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Whole Blood Reveals More Metabolic Detail of the Human Metabolome than Serum as Measured by ^{1}H-NMR Spectroscopy: Implications for Sepsis Metabolomics

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Abstract

Serum is a common sample of convenience for metabolomics studies. Its processing time can be lengthy and may result in the loss of metabolites including those of red blood cells (RBC). Unlike serum, whole blood (WB) is quickly processed, minimizing the influence of variable hemolysis while including RBC metabolites. To determine differences between serum and WB metabolomes, both sample types, collected from healthy volunteers, were assayed by ^{1}H-NMR spectroscopy. A total of 34 and 50 aqueous metabolites were quantified from serum and WB, respectively. Free hemoglobin (Hgb) levels in serum were measured and the correlation between Hgb and metabolite concentrations was determined. All metabolites detected in serum were at higher concentrations in WB with the exception of acetoacetate and propylene glycol. The 18 unique metabolites of WB included adenosine, AMP, ADP and ATP, which are associated with RBC metabolism. The use of serum results in the underrepresentation of a number of metabolic pathways including branched chain amino acid degradation and glycolysis and gluconeogenesis. The range of free Hgb in serum was 0.03-0.01 g/dL and 8 metabolites were associated ($p \leq 0.05$) with free Hgb. The range of free Hgb in serum samples from 18 sepsis patients was 0.02-0.46 g/dL. WB and serum have unique aqueous metabolite profiles but the use of serum may introduce potential pathway bias. Use of WB for metabolomics may be particularly important for studies in diseases like sepsis in which RBC metabolism is altered and mechanical and sepsis-induced hemolysis contributes to variance in the metabolome.

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Keywords

metabolomics; hemolysis; sepsis; critical care

Introduction

Metabolomics is a rapidly advancing field of discovery science aimed at furthering knowledge of the biological consequences of metabolic changes in an organism or cell (1). In human studies, blood samples are used to capture a physiological average of the host's metabolic status and serum is routinely collected, which makes it a frequent sample of convenience for metabolomics studies. However, the processing time for serum is lengthy (30-60 min) (2) and can be variable, which may lead to changes in metabolite concentrations. In addition, the analysis of the serum metabolome does not take into account the contribution of erythrocytes (RBC). The reliability of serum as a biofluid for metabolomics can be further compromised by *ex vivo* mechanical RBC hemolysis that can occur due to suboptimal blood collection technique or technical difficulties and centrifugation (3-5). The extent of hemolysis can vary tremendously leading to a broad range of free hemoglobin (Hgb) levels in serum samples (6). This phenomenon is known to impact the reliability of a number of clinical tests including the measurement of lactate dehydrogenase and liver function tests such as ALT and AST (4). Until recently, RBC were considered relatively unimportant in critical illnesses such as sepsis with their oxygen carrying capacity being viewed as their primary function. New knowledge and understanding of RBC function shows that their metabolic activity extends beyond glycolysis (7-9) and they play an essential role in transporting amino acids (10). In sepsis, RBC have gained recognition because they modulate the microcirculation, the RBC membrane participates in the regulation of blood rheology, and RBC width distribution is associated with survivorship (11, 12). In addition, pathogen (6, 13) and complement-induced (14) hemolysis as well as sepsis- induced RBC fragility, enhances the likelihood of *in vivo* hemolysis. The influence of RBC in sepsis is furthered by the recommendation that patients with septic shock receive RBC transfusion (15), which can lead to hemolysis.

Red blood cells are metabolically active, producing millimolar amounts of ATP and transporting a number of amino acids (10). As such, it is reasonable to expect that RBC make a significant contribution to the blood metabolome that could provide additional insight into sepsis-induced changes. In addition, the quicker processing of WB likely minimizes changes in metabolite levels, including those contributed by the RBC. In this study, we hypothesized that whole blood (WB), which can be quickly and consistently processed, is an alternative metabolomics test material to serum that will provide more metabolic detail and less variation related to processing than serum. To test this hypothesis, we aimed to determine the extent of the differences between the serum and WB metabolomes by comparing the quantified metabolic profiles using ^{1}H-NMR spectroscopy. We also assessed the extent of the association between free hemoglobin (Hgb) levels (as a surrogate of hemolysis) and serum metabolites from samples collected from healthy volunteers and conducted pathway analysis of the two datasets. Finally, in order to assess whether these findings had real world relevance and the potential to affect clinical research

conclusions, we measured the range of hemolysis present in representative serum samples from patients with sepsis.

Materials and Methods

Healthy Subjects

Normal, healthy volunteers were identified and recruited for study participation via the Claude D. Pepper Older Americans Independence Center (OAIC) Research Participant Program at the University of Michigan's Geriatric Center and the Michigan Institute of Clinical and Health Research (MICHR) clinical studies website (UMClinicalStudies.org) (16). The study and its associated informed consent form were approved by the University of Michigan's Institutional Review Board approved study (UM IRB; protocol number, HUM00038122).

For study eligibility, subjects had to be non-smoking, and non-obese with no known medical conditions that required chronic drug therapy. On the day of sample collection, volunteers presented to MICHR's clinical research unit (http://www.michr.umich.edu). Following the acquisition of written informed consent, fasting (12 h) blood samples (serum and WB) were collected between 08:30AM and 09:30AM by a single direct venipuncture. Upon collection, Vacutainer® (Becton Dickinson, Franklin Lakes, NJ USA) serum separator tubes (SST), were stored upright, at room temperature, for at least 30 min (but no more than 2 hr) until a visible clot formed. Serum was acquired by centrifugation (1000 × *g*, 25°C for 10 min), after which aliquots (500μL) were generated and immediately transferred to the freezer (-80°C). Blood for the generation of WB samples was collected into tubes containing sodium heparin (Vacutainer®, Becton Dickinson), which were immediately placed in an ice-water bath for 10 min during which aliquots (500μL) were generated; the samples were then promptly transferred to the freezer (-80°C). Blood was also collected on two separate occasions (approximately two weeks apart) from one donor between 0830-0930 via a single venipuncture to generate five WB and serum samples to test the impact of time on sample processing. These samples were processed as described above except that one of each sample was designated as time (T) 0 (immediately processed upon collection), T 30, 60, 120, and 180 minutes. To mimic serum processing conditions, the T30-T180 samples for both WB and serum were kept at room temperature until the time of allocation into separate tubes and centrifugation, respectively, after which aliquots were transferred to the freezer (-80°C).

