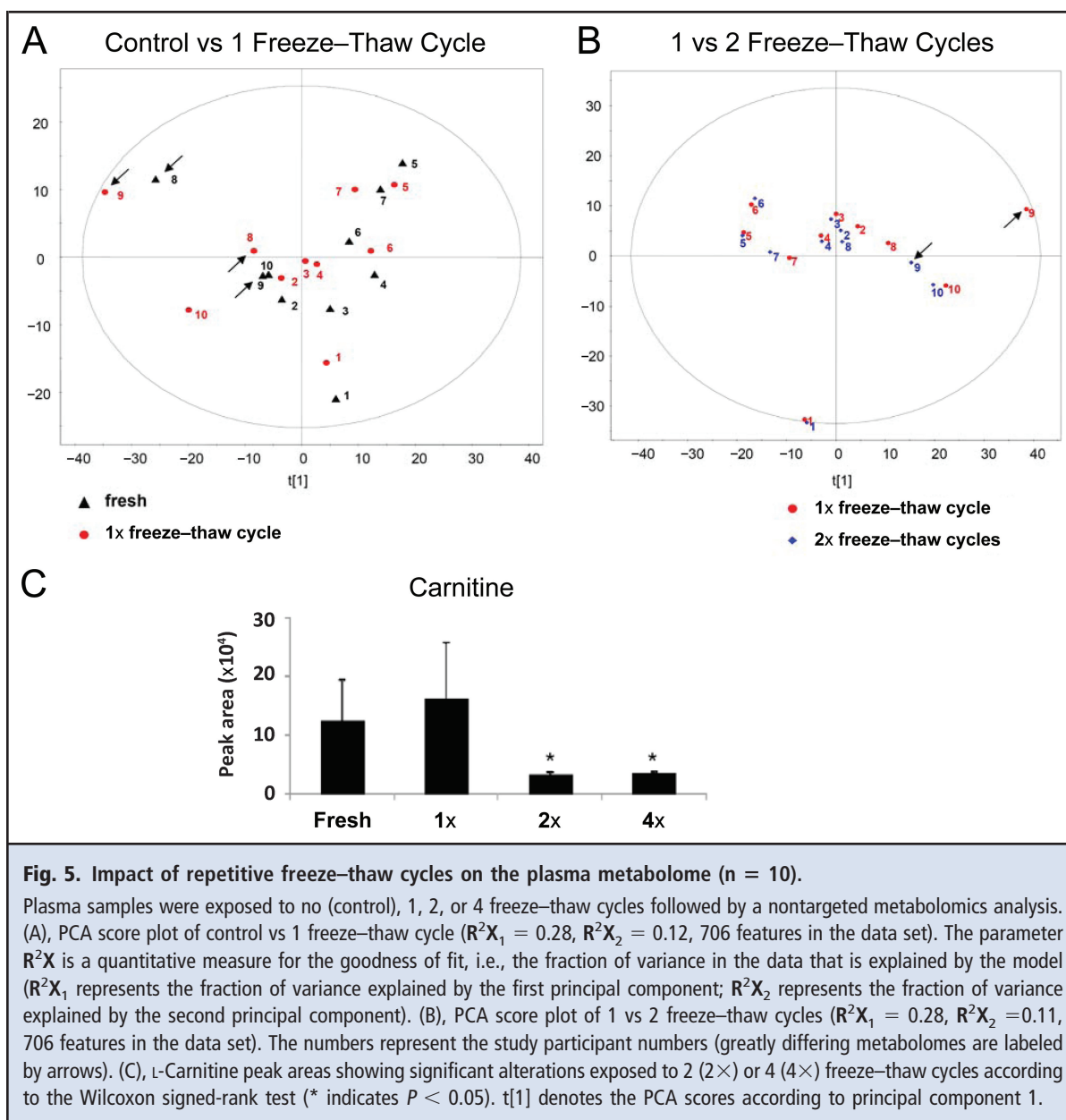


Fig. 4. Impact of placing freshly drawn blood in ice water for 2 and 4 hours (n = 10).

(A), PCA score plot of the nontargeted metabolomics analysis of plasma generated from whole blood stored in ice water for 2 and 4 h compared to control samples ($R^2X_1 = 0.37$; $R^2X_2 = 0.18$; 810 features in the data set). The parameter R^2X is a quantitative measure for the goodness of fit, i.e., the fraction of variance in the data that is explained by the model (R^2X_1 represents the fraction of variance explained by the first principal component, R^2X_2 represents the fraction of variance explained by the second principal component). The metabolite fingerprints of the 3 time points studied for every individual are circled and the number of the study participant is given. (*): controls, (▲): 2 h, (◆): 4 h. (B), Peak areas of hypoxanthine and sphingosine 1-phosphate in a set of 10 plasma samples (blinded to the analyst). The sample set contained 8 blood samples collected under standard clinical conditions, i.e., stored for 1 h in ice water until further processing by centrifugation, and 2 samples (nos. 3 and 7) that were incorrectly collected (exposed to room temperature for 4 h). The mean + 3 SD of the hypoxanthine and sphingosine 1-phosphate peak areas of the samples stored for 1 h in ice water are shown as a red line.



