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ORIGINAL ARTICLE

Metabolomic Quality Assessment of EDTA Plasma and Serum Samples

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Handling and processing of blood can significantly alter the molecular composition and consistency of biobank samples and can have a major impact on the identification of biomarkers. It is thus crucial to identify tools to determine the quality of samples to be used in biomarker discovery studies. In this study, a non-targeted gas chromatography/time-of-flight mass spectrometry (GC-TOFMS) metabolomic strategy was used with the aim of identifying quality markers for serum and plasma biobank collections lacking proper documentation of pre-analytical handling. The effect of postcentrifugation delay was examined in serum stored in tubes with gel separation plugs and ethylenediaminetetraacetic acid (EDTA) plasma in tubes with or without gel separation plugs. The change in metabolic pattern was negligible in all sample types processed within 3 hours after centrifugation regardless of whether the samples were kept at 4°C or 22°C. After 8 and 24 hours post-centrifugation delay before aliquoting, there was a pronounced increase in the number of affected metabolites, as well as in the magnitude of the observed changes. No protective effect on the metabolites was observed in gel-separated EDTA plasma samples. In a separate series of experiments, lactate and glucose levels were determined in plasma to estimate the effect of precentrifugation delay. This separate experiment indicates that the lactate to glucose ratio may serve as a marker to identify samples with delayed time to centrifugation. Although our data from the untargeted GC-TOFMS analysis did not identify any specific markers, we conclude that plasma and serum metabolic profiles remain quite stable when plasma and serum are centrifuged and separated from the blood cells within 3 hours.

Introduction

BIOBANKS CONSTITUTE AN INVALUABLE asset for medical research, providing access to millions of samples useful for analyzing the interplay between genes and environment, biomarker identification, and development of new diagnostic methods. The European strategy for research using samples stored in biobanks is now moving toward transnational infrastructural networks to enable large-scale multicenter research[1–4] projects. One challenge in using samples collected at multiple sites, either within one country or internationally, is the occurrence of preanalytical variations, for example, the effects of different collection tubes, time, and temperature before centrifugation, after centrifugation but before aliquoting, as well as the conditions during storage. Another challenge is to use archived samples that are already collected and stored within the healthcare system. Such biobanks may contain unique and important collections from different clinical studies, rare diseases, children, or different health screening programs. However, samples not collected for a specific research project may lack proper documentation of preanalytical handling and conditions of storage. Analytical results from such sample collections might then be difficult to evaluate. A quality standard, dependent on the details of a particular research design, the biology of the biomarkers, and the choice of technology, is a prerequisite for successful research projects using expensive large-scale ''omic'' analysis.[5] Quality control (QC) tools and requirements of sample quality have been well integrated into clinical diagnostics for several decades, driven by the demands of accreditation authorities. However, these standards have been set up for specific diagnostic assays in freshly collected samples and do not necessarily meet the demands of biobanking and modern omic analyses applied to samples already stored in biobanks. Thus, we wanted to explore the possibility to perform a robust and simple metabolomic profiling to evaluate the quality of stored samples.

The lack of quality assurance in the field of biobanking is now a well-recognized issue and there are several initiatives

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aimed at driving the development toward implementation of QC tools in biobanking, such as ISBER (International Society for Biological and Environmental Repositories) and the EU funded SPIDIA project (Standardization and Improvement of Generic Preanalytical Tools and Procedures for *In Vitro* Diagnostics). Both initiatives are concerned with evidence-based guidelines for the preanalytical phase, as well as development of QC assays. The large European initiatives in biobank infrastructure, BBMRI-ERIC (Biobanking and Biomolecular Resources Research Infrastructure–European Research Initiative Consortium) and BBMRI-LPC (BBMRI-Large Prospective Cohorts), have sample management as a key point on their agendas. Furthermore, there are now many organizations involved in the work of preparing international standards for an ISO certification of biobanks.

A number of recent publications in metabolomics and proteomics describe major effects on analytical results caused by preanalytical variation, emphasizing the importance of validation of the sample handling protocols for every analytical method.[1,6–9] Boyanton and Blick in 2002[10] reported that storage of centrifuged blood specimens beyond 24 hours caused significant changes in glucose, among other analytes. Since then, numerous studies have demonstrated the complexity of the plasma metabolome, with a pronounced variation in stability and a high interindividual variability in biochemical activities.[11] Fliniaux et al.,[12] reported the influence of different precentrifugation delay and temperature conditions in serum samples using proton nuclear magnetic resonance (NMR) spectroscopy. Bernini et al., proposed that prospectively collected serum and plasma samples should be processed within 2 hours.[13] Anton et al.[14] recently showed that the concentration ratios between lysophosphatidylcholines and phosphatidylcholines could serve as a measure to distinguish samples with a delayed processing time of 12 hours after centrifugation. A better understanding of how sample quality is defined is of fundamental importance before undertaking large-scale omic analysis. Equally important is a requirement that the sample conditions are standardized and documented and taken into consideration, both when interpreting results and comparing data from different studies, or when samples from different biobanks are combined into one study.[15]

In this study, we have applied an untargeted gas chromatography/time-of-flight mass spectrometry (GC-TOFMS) approach to identify metabolites in K2-EDTA plasma and serum that could potentially be used as QC markers in biobank sample collections where proper documentation of the preanalytical handling is absent. Furthermore, in a separate experiment, we have examined the possibility of using the ratio of lactate to glucose concentration in ethylenediaminetetraacetic acid (EDTA) plasma to evaluate precentrifugation delay.