NMR Spectroscopy Metabolomics

At the time of assay, frozen samples were thawed on ice and macromolecules were removed by the addition of methanol:chloroform (1:1) as previously described (17). Following extraction, the resulting aqueous phase of each sample was assayed by ^{1}H-NMR spectroscopy. The NMR spectra were acquired on a 500 MHz Agilent spectrometer with a 5mm probe housed at the University of Michigan College of Pharmacy's Biochemical NMR core laboratory. The internal standards of known concentration, formate and deuterated 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS-d_6; IS-2, Chenomx Internal Standard), were used for quantification of metabolites for serum and WB, respectively. Spectra were

processed and compounds were identified and quantified using Chenomx NMR Suite 7.6 (Chenomx, Inc., Edmonton, Alberta, Canada) by two skilled NMR spectroscopists (CM and LY) with Chenomx software experience. In order for a compound to be categorized as detectable, it needed to be present in at least 70% of the samples (WB or serum) at a concentration of greater than or equal to 5μM.

Serum samples from patients with sepsis

Serum samples that were generated from blood collected via indwelling catheters as part of a clinical trial ((18); clinicaltrials.gov NCT 00372502) from patients with sepsis in the emergency department or intensive care unit. The clinical indices for the diagnosis of sepsis were consistent with consensus definitions for severe sepsis or septic shock: confirmed or suspected infection, two or more systemic inflammatory response criteria (19), and hypoperfusion evidenced by either a systolic blood pressure of < 90 mmHg after 20 min of fluid resuscitation or a blood lactate level of at least 36 mg/dL (18). Blood was drawn into a SST, was allowed to clot at least 30 minutes, and was subsequently centrifuged (10 min at 1,800 × *g* at 15°C) at which time serum was aliquoted in 0.5 mL increments and stored (-80°C) until they were assayed for free hemoglobin.

Free Hemoglobin Assay

Free Hgb levels in serum samples from healthy controls and sepsis patients were measured using a commercially available colorimetric assay kit (700540; Cayman Chemical, Ann Arbor, MI) using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). The assay was performed in accordance with the manufacturer's instructions and sample Hgb concentrations were calculated from a standard curve constructed with known concentration standards provided in the kit using the plate reader's software (Softmax, version 4.1, Molecular Devices).

Data Analysis

Statistical Analysis

The metabolite data set from WB and serum were range scaled and cube root transformed in preparation for parametric statistical analysis. Principal component analysis of the normalized data was performed using SIMCA (SIMCA software, Umetrics, Umeå, Sweden). For quantitative analysis, the mean normalized value of each metabolite common to both WB and serum were compared using a unpaired Student's t-test with Welch's correction for different variances, if applicable, and each resulting p value was corrected for multiple comparisons by calculating a corresponding false discovery rate (FDR) (20). The extent of the association between free Hgb and concentration of metabolites that were common to, but different between serum and WB, was determined by Spearman correlation. To quantitatively compare changes in the total WB and serum metabolomes over time, the median (IQR) concentrations at each time point (0-180 min) of all 32 common metabolites for serum and WB from the single donor experiment were calculated and compared by a Mann Whitney test. All statistics were performed using GraphPad Prism (version 6 for Mac; GraphPad software, La Jolla, CA., www.graphpad.com).

Pathway analysis

Bioinformatics aids in the interpretation and allows for the visualization of metabolomics data. The serum and WB pathway analysis was done with Metscape 2, a plugin for Cytoscape (http://apps.cytoscape.org/apps/metscape) (21). To perform the analysis we uploaded the list of compounds that were significantly underrepresented in serum plus those that were unique to WB and generated metabolic networks. The networks were examined using Metscape pathway filter to identify representative pathways that included input compounds.

RESULTS

The Serum and Whole Blood Metabolomes are Distinct

A total of 20 volunteers were enrolled in the study of which 50% were female and the mean (± S.D.) age was 54.9±8.2 years. Metabolite data were acquired for 19 WB and 20 serum samples; one WB sample did not properly extract and the NMR spectrum was uninterruptable. The urine metabolomics data from this cohort has been previously published (16).

A total of 34 and 50 aqueous metabolites were detected and quantified in serum and WB, respectively. All metabolites that were detected in serum were detected in WB with the exception of acetoacetate, which was only detectable in 45% of the WB samples, and propylene glycol, which may be a contaminant from the SST blood collection tube but has been reported as a human serum metabolite (22), resulting in a total of 32 common metabolites between the two biofluids (**Figure 1A**). Principal component analysis showed that the metabolomes of the two sample types were distinct (**Figure 1B**). The 18 unique WB metabolites are shown in **Table 1**. Representative ^{1}H-NMR spectra of examples of metabolites present in WB and absent in serum or at different concentrations in the two sample types are shown in **Figure 2**.

Using a FDR of ≤ 5%, statistical comparison of the normalized concentrations of the 32 metabolites common to both sample types identified 18 metabolites that were different between WB and serum, all of which, with the exception of glycerol, had median concentrations that were higher in WB (**Table 2**). Of the 18 differentiating metabolites, 8 were associated with free Hgb levels (**Figure 3A-H**).

