Fission yeast and human blood metabolomic comparison with focus on age related compounds

分裂酵母とヒト血液のメタボローム比較

SUMMARY

Romanas Chaleckis

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ABSTRACT

Metabolomics, a modern branch of chemical biology, provides qualitative and quantitative information about the metabolic states of organisms or cells at the molecular level. Here I report non-targeted, metabolomic analyses of human blood, using liquid chromatography-mass spectrometry (LC-MS). I compared the blood metabolome to the previously reported metabolome of the fission yeast, *Schizosaccharomyces pombe*. The two metabolomic datasets were highly similar: 101 of 133 compounds identified in human blood (76%) were also present in *S. pombe*, and 45 of 57 compounds enriched in red blood cells (RBCs) (79%), were also present in yeast. The most abundant metabolites were ATP, glutathione, and glutamine. Apart from these three, the next most abundant metabolites were also involved in energy metabolism, anti-oxidation, and amino acid metabolism. I identified fourteen new blood compounds, eight of which were enriched in RBCs: citramalate, GDP-glucose, trimethyl-histidine, trimethyl-phenylalanine, trimethyl- tryptophan, trimethyl-tyrosine, UDP-acetyl-glucosamine, UDP-glucuronate, dimethyl-lysine, glutamate methyl ester, *N*-acetyl-(iso)leucine, *N*-acetyl-glutamate, N_2-acetyl-lysine, and N_6-acetyl-lysine. Ten of the newly identified blood metabolites were also detected in *S. pombe*, and ten of them were methylated or acetylated amino acids. Trimethylated or acetylated free amino acids were also abundant in white blood cells. It may be possible to investigate their physiological roles using yeast genetics.

INTRODUCTION

1.1 Metabolomics

Metabolomics, a modern branch of chemical biology, provides qualitative and quantitative information about the metabolic states of organisms or cells at the molecular level (Fernie et al. 2004; Kell 2004; Goodacre et al. 2004; Nicholson & Lindon 2008; Patti et al. 2012). It aims to profile small molecules present in living organisms, and is now recognized as an important tool for studying metabolic regulation in a synthetic way, together with transcriptomic and proteomic analyses (Hirai et al. 2004). From the "-omics" sciences metabolomics is closest to the phenotype (Figure 1 p.1), as the metabolites are the end products of cellular processes, and their levels can be regarded as the ultimate response of the biological systems to genetic and environmental changes (Fiehn 2002).

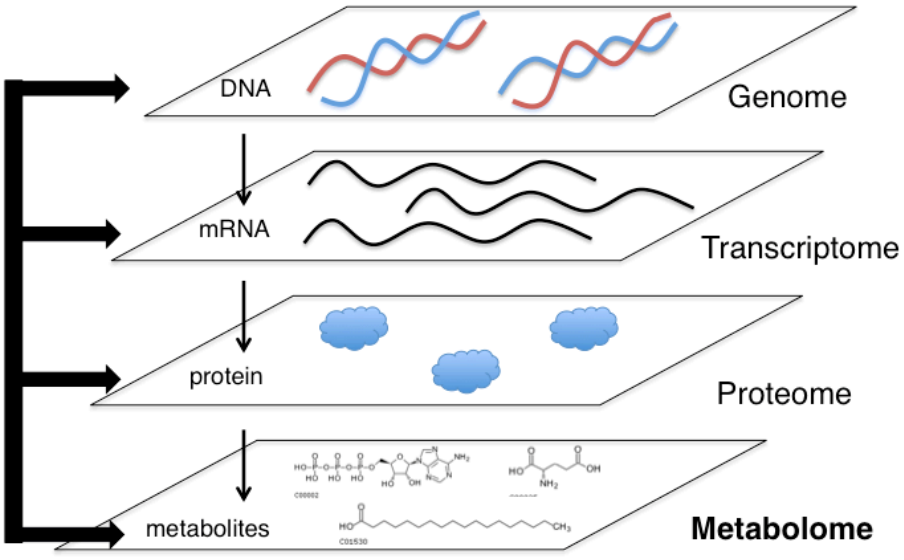


Figure 1. "Omics" Sciences: Genomics, Transcriptomics, Proteomics, and Metabolomics

Thousands of diverse intracellular metabolites (Figure 2 p.2) over a very broad range of concentrations can be detected in biological samples such as human blood (Rappaport et al. 2014). In metabolomics, two detection techniques are commonly used – Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). A number of approaches are used to separate metabolites prior to detection in MS, such as gas chromatography (GC-MS), liquid chromatography (LC-MS) and capillary electrophoresis (CE-MS) (Fernie et al. 2004). The advantages and disadvantages of analytical techniques used in metabolomics research are summarized in Table 1 p.3 (Chaleckis et al. 2013). While both NMR and MS have their advantages and disadvantages, one of the major differences is that MS is a more sensitive molecular detection method than NMR. MS allows the detection of molecules present at low concentrations in biological samples.

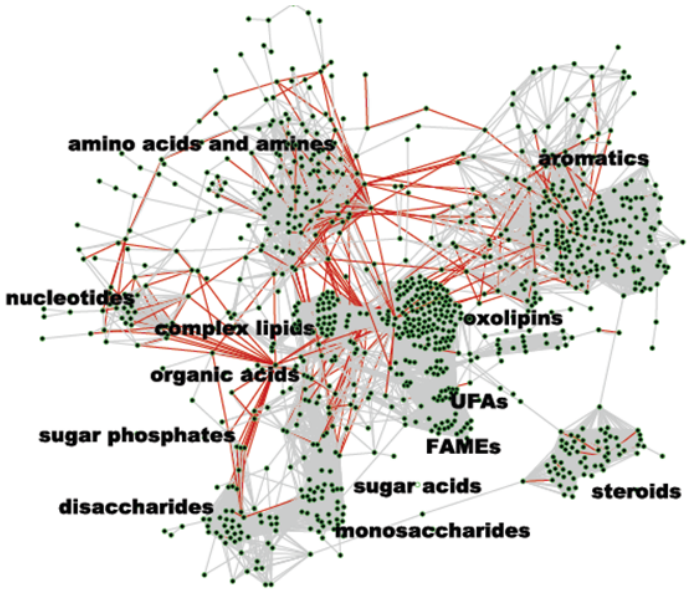


Figure 2. Biological molecules detectable in metabolomics experiments
Over thousand metabolites belonging to different groups can be detected in NIH West Coast Metabolomics Center.
(Source: http://metabolomics.ucdavis.edu/core-services/metabolites)

Table 1. Advantages and disadvantages of analytical techniques used in metabolomics research

Technique	Advantages	Disadvantages
GC-MS	・ Relatively low-cost method ・ No ion suppression ・ Long-term stability and robustness ・ Large spectral libraries available	・Requires derivatization ・ Not suited for thermo labile compounds ・ Impractical for identification of unknowns
LC-MS	・ Compatible with a large number of compound classes, both charged and uncharged ・ Utilizes organic solvents well-suited for MS ・ Good sensitivity	・ Poor reproducibility of retention times ・ Different classes of compounds require different columns/solvents to achieve optimal separation
CE-MS	・ Ideal for ionic or very polar metabolites ・ High resolution separation ・ High peak capacity ・ Low cost of operation due to minimal consumption of solvents ・ Low sample consumption	・ Sensitive to sample ion strength ・ Higher detection limits due to small injection volumes
NMR	・ High analytical reproducibility ・ Non-destructive method ・ High speed of analysis ・ Ability to study living systems	・ Unable to identify individual metabolite signals in complex NMR spectra ・ Low sensitivity compared to MS

(Chaleckis et al. 2013)

However, the number of identified and named compounds in metabolomics experiments is surprisingly small. One reason is the paucity of commercially available standards. Second, it is difficult to identify unknown ions, due to the limited structural information that can be obtained using MS. Third, the concentrations of many metabolites present in biological samples are very low, and they tend to undergo changes during sample preparation. Hence, fewer than several hundred relatively abundant compounds are usually reported in comprehensive metabolomic studies (Brauer et al. 2006; Pluskal, Nakamura, et al. 2010; Soga et al. 2003).

