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Quality Markers Addressing Preanalytical Variations of Blood and Plasma Processing Identified by Broad and Targeted Metabolite Profiling

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BACKGROUND: Metabolomics is a valuable tool with applications in almost all life science areas. There is an increasing awareness of the essential need for high-quality biospecimens in studies applying omics technologies and biomarker research. Tools to detect effects of both blood and plasma processing are a key for assuring reproducible and credible results. We report on the response of the human plasma metabolome to common preanalytical variations in a comprehensive metabolomics analysis to reveal such high-quality markers.

METHODS: Human EDTA blood was subjected to preanalytical variations while being processed to plasma: microclotting, prolonged processing times at different temperatures, hemolysis, and contamination with buffy layer. In a second experiment, EDTA plasma was incubated at different temperatures for up to 16 h. Samples were subjected to GC-MS and liquid chromatography–tandem mass spectrometry–based metabolite profiling (MxP™ Broad Profiling) complemented by targeted methods, i.e., sphingoids (as part of MxP™ Lipids), MxP™ Catecholamines, and MxP™ Eicosanoids.

RESULTS: Short-term storage of blood, hemolysis, and short-term storage of noncooled plasma resulted in statistically significant increases of 4% to 19% and decreases of 8% to 12% of the metabolites. Microclotting, contamination of plasma with buffy layer, and short-term storage of cooled plasma were of less impact on the metabolome (0% to 11% of metabolites increased, 0% to 8% decreased).

CONCLUSIONS: The response of the human plasma metabolome to preanalytical variation demands implementation of thorough quality assurance and QC measures to obtain reproducible and credible results from metabolomics studies. Metabolites identified as sensitive to preanalytics can be used to control for sample quality.

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Research in healthcare areas, such as identification and validation of diagnostic biomarker candidates and new drug targets, is often based on clinical studies lacking accurate sampling protocols, e.g., the analysis of existing blood samples from either biobanks or sample collections performed for other purposes. The quality of these biospecimens may be impacted by various preanalytical sample processing steps *(1)* or storage effects that confound the analytical results and decrease the credibility of the research outcomes if not identified and addressed properly. It is well known in the field of routine clinical chemistry that preanalytics are considered to account for approximately 68% of diagnostic misinterpretations *(2)*. Defined preanalytical protocols are a prerequisite for reproducible and reliable results *(3–4)*. There is an increasing awareness of the essential need for securing high-quality biospecimens and implementing QC tools for assuring reproducible and credible results in biomarker studies *(5)*. For example, in the area of transcriptomics, preanalytical variations led to the formation of the SPIDIA (Standardization and Improvement of Generic Preanalytical Tools and Procedures for in Vitro Diagnostics) consortium *(6)*. In the emerging field of proteomics and peptidomics, preanalytics *(7)* were the subject of specially designed studies *(8)* that identified new biomarker candidates in different biomedical applications of high sensitivity to preanalytical variations *(9)*.

Metabolomics complements transcriptomics and proteomics as a physiological "down-stream result" and is applicable to a broad range of life science areas *(10–11)*. Because of its broad coverage of physiological and chemical processes, metabolomics is well-suited to

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identify biomarkers for the quality assessment of biospecimens.

The aim of our experiments was to analyze the impact of common preanalytical variations of blood and plasma processing on the comprehensive human EDTA plasma metabolome to describe and aim to better understand the biochemistry of the preanalytical phase and its consequences for quality assurance, QC, biomarker discovery, drug target discovery, and other applications in biomedical metabolomics-based research. Recent publications have emphasized the impact of preanalytics on metabolomics *(12–16)*. Here we show, in a comprehensive manner, the response of the human plasma metabolome on common preanalytical variations using a GC-MS and liquid chromatography–tandem mass spectrometry (LC-MS/MS)[3]-based approach (MxP™ Broad Profiling) complemented by targeted methods, i.e., sphingoids (as part of MxP™ Lipids), MxP™ Catecholamines, and MxP™ Eicosanoids.

Materials and Methods

The study was conducted in adherence to the Declaration of Helsinki and was reviewed by the local ethical committee. All study participants provided written informed consent.

STUDY DESIGN

All study participants were self-reported healthy volunteers (13 females, 7 males). Inclusion criteria were age from 18 to 40 years, overnight fasting status, and body mass index of 18–30 kg/m^2. Exclusion criteria included presence of known acute or chronic diseases, anemia, pregnancy (second or third trimester), medication with heparin, nonsteroidal or steroidal anti-inflammatory drugs within the last 10 days, and medication with antihistamines or selective serotonin reuptake inhibitors within the last 4 weeks. The blood collection system and all disposable laboratory materials for the blood processing groups were purchased from Sarstedt. EDTA was used in the form of K_3EDTA salt.

Approximately 64 mL of blood was collected from each volunteer by peripheral venipuncture using a 20-gauge safety-fly blood collection system. The volunteers were placed in an upright sitting position and the tourniquet released after blood flow started. If the first venipuncture was unsuccessful the procedure was repeated once on the other arm. First, three 9-mL-EDTA S-Monovette® tubes were filled, and then 1 mL blood was collected into a neutral S-Monovette® and discarded before the collection of one 9-mL neutral S-Monovette and an additional three 9-mL-EDTA S-Monovette tubes. The blood was gently mixed with EDTA by inverting the S-Monovette tubes 5 times immediately after blood draw. The EDTA S-Monovette tubes from each study participants were opened and the blood was pooled and processed within the different processing groups as described below. Plasma was prepared by centrifugation at 1500*g* for 15 min at 4–8 °C. The plasma supernatant was removed, mixed in a new centrifugation tube by inverting, split into 0.5-mL aliquots, and stored at −80 °C in 2-mL screw-cap tubes until analysis. Samples were shipped on dry ice to the analytical laboratory. MxP analysis was performed within 4 months of storage.

SHORT-TERM STORAGE OF BLOOD

We incubated 3 × 5 mL of pooled blood at room temperature of 19–22 °C for 2 h or kept pooled blood on wet ice for 2 h or 6 h before processing to plasma as described above.