Free Hemoglobin Levels in Sepsis Patients are Highly Variable

In the serum of sepsis patients (n=18; mean±S.D. age: 54.7±17.3 years), the median Hgb level was 0.05 g/dL (range: 0.02-0.46 g/dL), which was, as expected, associated with a range of visible but subtle differences in hemolysis (**Figure 4**). Serum free Hgb levels were available from 19 of the 20 healthy control serum samples (median, range: 0.04, 0.03-0.10 g/dL). but there was no difference between them and those of sepsis patients (Mann Whitney test, p = 0.138), suggesting that this level of hemolysis is common to sample processing and not necessarily a result of underlying pathology.

Both the Serum and Whole Blood Metabolomes Change Over Time

Temporal changes in serum and WB were assessed using blood from a single donor. During the time course, WB was left at room temperature to mimic the conditions of serum processing. All metabolites in both serum and WB changed over time (**Figure 5**), but the difference between serum and WB metabolomes was not different until T180 (Sidak's p value < 0.001). The median (IQR) percent change at T30 was 4.6% (16.1%) and 16.2% (37%) for serum and WB, respectively. The metabolite with the greatest percent decline in serum was creatine (72% at T120) and the metabolite with the greatest percent increase was taurine (152%) at T180. In WB, arginine increased the most (271% at T180) and glycerol declined the most (51% at T30). Over the course of time, glycerol increased in serum by an average of 52% and in WB, decreased by an average of 28% (data not shown). This may contribute to the higher detected glycerol levels in serum compared with WB.

Pathway Analysis Reveals Underrepresented Metabolism in Serum Samples

Since our NMR analysis found marked differences in the WB and serum metabolomes, we used Metscape 2 (21) to conduct pathway analysis of metabolites that differentiated the two biofluids. This type of analysis aids in the understanding of the metabolic significance of the found differences between WB and serum. The metabolic pathways containing WB-specific metabolites and the most significant metabolites (FDR < 5%) are shown in **Table 3**. These include amino acid metabolism, bile acid biosynthesis and the urea cycle. These results underscore the importance of the differences we found such that these pathways will be underrepresented in metabolomics data derived from serum samples.

DISCUSSION

Metabolomics is a rapidly evolving field of discovery science for which the generation of reliable data hinges on the proper collection and processing of samples as well as accurate interpretation. The aim of this study was to determine the extent of the differences between the serum and WB metabolomes and influence of hemolysis on metabolite concentrations. The latter was accomplished by assessing the extent of the associations between free Hgb levels and serum metabolites. Here we demonstrate that the WB metabolome is distinct compared to that of serum when measured by ^{1}H-NMR spectroscopy. This is supported by two primary pieces of evidence: 1) a greater number of metabolites were detected in WB than in serum; and 2) there were significant differences in metabolite concentrations between WB and serum samples. Specifically, of the 32 common metabolites of WB and serum, 17 of them had higher concentrations in WB. In addition, we showed that variability is introduced into the serum metabolome through prolonged (30-180 min) sample processing (**Figure 5**) and by RBC hemolysis, as the concentrations of multiple metabolites (valine, glutamate, phenylalanine, leucine, creatine phosphate, alanine, isoleucine and 2-hydroxybutyrate) were associated with free hemoglobin levels (**Figure 3A-H**). Collectively, the impact of using serum rather than WB for metabolomics studies (regardless of the presence of disease) is demonstrated by our pathway analysis, which showed that the absence or underrepresentation of metabolites in serum samples introduces potential bias into the biological interpretation of metabolomics data. A number of recently published tools in addition to pathway mapping calculate statistical significance of pathways or other

predefined groups of metabolites based on whether they are enriched with differentiating metabolites (23, 24). Application of such methods may further compound this bias.

Implications for Sepsis Metabolomics

The application of untargeted metabolomics to critical care is a rapidly emerging field that holds great promise for lending insight to the mechanistic underpinnings of complex critical illnesses such as sepsis (25, 26). Studying the metabolome in this group is complicated by a number of pathophysiological and logistical hurdles. These include the fact that metabolism in critically ill patients is rapidly dynamic, and its measurement, as demonstrated in this study, is highly likely to be confounded by RBC and leukocyte lysis in circulating blood, which is well known to be part of the illness. In addition, a major logistical challenge in studying critical ill patients is their unscheduled presentation into relatively poorly controlled areas of the health care systems (e.g., the emergency department, the operating room, the ICU), which may contribute to delays, and technical variation inherent in sample handling and processing even in a clinical research setting. Nevertheless, metabolomics holds promise to inform of underlying etiology and may be a more sensitive gauge of illness severity and outcome than traditional biomarkers (e.g., SOFA scores) (17). This makes the implications of our findings particularly relevant to sepsis because the employment of sound metabolomics approaches are critical for the generation of reliable metabolomics data, though these findings are also likely to be relevant in any number of disease states notable for alterations in blood rheology. In this regard, WB will be more informative than serum because it can be processed more quickly, minimizing time-dependent changes in metabolite levels and it consistently reflects the contribution of RBC. The use of serum, with its lengthy processing time and highly variable free Hgb levels, which is indicative of the presence of the RBC metabolome, will introduce bias into the metabolomics analysis.