1.2 Fission yeast

The fission yeast, *Schizosaccharomyces pombe,* a eukaryotic microbe with a genome encoding only ~5,000 genes (Wood et al. 2002; Wood et al. 2012), is an excellent model organism to study nutritional control of the transition between proliferation and quiescence (Yanagida et al. 2011).

Metabolomic analysis of *S. pombe* cells has been previously used in order to understand cellular metabolic states under different nutritional regimes and as governed by different genotypes. Initially, our laboratory reported the identification of 123 compounds using liquid chromatography-mass spectrometry (LC-MS) and their changes following heat stress and genetic perturbations (Pluskal, Nakamura, et al. 2010). Our laboratory described the accumulation of antioxidants, glutathione and ergothioneine, in a proteasome regulatory subunit mutant *mts3-1* (Takeda et al. 2010). Also, our laboratory performed targeted measurements of intermediates of the coenzyme A biosynthetic pathway from pantothenate (Nakamura et al. 2012), investigated changes in the cellular metabolome upon glucose (Pluskal et al. 2011) and nitrogen starvation (Sajiki et al. 2013), and accumulation of various metabolites in the quiescence-defective *Δklf1* mutant (Shimanuki et al. 2013). These studies showed that *S. pombe* may be used as a model organism to study comprehensive metabolic patterns under defined genetic and physiological conditions.

1.3 Human blood

In humans, approximately 5 liters (~7% of body mass) of blood circulates throughout the body per min (Allison 1960). As shown in Figure 3 p.5, in a

healthy individual 45% of the blood volume is occupied by RBCs, while only 1% comprises platelets and leucocytes, white blood cells (WBC), the rest being plasma (Alberts et al. 2002).

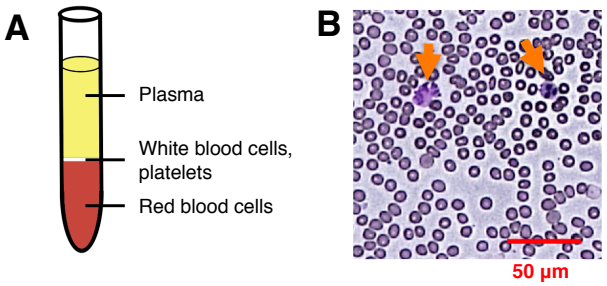


Figure 3. Blood components
(A) Blood components plasma, WBC, RBC separated by centrifugation. **(B)** Blood under the microscope. Most of the cells are RBC; WBCs are indicated by orange arrows.

Plasma contains proteins (e.g. albumins, globulins, fibrinogen, etc.), electrolytes, hormones, glucose, and other metabolites. Nutrients are distributed throughout the body while waste products are brought to the kidneys for excretion.

Blood cells differ in their size and abundance (Table 2, p.6). Platelets are cytoplasm fragments (2-3 µm diameter) of megakaryocytes and contain no nucleus. The main function of platelets is the prevention of bleeding through clot formation. Their number is 10-20 times smaller compared to that of the RBC. WBCs, also called leukocytes, are a group of diverse cells involved in the body's defense against infections. These cells have a nucleus, and are fully biochemically active. WBCs are larger by volume than the RBCs, but since they are about 100 times less numerous than RBCs, they constitute only a minor portion of blood. RBCs are most abundant by number and total cell volume in blood (Alberts et al. 2002).

Table 2. Comparison of blood cells in healthy individual

	Platelets	Red blood cells	White blood cells
Number of cells (cell/mL)	5×10^8	5×10^9	1×10^7
Cell volume (fL)	10	90	200[a]

[a] lymphocytes (Segel et al. 1981)

Red blood cells (RBC), alternatively called erythrocytes, serve to deliver oxygen to tissues via the blood flow in vertebrates. Virtually all vertebrates have RBCs (with exception of some deep ocean fish (Ruud 1954)). The shape of RBCs in humans is a biconcave disk with a diameter 6-8 μm (thickness, 2-2.5 μm). A typical RBC is flattened and depressed in the center, with a dumbbell-shaped cross section. This shape is believed to optimize the flow properties of blood and provides the basis for the remarkable flexibility of RBCs. The structural integrity of RBC is obvious from its unique shape. RBC shows extensive deformability. The capability of changing the shape when in narrow capillaries (5-10 μm in diameter) should be an essential property of RBC (Mohandas & Gallagher 2008). Roughly half of the membrane mass of human RBCs is proteins, and the other half is carbohydrate and lipids, which consists of phospholipids and cholesterol (Steck 1974). While proteins and nucleic aids are not synthesized, membrane phospholipid fatty acid turnover appears to occur in mature RBCs (Arduini et al. 1992). The membrane of the RBC aids in regulating the surface flexibility and deformability. It is composed of three layers, the exterior one rich in carbohydrates, while the lipid bilayer contains

many membrane bound proteins; in addition, there exists a membrane skeleton, a network of proteins that is situated on the inner surface of the lipid bilayer.

Mammalian RBCs exclude nuclei, mitochondria, and other prominent cell organelles, such as lysosomes, endoplasmic reticulum, and Golgi bodies during erythropoiesis in the bone marrow. Mature RBCs, simplified and specialized for gas transfer, have no demonstrable protein synthesis, nor tricarboxylic acid (TCA) cycle activity (Rapoport et al. 1990). Nevertheless, RBCs produce adenosine triphosphate (ATP) glycolytically, maintain redox homeostasis, and osmoregulate (van Wijk & van Solinge 2005). Human RBCs have a relatively long life span of about 120 days (Franco 2009). When senescent, they are captured by the spleen for degradation.

To carry out its function throughout its relatively long lifespan an RBC needs to maintain cellular homeostasis. Relatively simple RBCs, without a nucleus, transcription and protein synthesis, are a model system for metabolic simulations (Joshi & Palsson 1990; Nakayama et al. 2005). To maintain structural integrity RBCs need energy, redox, and biosynthesis of key metabolites (Table 3 p.7).

Table 3. Major metabolic pathways in RBC

Pathway	**Function**
Glycolysis	Energy
Pentose phosphate pathway and glutathione metabolism	Redox
Nucleotide metabolism	Purine synthesis

Many membrane proteins have been identified, including transporters for water, glucose, ions, and gases, and ATPases such as Na^+/K^+-ATPase and Ca^{++}-ATPase (Pasini et al. 2006). These consume ATP and may act to create

the ionic inner environment appropriate for the function of RBCs. ATP is the main source of cellular energy for RBCs, which is used for sugar metabolism, for membrane transport and the cytoskeletal-membrane movements. ATP is produced by glycolysis of glucose, so it follows that glycolytic enzymes and coenzymes are present in RBCs.

The 'cytoplasmic' part of the RBC is rich in highly dense haemoglobin that binds to oxygen. The pentose phosphate pathway (PPP) accounts for only approximately 8% of glucose metabolism in RBCs under normal steady-state conditions, since 92% of glucose is metabolized through glycolysis (D'Alessandro et al. 2013). When faced with oxidative stress, RBCs respond by diverting as much as 90% of glucose metabolism toward the PPP (D'Alessandro et al. 2013). The principal role of RBCs is obviously as a carrier of haemoglobin, as 33% of RBC weight including water is haemoglobin. Although the contents are smaller than haemoglobin, a number of proteins and enzymes required for metabolism to maintain RBCs are present in the cytoplasm (Roux-Dalvai et al. 2008).