HEMOLYSIS

A total of 2 × 6 mL of pooled blood was passed through a syringe with a 25-gauge (grade 1 hemolysis) or 27-gauge needle (grade 2 hemolysis), respectively.

CONTROL AND CONTAMINATION WITH BLOOD CELLS

The remaining pooled blood was processed to plasma as described above. The first fraction of the plasma (upper plasma supernatant) was withdrawn to serve as the control. The second plasma fraction (lower plasma supernatant) was contaminated with blood cells of the centrifugation tube by touching the buffy layer with the pipette (2 grades). The time between blood draw and centrifugation was monitored and was ≤16 min for the control group.

MICROCLOTTING

Blood from the 9-mL neutral S-Monovette was carefully decanted into a 9-mL EDTA S-Monovette. After 5 min at room temperature the plasma was prepared as described above.

PLASMA PROCESSING PROTOCOL

An EDTA human plasma pool was split into 1-mL aliquots and incubated at 3 different temperatures with each 23 aliquots: 4 °C (laboratory refrigerator, Liebherr), 12 °C (Eppendorf Thermomixer Comfort), and room temperature. At the time points 0, 0.5, 2, 5, and 16 h, aliquots were taken out of the samples to allow for each 10–12 replicates of MxP Broad Profiling, MxP

[3] Nonstandard abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; SPE, solid phase extraction; UHPLC, ultra-HPLC; MRM, multiple reaction monitoring; add, additional; PCA, principal component analysis; FDR, false discovery rate; SOP, standard operating procedure.

Catecholamines, and MxP Eicosanoids as described below.

FREE HEMOGLOBIN ANALYSIS

Free hemoglobin was analyzed in the samples of the blood processing experiment by using the 2-wavelength (540/680 nm) cyanhemoglobin method *(17)*. One study participant was excluded from the hemoglobin data analysis because of turbid plasma samples in all processing groups that interacted with the assay.

METABOLITE PROFILING ANALYSIS

Four types of MS analyses were applied to all samples. GC-MS (Agilent 6890 GC coupled to an Agilent 5973 MS-System) and LC-MS/MS (Agilent 1100 HPLC system coupled to an Applied Biosystems API4000 MS/MS-System) were used for MxP Broad Profiling *(18)*. Solid phase extraction (SPE)–LC-MS/MS (Symbiosis Pharma, Spark, coupled to an Applied Biosystems API4000 MS/MS system) was used for the determination of catecholamine (MxP Catecholamines) and eicosanoid (MxP Eicosanoids) concentrations. For the determination of the sphingoids (as part of MxP Lipids), an Agilent 1290 ultra-HPLC (UHPLC) system coupled to an Applied Biosystems API5500 MS/MS system was used.

Fractionation and derivatization of samples and details of the detection technologies were as previously described *(18–20)*. Proteins were removed from plasma samples (60 μL) by precipitation. Subsequently, polar and nonpolar fractions were separated for both GC-MS and LC-MS/MS analyses by adding water and a mixture of ethanol and dichloromethane. For GC-MS analyses, the nonpolar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The polar and nonpolar fractions were further derivatized with *O*-methylhydroxylamine hydrochloride (20 g/L in pyridine, 50 μL) to convert oxo groups to *O*-methyloximes and with a silylating agent [*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide, 50 μL] before GC-MS analysis. For LC-MS/MS analyses, both fractions were reconstituted in appropriate solvent mixtures. HPLC was performed by gradient elution using methanol/water/formic acid on reversed-phase separation columns. A special MS detection technology was applied that allowed for targeted and high sensitivity multiple reaction monitoring (MRM) profiling in parallel with a full-screen analysis. For the polar fraction, the instrument is operated in negative ionization mode, for the lipid fraction in positive ionization mode. MS detection was performed with repetitive cycles of MRM transitions for important preselected metabolites followed by a full scan from *m/z* 100 to 1000. Catecholamines and eicosanoids were measured by offline and online SPE-LC-MS/MS *(21–22)*. Absolute quantification was performed by means of stable isotope-labeled standards. Sphingoids were measured by offline SPE cleanup of the sample and subsequently determined semiquantitatively by UHPLC-MS/MS. An Oasis® hydrophilic-lipophilic-balanced μElution SPE cartridge (Waters) was conditioned with n-hexane, methanol, and methanol/phosphoric acid. After application of the plasma sample, the cartridge was washed with methanol/phosphoric acid before elution of the sphingoids with acetonitrile/isopropanol. The sample was directly injected into the UHPLC-MS/MS system.

DATA NORMALIZATION, DATA SET ALIGNMENT, METABOLITE CONCENTRATIONS AND NOMENCLATURE

Metabolite profiling based on a semiquantitative analytical platform results in relative metabolite concentrations (ratios) referenced to a defined control group. To support this concept and to allow an alignment of different analytical batches, 2 different reference sample types were run in parallel throughout the whole process. First, a project pool was generated from aliquots of all samples and measured with 4 replicates within each analytical sequence, which comprised 24 samples. For all semiquantitatively analyzed metabolites, the data were normalized against the median in the pool reference samples within each analytical sequence to provide pool-normalized ratios (performed for each sample per metabolite). This process step compensated for inter- and intrainstrumental variation, i.e., normalized the device variability that occurs when different analytical sequences are analyzed by different devices. Second, to allow an experiment-to-experiment alignment of semiquantitative data, MxPool™ (a large pool of a commercial human EDTA plasma suited for alignment of MxP studies) was analyzed with 12 replicated samples in each experiment, and the pool-normalized ratios were further normalized to the median of the MxPool samples, i.e., ratios from this study are on the same level and therefore comparable with data from other studies normalized to other aliquots of the same MxPool. Quantified data from targeted methods (eicosanoids, catecholamines) remained with their absolute quantification data and were not transformed to ratios.