The contribution of RBC metabolites to the variance of the serum metabolome is particularly relevant because there is growing evidence that *in vivo* hemolysis contributes to the pathogenesis and severity of sepsis (6, 13, 27). Hemolysis can be a consequence of sepsis that perpetuates inflammation and it has been recently shown that free Hgb is associated with mortality in patients with sepsis (6). We found multiple components of the metabolome to be associated with free Hgb levels, which has implications for the interpretation of data regarding the underlying pathophysiologic changes in metabolism in the setting of sepsis, and ultimately in the design of interventions to affect these processes. For example, the concentration of glutamate (**Figure 3B**) was more than 3X higher in WB than in serum (Table 2) and is known to be 10X higher in RBC than in plasma (28). Glutamate is integral in glucose metabolism and amino acid transport and utilization, particularly in skeletal muscle, and has also been identified as a potential differentiating metabolite of sepsis (26) as have phenylalanine (**Figure 3C**) and alanine (**Figure 3F**), both of which are transported by RBC (10). Also, creatine phosphate has been reported to decline in sepsis (26). The association between creatine phosphate, a low abundant serum metabolite, and free Hgb (**Figure 3E**) warrants further investigation because even though RBC are known to contain creatine (29), it is generally accepted that RBC lack creatine kinase (30) (EC 2.7.3.2), the enzyme responsible for phosphorylating creatine to creatine phosphate. Of further relevance, RBC possess lactate dehydrogenase (LDH) (31) (EC

1.1.1.27) which catalyzes the conversion of α-ketobutyrate to 2-hydroxybutyrate (**Figure 3H**), a pathway that may be involved in the human inflammatory response to endotoxin (32). Finally, red cells are also a microcosm of branched chain amino acid (BCAA) metabolism and transport (7, 10) so an association between the concentrations of these amino acids and free Hgb is not surprising (**Figure 3A, 3D, 3G**). Nevertheless, this finding is relevant to sepsis because studies that have utilized serum or plasma samples have shown that BCAA metabolism is disrupted in critical illness (26, 32, 33). A recent metabolomics study of plasma collected from healthy volunteers that received endotoxin likewise showed a shift in amino acid (including BCAA) metabolism and elevated 2-hydroxybutyrate, the levels of which are all influenced by free Hgb as shown here (32).

The differences between the serum and WB NMR metabolomes however, are not completely explained by RBC hemolysis because only 8 of the 18 differentiating WB metabolites were associated with Hgb (**Figure 3A-H**). The median creatine concentration was nearly 9 times higher in WB than in serum. Despite the lack of association with Hgb, transport and exchange of creatine is an important function of RBC, particularly in skeletal muscle (29). This points to differences in sample processing between the two sample types as a possible explanation for the higher creatine levels in WB compared with serum (5). As a whole, both the serum and WB metabolomes changed over time but did not differ from each other within the first two hours at room temperature, which mimicked serum processing conditions (**Figure 5**). However, these sampIes remained metabolically active as evidenced by changes in individual metabolite levels (e.g., creatine, taurine) over time as reported here and by others (34). This illustrates the importance of timely and consistent sample processing for the generation of reliable and consistent metabolomics data.

There were also 18 metabolites that were only detected in WB (**Table I**). Some of these have been previously reported in serum (22) but in our study, they did not meet our detection criteria. A number of these metabolites, however, have not been reported in serum including adenosine, AMP, ADP and ATP which are known to be abundant in RBC. Also, RBC have a reservoir of glutathione, the most prevalent anti-oxidant in humans and one that is essential for the protection of hemoglobulin (7, 8). The distinct presence of these metabolites in WB further highlights the contribution RBC makes to the NMR detected blood metabolome. Collectively, the implications of these results are illustrated by our pathway analysis which shows that several metabolic pathways including BCAA metabolism, glycolysis, and gluconeogenesis, which are important pathways in sepsis, are underrepresented in the serum metabolome.

Limitations and Conclusions

One limitation of our study is that healthy controls were used to illustrate the differences in WB and serum NMR-based metabolomes. We elected to utilize this approach for this preliminary study because it permitted collection of samples under controlled conditions and the simultaneous acquisition of a WB and serum sample from a single venipuncture of the same subject. To determine whether the changes found in this controlled setting were likely relevant to clinical sepsis research, we measured free Hgb levels in serum from both control and sepsis patients with a representative visible range of hemolysis. *In vivo* hemolysis can

occur under a number of conditions (6, 13-15), though can commonly occur in sepsis and is increasingly recognized as contributing to the pathology of the condition, forming some of the motivation for this investigation. We do acknowledge, however, that in our study, the free Hgb levels in serum collected from sepsis patients were not different from those of healthy controls. This does not necessarily mean that sepsis patients and healthy subjects are equally prone to *in vivo* hemolysis. We collected a single sample from each healthy control under well-controlled conditions (e.g., single direct venipuncture by a skilled phlebotomist) between 0830AM and 0930AM in the morning. Samples from sepsis patients were collected at all times of the day and at various points during the course of illness. So, while mechanical hemolysis may contribute to the absence of a detected difference between the two groups, we cannot draw confident conclusions about changes in free Hgb that may have occurred over time in sepsis patients because we only collected a single sample from each patient (6). However, this lack of difference between groups does provide reassurance that the findings in healthy controls is highly likely to be relevant in clinical samples and for data interpretation.

In summary, our results highlight the metabolic relevance of the contribution of RBC to the blood metabolome. These cells survey all regions of the human body but lack of internal membrane bound organelles has left them with a reputation of mere oxygen transporters. These cells are pivotal to the transport and tissue exchange of amino acids and are a reservoir of anti-oxidants like glutathione and a number of enzymes such as LDH. The metabolic capabilities of RBC, which remain underappreciated, extend beyond glycolysis. Exclusion of RBCs from blood metabolomics studies causes important information about a number of metabolites to be lost, many of which are involved in metabolic pathways relevant to sepsis. Although some work has been done in RBC metabolomics (6, 35), to the best of our knowledge, this is the first study to directly compare the WB and serum metabolomes. The use of serum or plasma without accounting for free Hgb levels or sample processing time likely enhances metabolomics data variance and may lead to misinterpretation of concentration data, both of which have implications in sepsis metabolomics. Given our findings, we believe WB metabolomics may have unique advantages as the choice biofluid, particularly in disease states characterized by changes in RBC fragility. As such, we recommend that when serum or plasma is chosen for metabolomics studies, free Hgb levels be measured in order to gauge the potential contribution of RBC hemolysis to metabolite measurements. Furthering knowledge of the metabolic importance of RBC in critical illness will lend new insight into the molecular mechanisms that underlie sepsis and sepsis severity and expand the scope of drug target opportunities.