Numerous blood metabolomic analyses have been previously reported, mostly of serum and plasma (A et al. 2005; Lawton et al. 2008; Bruce et al. 2009; Kimura et al. 2009; Serkova et al. 2011; Psychogios et al. 2011). However, metabolomics of whole blood or RBCs have been less well investigated (summarized in Table 4 p.9 (Chaleckis et al. 2013)), except for several reports on long-term stored blood (D'Alessandro et al. 2012; D'Alessandro et al. 2013; Nishino et al. 2009), blood of disease patients (Darghouth, Koehl, Heilier, et al. 2011; Darghouth, Koehl, Madalinski, et al.

2011), or blood marker compounds of food intake (Catalán et al. 2013). Reports on the WBC, platelet metabolomes are scarce (Sze & Jardetzky 1990; Lee & Britz-McKibbin 2009; Paglia et al. 2012).

Table 4. Recent reports covering RBC metabolome

Study	Metabolite extraction	Method	Number of metabolites	Reference
Metabolic signature of RBCs from sickle cell disease patients, overhydrated hereditary stomatocytosis patients	Boiling	LC-MS	89（46 confirmed by standards）	(Darghouth, Koehl, Madalinski, et al. 2011; Darghouth, Koehl, Heilier, et al. 2011; Darghouth et al. 2010)
Metabolic analysis of RBCs during prolonged storage	Methanol, chloroform, acetonitrile	LC-MS	Around 40	(D'Alessandro et al. 2013; D'Alessandro et al. 2012; D'Alessandro et al. 2011)
Metabolite extraction efficiency with various solvents	Methanol, chloroform, water	GC-MS, LC-MS	85	(Zhang et al. 2009)
Verification of erythrocyte metabolism by metabolome analysis	Methanol	CE-MS	32	(Kinoshita et al. 2007; Nishino et al. 2009)
Metabolic studies of *P.falciparum* in red blood cell culture	Methanol	LC-MS	90	(Olszewski & Llinás 2013)
Determination of metabolite and nucleotide intracellular concentration	Perchloric acid	NMR	52	(Sze & Jardetzky 1990)

(Chaleckis et al. 2013)

In the human body about 90% of the 10^{14} cells are microbes in the gut (Savage 1977). In addition to the human 20,000 protein-coding genes, there are about 500,000 microbial protein-coding genes (Qin et al. 2010). Thus, human biospecimens contain a plethora of bioactive molecules generated from microbial metabolism (Nicholson, Holmes, J. Kinross, et al. 2012) together with chemicals introduced by the diet, drugs, infectious organisms, pollution, and lifestyle factors (Nicholson & Wilson 2003; Rappaport & Smith 2010; Rappaport et al. 2014). Because blood transports chemicals to and from tissues and

represents a reservoir of all endogenous and exogenous chemicals in the body at a given time (Nicholson, Holmes, J. M. Kinross, et al. 2012), the blood metabolome offers a convenient means for interrogating biologically relevant questions such as human aging.

1.4 Comparison of fission yeast and human red blood cells

S. pombe is a small eukaryotic microorganism containing the nucleus, mitochondria and other cellular organelles, while human RBCs are highly specialized in oxygen transport, do not have cellular organelles and hence do not grow or divide. The cell shape of growing and dividing *S. pombe* is cylindrical, and distinct from RBCs. However, the cell volume of RBCs and *S. pombe* is rather similar (Figure 4 p.10). The RBC is around 90 fL (Mohandas & Gallagher 2008) and fission yeast 120 fL [3.5 µm diameter (Mitchison 1957) and 13 µm cell length at 4.4 mM glucose (Pluskal et al. 2011), with the volume calculated using formula from Mitchison (1957)].

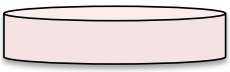
	Fission yeast	**Red blood cell**
Shape	 Cylinder	 Disc
Dimensions (µm)	3.5 (Diameter) 12 (Length)	6-8 (Diameter) 2 (Height)
Volume (fL)	120	90

Figure 4. Fission yeast and RBC

Furthermore, *S. pombe* cells can grow and divide in the culture medium with glucose concentrations equivalent to that of human blood. In plasma the

glucose concentrations in healthy persons are reported to be around 4-7 mM (Saltiel & Kahn 2001). Surprisingly, the rate of *S. pombe* cell division in low (4.4 mM, 0.08%) glucose medium (the concentration similar to blood glucose) is identical to that in high (111 mM, 2%) glucose medium (Pluskal et al. 2011). Thus the glucose environment is appropriate for both RBCs and *S. pombe*.

1.5 The aim of work

To understand the small molecule diversity and conservation in the human red blood cell (RBC), I compared the metabolomes of human blood with that of fission yeast cells (Pluskal, Nakamura, et al. 2010; Pluskal et al. 2011) and determined metabolites in common, or unique to RBCs. The reason to employ *S. pombe* cells for comparison is that compounds commonly present in RBCs and *S. pombe* may represent the necessary molecular functions to maintain RBCs and *S. pombe* cells. In addition, the powerful genetics of *S. pombe* may allow future investigations of the functional roles of the compounds whose functions are obscure.

RESULTS

2.1 Isolation of metabolites from human blood

Blood was taken from the volunteers (Figure 5 p.12) in the morning before breakfast after the overnight fast. Blood samples were taken directly in the laboratory and immediately used for the experiments. For the metabolome sample we took 5 mL of blood, which is an acceptable amount for healthy persons, including the elderly.

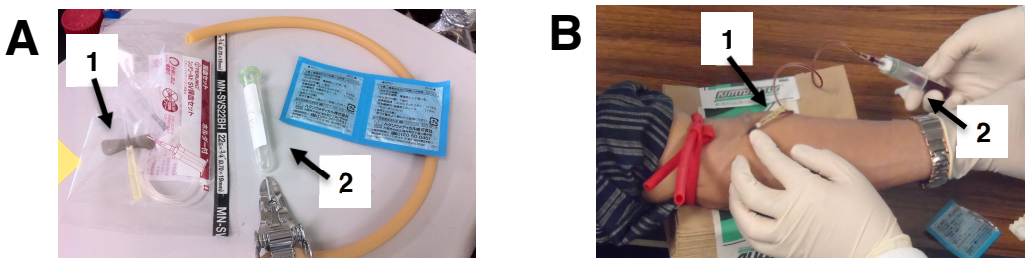


Figure 5. Blood donation
(A) Blood donation set. (**B)** Blood donation. Indicated by arrows (1) needle and (2) 5 mL heparin tube.

2.1.1 Methods for separation of blood components

Whole blood consists of plasma and blood cells, such as RBC, WBC, and platelets. For the metabolome experiments, I tried isolation of RBC by leukocyte filter, cellulose column, Ficoll gradient, and centrifugation.

An easy and straightforward way to fractionate plasma and blood cells is centrifugation. After centrifugation of a whole blood sample, the plasma is on top, RBCs on the bottom, with a thin milky layer of WBCs ("buffy coat") in between. To retrieve the RBCs without contamination with the "buffy coat" is challenging (Figure 6 A p.14, RBC fraction still contains WBCs). Even with specialized centrifugation equipment only about 80% of WBCs can be depleted from the RBC fraction, due to the very similar densities of some of the WBCs

subtypes and the RBCs (Singh & Kumar 2009). While centrifugation of the blood sample is quick and easy, leukodepletion of RBCs is only partial (Figure 6 A p.14 and Table 5 p.16).