The limits of detection and the dynamic ranges of the semiquantitative measurements were determined by dilution and spiking experiments during method development. A signal-to-noise ratio threshold equal to 15 was used for a metabolite to be considered semiquantitative. For quantitative catecholamine and eicosanoid determination, the limit of detection or quantification was determined from the daily calibration curve, and absolute quantification was performed with stable isotope-labeled

Table 1. Number and percentage of statistically significant metabolite changes after applying defined preanalytical confounders out of 267 metabolites for the blood and 262 metabolites for the plasma processing experiment.[a]

		Significantly changed metabolites (increase/decrease)[b]	
Material	Preanalytical variable applied	Number	Percent change
Blood	Microclotting	31 (3/28)	12 (1/10)[c]
	Room temperature, 2 h	59 (27/32)	22 (10/12)
	Wet ice, 2 h	44 (12/32)	16 (4/12)
	Wet ice, 6 h	46 (17/29)	17 (6/11)
	Hemolysis, grade 1	47 (15/32)	18 (6/12)
	Hemolysis, grade 2	81 (50/31)	30 (19/12)[c]
	Contamination with buffy layer, grade 1	0 (0/0)	0 (0/0)
	Contamination with buffy layer, grade 2	8 (8/0)	3 (3/0)
EDTA plasma	4 °C, 0.5 h	0 (0/0)	0 (0/0)
	4 °C, 2 h	7 (7/0)	3 (3/0)
	4 °C, 5 h	16 (12/4)	6 (5/2)[c]
	4 °C, 16 h	30 (24/6)	11 (9/2)
	12 °C, 0.5 h	1 (1/0)	0 (0/0)
	12 °C, 2 h	7 (7/0)	3 (3/0)
	12 °C, 5 h	14 (11/3)	5 (4/1)
	12 °C, 16 h	37 (29/8)	14 (11/3)
	Room temperature, 0.5 h	4 (4/0)	2 (2/0)
	Room temperature, 2 h	28 (25/3)	11 (10/1)
	Room temperature, 5 h	47 (27/20)	18 (10/8)
	Room temperature, 16 h	61 (41/20)	23 (16/8)[c]

[a] Statistical analysis was done via ANOVA, the significance level was set to $P < 0.05$ and FDR < 0.2. Details are given in online Supplemental Tables 1 and 2.
[b] Including free hemoglobin.
[c] All 3 percentages are rounded to an integer, so the values in brackets do not necessarily add up to the total.

standards. When these criteria were applied, 262 and 266 metabolites were analyzed for the plasma and blood processing experiment, respectively.

Details on metabolite nomenclature are available *(19)*, but in short, the term additional (add) was applied to indicate that quantification can be disturbed by metabolites exhibiting identical analytical characteristics with respect to the quantification method.

STATISTICAL ANALYSIS AND DATA VISUALIZATION

Before statistical analysis, log10 transformation of ratios was conducted to approach a normal distribution of the data. The software packages R 2.8.1 (package nlme), SIMCA 13.0 (Umetrics), and TIBCO® Spotfire® 3.3.1 were used for data analyses and visualizations. Statistical analysis of the blood processing experiment was done via a mixed linear model (ANOVA) with "subject" as random intercept and "gender" and "processing group" as fixed effects. Residuals from another mixed linear model without "processing group" as a fixed effect were used for principal component analysis (PCA). Statistical analysis of the plasma processing experiment was done by a simple linear model (ANOVA) with "time" and "temperature" as fixed effects. Significance level was set to an α error of 5%. The multiple test problem was addressed by calculating the false discovery rate (FDR) using the Benjamini and Hochberg method *(23)*.

Results

PREANALYTICAL VARIATION AFFECTS THE PLASMA METABOLOME

An MS-based metabolomics MxP Broad Profiling approach supplemented by the targeted methods MxP Eicosanoids, MxP Catecholamines, and sphingoids (as part of MxP Lipids) on human EDTA plasma samples revealed that a high number of metabolites was significantly increased or decreased by preanalytical variation during blood and plasma processing (Table 1). Short-term storage of blood either at room temperature or cooled on wet ice, hemolysis, and short-term

storage of plasma for 5 or 16 h at room temperature resulted in statistically significant changes (4% to 19% of metabolites increased and 8% to 12% of metabolites decreased) of the human EDTA plasma metabolites. Microclotting induced by delayed addition of EDTA to blood, contamination of plasma with buffy layer, and short-term storage of plasma if cooled at 4 °C or 12 °C resulted in a lower number of statistically significant differences (0% to 11% of metabolites increased and 0% to 8% of metabolites decreased). Statistical details of the blood processing experiment are provided in Table 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol60/issue2, and statistical details of the plasma processing experiment are provided in online Supplemental Table 2. A PCA of the blood processing experiment metabolomics data (Fig. 1A) showed that different temperatures of blood processing had different metabolic consequences, with samples cooled on wet ice being more similar to the hemolysis group. A PCA of the plasma processing experiment metabolomics data (Fig. 1B) demonstrated that time and temperature of plasma processing drove the score plot in the same direction, thus samples stored for 16 h at 12 °C clustered with samples incubated for 5 h at room temperature.

SIGNALING METABOLITES (CATECHOLAMINES, EICOSANOIDS, SEROTONIN)

Table 2 gives an overview of signaling metabolites that are commonly and specifically affected by preanalytical variations. Various prostaglandin and thromboxane concentrations, e.g., adrenaline (epinephrine; Fig. 2A), noradrenaline (norepinephrine), metanephrine, dopamine, and eicosanoids (Fig. 2B) were strongly affected by prolonged processing of blood and/or plasma. For reproducible and reliable analysis of catecholamines and eicosanoids, sample preprocessing is a critical step and should be performed as quickly as possible. Serotonin was affected only by blood processing variables (Fig. 3A) and was stable in plasma in this experimental setting. Recent literature has indicated that serotonin in blood is located almost exclusively in platelets *(24)*. Therefore, blood processing steps that change platelet metabolism, induce platelet activation, or strive for platelet separation from plasma (platelet-rich plasma vs platelet-poor-plasma) would also be critical for reproducible serotonin analysis from blood *(25)*.

