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The Influence of Preanalytical Biospecimen Handling on the Measurement of B Vitamers, Amino Acids, and Other Metabolites in Blood

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Introduction: Sample handling can influence biomarker measurement and introduce variability when combining data from multiple studies or study sites. To inform the development of blood collection protocols within a multisite cohort study, we directly quantified concentrations of 54 biomarkers in blood samples subjected to different handling conditions.
Materials and Methods: We obtained serum, lithium heparin plasma, and EDTA plasma from 20 adult volunteers. Tubes of chilled whole blood were either centrifuged and processed within 2 hours of collection (the ''reference standard'') or were stored with cool packs for 24 or 48 hours; centrifuged before and/or after this delay; or collected in tubes with/without gel separators. We used linear mixed models with random intercepts to estimate geometric mean concentrations and relative percent differences across the conditions.
Results: Compared to the reference standard tubes, concentrations of many biomarkers changed after processing delays, but changes were often small. In serum, we observed large differences for B vitamers, glutamic acid (37% and 73% increases with 24- and 48-hour delays, respectively), glycine (12% and 23% increases), serine (16% and 27% increases), and acetoacetate (−19% and −26% decreases). Centrifugation timing and separator tube use did not affect concentrations of most biomarkers.
Conclusion: Sample handling should be consistent across samples within an analysis. The length of processing delays should be recorded and accounted for when this is not feasible.

Keywords: epidemiologic studies, biomarkers, specimen handling, B vitamers, amino acids

Introduction

Understanding the influence of biospecimen handling on the measurement of biomarker concentrations is critical for making informed inferences about causal relationships in biomedical research, especially when data are pooled across multiple studies. Many studies examine the stability of biomarkers in blood, particularly after samples undergo multiple freeze-thaw cycles or are stored for long periods of time.[1–3] Comparisons across matrix type are also common.[4–7] Some studies assess the effect of handling procedures before processing and freezing, but among these studies, the designs and analytic approaches vary considerably.[2,4,8–21] Furthermore, many of these studies present results collectively or visually (e.g., grouped by biologic pathway or using principal components analyses), rather than

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report concentrations of biomarkers individually—which makes comparisons with prior research difficult.

The Connect for Cancer Prevention Study (Connect) is a cohort study initiated by the United States National Cancer Institute (NCI) in partnership with nine integrated health care systems serving 11 U.S. states.[22] Biospecimens will be collected in clinical facilities at or near the study sites. To ensure standardized processing, all biospecimens for Connect will be shipped in chilled containers to a central NCI laboratory in Maryland after collection.

Most researchers agree that immediately processing and freezing blood samples is ideal, but this is rarely feasible and may be cost-prohibitive—especially in large multisite epidemiologic studies. To inform the development of protocols for blood collection within Connect, we conducted a pilot study to evaluate changes in biomarker concentrations associated with delayed processing (storing samples with cool packs for 24 or 48 hours to simulate shipment times); type of collection tube (standard or containing a gel separator); and timing of centrifugation (both before and after the processing delays, or only after). We examined the influence of these handling conditions in three matrix types: serum, lithium heparin plasma, and ethylenediaminetetraacetic acid (EDTA) plasma. We then directly quantified concentrations of 54 biomarkers, including B vitamers, amino acids, and other metabolites which may be of interest in cancer or diabetes-related research.

Materials and Methods

Study population

For this pilot study, nonfasting blood samples were collected from 20 participants in the Research Donor Program at the Frederick National Laboratory for Cancer Research. Each volunteer donated 24 tubes of blood (seven tubes each of serum, lithium heparin plasma, and K2 EDTA plasma were included in this analysis, $n=21$). In brief, 10 men and 10 women, 34–60 years of age, and two of whom were current smokers, comprised the study population. To preserve confidentiality, further characteristics are not reported due to the small sample size. All participants provided written informed consent.

The specimens were collected as part of clinical research protocol number OH99CN046, Collection and Distribution of Samples from Healthy Donors for *In Vitro* Research at NCI-Frederick. The protocol was reviewed and approved by the National Institutes of Health Institutional Review Board. The data generated in this study are available upon reasonable request (contact: Dr. Stephanie Weinstein, weinstes@mail.nih.gov).

Sample collection and handling

Blood draws occurred between 8:00 AM and 2:00 PM through standard forearm venipuncture. All tubes were collected as whole blood. Tubes designated for serum were allowed to clot for 30 minutes at room temperature before any centrifugation or further processing. For each matrix type, one standard tube was centrifuged and aliquoted within 2 hours of collection (''immediately''); these tubes did not contain gel separators and are considered the reference standard tubes for purposes of comparison (''RST''/tube 0). Tubes without gel separators are referred to as standard tubes. Each of the standard tubes was 6 mL; the serum and EDTA plasma separator tubes were 5 mL, and the lithium heparin plasma separator tubes were 4.5 mL. All tubes were manufactured by Becton, Dickinson and Company.

In addition to the RST, for each matrix type, another six tubes underwent different handling procedures to simulate conditions that occur in typical epidemiologic studies (Fig. 1). All whole blood tubes were stored in shipping containers with cool packs and placed within a walk-in refrigerator until the final processing step, which consisted of centrifugation, separating the matrix from the cell pellet, aliquoting, and freezing.

The conditions we assessed were as follows: centrifuging a standard tube immediately and then storing for 24 hours before the final processing step (tube type 1); storing a standard tube for 24 hours before the final processing (tube 2); centrifuging a gel separator tube immediately and storing for 24 hours before final processing (tube 3); centrifuging a standard tube immediately and then storing for 48 hours before final processing (tube 4); storing a standard tube for 48 hours before the final processing (tube 5); and centrifuging a gel separator tube immediately and storing for 48 hours before final processing (tube 6).

Tubes that were centrifuged immediately were also centrifuged again during the final processing step (i.e., a second time), with the exception of the immediately processed RST. All centrifugations were 1200*g* for 10 minutes. The final aliquots were frozen at −80°C before being shipped on dry ice for biochemical analysis.