For blood transfusion, approximately 99% leukocyte depleted RBCs are needed to avoid immunological reactions (Singh & Kumar 2009). Various designs of leukodepletion blood filters are used in practice (Dzik 1993). For the preparation of RBC transfusion units, kits with leukocyte filters are commercially available. I tried the PALL Purecell® Neo filter (Pall Corporation, USA). These kits are designed for collection of over 100 mL of blood, have a large dead volume (~20 mL) and thus are less suitable for processing small amounts of blood, as in my case (5 mL). To obtain the necessary volume (over 20 mL), I had to dilute the blood. I also tried a smaller scale commercial filter, Plasmodipur (Euro Diagnostica BV, Arnhem, The Netherlands) that is used for malaria research (Janse et al. 1994), but there, too, at least 10 mL of blood is required, thus again the dilution was necessary. As an alternative for the expensive filter, self-made cellulose columns are used for the leukodepletion in malaria research (Sriprawat et al. 2009). However for the self-made cellulose column, a blood volume of over 20 mL was necessary. The relatively small volume (5 mL) of donated blood was not suitable for leukodepletion by leukocyte filters or cellulose columns without modifications to the original protocol. I could obtain leukodepleted RBCs by using leukocyte filter and cellulose column (Figure 6 B and C p.14), but the cells after the dilution with PBS and the lengthy procedure were clearly different from the fresh or centrifuged blood (Figure 6 p.14). Blood taken from veins is dark red, but

during processing through filters or columns it turns bright red, possibly due to binding oxygen. Furthermore, I frequently experienced column and filter clogging, which prolonged the procedure. Thus, I decided to work with the whole blood and RBC samples, containing leukocytes. To assess the contribution of leukocytes to the blood metabolome, I also isolated leukocytes.

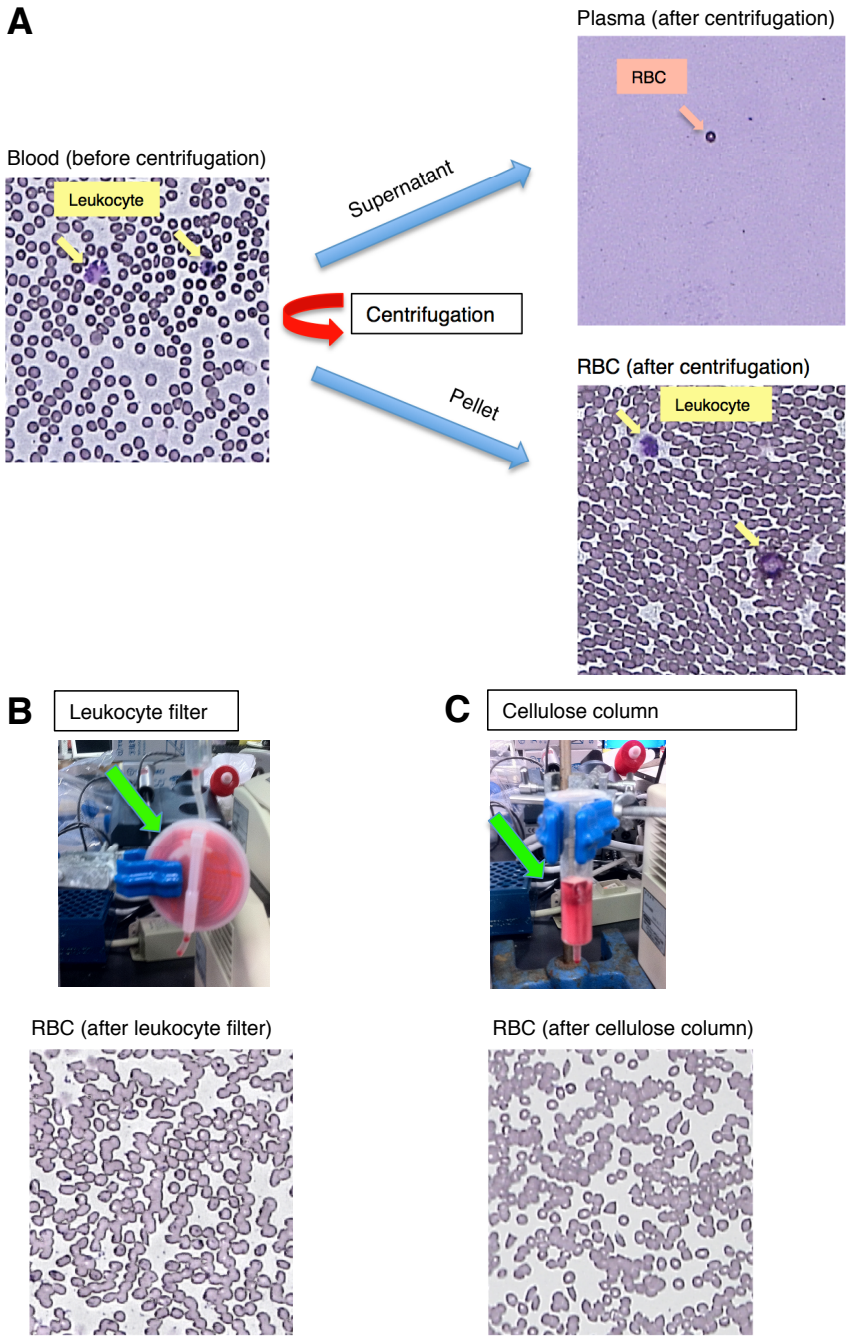


Figure 6. Methods for isolation of RBCs
RBCs were isolated by (**A**) centrifugation, (**B**) leukocyte filter and (**C**) cellulose column. Blood cells stained with Giemsa solution under a microscope.

For the leukocyte isolation I used the standard Ficoll gradient technique (Figure 7 p.15, Materials and Methods). To isolate a sufficient amount of the leukocytes for the metabolome sample, I had to use at least 20 mL of blood. I also could obtain plasma and RBCs after Ficoll centrifugation (Figure 7 B p.15). To remove the Ficoll solution, the cells had to be washed three times with a PBS buffer, adding to the lengthy processing time (Table 5 p.16). I also isolated leukocytes for the metabolome analysis for semi-quantitative comparison with the whole blood and RBC samples.

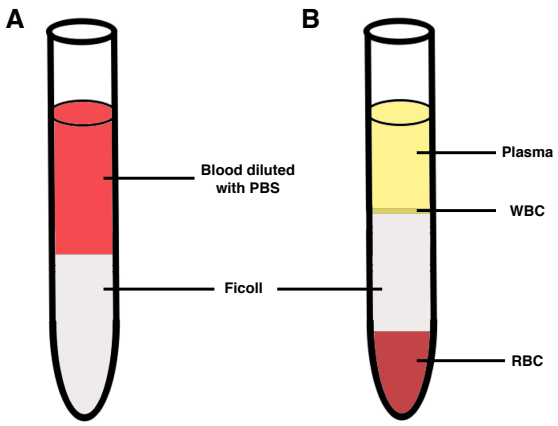


Figure 7. Isolation of blood cells by Ficoll gradient
(**A**) Blood diluted with PBS is layered above Ficoll solution and (**B**) after centrifugation the blood components are separated into fractions (see Materials and Methods).

The RBC and leukocyte isolation methods are summarized in the Table 5 p.16. Use of whole blood is easy and quick, but leaves the WBC inside the sample. Methods to isolate leukodepleted RBCs worked, but required larger blood volumes (20 mL and more) than I could obtain (5 mL), as well as longer processing times. Thus, to keep the protocol simple, reproducible and feasible for larger scale studies, I decided to use the procedure described below.