AMINO ACIDS

The concentrations of amino acids differ in plasma and serum *(26)*. Here we report their changes when applying preanalytical variations on plasma preparation (Table 2). Glutamate concentrations in plasma were influenced by both blood and plasma (Fig. 2C) processing time, whereas taurine was influenced by blood processing (Fig. 3A) but not by plasma processing time. The sulfur-containing amino acids cysteine and cystine decreased during incubation of plasma (Fig. 2D and online Supplemental Table 2), whereas methionine was stable under the experimental conditions.

CARBOHYDRATES, COFACTORS, ENERGY METABOLISM, AND OTHERS

Preanalytical variation affected carbohydrates, cofactors, and energy metabolism, as shown in Table 3. In contrast to other metabolite classes, carbohydrate and energy metabolites were more affected by blood than by plasma incubation, indicating that these catabolic activities are related to blood cells. Glucose concentrations in blood decreased and lactate concentrations increased (Table 3) despite the presence of EDTA. Maltose increased during plasma incubation at any of the tested temperatures (Fig. 2E). Threonic acid is an oxidation product of ascorbic acid and increased when plasma was incubated for a prolonged time (Fig. 2F). Hemoglobin was increased 14-fold in the hemolysis grade 1 group and 36-fold in the hemolysis grade 2 group, indicating efficacy of shear-force–induced hemolysis (see online Supplemental Table 1). The increase of hypoxanthine upon blood incubation (see online Supplemental Table 1) was probably related to platelet metabolism of AMP *(27)*.

LIPID METABOLISM

Preanalytical variation of both blood and plasma processing strongly affected lipid metabolism (Table 3). Affected were several classes of lipids, e.g., cholesterol metabolites, triacylglycerides, phosphatidylcholines, lysophosphatidylcholines, and sphingolipids. The plasma concentrations of sphingosine-1-phosphate increased upon blood processing time probably due to reported erythrocyte activities *(28)*. The ratios of a phosphorylated to dephosphorylated sphingobase [sphingadienine-1-phosphate (d18:2)/sphingadienine (d18:2); Fig. 3B] showed unexpected and interesting responses upon temperature differences by increasing upon incubation at room temperature and decreasing upon incubation on wet ice.

Discussion

The responsiveness of the human metabolome coupled with the high analytical sensitivity of modern technologies offers a broad biomedical application for metabolomics of minimally invasive accessible matrices like plasma, serum, and urine.

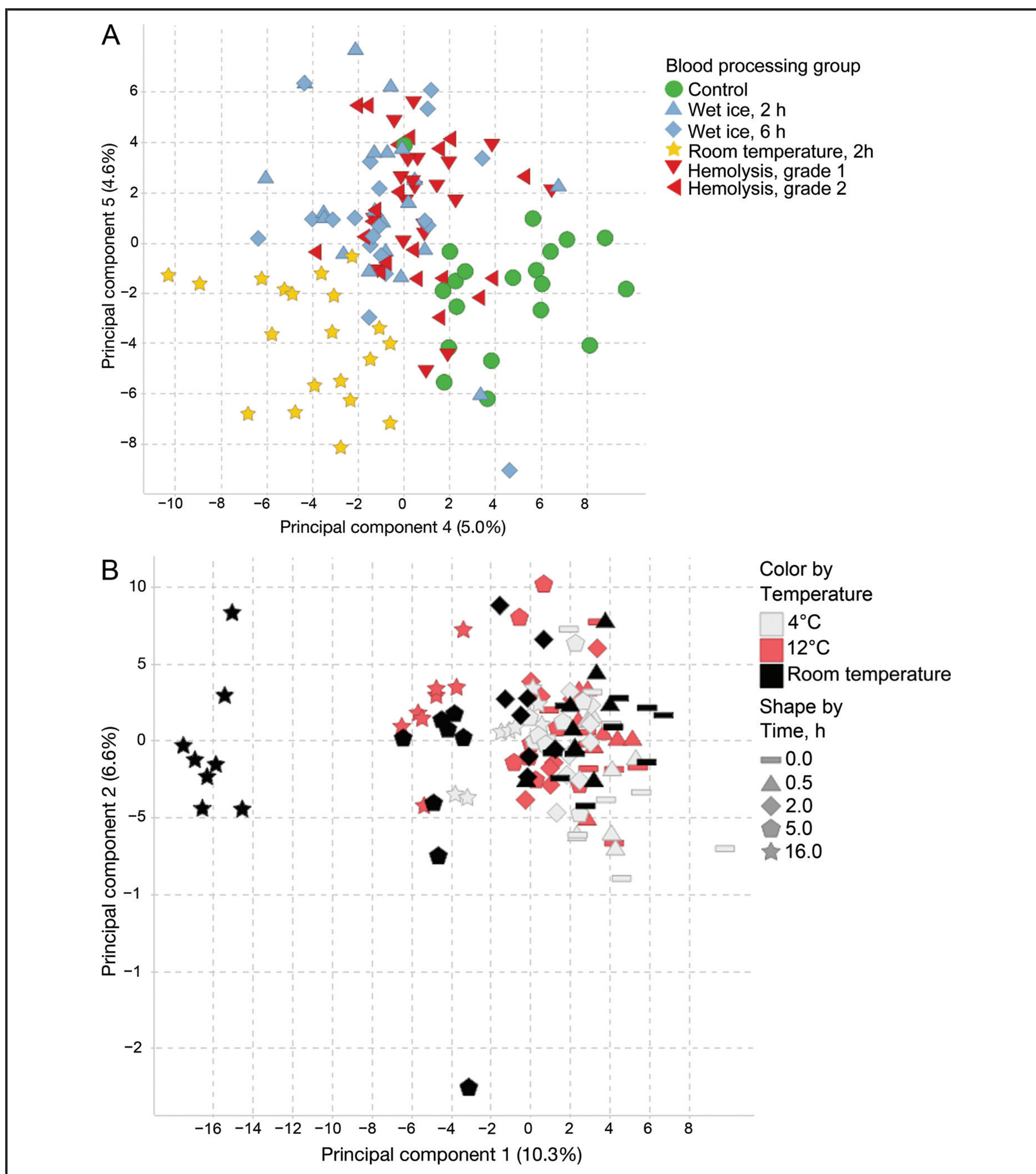

Fig. 1. Scores plots of PCA on metabolomics data after applying preanalytical confounders.
Blood processing experiment, principal components 4 and 5 of centered residuals of log10-transformed data after ANOVA correction for study participant and sex (A) and plasma-processing experiment PCA on log10-transformed data scaled to unit variance (B). Numbers within the axis labels give the fraction of variance captured.