Materials and Methods

Sample collection

Blood samples were collected from 16 healthy donors, 8 males and 8 females, in the age range of 24–60 years (sample set A). Blood was drawn by venipuncture into two 9 mL standard K2-EDTA tubes (Cat. No. 367525; Becton Dickinson [BD]), two K2-EDTA PPT tubes with a gel separation plug (Cat. No. 362799; BD), and two serum tubes with a gel separation plug (Cat. No. 366644; BD). All samples were gently mixed and the serum tubes were placed at room temperature (RT) for coagulation for 30 minutes. All samples were then centrifuged at 2000 *g* for 10 minutes at 22°C and stored for 1, 3, 8, and 24 hours at 4°C or 22°C. At the indicated time points, plasma and serum were aliquoted into 100 μL fractions into two-dimensional barcoded microtubes (REMP-96–300; Brooks Life Science System) and placed at −80°C until analysis. In a consecutive analysis designed to monitor the time- and temperature-dependent effects on glucose and lactate levels, EDTA blood samples from eight healthy donors (sample set B) were collected in K2-EDTA standard nongel tubes essentially as described above, but with a precentrifugation delay at five different time points (1, 3, 8, 24, and 36 hours) at two different temperatures, 4°C and 22°C.

The study was approved by the Regional Ethical Review Board in Stockholm.

Metabolite profiling analysis

Chemicals. The stable isotope-labelled internal standard compounds (IS) [2H4]-succinic acid, [13C5,15N]-glutamic acid, [1,2,3-13C3]-myristic acid, and [2H7]-cholesterol were from Cambridge Isotope Laboratories (Andover, MA); [13C12]-sucrose, [13C4]-palmitic acid, and [2H4]-butanediamine·2HCl were from Campro (Veenendaal, Netherlands); [2H6]-salicylic acid was from Icon (Summit, NJ); N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), 1% trimethylchlorosilane (TMCS), and pyridine (silylation grade) were from Pierce Chemical Co. Stock solutions of the reference compounds, and IS were prepared at a concentration of 0.5 μg/μL in either Milli-Q water or analytical grade methanol.

Sample preparation. Metabolites in serum and plasma samples were extracted and derivatized for metabolomic measurements using GC-TOFMS essentially according to A J et al.[16] In brief, the plasma and serum samples were thawed at 22°C. A solution of 450 μL methanol and water 9:1, spiked with eight stable isotope-labelled internal standards at a concentration of 7 ng per μL, was added to 50 μL of sample. Metabolites were extracted using a bead mill (MM301 vibration Mill; Retsch GmbH & Co. KG), without beads, at a frequency of 30 Hz for 2 minutes at RT. Before extraction, the samples holders were cooled.

Samples were then incubated on ice for 2 hours followed by centrifugation for 10 minutes (4°C, 14,000 rpm). Two hundred microliters of the supernatant was transferred into GC-vials and dried by means of a SpeedVac without heating. The dried extracts were frozen and stored at −80°C before derivatization and GC-TOFMS analysis.

Derivatization. The extracted samples were thawed and dried in a SpeedVac for 10 minutes before derivatization with methoxyamination and silylation as described earlier.[17] In brief, 30 μL of methoxyamine dissolved in pyridine (15 μg/μL) was added to each sample. Samples were shaken vigorously for 15 minutes at 22°C and incubated for 1 hour at 70°C, followed by an additional incubation for 16 hours at 22°C. Thirty microliters of MSTFA 1% TMCS was added to each sample and incubated at 22°C for 1 hour followed by addition of 30 μL of heptane (containing 0.5 μg of methyl stearate as injection IS).

GC-TOFMS analysis. The metabolites in the samples were analyzed by GC-TOFMS, as described previously.[16] One microliter of sample was injected in splitless mode by an Agilent 7683 Series autosampler (Agilent) into an Agilent 6980 GC equipped with a 10 m × 0.18 mm inner diameter, fused-silica capillary column, and chemically bonded 0.18 μm DB5-MS

stationary phase (J&W Scientific). The ion source was a Pegasus III TOFMS (Leco Corp.). The front inlet temperature was set to 270°C. The helium carrier gas was applied at a constant flow rate of 1 mL/min through the column. Purge time was set to 1 m with a flow rate of 20 mL/min and set to equilibrate for 1 minute. The column temperature started at 70°C and was held at this temperature for 2 minutes and then increased to 320°C, increasing by 40°C/min. The maximum temperature was held for 2 minutes. Transfer line temperature was set to 250°C and ion source temperature to 200°C. Ions were generated using a 70 eV electron beam with a current of 2.0 mA.