Table 5 Comparison of RBC isolation methods

	Centrifugation	Leukocyte filter	Cellulose column	Ficoll gradient
Required sample volume	1 mL	20 mL	20 mL	20 mL
Approx. time required	15 min	20 min	60 min	60 min
Final sample	RBC in plasma, containing leukocytes	RBC in PBS, no leukocytes	RBC in PBS, no leukocytes	Separated RBC, WBC in PBS, plasma and Ficoll solution

Blood samples (4-5 mL) for metabolomic analysis were collected from healthy volunteers before breakfast (schematized in Figure 8 p.17, Materials and Methods). WBCs stained with Giemsa (Figure 6 p.14) were infrequently observed (<1%) in blood. WBCs were not removed, since rapid quenching of metabolic reactions was necessary to obtain reproducible, quantitative data (Ficoll centrifugation to separate WBCs is a time-consuming step.). I employed low speed centrifugation (120 g) for 15 min at room temperature for obtain plasma and RBC suspension, which were immediately quenched at –40°C followed by the metabolite extraction by 50% methanol. Similar to the fission yeast metabolome sample preparation (Pluskal, Nakamura, et al. 2010), low-molecular-weight metabolites were isolated by filtration with a 10 kDa cut-off filter at 4° C, concentrated on a rotary evaporator, re-suspended in 40 μL 50% acetonitrile, and stored at -80°C until analysis The whole blood, plasma (supernatant) and RBC (pellets), were employed for metabolomics analysis.

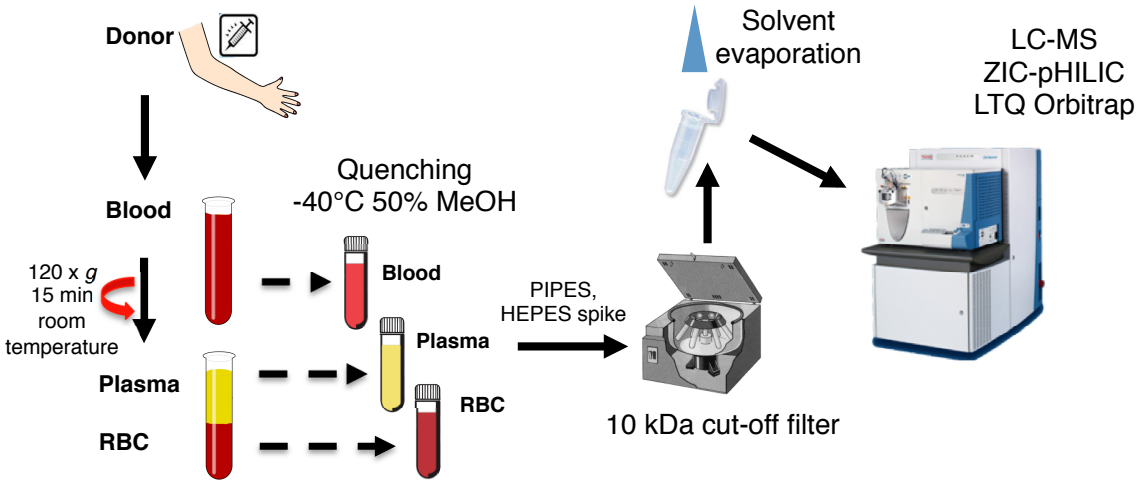


Figure 8. Preparation and analysis of blood metabolome samples
Metabolic compounds were extracted in 50% MeOH at -40° C from whole blood, plasma, and RBCs (Materials and Methods). Extracted metabolites were isolated with a 10-kDa cut-off filter, concentrated by a rotary evaporator, and analyzed on an LC-MS system, as illustrated.

2.1.2 Peak detection and identification of the compounds

For LC-MS analysis, metabolites were first separated by hydrophilic interaction chromatography (ZIC-pHILIC column; Merck SeQuant (Guo & Gaiki 2005)), and detected using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific), in full scan mode (100-1000 *m/z*, ratio of mass-to-charge) with both positive and negative electrospray ionization. LC-MS data contain semi-quantitative information about thousands of compounds in human blood. For compound analysis and quantification, I employed basically the same procedures used in previous analyses of *S. pombe* metabolites (Pluskal, Nakamura, et al. 2010) (Figure 9 p.18). For quantification, I integrated peak curves, obtaining peak areas in arbitrary AU units. ATP and glutathione are RBC-enriched, meaning that peak areas in the RBC-fraction were at least 2-fold higher than corresponding peaks in plasma (See 'Fifty-seven RBC-enriched compounds' below). It is difficult to obtain reproducible quantitative data on reduced glutathione (GSH) due to its auto-oxidation during sample

preparation. For that reason, only levels of total oxidized glutathione (GSSG) are reported in the present study.

For standardization of the data, a fixed amount of PIPES and HEPES were always injected into the samples analyzed. The peak heights and areas of these standard compounds were used to compare and calibrate data obtained from different experiments when necessary.