However, the effects of blood-related biochemical activities from preanalytical processes demand specially designed experiments to (*a*) identify the most affected metabolites, (*b*) determine the most important sources of preanalytical variability, (*c*) define reasonable standard operating procedures (SOPs) for plasma and serum processing for minimizing preanalytical effects (quality assurance), and (*d*) establish tools for

Table 2. Metabolites and fold changes of signaling molecules and amino acids that are significantly affected by preanalytical short-term storage of blood and plasma with a change of $P < 0.05$ and FDR < 0.2.[a]

Metabolite	Statistically significant metabolite fold changes		
	Blood, room temperature, 2 h	Blood, wet ice, 6 h	Plasma, room temperature, 16 h
Signaling molecules			
12-Hydroxyeicosatetraenoic acid (C20:*cis*[5,8,10,14]4)	0.48	0.66	10.47
12-Hydroxyheptadecatrienoic acid (C17:[5,8,10]3)	0.67	0.27	1.80
5-Hydroxyeicosatetraenoic acid (C20:*trans*[6]*cis*[8,11,14]4) (5-HETE)	1.25	1.17	7.80
9-Hydroxyoctadecadienoic acid (C18:*trans*[10]*cis*[12]2) (9-HODE)	1.52	1.14	1.24
Noradrenaline (norepinephrine)	0.76	0.79	0.35
Thromboxane B2	0.52	0.15	1.64
Dopamine	0.65	0.65	
Normetanephrine	1.56	1.67	
Serotonin (5-HT)	0.15	0.03	
13-Hydroxyoctadecadienoic acid (C18:*cis*[9]*trans*[11]2) (13-HODE)	1.15		1.38
3,4-Dihydroxyphenylacetic acid (DOPAC)	0.83		0.04
3,4-Dihydroxyphenylglycol (DOPEG)	0.72		0.28
Adrenaline (epinephrine)	0.87		0.47
Metanephrine	0.86		
11-Hydroxyeicosatetraenoic acid (C20:*cis*[5,8,12,14]4)		0.87	2.99
14,15-Dihydroxyeicosatrienoic acid (C20:*cis*[5,8,11]3)			1.24
15-Hydroxyeicosatetraenoic acid (C20:*cis*[5,8,11,13]4)			2.89
3,4-Dihydroxyphenylalanine (DOPA)			0.29
8,9-Dihydroxyeicosatrienoic acid (C20:*cis*[5,11,14]3)			1.99
8-Hydroxyeicosatetraenoic acid (C20:*trans*[5]*cis*[9,11,14]4) (8-HETE)			2.77
Δ-12-prostaglandin D2			60.76
Prostaglandin D2			13.72
Prostaglandin E2			3.78
Amino acids and related			
Glutamate	0.82	0.70	5.03
Taurine	0.49	0.54	
Ornithine (add: arginine, citrulline)	1.33		1.04
Ketoleucine	0.91		
Leucine	1.03		
Proline	1.03		
3-Hydroxyindole (add: 3-indoxylsulfate)		0.83	
Tryptophan		1.07	
Alanine			1.04
Cysteine (add: cystine)			0.61
Cystine			0.66
Glutamine			0.60

[a] Fold changes are relative to control for the blood-processing groups and relative to the time point 0 h for the plasma-processing group.