Measurements acquired m/z were between 50 and 800 at a rate of 30 spectra/s and the acceleration voltage was turned on after a solvent delay of 150 seconds.

To minimize drifting effects, the order of samples for GC-MS analysis was randomized. A mixture of standardized alkanes (C8-C40) was run at the beginning and the end of the GC-TOFMS measurement allowing determination of the retention index (RI) for each metabolite. After every 15th sample, a methyl stearate control sample was run to monitor the instrument sensitivity.

Analytical data were exported as NetCDF files and processed and analyzed using hierarchical multivariate curve resolution[18] and in-house scripts for MATLAB 7.14.739 (R2012a) (Mathworks).

Quantification of glucose and lactate

The concentrations of glucose and lactate in K2-EDTA plasma in sample set B were measured at Karolinska University Hospital following protocols used for routine diagnostic analysis (accreditation according to ISO 15189). Analysis was performed on Roche Modular P instruments (Roche Diagnostics) using the Gluco-quant Glucose/HK and Lactate (Cat. No. 11822837) assays, respectively. Since EDTA plasma is not the preferred sample type for those methods when used in a clinical routine, the stability of thawed plasma was estimated in aliquots from eight EDTA plasma samples, which had been kept frozen before thawing at 22°C. Results from six measurements distributed over 2 hours after thawing demonstrated no significant trend and the intraindividual coefficient of variation for the different samples ranged from 1.5% to 2.9% for glucose and 1.8% to 2.4% for lactate, respectively.

Statistics and data analysis

All multivariate analyses were performed by principal component analysis[19] and orthogonal projection to latent structures (OPLS)[20] using Simca v.13.0.2 (Umetrics). The univariate analysis was performed by either Student's *t*-test or Mann–Whitney, where appropriate, using SPSS v.21 (IBM). Software package R (3.0.1) was used for repeated measures for analysis of variance (ANOVA), with two-factor design using time and temperature as fixed variables for glucose and lactate measured separately at the clinical diagnostic laboratory. Friedman ANOVA, used for the glucose lactate concentration ratio, was calculated using Statistica v. 17 (StatSoft).

Results

Multivariate analysis of the effect of delayed sample handling on metabolic profile

We evaluated the effect of postcentrifugation storage delay at two different temperatures on the metabolite profile using an untargeted GC-TOFMS analysis. Different sample types of plasma and serum were kept for 1, 3, 8, and 24 hours after centrifugation at either 4°C or 22°C before aliquoting and freezing at −80°C in the SST (serum) and PPT (EDTA plasma) sample collection tubes. A gel barrier was used to separate serum and plasma from the blood cell fraction, while plasma in the standard EDTA tubes was in direct contact with blood cells during the processing delay. A total number of 72 metabolites were identified and quantified in these samples. To normalize the interindividual variations in metabolite level, the fold change (FC) of each metabolite was calculated by dividing the measured level at the time points 3, 8, and 24 hours with the corresponding level at 1 hour.

The OPLS plots in Figure 1 illustrate the FC in metabolite profiles after 3, 8, and 24 hours postcentrifugation delay in relation to the 1 hour time point. A clear separation is seen in samples stored for up to 8 hours compared to 1 and 3 hours delay, although the difference was less pronounced in plasma stored at 4°C. However, the 24 hour cluster was clearly separated from the 8 hour time points regardless of whether samples were kept at 4°C or at 22°C. Table 1 shows the statistical parameters in the multivariate analysis, that is, lower cumulative modeled variation in X (R2X), and lower cumulative predicted variation (Q2), which according to cross-validation suggest that EDTA plasma processed at +4°C showed the most stable conditions in this study.

Univariate analysis of metabolite changes

Figure 2 gives an overview of significant changes in metabolite levels after 3–24 hours in relation to the first 1 hour (statistical details are provided in the Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/bio).

Out of 72 metabolites, 44 were identified as showing a significant increase or decrease in relative levels in any of the sample types after the processing delay. Many of these are amino acids (Fig. 2) and as expected, the most significant effects were seen after 24 hours in most samples kept at 22°C. In the two different plasma samples, most amino acids decreased after 24 hours, while a more pronounced increase was seen in serum. It is worth noting that aspartic acid and glutamic acid increased in all sample types and temperatures. Even at 4°C, serum aspartic acid and glutamic acid showed a strong increase after 24 hours, with FC = 1.6 and 2.0, respectively, while cysteine levels were stable at 4°C, but decreased in all sample types kept at 22°C. The most likely explanation for increased levels is *in vitro* enzymatic activity and release of metabolites from cells.