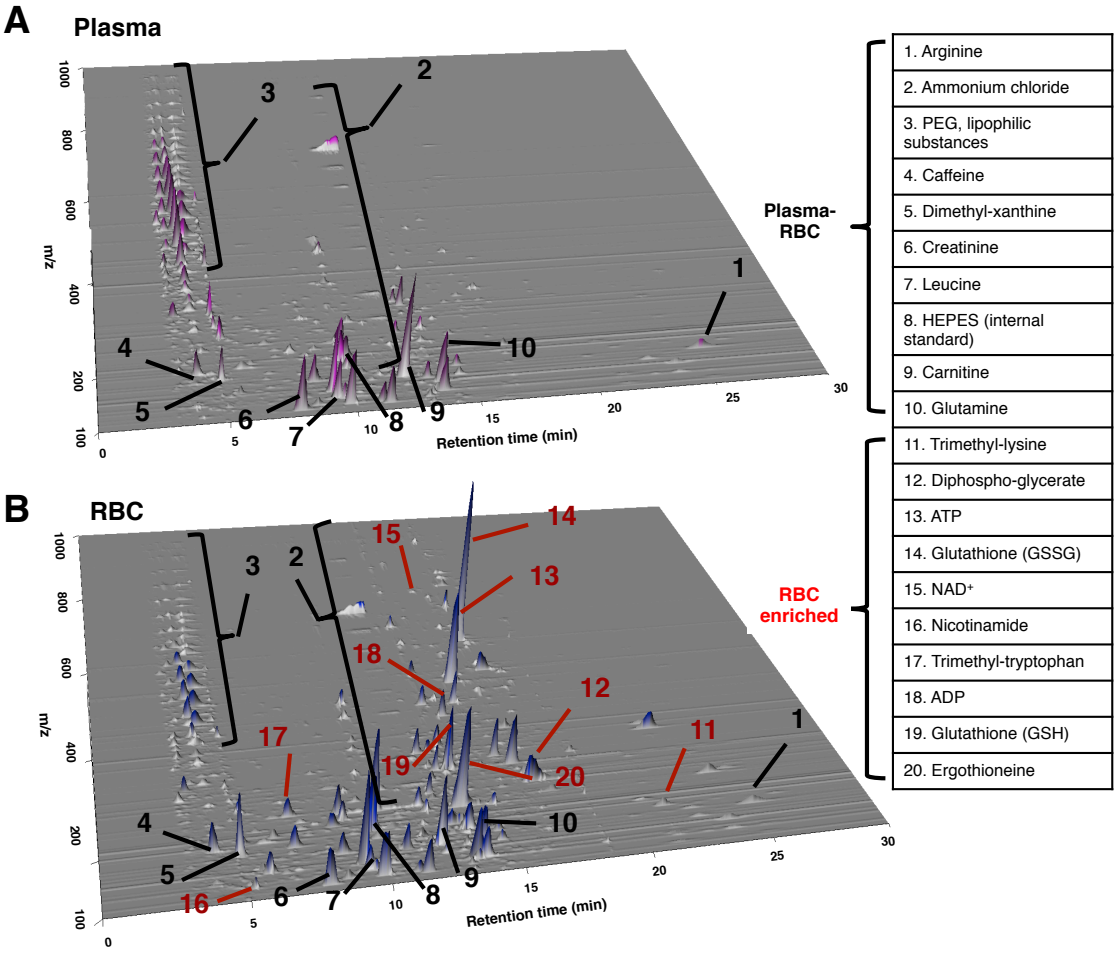


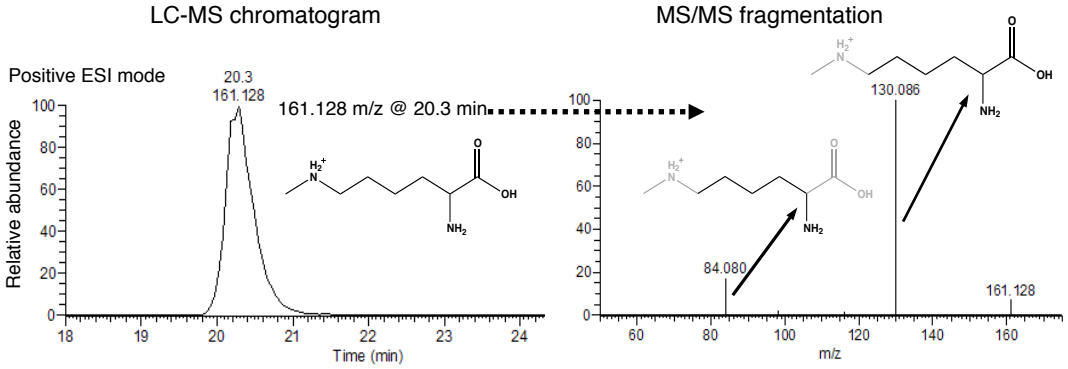
Figure 9. Raw LC-MS data 3D plots of plasma and RBC fractions
Raw LC-MS data 3D plots of plasma fraction (top) and RBC fraction (bottom) obtained in positive ionization mode are shown: X-axis, retention time (RT, min); Y-axis, *m/z*; Z-axis, signal intensity. Twenty identified peaks are shown as examples. Peaks 1-10 are detected in both plasma and RBCs. These are amino acids, creatine, carnitine, dietary metabolites (caffeine, dimethyl-xanthine), and compounds introduced during sample preparation (HEPES as internal standard, NH_4Cl formed in the LC-MS system). Peaks 11-20 are enriched in RBC samples.

2.2 Comparison of red blood cell and fission yeast metabolomes

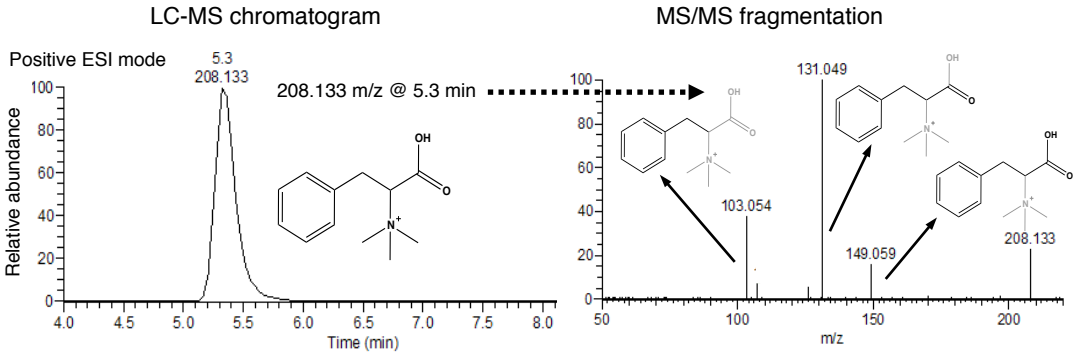
2.2.1 Hundred thirty-three compounds identified in human blood

MZmine 2 software (Pluskal, Castillo, et al. 2010; Pluskal et al. 2012) was used for data processing and identification of blood metabolites. I employed an in-house database of *m/z* and RT values of compounds previously identified in fission yeast studies (Pluskal, Nakamura, et al. 2010; Pluskal et al. 2011; Sajiki et al. 2013; Shimanuki et al. 2013; Takeda et al. 2010; Nakamura et al. 2012). For peaks not in the database, I performed a search using online databases HMDB (Wishart et al. 2013), KEGG (Kanehisa & Goto 2000), or ChemSpider (Pence & Williams 2010). Whenever possible, identified compounds were verified using purchased standards. In some cases, isomers (e.g. *N*-acetyl-leucine, *N*-acetyl-isoleucine; paraxanthine, theobromine, theophylline) could not be clearly distinguished by LC and were designated by more general names (*e.g., N*-acetyl-(iso)leucine or dimethyl-xanthine, respectively). To identify metabolites for which standards were not available, I performed MS/MS analysis. Methyl-lysine, *N*-trimethyl-phenylalanine, and *N*-trimethyl-tyrosine were tentatively identified and described (Figure 10 A-C p.20). I was able to identify 133 compounds in blood, representing 14 categories (Figure 11 p.21 and Table 6 p.22).

A Methyl-lysine MS/MS analysis



B *N*-Trimethyl-phenylalanine MS/MS analysis



C *N*-Trimethyl-tyrosine MS/MS analysis

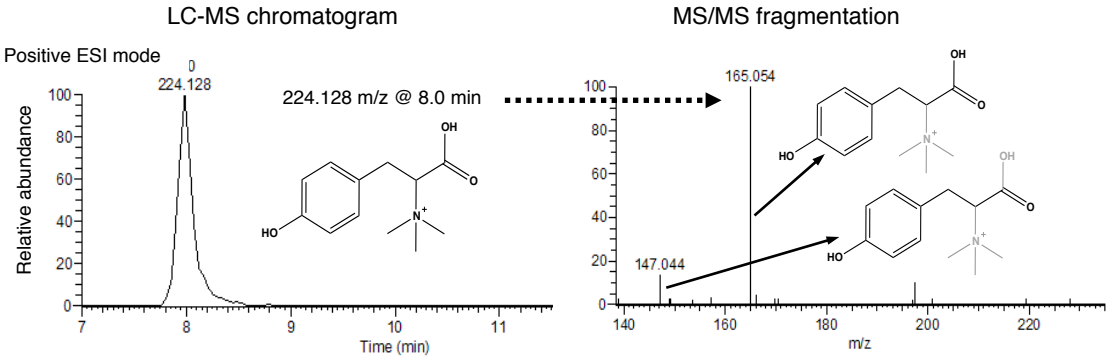


Figure 10. MS/MS analysis of peaks identified as methyl-lysine, *N*-trimethyl-phenylalanine and *N*-trimethyl-tyrosine