metabolomics study (13). In contrast, our nontargeted metabolomics analysis revealed undesirable effects after a room temperature exposure of only 2 h. In a recent study of nuclear magnetic resonance–driven metabolomics investigators also detected marked plasma metabolome alterations after exposure of blood to room temperature for 24 h and significant changes of 7 metabolites, which were most pronounced for glucose, lactate, and pyruvate (19). In our LC-MS–driven approach we detected a pattern of 64 significantly altered features in EDTA plasma after exposure to room temperature ($P < 0.05$, FDR < 0.01). The identified pre-

analytical biomarkers hypoxanthine, S-1-P, and linole-nyl carnitine may enable investigators using MS to identify (a) systematic inaccuracies that arise during processing of whole blood that affect the quality of many samples and (b) random errors leading to particular outliers. The first indication of a problem for the analyst occurs if hypoxanthine (monoisotopic mass, 136.0385) and S-1-P (monoisotopic mass, 379.2487) in individual samples exceeds the corresponding mean intensity + 3 SD of the sample set. The usefulness of this approach was demonstrated in a blinded set of samples in our study. The analyst and/or the clinical investigator may

thus consider excluding results from samples for which the exposure time of whole blood to room temperature before processing was unusually long.

These pronounced *in vitro* metabolome alterations at room temperature during the time period between blood drawing and plasma separation could primarily result from the action of the cells inside the blood collection tube rather than spontaneous chemical reactions. For example, a 9-mL sample of EDTA blood from an adult contains around 5×10^{10} red blood cells, 3×10^9 platelets, and 8×10^7 leukocytes. Hypoxanthine is a final product of adenosine monophosphate and inosine monophosphate catabolism that reaches the plasma by diffusion (27). S-1-P can be released by platelets (28) and erythrocytes (29).

Serum has been the primary sample material used in clinical studies for many decades and is widely available in biobanks. But is serum also appropriate for metabolomics studies? A targeted and a nontargeted metabolomics study reported an acceptable correlation of the metabolic pattern between serum and plasma (16, 17). However, in serum the concentrations of 104 of 121 metabolites were higher (around 10%), and 9 metabolites associated with the clotting process during serum generation showed obvious differences (20%–50%) (16). Notably, several studies demonstrated the suitability of serum for metabolomics investigations when standardized collection conditions were used (12, 30–33). Still, we suggest that using EDTA plasma can further minimize the risk of poor sample quality for several reasons: (a) EDTA plasma tubes can be immediately placed in ice water, whereas serum tubes must be exposed to room temperature for at least 25 min to allow proper clotting; (b) as a chelating agent EDTA inhibits several enzymes; (c) clotting in serum tubes activates additional processes, including the release of metabolites and enzymes from activated platelets; and (d) extremely low or high platelet counts may also affect the metabolite pattern in serum samples.

Because sample preparation and metabolomics analysis immediately after blood drawing without intermediate freezing are often not practical, we also studied the effects of repeated freezing in a -80°C freezer. The limited number of sample aliquots in biobanks inevitably leads to repetitive freeze–thaw cycles of the samples to preserve sample material for future investigations. Interestingly, we detected only minor, nonsignificant metabolome alterations of singly thawed compared to freshly prepared samples. As an alternative, samples from the same freeze–thaw cycle may be used, but use of biobank samples from different freeze–thaw cycles should be avoided. No systematic effects were detected; instead we detected increased interindividual variability of the plasma metabolome. These individual differences

in sample stability were also reported for whole blood exposed to room temperature for up to 48 h (13).

Hemolysis is a frequent occurrence in clinical laboratories and hemolytic specimens account for up to 40%–70% of all unsuitable specimens detected (34). MS metabolite fingerprints from total blood lysates, *i.e.*, dried blood spots, have a long history in pediatric diagnostics of inborn metabolic diseases (35), and several targeted metabolomics approaches that use lysates from whole blood or dried blood spots have been reported (36, 37). However, for nontargeted metabolomics studies the use of results from hemolyzed samples should absolutely be avoided.

In conclusion, the results of LC-MS–driven nontargeted metabolomics projects are affected by sample quality. Beyond the careful instruction and preparation of study participants (*e.g.*, standardized fasting), our data suggest the following line of action for planning and performing clinical metabolomics research projects that analyze blood:

1. Test the suitability of the blood collection tubes planned for use. We recommend EDTA plasma as the favored sample material.
2. Exclude results obtained from hemolyzed samples.
3. Place blood immediately in ice water after collection until further processing (for a fixed time; ideally not longer than 2 h).
4. Nonrefrozen plasma aliquots of biobank samples are recommended.
5. Carefully inspect MS data for unexpected signal intensities of preanalytical biomarkers to detect potential preanalytical shortcomings.

These recommendations may be helpful for clinical investigators, clinical and analytical chemists, and bioinformaticians in the planning and evaluation of metabolomics studies. The identified preanalytical biomarkers might be useful for the assessment of sample quality not only in metabolomics projects but also in other clinical studies.

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