Biomarker measurement

The serum, lithium heparin plasma, and EDTA plasma samples were each divided into two batches per matrix type. All frozen samples were shipped together to BEVITAL (Bergen, Norway; www.bevital.no) to directly quantify concentrations of the biomarkers; all biochemical analyses were based on validated mass-spectrometry assays that included stable isotope-labeled internal standards for each analyte. The B vitamers and markers of tryptophan metabolism were measured by liquid chromatography-tandem mass spectrometry (referred to as Platform D, 23 biomarkers).[23] Remaining sample volumes were re-frozen and then thawed in a cooler for a second time to measure amino acids and diabetes-related biomarkers by gas chromatography-tandem mass spectrometry (Platform B, 31 unique biomarkers).[24]

Tryptophan, kynurenine, and cystathionine were assessed on both platforms and measurements were comparable across the platforms (biomarker concentrations are reported for both platforms throughout our tables). Arginine is not offered for measurement on the second analytic platform; aspartic acid concentrations were measured, but not evaluated, due to known instability in samples after multiple freeze/thaw cycles or when left at room temperature.[25] Sarcosine was measured in serum and lithium heparin plasma, but not in EDTA plasma, as these tubes contain sarcosine.[8] The laboratory did not observe significant hemolysis in any sample.

Two identical quality control samples were placed in each batch; we estimated coefficients of variation (CV) within batch and matrix type. Most CVs within batch and within matrix type were excellent (<10% for both untransformed

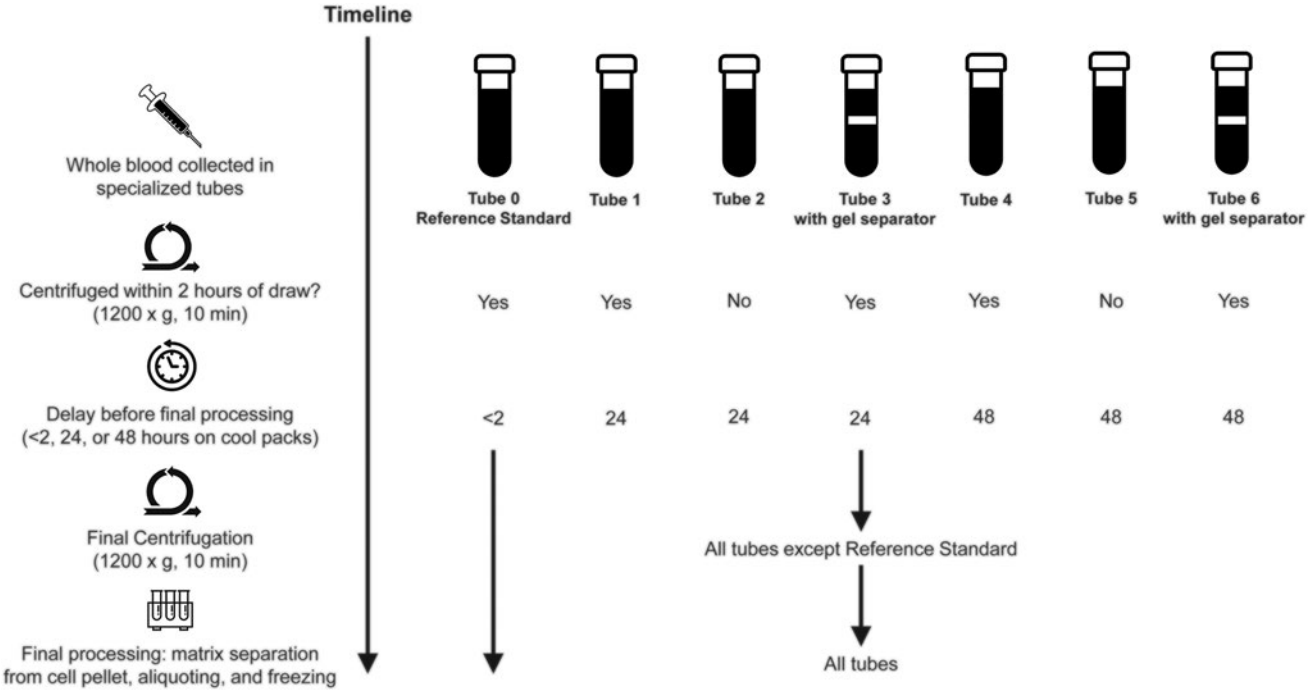

FIG. 1. Overview of blood samples collected and the preanalytical handling conditions assessed in this analysis.

and log-transformed concentrations). Within matrix types, we noted higher CVs for the following biomarkers (log-transformed): 3-hydroxybutyrate within the serum batches (24%); glutamic acid in lithium heparin plasma (17%); and 2-aminoadipic acid within lithium heparin (18%) and EDTA plasma (18%).

Statistical analysis

Biomarker concentrations were log transformed. In addition to measuring multiple B vitamers, we also calculated prespecified summary measures of B vitamin status that show some stability under a range of processing conditions: thiamine plus thiamine monophosphate (vitamin B1), riboflavin plus flavin mononucleotide (B2), and pyridoxal 5-phosphate plus pyridoxal (B6).[1,2]

We then used linear mixed models to estimate least-squares geometric mean concentrations and 95% confidence intervals (CIs) for each biomarker within the tube types described above, or for groups of tubes. CIs were constructed without adjustment for multiple comparisons. Models accounted for correlation among each participant's repeated measurements using a random intercept and equal covariance between repeated measures (proc mixed modeling in SAS with a ''random'' statement). Model residuals were examined for normality and heteroskedasticity; no major deviation was observed. Percent differences between geometric means were calculated from the model beta estimates and CIs using the formula ((*e*^(estimate))-1)*100.

Our primary objective was to quantify the effects of delayed processing on biomarker concentrations (i.e., storing with cool packs for 24 or 48 hours before the final centrifugation and separation from the cell pellet). Within each matrix type, we calculated geometric mean concentrations of biomarkers for each processing condition/in each of the tubes and then estimated percent differences for tubes 1–6 compared to the immediately processed RST (tube 0).