The levels of lactate increased in both plasma sample types, while no consistent pattern was observed in serum. A corresponding decrease of glucose was observed in both plasma types when samples were kept at 22°C for more than 3 hours.

The two purine nucleosides, inosine and adenosine-5-monophosphate, were both sensitive to delayed processing. Inosine decreased in serum samples at both tested temperatures, while in EDTA plasma a large increase was observed when kept at 4°C. Adenosine-5-monophosphate displayed no significant changes in concentration in serum, but in both plasma types large increases were observed after 24 hours at 22°C.

Sugar acids were found to be sensitive to delayed preprocessing. Glyceric acid increased in all sample types at 22°C, but in serum the observed increase was lower

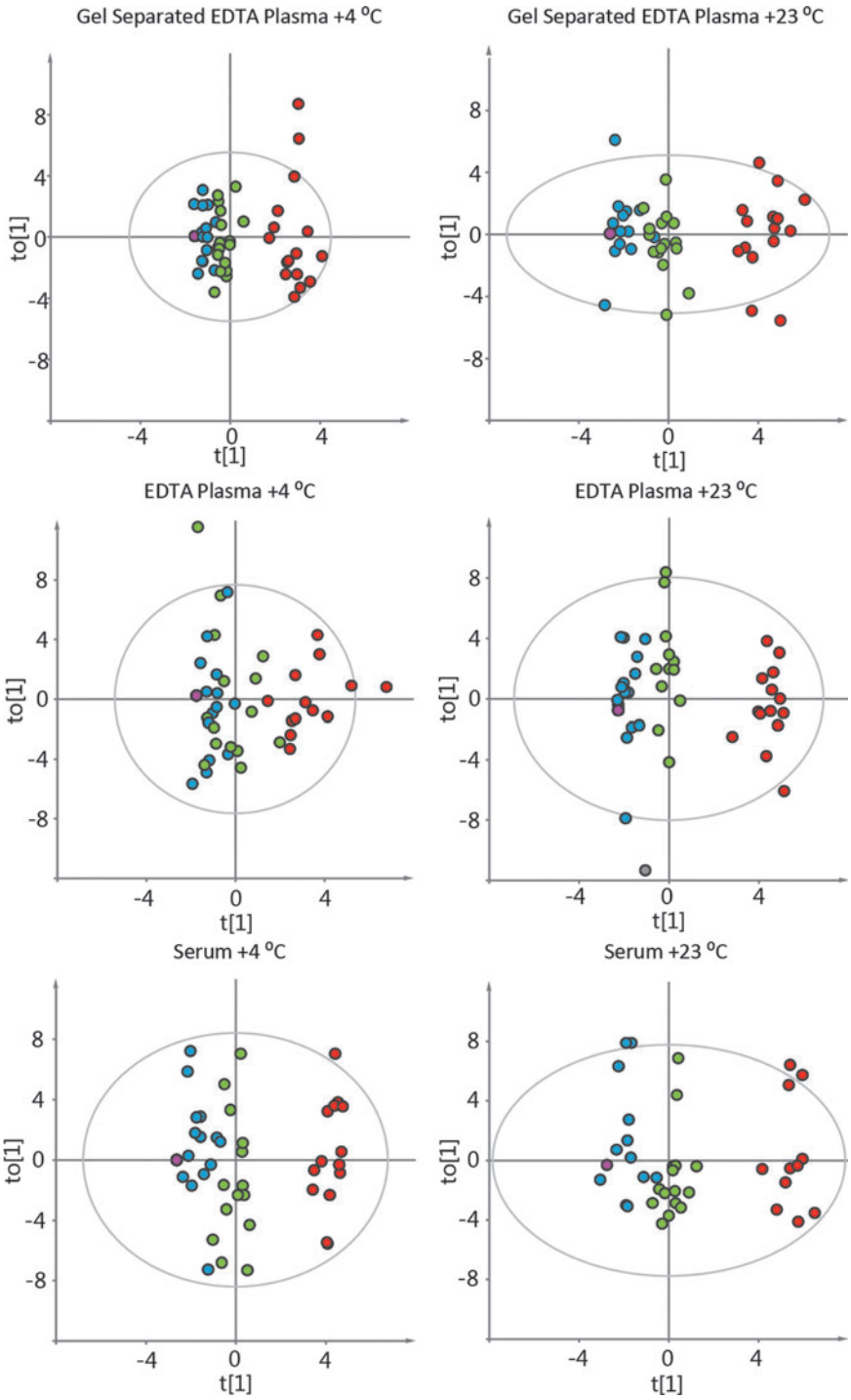

FIG. 1. OPLS score plots based on metabolomic measurements of plasma in gel separated EDTA plasma, standard EDTA plasma, and serum kept at either 4°C or 22°C between 1 and 24 hours before freezing at −80°C. The predictive component (t[1]) show time-dependent differences in metabolite profile between samples stored for 1 hour (*purple*), 3 hours (*blue*), 8 hours (*green*), and 24 hours (*red*) at either 4°C (*left*) or 22°C (*right*). EDTA, ethylenediaminetetraacetic acid; OPLS, orthogonal projection to latent structures. Color images available online at www.liebertpub.com/bio

compared to plasma. Keeping samples at 4°C resulted in a smaller increase of glyceric acid in EDTA plasma and serum. Aldopentose was greatly increased even after 3 hours in the serum samples at both temperatures, while the level was stable for at least 8 hours in plasma samples. A considerable increase was also observed for the sulfonic acid taurine in plasma but not in serum samples.