See next page for description

Identification of any peak by MS/MS analysis in the absence of a standard requires the *m/z* value, fragmentation pattern, and retention time (RT). Similar RTs were found in cases of amino acids and their methylated counterparts confirmed by standard compounds (e.g. *N*-trimethyl-tryptophan 6.0 min and tryptophan 8.7 min). **(A)** A peak with an *m/z* value of 161.128 Da in positive ionization mode matched the calculated value for the methyl-lysine positive ion with a hydrogen adduct (161.129 Da). The RT is 20.3 min while that of lysine is 23.3 min. The MS/MS fragmentation pattern matches the methyl-lysine structure; the 130.086 Da fragment corresponds to loss of $NH_2(CH_3)$ (31.043 Da), the 84.080 Da fragment to loss of $NH_2(CH_3)$ (31.043) and CO_2H_2 (46.005 Da). While lysine has two amino-groups, I could not determine which of the amino groups is methylated; thus, I tentatively identified the compound as methyl-lysine. **(B)** A peak with an *m/z* value of 208.133 Da in positive ionization mode matched the calculated value for the trimethyl-phenylalanine positive ion with a hydrogen adduct (208.133 Da). The RT is 5.3 min while that of phenylalanine is 7.3 min. The MS/MS fragmentation pattern matches the *N*-trimethyl-phenylalanine structure; the 149.059 Da fragment corresponds to loss of $N(CH_3)_3$ (59.074 Da), the 131.049 Da fragment to loss of $N(CH_3)_3$ (59.074 Da) and H_2O (18.010 Da), and the 103.054 Da fragment to loss of $N(CH_3)_3$ (59.074 Da) and CO_2H_2 (46.005 Da). Therefore I tentatively identified the peak as *N*-trimethyl-phenylalanine. **(C)** A peak with an *m/z* value of 224.128 Da in positive ionization mode matched the calculated value for the trimethyl-tyrosine positive ion with a hydrogen adduct (224.128 Da). The RT is 8.0 min while that of tyrosine is 10.3 min. MS/MS fragmentation pattern matches the *N*-trimethyl-tyrosine structure; the 165.054 Da fragment corresponds to loss of $N(CH_3)_3$ (59.074 Da), the 147.044 Da fragment to loss of $N(CH_3)_3$ (59.074 Da) and H_2O (18.010 Da). I thus tentatively identified the peak as *N*-trimethyl-tyrosine.

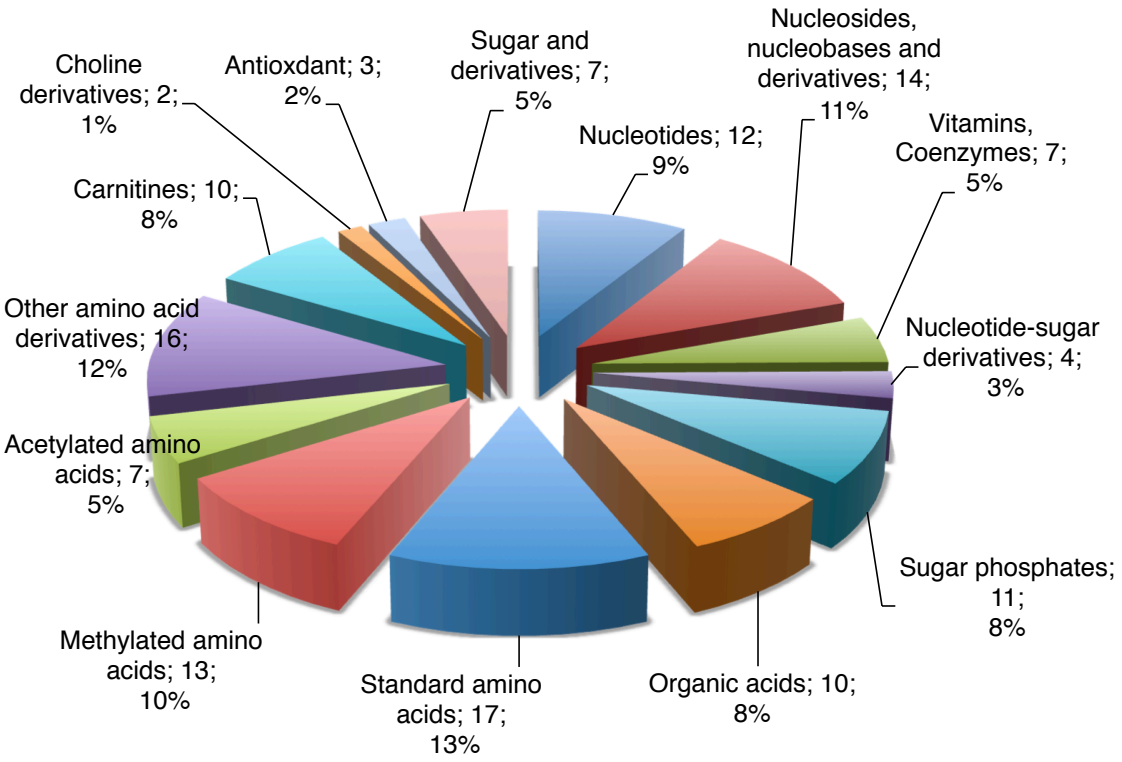


Figure 11. Overview of 133 compounds detected in blood by HILIC LC/MS. Shown are number of compounds in each category and percentages.

Table 6. 133 compounds detected in blood metabolome samples

Category / Compound	**Status**	**Not in fission yeast**	**New blood component**	**RBC enriched**[b]	**Ratio RBC:plasma ± standard deviation**[c]	**Peak area**[d] **Blood**	**Peak area**[d] **Fission yeast**
Nucleotides							
ADP	STD			**R**	**64.0 ± 15.0**	M	M
AMP	STD			**R**	**52.0 ± 6.8**	M	M
ATP	STD			**R**	**81.0 ± 13.0**	H	H
CDP	STD			**R**	**4.6 ± 2.1**	L	L
CTP	STD			**R**	**2.2** ± 0.48	L	M
GDP	STD			**R**	**32.0 ± 5.5**	L	M
GMP	STD			**R**	**3.5** ± 0.29	L	L
GTP	STD			**R**	**84.0 ± 14.0**	M	M
IMP	STD			**R**	**180.0 ± 48.0**	L	L
UDP	STD			**R**	**2.3** ± 0.5	L	M
UMP	STD			**R**	**19.0 ± 3.6**	L	L
UTP	STD			**R**	**4.1** ± 1.6	L	H
Nucleosides, nucleobases and derivatives							
1-Methyl-adenosine	STD				1.3 ± 0.14	M	M
1-Methyl-guanosine	STD				1.1 ± 0.36	L	L
Adenine	STD				**3.4** ± 0.055	L	M
Adenosine	STD				0.045 ± 0.012	L	L
Caffeine	STD	o			0.77 ± 0.057	M	ND
Cytidine	STD				**2.1** ± 0.22	L	L
Dimethyl-guanosine	STD				0.77 ± 0.1	L	L
Dimethyl-xanthine	STD	o			0.88 ± 0.044	M	ND
Guanosine	STD				0.12 ± 0.077	L	L
Hypoxanthine	STD				1.4 ± 0.22	L	L
Inosine	STD				0.059 ± 0.12	L	L
Uracil	STD				1.0 ± 0.041	L	L
Urate	STD	o			0.69 ± 0.22	M	ND
Uridine	STD				1.0 ± 0.027	L	L
Vitamins, Coenzymes							
4-Aminobenzoate	STD	o			**2.5** ± 0.86	L	ND
NAD^+	STD			**R**	**130.0 ± 30.0**	M	H
NADH	STD			**R**	**80.0 ± 68.0**	L	M
$NADP^+$	STD			**R**	**7200.0 ± 3000.0**	L	L
NADPH	STD			**R**	**340.0 ± 220.0**	L	L
Nicotinamide	STD			**R**	**26.0 ± 5.7**	M	M
Pantothenate	STD				**3.3** ± 1.1	L	M
Nucleotide-sugar derivatives							
GDP-glucose	STD		o	**R**	**20.0 ± 3.6**	L	M
UDP-acetyl-glucosamine	STD		o	**R**	**11.0** ± 1.4	L	M
UDP-glucose	STD			**R**	**35.0 ± 6.6**	M	H
UDP-glucuronate	STD	o	o	**R**	**37.0 ± 6.2**	L	ND
Sugar phosphates							
6-Phosphogluconate	STD			**R**	**150.0 ± 25.0**	L	L
Diphospho-glycerate	STD			**R**	**1700.0 ± 350.0**	H	L
Fructose-1,6-diphosphate	STD			**R**	**1400.0 ± 360.0**	M	M
Fructose-6-phosphate	STD			**R**	**23.0 ± 2.2**	L	M
Glucose-6-phosphate	STD			**R**	**32.0 ± 4.1**	M	M
Glyceraldehyde-3-phosphate	STD			**R**	**910.0 ± 1100.0**	L	L
Glycerol-2-phosphate	STD			**R**	**3.7** ± 1.1	L	M
Pentose-phosphate	STD			**R**	**39.0 ± 12.0**	L	M
Phosphoenolpyruvate	STD			**R**	**1000.0 ± 780.0**	L	M
Phosphoglycerate	STD			**R**	**150.0 ± 17.0**	M	M
Sedoheptulose-7-phosphate	STD			**R**	**3.6** ± 0.76	L	M
Sugars and derivatives							
1,5-Anhydroglucitol	STD	o			0.95 ± 0.14	M	ND
Gluconate	STD			**R**	**16.0** ± 1.8	M	L
Glucosamine	STD				0.89 ± 0.13	M	L
Glucose	STD				0.87 ± 0.12	M	L
myo-Inositol	STD				1.3 ± 0.39	L	L
N-Acetyl-D-glucosamine	STD			**R**	**35.0** ± 1.4	M	L
Quinic acid	STD				**2.1** ± 2.0	L	M
Organic acids							
2-Oxoglutarate	STD				0.94 ± 0.16	L	L
Chenodeoxycholate	STD	o			0.49 ± 0.14	M	ND
cis-Aconitate	STD				0.33 ± 0.11	L	L
Citramalate	STD		o	**R**	**3.2** ± 0.87	L	M
Citrate	STD				0.28 ± 0.069	M	M
Fumarate	STD			**R**	**5.6** ± 0.9	L	L
Glutarate	STD				0.94 ± 0.072	L	L
Glycerate	STD	o			0.69 ± 0.18	L	ND
Malate	STD			**R**	**4.6** ± 0.6	L	L
Succinate	STD				0.92 ± 0.21	L	L