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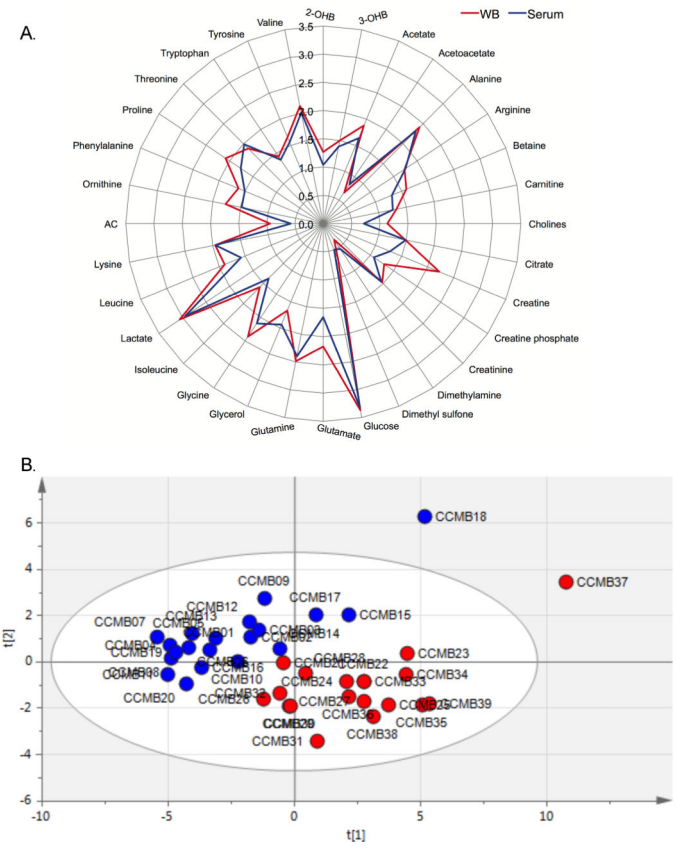

Figure 1.

Differences in serum (blue) and whole blood (WB, red) metabolomes as detected by ^{1}H-NMR (500mHz) spectroscopy are illustrated by (**A**) a radar plot of the 32 common aqueous metabolites of serum and WB and (**B**) principal component analysis of these metabolites. Samples CCMB 18 and CCMB 37 were acquired from the same study volunteer. The first two principal components (t[1] and t[2]) explain 54% of the variability in the data. Data were generated from 20 serum and 19 WB samples and in the radar plot, data are the median Log10 concentration (μM). 2-; 3-OHB = hydroxybutyrate; AC = acetylcarnitine.

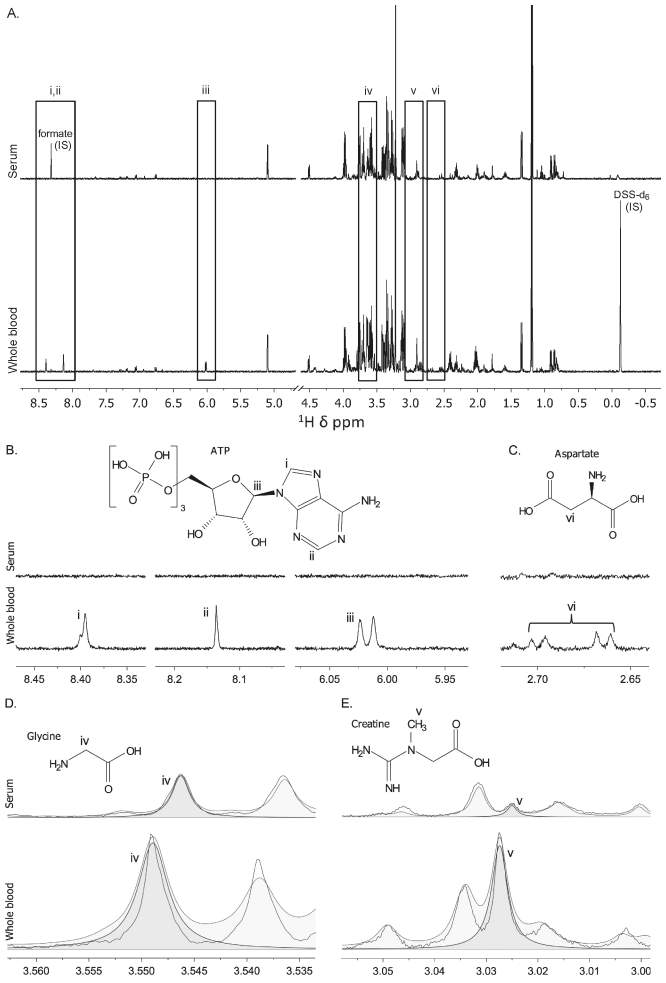

Figure 2.

Differences in the ^{1}H-NMR spectra of whole blood and serum. (**A**) A representative serum and whole blood spectrum showing regional differences (i-vi) in the spectra that are illustrated by two metabolites, (**B**) adenosine tri-phosphate (ATP) and (**C**) aspartate both of which are absent in the ^{1}H-NMR spectrum of serum but present in that of whole blood. The spectral peaks of the associated protons of ATP (i-iii) and aspartate (vi) are shown in the whole blood spectrum. (**D**) Glycine and (**E**) creatine are examples of metabolites that are common to serum and whole blood but are higher in concentrations in whole blood than in serum. The spectral peaks of the associated protons of glycine (iv) and creatine (v), as visualized in Chenomx software that is used for metabolite identification and quantification, are shown to illustrate concentration differences. The spectra are not aligned because of the use of different internal standards (chemical shift references), formate for serum and deuterated 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS-d_6; IS-2, Chenomx Internal Standard).