To examine the overall influence of processing delays, we also estimated geometric means and percent differences for standard tubes that were held for 24 hours (grouping tubes 1 and 2 in the same category) or 48 hours (tubes 4 and 5) and compared to the RST. These models were adjusted for timing of centrifugation (i.e., immediately and during the final processing or only during the final processing). Separator tubes were not included in this analysis.

To estimate the independent effect of centrifugation timing on biomarker concentrations, we also compared tubes that were only centrifuged after a delay (i.e., during their final processing; tubes 2 and 5) to tubes that were centrifuged both immediately and after a delay (tubes 1 and 4). These models were adjusted for length of delay. RST and separator tubes were not included in this analysis.

All separator tubes were centrifuged immediately and underwent a delay before final processing. We assessed the independent effect of using separator tubes (tubes 3 and 6) by making comparisons to standard tubes that were also centrifuged immediately and had a processing delay (tubes 1 and 4). These models were adjusted for length of processing delay. RST and standard tubes centrifuged only during final processing were not included.

A priori, we expected measurement differences by matrix type and stratified all results. We also calculated the geometric mean biomarker concentrations within all serum, lithium heparin plasma, and EDTA plasma tubes and then estimated percent differences in concentrations by comparing to serum. These models were adjusted for processing condition (i.e., tube type), but separator tubes were not included because separator types differ by matrix.

We assessed statistical interaction between the processing conditions and matrix type by Type III/F-test *p*-values for interaction terms. We also calculated the absolute values of

all percent differences across the biomarkers or groups of biomarkers and report the ranges and interquartile ranges (IQRs) of these effect magnitudes. An overview of observations included and comparisons made in each analysis is presented in Supplementary Figure S1. SAS version 9.4 was used for statistical analyses (SAS Institute, Inc., Cary, NC).

Results

Processing delays

While most differences were small (<15%), the geometric mean concentrations for several biomarkers consistently differed relative to the immediately processed RST (Figs. 2–4; Supplementary Tables S1–S3). In serum, B vitamers and amino acids showed the strongest differences associated with delayed processing (Fig. 2, Supplementary Table S1). For example, geometric mean riboflavin concentrations increased by 15% or more in tubes with a 24-hour processing delay and by 23% or more in tubes with a 48-hour delay, relative to concentrations in the RST. Concentrations of its metabolic precursor, flavin mononucleotide, decreased by at least −12%, regardless of processing delay length. Of all B vitamers, we identified the largest differences compared to the RST for nicotinamide (a B3 vitamer; 56%–220% increases across serum tubes). For the amino acids, glutamic acid showed the strongest differences in concentration (34%–78% increases across the serum tubes), but changes were demonstrable for glycine, phenylalanine, and serine.

In general, biomarker concentrations that differed when compared to the RST in serum also differed when measured in lithium heparin plasma, with the strongest differences for this matrix type also observed among the B vitamers (Fig. 3, Supplementary Table S2). Within EDTA plasma, there were fewer and smaller differences for B vitamer concentrations when compared to the RST, although increases in riboflavin were visible after 48 hours (increases of 7%–12% across tubes with a 48-hour delay) and nicotinamide continued to show poor reproducibility relative to the RST (Fig. 4, Supplementary Table S3). Unlike serum, for both plasma types, the concentrations of many proteinogenic amino acids, except glutamic acid, seemed relatively stable across tube types when compared to the RST.

When we estimated the overall effects of delayed processing (i.e., grouping tubes by length of processing delay [24 or 48 hours]; Supplementary Table S4), many biomarkers seemed stable, but we observed differences for: nicotinamide (after 48 hours: 163% increase in serum, 117% in lithium heparin plasma, and 105% in EDTA plasma), neopterin (48 hours: −14% decrease in serum, −12% in heparin, and −34% in EDTA), glutamic acid (48 hours: 73% increase in serum, 36% in heparin, and 24% in EDTA), and acetoacetate (48 hours: −26% decrease in serum, −27% in heparin, and −32% in EDTA). Delay-associated increases in homocysteine and ornithine were more modest in plasma than in serum. B vitamers and amino acids showed the strongest overall concentration differences associated with delayed processing, especially in serum and lithium heparin plasma.

Across all biomarkers in serum, the IQR of percent differences (absolute magnitudes) was 1%–7% for tubes with a 24-hour delay and 2%–10% for tubes with a 48-hour delay (Supplementary Table S5). The absolute percent differences associated with delayed processing were smaller in both lithium heparin (IQR <1%–3% for a 24-hour delay and IQR 2%–8% for a 48-hour delay) and EDTA plasma (IQR <1%–2% for a 24-hour delay and IQR <1%–4% for a 48-hour delay).

Timing of centrifugation

When compared to the RST, most serum biomarker concentrations differed to a similar extent, regardless of whether they were centrifuged both before and after the processing delays, or only after the delays, during final processing (Fig. 2, Supplementary Table S1). Concentrations of nicotinamide, homocysteine, and ornithine increased more strongly in tubes centrifuged only after the delays, while α-ketoglutaric acid decreased. Centrifuging only after the delays made measurement of serum neopterin more comparable to the reference standard tubes (centrifuging before and after a 48-hour delay: −15% decrease and centrifuging only after: −3%, not statistically significant). Similar patterns were noted in both plasma types.

When we compared the standard tubes that were only centrifuged after a processing delay to those that were centrifuged both before and after a delay (as opposed to the RST, which had no delay), we confirmed that most biomarkers were not strongly influenced by timing of centrifugation, except the above-mentioned biomarkers (Supplementary Table S6). For neopterin, the percent differences associated with only centrifuging after the delays changed direction when we used the alternate reference group (increases of 8%–23% across the matrix types). Overall, the IQRs of the absolute percent differences associated with centrifuging only after the processing delays were similar by matrix type, platform, and biomarker group (Supplementary Table S5).