Quantification of glucose and lactate in EDTA plasma

The untargeted GC-TOFMS analysis indicated a significant increase in lactate in EDTA plasma during the time span 8–24 hours with a more pronounced effect when stored at 22°C. To explore the usefulness of glucose and lactate as QC markers, we applied an accurate analytical assay to obtain more quantitative data compared with the untargeted analysis. EDTA blood was collected in standard nongel tubes from eight nonfasting nondiabetic individuals, and glucose and lactate were quantified using a diagnostic method as described in Materials and Methods section. The processing of these eight EDTA samples (sample set B) differed from the protocol used for sample set A in that for sample set B the precentrifugation conditions were varied. As expected, both degradation of glucose and formation of lactate were strongly increased by delayed precentrifugation,

Table 1. Statistical Summary of Orthogonal Projection to Latent Structure Models

	Gel separated plasma		*EDTA plasma*		*Serum*	
	+4°C	*+22°C*	*+4°C*	*+22°C*	*+4°C*	*+22°C*
Components	1+4	1+2	1+1	1+3	1+3	1+4
R2X	0.50	0.45	0.23	0.55	0.45	0.59
Q2	0.81	0.91	0.61	0.94	0.88	0.85

Multivariate analysis of the relationship between metabolomic profiles and preprocessing time of different sample types at two conditions show that the most stable conditions tested in this study are EDTA plasma preprocessed at 4°C. R2X is the cumulative modeled variation in X, and Q2 is the cumulative predicted variation in Y, according to cross-validation. The range of these parameters is 0–1, where 1 indicates a perfect fit.
EDTA, ethylenediaminetetraacetic acid.

with a less pronounced change in samples kept at 4°C compared to 22°C (Figs. 3 and 4). Statistical analysis using two factor repeated measures ANOVA showed that time and temperature, but not individual variability, significantly contributed to the effect ($p < 2.0\,e^{-16}$), see Table 2. The EDTA plasma concentrations of glucose and lactate were within the expected concentration levels for healthy individuals after 1 hour processing. The lactate/glucose concentration ratios were also evaluated. As indicated in Figure 5, the ratio of lactate to glucose concentration can be useful in distinguishing at least EDTA samples that have been stored for >8 hours at 22°C before centrifugation. The Friedman ANOVA test, comparing multiple depended samples, revealed statistically significant differences ($p < 0.05$) between the time points at both