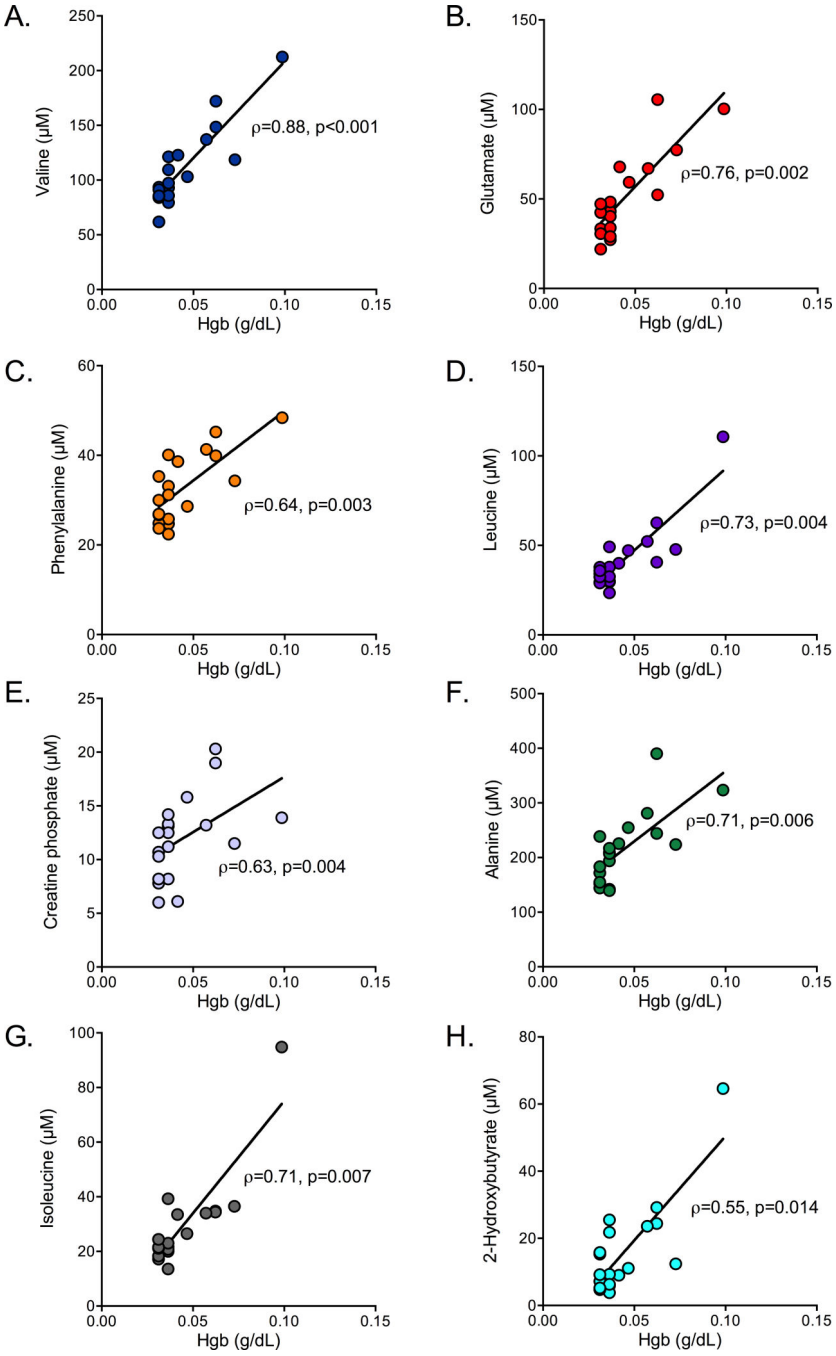

Figure 3.

Serum metabolites associated with free hemoglobin levels. (**A**) Valine, a branched chain amino acid; (**B**) glutamate, which is integral in glucose metabolism and amino acid transport and utilization, particularly in skeletal muscle; (**C**) phenylalanine, which is transported by RBC; (**D**) leucine, a branched chain amino acid; (**E**) creatine phosphate (see text); (**F**) alanine, which is transported by RBC; (**G**) isoleucine, a branched chain amino acid; and (**H**) 2-hydroxybutyrate, a by-product in the pathway of glutathione synthesis were all associated with free Hgb levels in serum. Metabolite and free Hgb levels were available from 19 of 20

serum samples obtained from healthy subjects. The Spearman correlation coefficients (ρ) and the associated p values are reported.

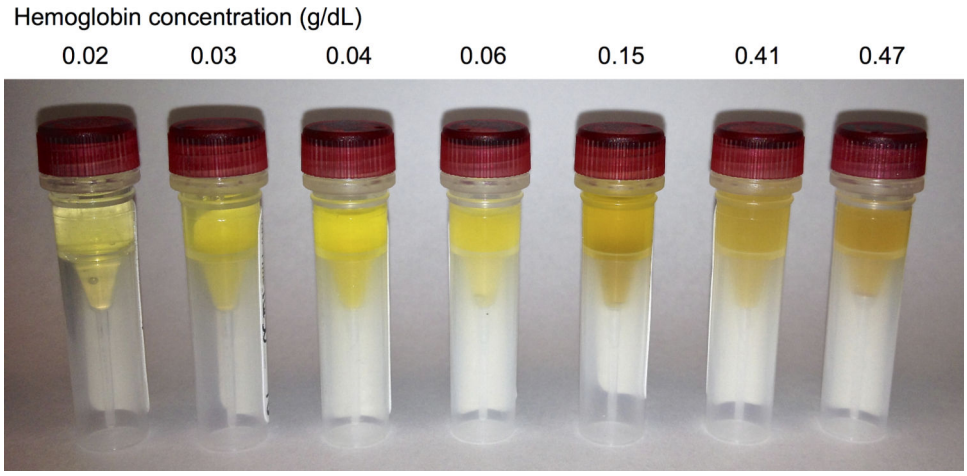

Figure 4.