Separator tubes

For many biomarkers measured in serum, concentrations in separator tubes were comparable to the RST; furthermore, if we observed concentration differences in standard tubes with delays, they were generally also observed in separator tubes (Fig. 2, Supplementary Table S1). Use of separator tubes exacerbated measurement differences for a few biomarkers compared to the RST. As the most extreme example, serum concentrations of α-hydroxyglutaric acid increased 14%–17% in standard tubes with a 24-hour processing delay, but increased by 132% in separator tubes with a 24-hour delay. This was similarly noted in lithium heparin and EDTA plasma (Figs. 3 and 4; Supplementary Tables S2 and S3). We found measurement differences for many amino acids were more visible within serum separator tubes than within separator tubes of either plasma type.

When we compared separator tubes to standard tubes that also had a processing delay (Supplementary Table S7), we identified fewer differences in amino acid concentrations associated with separator tube use—suggesting that differences relative to the RST may be attributable to the processing delays in separator tubes, rather than the separators. However, use of separator tubes still affected the measurement of several B vitamers, quinaldic acid, nicotinamide, and α-hydroxyglutaric acid across the matrix types. The IQR

Biomarker (Serum)	Percent Difference in Concentrations (vs. reference standard tube)					
	24-hour processing delay			48-hour processing delay		
	Centrifuged before and after (tube 1)	Only centrifuged after (tube 2)	Centrifuged before and after, separator tube (tube 3)	Centrifuged before and after (tube 4)	Only centrifuged after (tube 5)	Centrifuged before and after, separator tube (tube 6)
Platform D						
B1 vitamers						
Thiamine	26.5*	25.9*	30.0*	39.8*	36.6*	44.3*
Thiamine Monophosphate (TMP)	-49.4*	-51.4*	-45.3*	-70.1*	-68.4*	-67.2*
Sum of Thiamine and TMP	3.9*	2.1	7.9*	6.4*	4.1*	10.3*
B2 vitamers						
Riboflavin	17.0*	17.2*	15.2*	24.3*	27.0*	22.5*
Flavin mononucleotide (FMN)	-27.0*	-25.3*	-3.2	-33.3*	-25.7*	-12.1*
Sum of Riboflavin and FMN	4.6*	4.9*	9.4*	7.7*	12.0*	12.0*
B6 vitamers						
Pyridoxal 5 phosphate	-3.0*	-4.1*	-3.3*	-5.2*	-4.5*	-4.5*
Pyridoxal	6.7*	3.0	15.9*	7.9*	3.8*	20.8*
4 Pyridoxic acid	-1.1	-1.5	-2.8*	-2.6*	-1.0	-3.3*
Sum of pyridoxal 5 phosphate and pyridoxal	0.1	-2.0*	2.6*	-1.3	-2.2*	2.9*
Tryptophan and B3 metabolism						
Tryptophan	0.3	3.0*	-5.1*	1.1	3.3*	-6.0*
Kynurenine	-1.0	-3.1*	-4.7*	-2.1	-0.1	-1.8
Kynurenic acid	0.8	0.9	-1.3	0.4	0.7	-2.4
Anthranilic acid	5.9*	9.8*	3.2	8.9*	12.7*	5.3*
3 Hydroxykynurenine	-3.5	-2.9	-3.9*	-6.0*	-7.4*	-8.5*
Quinaldic acid	-3.7	-3.1	7.6*	-1.3	-2.1	11.8*
3 Hydroxyanthranilic acid	-1.1	-2.3	-1.8	-0.8	-1.6	-2.0
Xanthurenic acid	-0.8	-1.2	-0.6	-2.6	0.2	-1.4
Picolinic acid	3.7	3.1	3.6	4.1*	1.5	-2.7
Quinolinic acid	0.7	-0.6	-0.9	0.9	1.1	-1.8
Nicotinamide	99.3*	132.9*	56.1*	159.6*	220.2*	86.1*
N1 methylnicotinamide	0.0	-2.8	-2.1	-1.0	-2.6	-0.7
Trigonelline	-0.2	-0.7	-0.2	0.9	1.0	-0.6
Neopterin	-8.5*	-0.6	-15.0*	-15.4*	-3.4	-27.7*
Miscellaneous						
Cystathionine	0.8	1.0	-0.4	1.4	2.2	0.2
Trimethylamine N oxide	-0.2	0.3	-0.2	1.4	1.3	-0.1
Platform B						
Proteinogenic amino acids						
Alanine	5.2*	3.6*	4.4*	8.5*	9.5*	7.5*
Asparagine	2.2	4.5*	1.5	9.7*	12.6*	8.5*
Total Cysteine	1.5	1.6	1.1	2.2*	2.6*	-0.4
Glutamic Acid	34.4*	42.0*	39.5*	74.6*	77.9*	71.3*
Glutamine	0.9	-3.2*	-0.5	-3.3*	-4.9*	-2.5
Glycine	13.4*	10.7*	11.2*	22.0*	23.8*	20.9*
Histidine	3.8*	2.2*	2.6*	6.3*	6.9*	5.7*
Isoleucine	1.3	3.7*	3.0*	6.4*	8.5*	4.0*
Leucine	6.1*	6.8*	5.1*	10.0*	10.7*	8.6*
Lysine	3.0*	3.0*	2.8*	6.1*	7.3*	5.2*
Methionine	2.3*	0.8*	3.2*	5.2*	4.4*	5.9*
Phenylalanine	12.0*	14.1*	13.6*	22.2*	22.8*	21.5*
Proline	1.3	1.3	1.7	3.6*	3.8*	3.1*
Serine	15.6*	17.2*	17.1*	27.3*	28.6*	26.8*
Threonine	2.2	1.6	2.4	6.3*	6.3*	4.6*
Tryptophan (Second Platform)	3.3*	4.0*	-3.3*	2.9*	5.2*	-3.7*
Tyrosine	2.5*	1.3	2.5*	4.8*	4.9*	3.9*
Valine	2.8*	3.7*	2.7*	5.0*	6.1*	4.3*
Diabetes related markers						
2-Hydroxybutyrate	-0.2	5.3*	5.4*	5.7*	9.6*	4.9*
3-Hydroxybutyrate	-2.6	-0.8	4.2	6.7*	5.4*	2.1
3-Hydroxyisobutyrate	1.2	2.9*	2.3	3.0*	4.0*	4.9*
2-Aminoadipic acid	-0.4	-6.0	-5.2	-2.3	-7.3	-5.6
Acetoacetate	-21.7*	-16.9*	-6.7	-23.2*	-28.7*	-19.0*
ß-Aminoisobutyrate	1.7	1.3	0.2	2.8*	3.2*	1.6
α-Hydroxyglutaric acid	16.8*	13.8*	132.2*	30.6*	29.0*	215.3*
α-Ketoglutaric acid	-8.2*	-15.1*	-0.7	4.2	-7.4*	7.5*
Carboxyethyllysine	1.2	-0.7	0.9	0.7	0.2	1.4
Carboxymethyllysine	3.6	3.7*	1.5	4.2*	6.2*	1.7
Miscellaneous						
Cystathionine (Second Platform)	1.3	0.8	3.8	3.6*	3.4*	4.6*
Homocysteine	7.6*	11.6*	3.3*	13.4*	18.4*	6.0*
Kynurenine (Second Platform)	2.0*	0.3	-2.7*	0.5	1.1	-2.7*
Methylmalonic acid	0.1	-1.2	1.7	0.6	-1.3	4.8*
Ornithine	16.3*	23.8*	8.0*	29.6*	43.9*	16.0*
Sarcosine	1.5	0.9	0.5	2.7*	3.6*	1.0