		Gel Separated EDTA Plasma						EDTA Plasma						Serum					
		+4°C			+23°C			+4°C			+23°C			+4°C			+23°C		
Compound Class	Metabolite	3 hrs	8 hrs	24 hrs	3 hrs	8 hrs	24 hrs	3 hrs	8 hrs	24 hrs	3 hrs	8 hrs	24 hrs	3 hrs	8 hrs	24 hrs	3 hrs	8 hrs	24 hrs
Amino acid	4-Hydroxyproline	-	-	-	-	-	-	-	-	-	-	↓	-	-	-	-	↑	-	↑
Amino acid	Arginine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	↑	↑
Amino acid	Asparagine	-	-	-	-	↓	↓	-	-	-	-	↓	↓	-	-	-	↑	-	↑
Amino acid	Aspartic acid	-	-	↑	-	-	↑	-	-	↑	-	-	↑	-	↑	↑	↑	↑	↑↑
Amino acid	Beta-alanine	-	-	↑	↓	-	-	-	-	-	-	-	-	-	↓	-	↑	-	↑
Amino acid	Cysteine	-	-	↓	-	↓	↓	-	-	-	-	↓	↓	-	-	-	-	-	-
Amino acid	Cystine	-	-	↓	↓	↓	↓	-	-	-	↓	↓	↓↓	-	↓	↓	-	↓	↓↓
Amino acid	Glutamic acid	↑	↑	↑	-	↑	↑↑	-	-	↑	-	-	↑	-	↑	↑↑	↑	↑	↑↑
Amino acid	Glutamine	-	-	-	-	-	-	-	-	↑	-	-	↓	-	-	-	↑↑	↑	-
Amino acid	Glycine	-	-	↑	-	-	-	-	-	↑	-	-	-	-	-	-	↑	↑	↑
Amino acid	Histidine	-	-	-	-	-	-	-	-	-	-	-	↓	-	-	-	-	-	↑
Amino acid	Leucine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	-	↑
Amino acid	Lysine	-	-	-	↓	-	↓	-	-	-	-	-	↓	-	↓	-	↑	-	-
Amino acid	Methionine	-	-	↓	-	-	-	-	-	-	-	-	-	-	↓	-	-	↓	-
Amino acid	Ornithine	-	-	↑	-	-	-	-	-	-	-	↓	-	-	-	-	↑	-	↑
Amino acid	O-Phosphoetanolamine	-	-	↑	-	-	-	-	-	-	↑	-	-	-	-	-	↑	-	-
Amino acid	Phenylalanine	↑	-	-	-	↓	-	-	-	-	-	↓	↓	-	-	↑	↑	↑	↑
Amino acid	Serine	↑	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	↑	-	↑
Amino acid	Threonine	-	-	-	-	-	-	-	-	-	↓	↓	-	-	-	↑	-	-	↑
Amino acid	Tryptophan	-	-	↑	-	↓	-	-	-	-	-	↓	↓	-	-	-	-	-	-
Amino acid	Tyrosine	-	-	↑	-	↓	-	-	-	-	-	-	↓	-	-	-	-	↓	↑
Carboxylic acid	Citric acid	-	-	↑	↓	↓	↓	-	-	-	-	↓	↓	-	↓	-	-	↓	↓
Carboxylic acid	Malic acid	↑	-	-	-	-	↑	-	-	-	-	-	↑	-	-	↑	-	-	-
Carboxylic acid	Succinic acid	-	-	-	-	-	↑	-	-	-	↓	↓	↑	-	-	-	-	↓	↓
Cyclic alcohol	Inositol	-	-	↑	-	↓	-	-	-	-	-	↓	-	-	-	-	-	-	-
Fatty acid	Ketoleucine	-	-	-	-	-	-	-	↓	↓	-	↓	↓	-	-	-	-	-	-
Fatty acid	Oleic acid	-	-	↑	-	-	↑	-	-	-	-	-	↑	-	-	-	-	↑	↑
Fatty alcohol	Palmitic acid	-	-	↑	-	-	↑	-	-	-	-	-	↑	↑	↑	↑	-	-	↑
Hydroxy acid	Lactic acid	↑	↑	↑	-	↑	↑↑	↑	↑	↑↑	-	↑	↑↑	-	-	↑	↑	↓	-
Imidazopyrimidine	Uric acid	-	↑	-	-	↓	↓	-	-	↑	-	↓	↓	-	↓	↓	-	-	↓
Keto acid	Alpha-ketoglutaric acid	-	-	-	-	-	↑	-	-	↑	-	-	↑	-	-	-	-	-	↑
Lactams	Creatinine	-	-	↓	-	-	-	-	-	-	-	-	-	↑	-	-	-	-	-
Monosaccharide	1,5-Anhydro-D-glucitol	-	↑	↑	-	↓	-	-	-	-	-	↓	-	-	↓	-	-	-	-
Monosaccharide	Glucose	-	-	-	-	↓	↓	-	-	-	-	↓	↓	-	-	-	-	-	-
Monosaccharide	Glycerol-3-phosphate	-	-	-	-	-	↑	-	-	↑	-	-	↑↑	-	-	-	-	↑	↑
Monosaccharide	Pyruvic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓
Purine nucleotide	Adenosine-5-monophosphate	-	-	-	↑	-	↑↑	-	↑	↑	-	-	↑↑	-	-	-	-	-	-
Steroid	Cholesterol	-	-	-	-	-	↓	-	-	-	-	-	↓	-	↓	-	-	-	↓
Sugar acid	Glyceric acid	-	↑	↑↑	-	↑↑	↑↑	-	-	↑↑	-	↑↑	↑↑	↑	-	↑	↑	↑	↑
Sugar acid	Aldopentose	-	-	-	-	-	↑	-	-	↑	-	-	-	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Sulfonic acid	Taurine	↑↑	-	↑↑	-	↑↑	↑↑	↑↑	↑↑	↑↑	-	-	↑↑	-	-	-	-	-	-
Urea	Urea	-	-	-	-	-	↑↑	-	-	-	↓	-	-	-	↑↑	-	↓	-	↓

FIG. 2. Trends in metabolite concentration in plasma and serum samples with delayed preprocessing time under two conditions. Significant changes ($p < 0.05$) in 44 metabolites out of 72 identified metabolites are presented. Metabolites with an average fold change >1.0 are indicated by ↑ and an average fold change <1.0 is indicated by ↓. Metabolites with either a fold change >1.5 or <0.5 are indicated by ↑↑ or ↓↓, respectively. A delayed preprocessing of EDTA plasma at 4°C for 3 hours was shown to give the most stable metabolite profile, with only two metabolites, lactate and taurine, showing significant changes.