A range of subtle visual differences in red blood cell hemolysis in seven representative serum samples acquired from patients with sepsis. Samples are ordered from left to right in ascending order of free hemoglobin (Hgb) levels acquired from each sample, which reflects over a 20-fold difference in Hgb concentration.

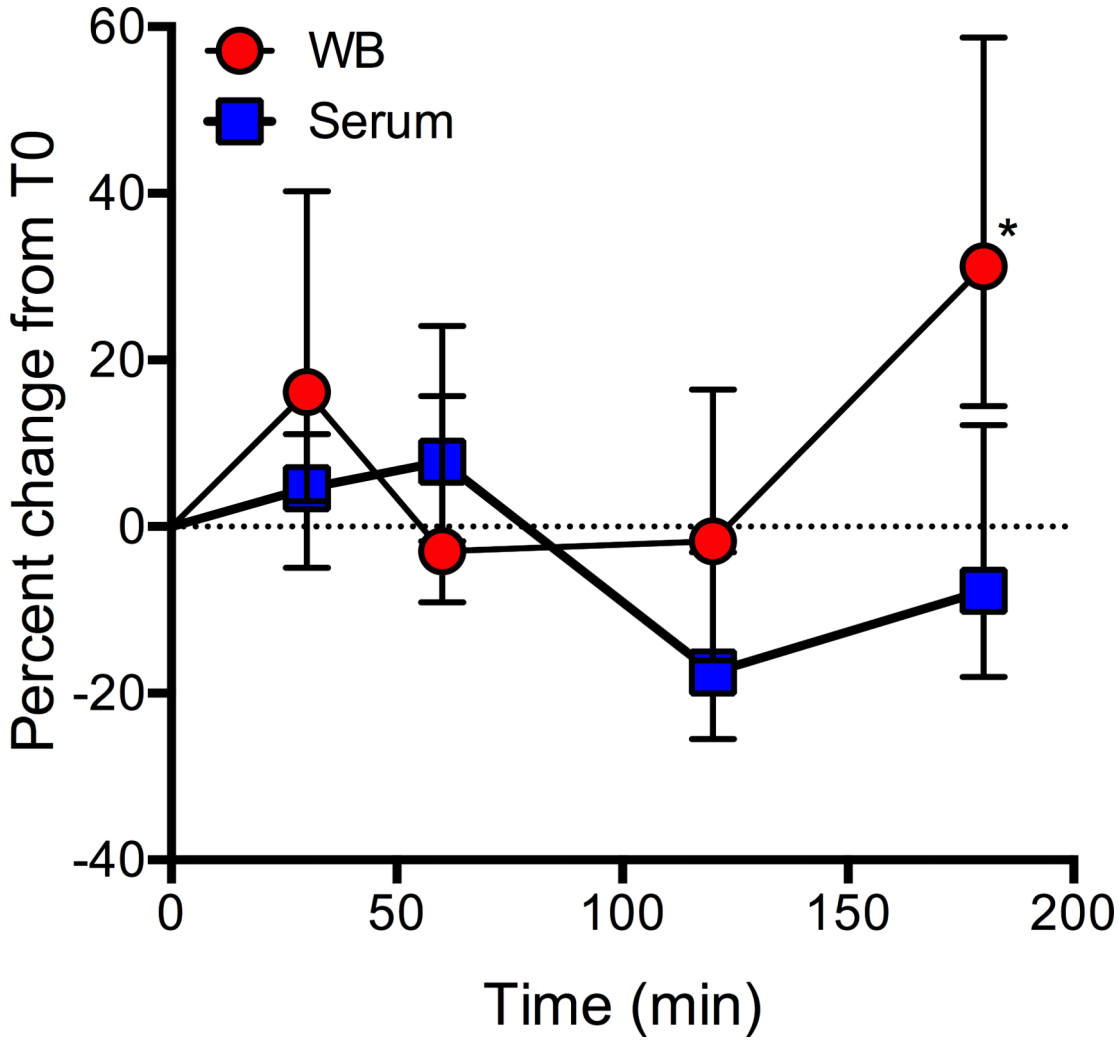

Figure 5.

Percent change over time in the whole blood (WB) and serum metabolomes. Data represent the median value (± IQR) of the concentrations of the 32 common metabolites of WB and serum at each time that were collected from a single donor. Both the WB and serum sample were kept at room temperature until the time of centrifugation (serum) or transfer into the freezer (-80°C). This graph is meant to illustrate the variance that may be introduced into metabolomics data due to prolonged (30-180 min) sample processing times. ANOVA Sidak post-test *$p < 0.0001$.