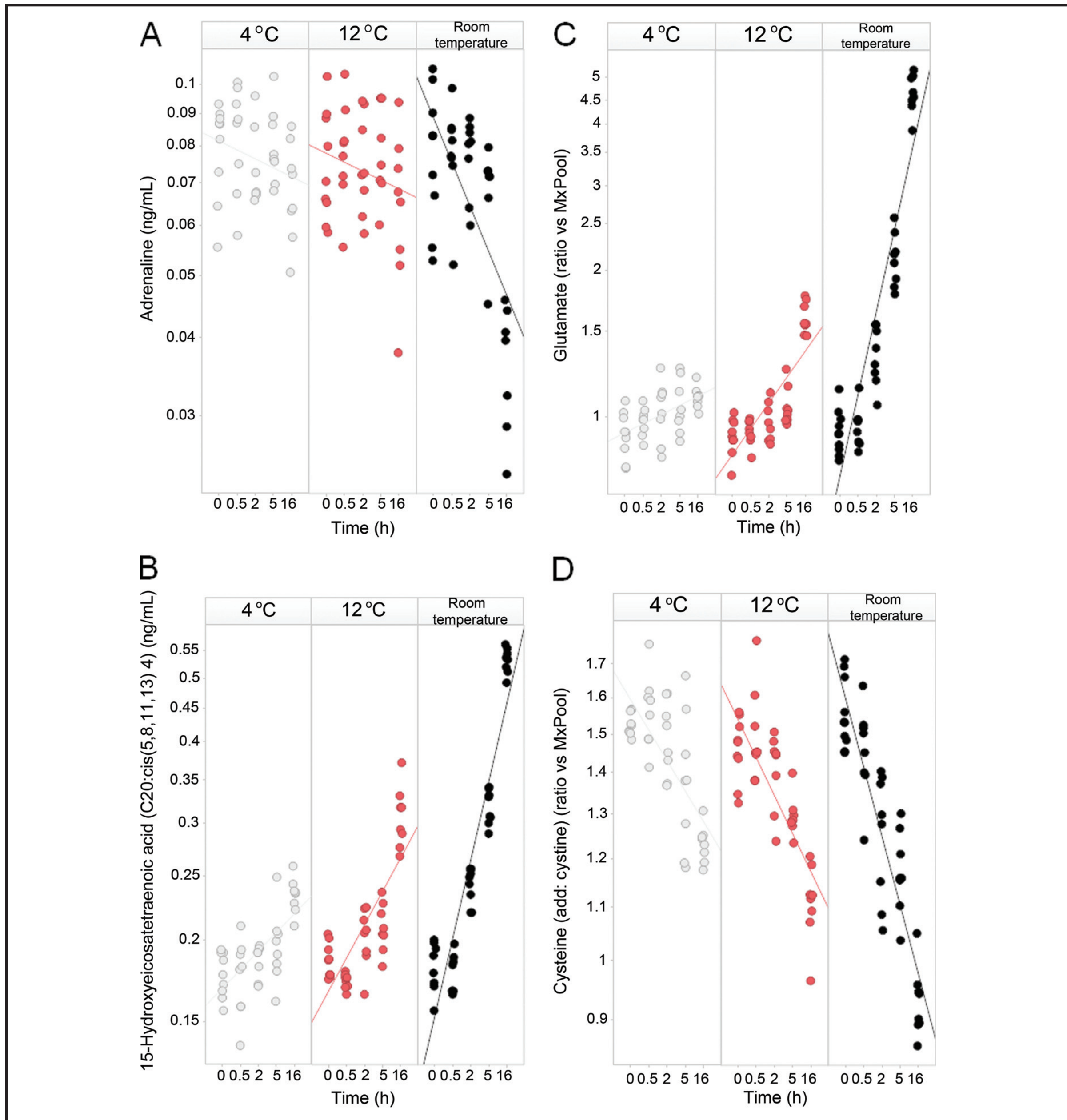

Fig. 2. Effects of plasma processing time and temperature on selected metabolites.
Straight line fits give the linear regression to indicate the direction of the change.

Continued on page 407

controlling preanalytical steps (QC). SOPs should include criteria beyond the common knowledge in clinical chemistry, such as defining processing temperature or specifying the upper limit of the time frame between sample collection and freezing.

Here we have shown, in a comprehensive study design based on broad metabolite classes, that preanalytics affect almost all ontological classes of metabolites in human plasma. From the high impact of blood processing time and temperature, we concluded that the metabolism of blood cells, which make up 37% to 50% of the blood volume, were the cause of the observed effects. Different blood activities of enzymes in study participants as a consequence of interindividual variability or various diseases drive the expectation that an individual response to preanalytics reported for tissue

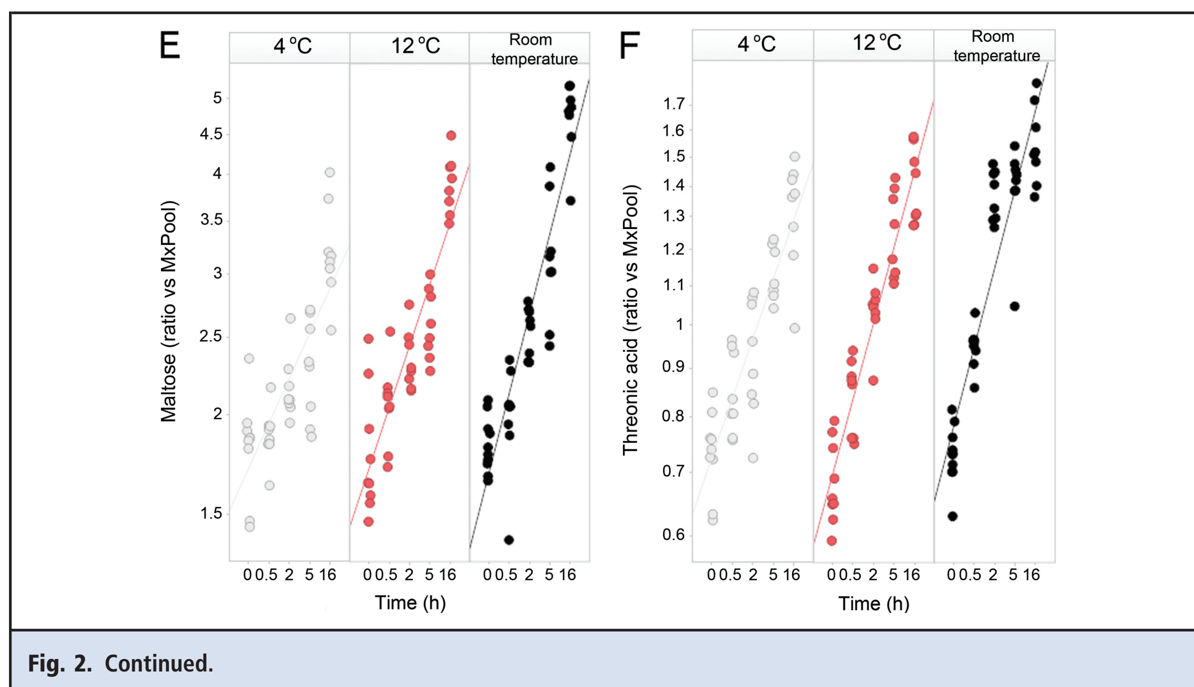

Fig. 2. Continued.

(8) also exists for blood preanalytics. Given this background on blood enzymes, EDTA plasma should be the preferred metabolomics matrix over heparin plasma or serum, because EDTA not only inhibits coagulation but also inhibits Mg^{2+}-dependent enzymes such as the first glycolytic enzyme hexokinase in erythrocytes. However, we also found that a significant decrease of glucose after prolonged incubation occurs in EDTA plasma, emphasizing the importance of rapid and standardized sample preparation for metabolomics. The removal of the blood cells by centrifugation of blood, the highly effective oxygen binding of hemoglobin, and the antioxidative system of erythrocyte glutathione oxidase system makes the plasma more susceptible to chemical-oxidative processes than blood. As a consequence, not only the blood processing time but also the preanalytical plasma processing time (the time from centrifugation to freezing aliquots and the time from thawing samples to analytics device) should be kept to a minimum *(14)*. To avoid contamination with blood cells that change the plasma metabolome, the plasma supernatant should be carefully removed after centrifugation without touching the buffy layer. Our observed differences between short-term blood storage at room temperature and on wet ice emphasize the importance of the definition of detailed and reasonable SOPs for plasma processing.