Color Key:
≤ -15.0%
> -15.0% and < 15.0%
≥ 15.0%

FIG. 2. Percent differences in biomarker concentrations for each tube type, relative to concentrations in the reference standard tube: serum. Percent differences in *bold* and marked with an *asterisk* are statistically significant ($\alpha < 0.05$). See Supplementary Table S1 for the associated CIs and geometric mean concentrations for each biomarker in each tube type. CIs, confidence intervals.

Biomarker (Heparin Plasma)	Percent Difference in Concentrations (vs. reference standard tube)					
	24-hour processing delay			48-hour processing delay		
	Centrifuged before and after (tube 1)	Only centrifuged after (tube 2)	Centrifuged before and after, separator tube (tube 3)	Centrifuged before and after (tube 4)	Only centrifuged after (tube 5)	Centrifuged before and after, separator tube (tube 6)
Platform D						
B1 vitamers						
Thiamine	**24.4***	**20.4***	**24.5***	**33.5***	**30.4***	**36.5***
Thiamine Monophosphate (TMP)	**-55.7***	**-51.2***	**-52.7***	**-76.4***	**-71.5***	**-70.8***
Sum of Thiamine and TMP	2.1	0.6	2.9	2.4	1.3	**6.3***
B2 vitamers						
Riboflavin	**11.6***	**16.6***	**8.6***	**16.9***	**22.0***	**14.2***
Flavin mononucleotide	**-33.2***	**-27.5***	**-20.2***	**-38.1***	**-28.9***	**-25.9***
Sum of Riboflavin and FMN	-0.7	**4.4***	0.5	2.1	**8.2***	3.0
B6 vitamers						
Pyridoxal 5 phosphate	**-3.0***	**-3.7***	**-4.4***	**-4.3***	**-4.0***	**-5.8***
Pyridoxal	**10.0***	**4.4***	**40.6***	**20.0***	**10.8***	**48.9***
4 Pyridoxic acid	1.6	0.0	0.5	0.5	0.9	0.4
Sum of pyridoxal 5 phosphate and pyridoxal	1.0	-1.0	**9.8***	**3.0***	0.5	**11.6***
Tryptophan and B3 metabolism						
Tryptophan	**2.7***	**4.2***	-0.5	1.7	**4.7***	0.0
Kynurenine	**2.9***	2.2	2.6	**3.4***	2.4	**3.0***
Kynurenic acid	-1.6	-0.6	-2.2	-1.4	0.8	-1.9
Anthranilic acid	2.0	**5.4***	**4.0***	3.7	**11.0***	**7.7***
3 Hydroxykynurenine	-1.7	-1.7	-1.1	**-3.1***	**-3.2***	**-4.1***
Quinaldic acid	1.6	0.1	**7.9***	1.7	**3.7***	**9.6***
3 Hydroxyanthranilic acid	0.9	1.6	**3.4***	1.1	1.2	**5.7***
Xanthurenic acid	-1.4	-0.8	-2.8	-1.0	0.4	0.3
Picolinic acid	-0.9	0.6	0.9	-0.5	**4.7***	1.1
Quinolinic acid	1.3	0.6	0.8	0.8	1.5	1.2
Nicotinamide	**71.3***	**73.5***	**61.8***	**116.4***	**123.5***	**114.5***
N1 methylnicotinamide	-0.2	3.3	2.0	**3.8***	**6.7***	1.8
Trigonelline	0.2	-0.4	0.9	0.7	1.0	0.5
Neopterin	**-8.7***	-2.6	**-6.7***	**-12.6***	**-4.9***	**-15.6***
Miscellaneous						
Cystathionine	1.5	0.4	0.5	**2.5***	1.7	1.1
Trimethylamine N oxide	2.6	2.6	2.1	**4.8***	**3.3***	**3.3***
Platform B						
Proteinogenic amino acids						
Alanine	0.4	-0.1	-0.6	**4.0***	**2.4***	1.6
Asparagine	-0.7	-0.1	-0.4	0.0	0.5	-1.3
Total Cysteine	1.3	-0.6	-0.3	**4.0***	**3.0***	1.3
Glutamic Acid	**16.8***	**18.0***	**16.6***	**36.6**	**35.2***	**42.9***
Glutamine	-1.5	-2.3	**-5.5***	-0.7	**-2.7***	**-5.8***
Glycine	**2.0***	1.7	1.5	**8.7***	**7.2***	**4.8***
Histidine	0.1	-0.2	-0.5	**3.2***	**3.1***	1.6
Isoleucine	-0.5	0.7	1.2	**3.7***	**2.7***	**2.8***
Leucine	**2.3***	**2.8***	1.7	**5.0***	**6.0***	**5.3***
Lysine	-1.0	-0.6	0.1	**2.6***	**2.5***	0.6
Methionine	0.2	-1.3	1.6	**4.2***	1.4	**7.0***
Phenylalanine	1.6	-0.1	-0.9	**5.8***	**4.7***	**4.3***
Proline	0.6	0.5	-0.1	**2.7***	**1.9***	**2.0***
Serine	1.8	**3.4***	1.5	**8.4***	**9.1***	**7.5***
Threonine	-0.7	-1.0	-0.8	**3.1***	**2.5***	**3.0***
Tryptophan (Second Platform)	0.6	**3.0***	-0.6	**2.6***	**4.4***	0.8
Tyrosine	0.2	-0.9	-0.4	**2.5***	1.1	**1.7***
Valine	-0.1	0.2	-0.6	1.6	1.0	1.3
Diabetes related markers						
2-Hydroxybutyrate	1.7	**5.0***	**2.5***	**4.7***	**5.6***	**6.3***
3-Hydroxybutyrate	1.9	**4.0***	**4.6***	**5.5***	**5.0***	**8.1***
3-Hydroxyisobutyrate	-0.3	0.3	0.8	**3.0***	1.0	**3.4***
2-Aminoadipic acid	-5.9	-4.2	**-8.3***	**-11.1***	-5.7	-2.3
Acetoacetate	**-16.3***	**-13.8***	**-13.7***	**-25.9***	**-27.1***	**-14.8***
ß-Aminoisobutyrate	-0.5	-0.9	-2.1	1.1	0.8	-1.0
α-Hydroxyglutaric acid	2.5	4.4	**90.9***	**9.8***	**12.2***	**155.5***
α-Ketoglutaric acid	**6.2***	-4.4	4.2	**11.6***	-1.3	**15.3***
Carboxyethyllysine	2.2	**3.2***	1.9	**3.0***	1.7	2.6
Carboxymethyllysine	0.1	-2.4	-1.4	3.1	-0.2	0.1
Miscellaneous						
Cystathionine (Second Platform)	0.8	**3.1***	2.3	**3.4***	**4.1***	**4.7***
Homocysteine	**3.0***	**8.2***	-0.2	**7.7***	**11.9***	1.3
Kynurenine (Second Platform)	-0.1	-0.1	-0.5	1.1	0.5	-0.7
Methylmalonic acid	-1.6	-0.5	0.7	0.6	-1.0	**3.8***
Ornithine	3.0	**10.9***	-1.1	**8.5***	**16.7***	1.3
Sarcosine	0.2	-0.1	-0.3	1.6	1.6	0.1