Table 1

Whole Blood (WB) Metabolites

Metabolite	KEGG[a] ID	WB Concentration (μM)[b]	Reported in the Human Serum Metabolome[c]
2-Oxoisocaproate	C00233	10.3, 2.7	No
3-Hydroxyisobutyrate	C06001	8.7, 4.9	No
3-Methyl-2-oxovalerate	C03465	11.2, 1.5	No
Adenosine	C00212	6.2, 5.0	No
Adenosine diphosphate (ADP)	C00008	75.9, 22.7	No
Adenosine monophosphate (AMP)	C00020	45.6, 48.3	No
Adenosine triphosphate (ATP)	C00002	249.5, 88.0	No
Asparagine	C00152/C01905	78.7, 17.0	Yes
Aspartate	C00049	69, 73.1	Yes
Formate	C00058	31.9, 22.4	Yes[d]
Glutathione	C00051	324.9, 172.7	No
Histidine	C00135	55.7, 8.8	Yes
Inosinic acid	C00130	30.1, 17.0	No
Malonate	C00383	17.4, 8.2	Yes[e]
Acetylcarnitine	C02571	8.8, 4.5	No
Pyruvate	C00022	23.4, 29.6	Yes[f]
Succinate	C00042	7.0, 2.6	No
Trimethylamine N-oxide	C01104	33.3, 12.6	No

[a] Kyoto Encyclopedia Genes and Genomes (KEGG) identification (ID) numbers (http://www.genome.jp/kegg/)

[b] concentration data are median, interquartile range of 19 and 20 healthy volunteers for whole blood (WB) and serum, respectively

[c] reported as an aqueous metabolite detected by NMR in reference (30) at an occurrence of 100% of samples unless otherwise noted

[d] reported as occurring in 48% of samples and is removed by MeOH:$CHCl_3$ extraction (24)

[e] reported as occurring in 14% of samples

[f] reported as occurring in 81% of samples

Table 2

Metabolites Common to Whole Blood and Serum as Measured by ^{1}H-NMR Spectroscopy

Metabolite	KEGG[a] ID	WB Concentration (μM)[b]	Serum Concentration (μM)[b]	p value	FDR[c] (%)
Choline	C00114	13.6, 4.3	5.2, 5.4	0.0001	0.14
Creatine	C00300	167.2, 60.3	19.2, 10.9	0.0001	0.14
Glutamate	C00217	149.7, 27.3	45.2, 33.2	0.0001	0.14
Glycine	C00037	254.7, 75.8	139.4, 38.2	0.0001	0.14
Leucine	C00123/C01570	77.8, 10.5	37.9, 15.4	0.0001	0.14
Ornithine	C00077/C00515	58,8, 19.0	30.4, 17.6	0.0001	0.14
Proline	C00148/C00763	121.0, 46.4	57.5, 27.8	0.0001	0.14
Serine	C00065/C007740	115.3, 63.4	69.1, 29.8	0.0001	0.14
Taurine	C00245	169.3, 48.5	79.8, 43.2	0.0001	0.14
Creatine phosphate	C02305	19.8, 10.2	12.0, 5.2	0.0004	0.14
Isoleucine	C00407	39.0,8.2	24,4, 13.1	0.0013	0.42
Methionine	C00073	24.3, 6.1	16.0, 8.5	0.0015	0.44
Phenylalanine	C00079	42.7, 11.8	32.2, 13.5	0.0021	0.56
Betaine	C00719	39.2, 21.0	20.7, 8.8	0.0028	0.66
Alanine	C00041/C00133	255.8, 77.2	208.4, 72.4	0.0029	0.66
Glycerol	C00116	46.4, 18.6	85.8, 131.5	0.0031	0.66
Valine	C00183	132.8, 27.8	100.2, 38.6	0.0230	4.50
2-Hydroxybutyrate	C05984	18.5, 8.0	11.0, 15.3	0.0239	4.50
Tyrosine	C00082	45.3, 13.6	36.2, 15.1	0.0342	6.08
Ethanol	C00469	29.7, 21.2	39.2, 31.7	0.0463	7.80
Glucose	C00031	2341.3, 636.4	2124.2, 428.1	0.0490	7.84
Acetate	C00033	75.2, 44.3	43.6, 19.8	0.1027	15.27
Threonine	C00188	75.8, 42.2	96.2, 36.5	0.1050	15.27
Glutamine	C00819/C00064	304.3, 73.3	257.2, 48.4	0.1101	15.32
Carnitine	C00318	20.9, 3.2	18.0, 5.4	0.1203	16.04
Lactate	C00186/C00256	1142.0, 741.0	973.6, 630.0	0.2341	29.96
Tryptophan	C00078/C00525	26.5, 6.6	22.7, 9.8	0.3058	37.64
Arginine	C00062/C00792	54.0, 22.8	56.2, 26.4	0.3982	47.19
Lysine	C00047/C00739	89.6, 29.3	89.3, 29.8	0.4538	51.86
3-Hydroxybutyrate	C01089	31.5, 31.9	24.5, 28.0	0.5377	59.33
Creatinine	C00791	29.6, 8.9	28.4, 10.4	0.7736	82.52
Citrate	C00158	30.1, 11.1	29.9, 8.4	0.9113	94.07

[a] Kyoto Encyclopedia Genes and Genomes (KEGG) identification (ID) numbers (http://www.genome.jp/kegg/)

[b] concentration data are median, interquartile range of 19 and 20 healthy volunteers for whole blood (WB) and serum, respectively

[c] false discovery rate

Table 3

Pathways involving metabolites underrepresented or absent from the serum metabolome

Pathways	Metabolites
Bile acid biosynthesis	Taurine
Glycine, serine, alanine and threonine metabolism	Creatine, Choline, Creatine phosphate, Glycine, Pyruvate
Glycolysis and Gluconeogenesis	Pyruvate, ADP, ATP
Purine metabolism	Adenosine, AMP, ADP, ATP, IMP
TCA cycle	Succinate
Urea cycle and metabolism of arginine, proline, glutamate, aspartate and asparagine	Glutamate, Ornithine, Glycine, Aspartate, Asparagine, Formate, Glutathione, Pyruvate, O-Acetylcarnitine, Succinate
Valine, leucine and isoleucine degradation	Leucine, 3-Hydroxyisobutyrate, 2-Oxoisocaproate
Vitamin B9 (folate) metabolism	Glycine, Formate