One published SOP for metabolomics recommends the cooling of blood during the time between collection and centrifugation *(14)*. Though this seems to be advantageous, a review of the preanalytical effects in proteomics analysis found that blood-derived samples should be processed only at low temperatures after platelet removal *(7)* due to the fact that platelets became activated and their metabolism was affected by cold *(29–31)*. Our observed reduction of the concentrations of the platelet-associated serotonin and eicosanoids after exposure to cold could be the consequence of platelet aggregation and therefore improved sedimentation during centrifugation. This hypothesis supports the need for further experiments to address the number of platelets and the serotonin concentration in plasma after applying different centrifugation protocols and different processing temperatures.

One of the most important preanalytical issues in clinical chemistry is in vitro hemolysis *(32)*. The procedure to induce hemolysis in our experiment was a shear-force–induced hemolysis followed by immediate centrifugation. The fact that more metabolite decreases than increases were observed in the hemolysis groups could have been due to dilution effects of the plasma. In a real clinical setting, hemolysis may occur at any time during sample processing (e.g., during phlebotomy) and therefore may be accompanied by a prolonged contact time between glycolytic enzymes from erythrocytes with plasma metabolites, resulting in other and even more severe effects.

Systematic studies like this one are relevant for interpreting of metabolomics results. Examples of metabolites that are highly affected by preanalytics and previously reported as biomarkers in various biomed-

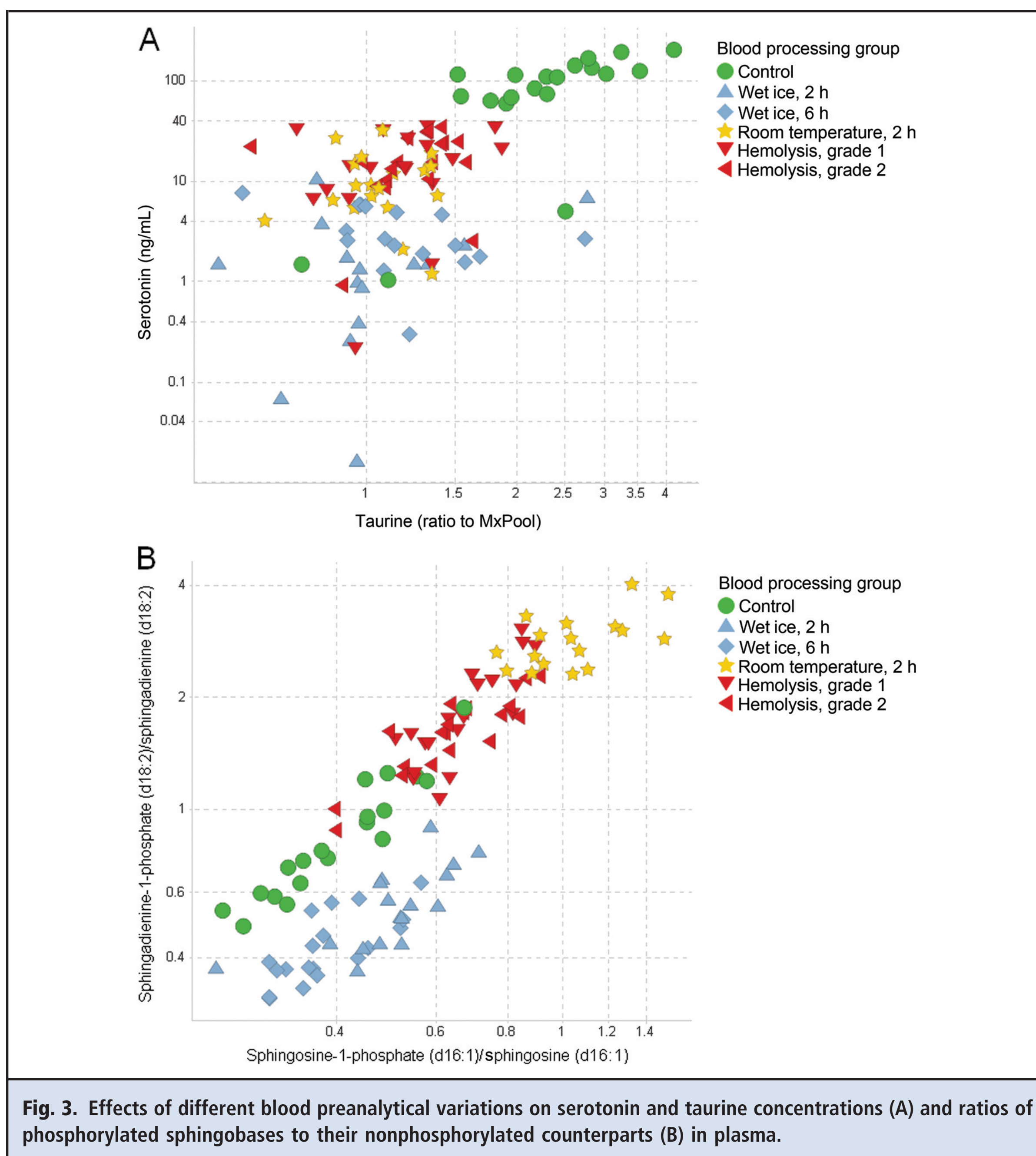

Fig. 3. Effects of different blood preanalytical variations on serotonin and taurine concentrations (A) and ratios of phosphorylated sphingobases to their nonphosphorylated counterparts (B) in plasma.

ical indications *(11, 33–39)* are serotonin, taurine, sphingosine-1-phosphate, glutamine, and glutamate (Fig. 2C, Fig. 3, A and B, and Table 2). Metabolites identified as sensitive to preanalytics should be nominated only carefully as biomarker candidates for disease diagnosis or prognosis, drug efficacy or adverse effects testing and must be thoroughly validated for robustness with respect to preanalytics before publication, product development, or any further application testing. Likewise, reported plasma concentrations of metabolites that are highly affected by preanalytics require careful interpretation, as has been reviewed for serotonin *(24)*.