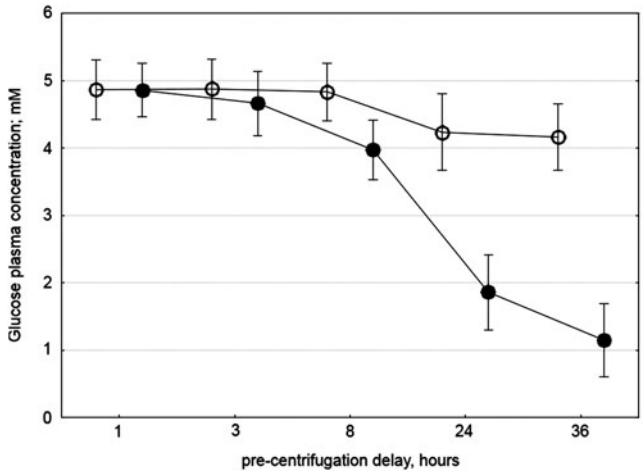

FIG. 3. Time- and temperature-dependent oxidation of glucose in EDTA plasma of eight individuals; mean ± SD. *Solid circles* refer to the concentration kinetics at 22°C and *open circles* at 4°C. SD, standard deviation.

temperatures. However, this conclusion needs to be further tested and validated in sample collections with a defined pre-centrifugation delay and samples containing different levels of plasma glucose and cell counts.

Discussion

The starting point of this study was to identify metabolites that could serve as quality markers in archived samples, as well as a simple and inexpensive test for fresh samples before storage. We therefore conducted a study on healthy volunteers to determine the effect of preanalytical variations on the levels of metabolites in different sample types. A nontarget GC-TOFMS method was used and applied to EDTA plasma and serum that were kept at 4°C and 22°C for 1, 3, 8, and 24 hours before centrifugation, which was immediately followed by aliquoting and freezing. In this study, we confirmed the results of Yin et al.,[9] Kamlage et al., and

Table 2. Two Factor Repeated Measures Analysis of Variance for Glucose and Lactate

	Df	*Sum sq.*	*Mean sq.*	F-*Value*	p-*Value*
Glucose					
Temperature	1	33.5	33.5	163.7	4.1e^{−6}
Time	4	67.2	16.9	174.4	2.0e^{−16}
Temp:Time	4	28.6	7.6	232.2	2.0e^{−16}
Lactate					
Temperature	1	182.4	182.4	228.1	1.3e^{−6}
Time	4	305.0	76.3	271.3	2.0e^{−16}
Temp:Time	4	139.6	34.9	180.1	2.0e^{−16}

Kang et al., that the plasma metabolome is quite stable if samples are handled within 3 hours at 4°C. Many metabolites and also proteins have been well characterized in these studies and it is clear that the identification of accurate quality markers is a considerable challenge. There are several explanations for this: the metabolome is very complex, with a high interindividual variability in plasma concentration and in biomarker stability; there is also a cascade of *in vitro* enzymatic activities, inhibition, and activation in the tube after the blood has been drawn from the vein. Another challenge is the analytical strategy, since different methods can detect different metabolites, which make it more difficult to compare analytical findings. Metabolomic profiling using GC-TOFMS has the advantage of covering a wide range of metabolic groups, and metabolites can be identified by comparing the extracted mass spectra and RI to those found in internal and external mass spectra libraries. By calculating the resolved peak areas for the detected metabolites, the relative concentrations can be compared between samples. The limitations of GC-TOFMS are that only volatile metabolites, and those made volatile by derivatization, can be measured. In addition, the use of an extraction procedure providing a large compound coverage might not be as efficient as extraction protocols optimized for specific compound classes.[16] While the use of specific derivatization and extraction methods would have provided some improvement

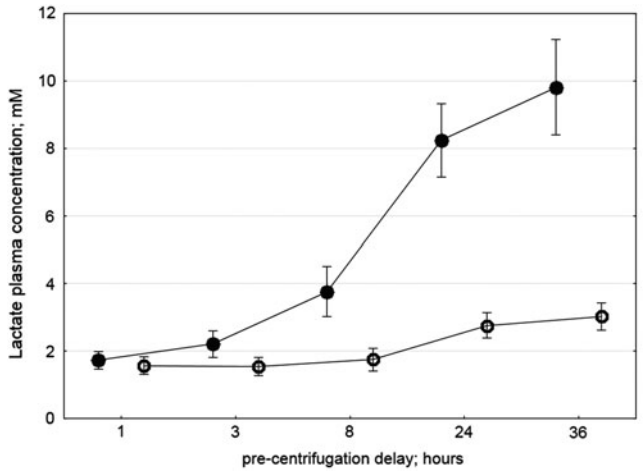

FIG. 4. Time- and temperature-dependent formation of lactate from glucose in EDTA plasma of eight individuals; mean ± SD. *Solid circles* refer to the concentration kinetics at 22°C and *open circles* at 4°C.