Color Key:

≤ -15.0%
> -15.0% and < 15.0%
≥ 15.0%

FIG. 3. Percent differences in biomarker concentrations for each tube type, relative to concentrations in the reference standard tube: heparin plasma. Percent differences in *bold* and marked with an *asterisk* are statistically significant ($\alpha < 0.05$). See Supplementary Table S2 for the associated CIs and geometric mean concentrations for each biomarker in each tube type.

Biomarker (EDTA Plasma)	Percent Difference in Concentrations (vs. reference standard tube)					
	24-hour processing delay			48-hour processing delay		
	Centrifuged before and after (tube 1)	Only centrifuged after (tube 2)	Centrifuged before and after, separator tube (tube 3)	Centrifuged before and after (tube 4)	Only centrifuged after (tube 5)	Centrifuged before and after, separator tube (tube 6)
Platform D						
B1 vitamers						
Thiamine	-0.7	**-4.9***	0.3	-2.1	**-5.8***	-0.3
Thiamine Monophosphate (TMP)	**5.2***	1.7	0.0	-1.8	2.0	0.4
Sum of Thiamine and TMP	**3.4***	-0.3	0.2	-1.8	-0.4	0.0
B2 vitamers						
Riboflavin	3.0	2.9	2.4	**12.3***	**10.0***	**7.0***
Flavin mononucleotide	-2.2	0.8	**-3.2***	-2.6	1.0	-2.1
Sum of Riboflavin and FMN	0.7	2.3	0.0	**5.3***	**5.2***	2.5
B6 vitamers						
Pyridoxal 5 phosphate	0.3	1.2	1.5	1.5	**5.7***	**5.0***
Pyridoxal	2.5	**-6.3***	**17.1***	**4.5***	-2.1	**22.6***
4 Pyridoxic acid	-0.6	1.2	-1.6	0.4	0.9	-0.7
Sum of pyridoxal 5 phosphate and pyridoxal	0.3	-0.5	**4.1***	1.8	**3.8***	**8.0***
Tryptophan and B3 metabolism						
Tryptophan	1.3	**3.8***	-1.4	0.0	**3.5***	**-2.0***
Kynurenine	0.7	1.6	0.3	0.2	-0.6	0.2
Kynurenic acid	-0.3	-0.4	-0.8	-0.6	-0.3	**-2.9***
Anthranilic acid	**2.8***	**5.7***	**5.0***	**4.3***	**10.3***	**8.9***
3 Hydroxykynurenine	**-3.1***	-2.1	**-2.9***	**-5.8***	**-7.2***	**-4.2***
Quinaldic acid	-1.3	-0.9	**10.6***	0.0	-0.9	**15.1***
3 Hydroxyanthranilic acid	0.3	**-3.9***	1.1	0.0	**-7.5***	1.8
Xanthurenic acid	0.5	-1.4	0.6	-0.2	-1.1	-0.4
Picolinic acid	-0.8	1.2	-0.6	-1.6	0.4	-1.8
Quinolinic acid	0.3	0.8	**1.3***	1.1	**1.7**	**1.3***
Nicotinamide	65.9	90.8	29.7	105.2	133.6	69.6
N1 methylnicotinamide	1.6	0.3	0.6	-1.4	-0.4	1.7
Trigonelline	-0.3	-0.2	1.2	0.6	1.1	0.2
Neopterin	**-25.2***	**-12.4***	**-34.3***	**-35.1***	**-16.3***	**-46.3***
Miscellaneous						
Cystathionine	**2.4***	**1.5***	1.2	**1.8***	**2.5***	1.5
Trimethylamine N oxide	0.5	-0.1	1.2	-1.2	-1.0	0.7
Platform B						
Proteinogenic amino acids						
Alanine	0.2	0.7	0.2	1.2	**1.7***	0.2
Asparagine	-0.3	-0.6	-1.2	**-3.5***	-0.8	**-2.5***
Total Cysteine	1.1	0.2	1.3	1.6	0.9	**2.9***
Glutamic Acid	**7.8***	**15.0***	**6.8***	**25.0***	**31.1***	**22.8***
Glutamine	-0.1	-1.8	-1.4	**-3.7***	**-5.0***	**-5.6***
Glycine	**1.8***	**1.9***	1.3	**3.2***	**4.0***	1.1
Histidine	0.2	0.2	0.5	0.2	0.7	0.0
Isoleucine	**3.0***	0.4	0.7	1.4	**3.2***	0.7
Leucine	**2.0***	**4.1***	**2.5***	**3.4***	**6.4***	**3.0***
Lysine	-0.3	-0.4	-0.3	0.3	2.2	0.9
Methionine	-0.7	**-1.9***	1.4	-1.0	**-2.4***	0.7
Phenylalanine	-0.1	-0.2	-1.0	-1.0	0.5	-1.0
Proline	1.1	1.1	**1.7***	**1.4***	**2.2***	1.0
Serine	0.7	**2.1***	-0.1	**1.6***	**4.1***	-1.5
Threonine	-0.2	0.6	0.3	0.1	1.5	0.2
Tryptophan (Second Platform)	1.1	**4.2***	0.7	1.1	**4.5***	-0.1
Tyrosine	0.4	-0.3	0.3	0.4	0.4	0.0
Valine	1.0	1.0	1.1	0.2	1.0	0.0
Diabetes related markers						
2-Hydroxybutyrate	1.9	**5.1***	**4.5***	**5.0***	**8.0***	**3.2***
3-Hydroxybutyrate	**3.3***	**3.7***	**6.4***	**7.0***	**8.0***	**8.3***
3-Hydroxyisobutyrate	-0.1	-1.0	1.1	0.4	1.8	**2.1***
2-Aminoadipic acid	-3.7	-2.7	-5.3	1.5	-3.3	-0.9