Systematic studies such as this one are key for designing blood sample handling procedures in prospective studies to avoid systematic metabolite concentration bias between study groups related to sample processing. Our data also support the general good practice of running omics studies, e.g., for biomarker identification and validation, in multicentered designs

Table 3. Metabolites and fold changes of carbohydrates, cofactors, energy, and lipid-related metabolism that are significantly affected by preanalytical short-term storage of blood and plasma with a change of $P < 0.05$ and FDR < 0.2.[a]

Metabolite	Statistically significant metabolite fold changes		
	Blood, room temperature, 2 h	Blood, wet ice, 6 h	Plasma, room temperature, 16 h
Carbohydrates, cofactors and energy metabolism related			
Maltose	0.34	0.27	2.61
Glucose	0.88	0.94	
Lactaldehyde	0.90	0.82	
Lactate	2.08	1.24	
myo-Inositol	0.93	0.91	
Nicotinamide	0.79	0.71	
Oleoylcarnitine	1.48	1.15	
Glucose-6-phosphate (add: fructose-6-phosphate, myo-inositol-1-phosphate, myo-inositol-2-phosphate, myo-inositol-4-phosphate)	0.64		1.56
Glycerate	1.20		2.39
Erythrol	0.93		
Fumarate	1.08		
Hexadecanoylcarnitine	1.16		
Hexadecenoylcarnitine	1.18		
Mannose	0.87		
Octadecanoylcarnitine	1.21		
Pentoses	1.45		
Pyruvate [(add: phosphoenolpyruvate (PEP)]		0.26	0.79
2-Hydroxybutyrate		1.05	
3-Phosphoglycerate (3-PGA)			2.46
Isocitrate			1.16
Threonic acid			2.13
Lipid and sphingolipid metabolism related			
Glycerol, polar fraction	0.77	0.84	1.16
Glycerol-3-phosphate, polar fraction	0.75	0.64	2.83
O-Phosphoethanolamine	0.30	0.32	
Sphingadienine (d18:2)[b]	0.47	1.77	
Sphingadienine-1-phosphate (d18:2)[b]	1.56	0.89	
Sphinganine (d18:0)[b]	0.73	0.64	
Sphingosine (d18:1)[b]	0.65	0.76	
Sphingosine-1-phosphate (d16:1)[b]	1.29	0.92	
Sphingosine-1-phosphate (d17:1)[b]	1.33	0.84	
Sphingosine-1-phosphate (d18:1)[b]	1.25	0.66	
Tetradecanol	0.70	0.72	
Lysophosphatidylcholine (C17:0)	1.13		1.81
Phosphatidylcholine (C16:0,C16:0)	0.96		0.96
Cholesterylester C18:1	1.11		
Phosphatidylcholine (C18:0,C22:6)	1.06		

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Table 3. Metabolites and fold changes of carbohydrates, cofactors, energy, and lipid-related metabolism that are significantly affected by preanalytical short-term storage of blood and plasma with a change of $P < 0.05$ and FDR < 0.2.[a] *(Continued from page 409)*

Metabolite	Statistically significant metabolite fold changes		
	Blood, room temperature, 2 h	Blood, wet ice, 6 h	Plasma, room temperature, 16 h
Phosphatidylcholine	1.07		
Sphingosine (d16:1)	0.51		
Tricosanoic acid (C23:0)	1.19		
Phosphatidylcholine (C16:0,C22:6) (add: phosphatidylcholine (C18:2,C20:4))		1.02	
Sphinganine-1-phosphate (d18:0)		0.59	
Sphingomyelin (d18:2,C16:0)		1.09	
Sphingomyelin (d18:2,C18:0)		1.05	
1-Hydroxy-2-amino-(*cis,trans*)-3,5-octadecadiene (from sphingolipids)			1.09
Ceramide (d18:1,C24:0)			1.19
Ceramide (d18:1,C24:1) [add: ceramide (d18:2,C24:0)]			1.14
Cholesta-2,4,6-triene			0.81
Cholesterol, free			0.94
Cholesterylester C18:2			1.08
Cholesterylester hydroperoxide (C18:2–9-OOH) [add: Cholesterylester hydroperoxide (C18:2–13-OOH), Cholesterylester hydroperoxide (C20:4-OOH)]			2.35
erythro-Dihydrosphingosine (d16:0)			1.11
erythro-Sphingosine (d18:1) (add: sphingolipids)			1.10
Lysophosphatidylcholine (C18:0)			1.48
Lysophosphatidylcholine (C18:1)			1.14
Lysophosphatidylcholine (C18:2)			0.81
Lysophosphatidylcholine (C20:4)			1.16
Phosphatidylcholine (C16:1,C18:2)			0.92
Phosphatidylcholine (C18:0,C18:1)			0.98
TAG (C18:1,C18:2)			0.96
TAG (C18:2,C18:2)			0.92
TAG(C16:0,C18:1,C18:2)			0.92

[a] Fold changes are relative to control for the blood processing groups and relative to time point 0 h for the plasma processing group.
[b] Sphingobases that were not analyzed in the plasma processing experiment.

that allow investigators to control for systematic differences between centers due to preanalytical conditions. In retrospective studies, balancing study groups with respect to different centers and preprocessing variables and conditions should be ensured. Additionally, all available data on sample preanalytics should be considered in statistical analysis and corrected for, if applicable. An example was reported by considering the sample storage time as a fixed effect in an ANOVA of a metabolomics data analysis *(19)*.

We strongly recommend QC applying a highly sensitive analytical method targeting the quality of preanalytics of samples for biomarker-related research to ensure reliability of the results and secure the investment in such projects.

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