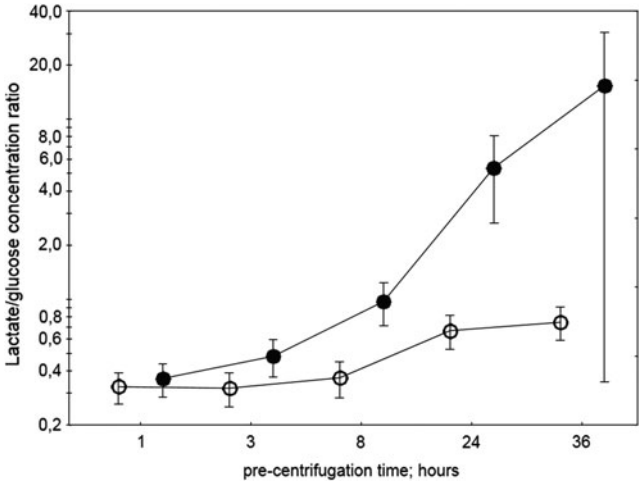

FIG. 5. Mean ± SD concentration ratio of lactate and glucose in EDTA plasma kept at +22°C (*solid circles*) and at 4°C (*open circles*) before centrifugation. The *Y*-axis represents real values in logarithmic scale.

in output for certain metabolite classes, such methods would be limited in coverage.

Taking this into consideration, untargeted metabolite profiling with GC-TOFMS is ideally suited as a first method in an explorative approach, with the aim of generating new hypotheses, to be further investigated and confirmed with targeted methods.

Definition of sample quality in biobanks is a balance between the demands of analytical platforms used for different downstream applications, practical issues in overall sample collection logistics, and the consecutive processes in the biobank, as well as cost. The metabolomic pattern is too complex to allow downstream analysis without a comprehensive validation of the stability of the metabolites, while preanalytical variables in specimen collection and storage that could influence the analytical findings are the most difficult to manage. Such problems are often not recognized in high-throughput analytical platforms.

Using previously collected samples with limited documentation is a challenging task of particular significance. For target analysis there are several approaches, of which the most powerful would be to apply an experimental protocol for validation of the metabolites before starting collection, as presented by Betsou et al.[6] Another approach presented in several publications is to find a feature based on a biomarker profile as discussed by Fliniaux et al.,[12] and Kamlage et al.[11] Anton et al.,[14] report the usefulness of using a metabolic ratio as an intrinsic marker to distinguish between ''good'' and ''bad'' samples. The advantage with this model is to minimize the interindividual differences in metabolite concentrations, which is significant and therefore limits the possibility to use concentration levels of specific metabolites as a QC marker. However, using advanced analytical methods in the evaluation of quality-related variations could be very costly and probably not applicable in academic research.

The results in this study confirm that metabolic pattern is affected in samples stored without separation from the cells and that temperature is an important factor. Tubes containing gel-separation plugs are used to limit the leakage of metabolites from red blood cells and their use is well established for clinical chemistry serum analytes. Nevertheless, in this study, we did not detect any protective effect of gel separation. This is probably explained by the choice of method applied in this study, for example, analytical precision may not have been high enough or important metabolites might have been undetected.

It is well known that glucose levels fall and lactate rises in stored blood samples due to reduced oxygenation, which activate the glycolytic anaerobic pathway. This effect was observed in the GC-TOFMS analysis, although not fully consistent between the sample types. In the follow-up study, a more accurate analytical assay was used to obtain more precise quantitative data in the time interval from 1 to 36 hours. We calculated the EDTA plasma concentration ratio of lactate and glucose to test the possibility of predicting the delay in processing time. Our data show pronounced changes in the concentration ratio that separate plasma samples processed within 8 hours from a longer processing time. This time point may be good enough for less sensitive biomarkers in the case where no information about the history of sample handling is available. This is consistent with several other studies,[12] but given the small number of individuals who are all healthy and nondiabetic, its not applicable as a quality signature on random archived samples.

Thus, while a golden QC standard for metabolomics has not yet been identified, it is possible to agree on general guidelines to support prospective collections ensuring their value in downstream molecular analysis applications. Based on recent publications, a general recommendation to obtain comparable samples would be first to ensure that standardized and well documented workflows are used, and second to use IT systems enabling the possibility of tracking all steps in the preanalytical chain from needle to freezer. Since temperature has a great impact on sample quality, samples should as far as possible be processed within 3 hours and kept frozen at −80°C until analysis. In cases where the sample collection logistics entail a longer processing time, the use of gel plug tubes with centrifugation of the samples within 60 minutes is one alternative. This will at least separate serum and plasma from the blood cell fraction before aliquotation.

Although there are many reports that demonstrate the stability of metabolites in different steps in the preanalytical phase, more studies are needed that can be applied on archived samples as a signature of how samples has been processed. In case preanalytical factor is a variable in a study, this should be included in a multivariate analysis when reporting metabolomic data[12,21]

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Author Disclosure Statement

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