Acetoacetate	**-18.6***	**-19.5***	**-12.7***	**-31.3***	**-36.1***	**-29.0***
ß-Aminoisobutyrate	1.3	**1.9***	0.2	**1.9***	**2.0***	0.0
α-Hydroxyglutaric acid	-1.4	2.3	**195.8***	2.9	**7.1***	**212.0***
α-Ketoglutaric acid	**9.3***	-2.9	**12.9***	**16.5***	2.0	**18.8***
Carboxyethyllysine	-0.7	-1.4	1.4	1.5	-1.1	-1.4
Carboxymethyllysine	2.0	1.5	0.5	3.1	3.5	-3.2
Miscellaneous						
Cystathionine (Second Platform)	-0.8	0.0	2.5	**3.4***	1.2	1.5
Homocysteine	**4.3***	**12.6***	1.4	**9.5***	**19.8***	**4.3***
Kynurenine (Second Platform)	1.2	**1.9***	0.4	1.1	1.5	0.2
Methylmalonic acid	1.9	-0.6	0.3	0.8	0.3	2.2
Ornithine	**4.4***	**16.3***	1.7	**9.7***	**23.1***	2.2
Sarcosine	*Not measured*					

Color Key:

≤ -15.0%
> -15.0% and < 15.0%
≥ 15.0%

FIG. 4. Percent differences in biomarker concentrations for each tube type, relative to concentrations in the reference standard tube: EDTA plasma. Percent differences in *bold* and marked with an *asterisk* are statistically significant ($\alpha < 0.05$). See Supplementary Table S3 for the associated CIs and geometric mean concentrations for each biomarker in each tube type. EDTA, ethylenediaminetetraacetic acid.

of the absolute percent differences associated with using separator tubes was modest (1%–4% in serum; <1%–3% in lithium heparin plasma; and <1%–2% in EDTA plasma; Supplementary Table S5).

Matrix type

As anticipated, biomarker concentrations differed by matrix, but most differences were modest (±15%; Supplementary Table S8). Many amino acid concentrations were lower in both plasma types relative to serum—several markedly so (glutamic acid, glycine, phenylalanine, serine, and ornithine). Measurements in lithium heparin plasma tubes were the most comparable with the serum tubes. Across all biomarkers, the range of absolute percent differences associated with using lithium heparin plasma versus serum was <1%–28% (IQR 2%–6%; Supplementary Table S5). The range of absolute percent differences associated with using EDTA plasma versus serum was <1%–306% (IQR 6%–16%).

Discussion

We examined the influence of biospecimen handling on the direct quantification of 54 biomarkers across three matrix types. Most biomarker concentrations measured in tubes with processing delays were statistically different from those measured in the immediately processed RST. Differences were often small in magnitude, but they typically grew stronger as the processing delay lengthened. Across all handling conditions assessed, several biomarkers showed poor reproducibility relative to the RST within at least two of the matrix types we assessed: B1 and B2 vitamers, pyridoxal, nicotinamide, glutamic acid, acetoacetate, and ornithine. Several other markers showed more modest, but consistent concentration changes across the handling conditions or large situation-specific changes: neopterin, serine, phenylalanine, α-hydroxyglutaric acid, and homocysteine. Accordingly, researchers may find the tables we provide useful for reference when one of these biomarkers is of interest.

Our findings are not directly comparable to many biomarker stability studies, owing to the wide range in methods used across these studies. Researchers may have used different assay types (e.g., immunoassays), relative versus absolute quantification, or may not have specified the assay type. Biomarkers are also often analyzed collectively, rather than reported on individually.[2,4,8–21] Despite this variation in study design, our results regarding the stability of specific biomarkers are supported by some of this research.

The potential instability of many B vitamers during sample storage is recognized, with increases or decreases in concentrations being vitamer specific. Using mixed modeling to estimate mean biomarker concentrations, Jones et al. tested delayed processing across matrix types from 16 participants and found no change in thiamin and riboflavin after a 24-hour delay in processing chilled whole blood; relative to our study, they found stronger decreases in pyridoxal 5-phosphate (B6) concentrations within serum and lithium heparin plasma associated with the processing delay.[10] Hustad et al. reported large concentration changes (often differences of ±50% or more) in riboflavin, flavin mononucleotide, pyridoxal 5-phosphate, and pyridoxal within serum and heparin plasma stored for a week at room temperature (23°C), while differences in EDTA plasma were less extreme.[1] However, when stored with ice packs for 24 or 48 hours, dramatic changes in biomarkers like pyridoxal (a B6 vitamer), homocysteine, anthranilic acid, and 3-hydroxyanthranilic acid can still be observed in EDTA plasma.[2]

We observed that within B vitamin groups (e.g., the vitamers comprising B1, B2, or B6), concentrations of individual vitamers sometimes changed diametrically with length of processing delay. Using serum vitamin B2 as an example, riboflavin concentrations increased, while its metabolic precursor, flavin mononucleotide, decreased relative to the reference standard tubes. This phenomenon is reported by other researchers and suggests a metabolic conversion of flavin mononucleotide to riboflavin may take place in stored samples.[1] For some B vitamins, measuring and combining concentrations of vitamers may aid in the estimation of total vitamin status.[26] However, the biologic role of the individual vitamers may be of research interest.

Using the same laboratory assays as our study, Midttun et al. reported decreases in EDTA plasma pyridoxal (a B6 vitamer) after a 24-hour processing delay among 12 participants (concentration losses of 1 to 2 standard deviations per hour)—which is in line with our finding for EDTA plasma tubes that were centrifuged only after the processing delays.[2] Pyridoxal can cross cell membranes; because our samples were first stored as whole blood, the concentration decrease may reflect cellular intake during the processing delays, before cell pellets were removed.[26] Indeed, Midttun et al. centrifuged their samples after processing delays, but the effect of centrifuging before and/or after these delays was not directly examined.[2] In agreement with our results within EDTA tubes centrifuged only after 24-hour delays, the researchers also identified increases in homocysteine, serine, and anthranilic acid, and a decrease in 3-hydroxyanthranilic acid compared to immediately processed samples.[2] For all these biomarkers, our results suggest that the timing (or number) of centrifugations may modestly affect concentrations in EDTA plasma (<10%).

Results from Breier et al. (20 participants) suggest that both serum and plasma concentrations of some amino acids may change in as little as 3–6 hours when stored with cool packs.[4] Although regression modeling was not used, they also found storage-related concentration changes were less pronounced in K^+EDTA^- plasma than in serum and they reported minimal measurement differences associated with using serum separator tubes.[4] Sotelo-Orozco et al. reviewed studies comparing metabolomics assays across matrix types and also assessed nuclear magnetic resonance spectroscopy-based metabolite profiles in serum and plasma from eight people; both their review and results support our observation that measurements in serum more closely resemble those in lithium heparin than EDTA plasma.[7] Multiple studies have also observed lower concentrations/peak areas for amino acids in EDTA plasma when compared to serum.[5–9,27,28] This being said, our study was not designed to contrast analyte recovery between the matrix types and should not be interpreted as evidence for preferring one matrix over another.

There are several aspects of our study design and analytic approach that must be considered when interpreting our results. It is important to remember that our samples were

stored as chilled whole blood before the final processing, at which point, the serum and plasma were separated from the cell pellet. We made this choice because a delay in cell pellet removal is not uncommon when collecting blood samples for clinical and epidemiologic studies. However, cells can continue to produce, release, or intake molecules during storage. Any difference in concentrations between tubes with different handling conditions (e.g., 24- vs. 48-hour processing delays) could be attributable, in part, to differences in cell vitality, lysis, or membrane transport before matrix separation. In addition to the pyridoxal example described above, homocysteine increases during whole blood storage, but storage on ice for ≤24 hours may mitigate dramatic concentration changes, relative to storage at room temperature.[29] Finally, with regard to our analytic approach, we did not correct for multiple comparisons—this is arguably a conservative approach because our goal was to broadly characterize potential biomarker instability. As such, some of our findings may be false positives, given the large number of comparisons made.

Each participant in our analyses served as their own control. We also used multilevel modeling to account for observation clustering by person—to our knowledge, an approach unique to our study and that of Jones et al.[10] Furthermore, our biomarker measurements were based on mass spectrometry and the inclusion of stable isotope-labeled internal standards for each analyte, which allows for the direct, absolute quantification of biomarkers with high precision, compared to semiquantitative or untargeted methods.[30] All these design considerations allowed us to report geometric mean concentrations for every biomarker, as well as the relative percent differences in concentrations across processing delay length, centrifugation timing, tube type, and matrix type.

Conclusions

While many of the biomarkers that we measured were affected by delayed processing to some degree, the changes in concentrations were typically small (<15%). For a few biomarkers, concentrations were dramatically different in samples that were subjected to 24- or 48-hour processing delays. Such delays could be expected in large multisite studies when samples are shipped to a central laboratory for processing. Taken together, our findings indicate that handling conditions should ideally be consistent across biospecimens collected for a study. Recognizing the difficulty in this, detailed collection information for each sample should be recorded—particularly the time between blood draw and processing before freezing. Researchers can then account for processing time in their analysis, as appropriate, and when interpreting results.

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Authors' Contributions

Conceptualization: S.J.W., P.S.A., A.B., M.B., N.A.D.M., W.Y.H., N.W., and C.C.A. Methodology: K.A.M., S.J.W., P.S.A., J.N.S., and C.C.A. Data curation: M.B., N.G., A.S., and K.W. Resources: N.A.D.M. Investigation: P.M.U. Formal analysis: K.A.M. Visualization: K.A.M., S.J.W., and C.C.A. Project administration: S.J.W. Supervision: N.W. and C.C.A. Writing-original draft: K.A.M. Writing-review and editing: all authors.

Author Disclosure Statement

No conflicting financial interests exist.

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Supplementary Material

Supplementary Figure S1
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