Table 6. (continued)

Category / Compound	**Status**	**Not in fission yeast**	**New blood component**	**RBC enriched**[b]	**Ratio RBC:plasma ± standard deviation**[c]	**Peak area**[d] **Blood**	**Peak area**[d] **Fission yeast**
Standard amino acids							
Arginine	STD				0.57 ± 0.072	H	H
Asparagine	STD				1.6 ± 0.18	L	L
Aspartate	STD			R	**6.3** ± 0.91	L	M
Glutamate	STD			R	**3.4** ± 0.74	M	H
Glutamine	STD				0.56 ± 0.053	H	H
Histidine	STD				0.93 ± 0.054	M	H
Isoleucine	STD				0.95 ± 0.14	M	L
Leucine	STD				1.1 ± 0.098	M	L
Lysine	STD				0.91 ± 0.15	L	L
Methionine	STD				0.76 ± 0.099	M	L
Phenylalanine	STD				0.93 ± 0.16	H	M
Proline	STD				0.92 ± 0.054	H	L
Serine	STD				1.1 ± 0.19	L	L
Threonine	STD				1.1 ± 0.12	M	M
Tryptophan	STD				0.47 ± 0.054	M	M
Tyrosine	STD				1.1 ± 0.083	M	M
Valine	STD				0.87 ± 0.07	M	L
Methylated amino acids							
Betaine	STD				1.2 ± 0.11	H	L
Butyro-betaine	STD	O		R	**5.3** ± 0.52	M	ND
Dimethyl-arginine	STD				0.95 ± 0.12	L	L
Dimethyl-lysine	STD		O		0.51 ± 0.09	L	L
Dimethyl-proline (stachydrine)	STD	O		R	**4.9** ± 0.66	H	ND
Methyl-histidine	STD				0.92 ± 0.056	L	M
Methyl-lysine	MS/MS				0.67 ± 0.11	M	L
S-Methyl-ergothioneine	STD	O		R	**1500.0** ± **2300.0**	L	ND
Trimethyl-histidine (hercynine)	MS/MS		O	R	**110.0** ± **140.0**	L	M
Trimethyl-lysine	STD			R	**6.3** ± 1.5	M	L
Trimethyl-phenylalanine	MS/MS	O	O	R	**14.0** ± **9.0**	L	ND
Trimethyl-tryptophan (hypaphorine)	STD	O	O	R	**6.1** ± 1.7	H	ND
Trimethyl-tyrosine	MS/MS	O	O	R	ND*	L	ND
Acetylated amino acids							
N-Acetyl-(iso)leucine	STD		O		1.8 ± 0.39	L	L
N-Acetyl-arginine	STD				0.34 ± 0.047	L	M
N-Acetyl-aspartate	STD				0.84 ± 0.2	L	L
N-Acetyl-glutamate	STD		O		0.82 ± 0.18	L	M
N-Acetyl-ornithine	STD				**2.6** ± 1.2	L	L
N_2-Acetyl-lysine	STD		O		**2.1** ± 0.3	L	L
N_6-Acetyl-lysine	STD		O		0.88 ± 0.26	L	M
Other amino acid derivatives							
2-Aminoadipate	STD				0.91 ± 0.073	L	L
Arginino-succinate	STD				0.69 ± 0.46	L	M
Citrulline	STD				0.97 ± 0.19	M	M
Creatine	STD	O		R	**7.0** ± 1.6	H	ND
Creatinine	STD	O			0.97 ± 0.079	H	ND
Glutamate methyl ester	STD		O		1.5 ± 0.28	L	L
Hippurate	STD	O			0.54 ± 0.083	M	ND
Histamine	STD				1.0 ± 0.5	L	L
Indoxyl-sulfate	STD	O			0.43 ± 0.092	M	ND
Kynurenine	STD	O			0.41 ± 0.081	L	ND
Ornithine	STD				1.2 ± 0.16	L	M
Phosphocreatine	STD	O		R	**3.0** ± 0.95	L	ND
Quinolinic acid	STD	O			0.35 ± 0.04	L	ND
S-Adenosyl-homocysteine	STD			R	**2100.0** ± **1600.0**	L	M
S-Adenosyl-methionine	STD			R	**57.0** ± **62.0**	L	L
Taurine	STD	O			0.92 ± 0.18	M	ND
Carnitines							
Acetyl-carnitine	STD	O		R	**4.0** ± 0.58	H	ND
Butyryl-carnitine	STD	O			0.95 ± 0.2	M	ND
Carnitine	STD	O			0.85 ± 0.1	H	ND
Decanoyl-carnitine	STD	O			0.42 ± 0.11	M	ND
Dodecanoyl-carnitine	STD	O			0.51 ± 0.072	L	ND
Hexanoyl-carnitine	STD	O			0.68 ± 0.087	L	ND
Isovaleryl-carnitine	STD	O			0.94 ± 0.25	L	ND
Octanoyl-carnitine	STD	O			0.45 ± 0.086	M	ND
Propionyl-carnitine	STD	O		R	**5.3** ± 0.82	M	ND
Tetradecanoyl-carnitine	STD	O		R	**11.0** ± **15.0**	L	ND
Choline derivatives							
CDP-choline	STD				0.98 ± 0.37	L	L
Glycerophosphocholine	STD				1.5 ± 0.24	M	H
Antioxidant							
Ergothioneine	STD			R	**100.0** ± **4.8**	H	M
Glutathione disulfide (GSSG)	STD			R	**1900.0** ± **430.0**	H	H
Ophthalmic acid	STD			R	**310.0** ± **150.0**	L	M

[a] One hundred thirty-three identified metabolic compounds detected in human blood metabolome samples by LC-MS. Status of the compounds was either confirmed by comercially available standard standard (STD) or MS/MS analysis (MS/MS). Compounds not detected in fission yeast (32 compounds) and compounds not reported as blood metabolites (14 compounds) are marked by **O** in respective columns. [b]on average in mutiple persons. Ratios between RBC and plasma samples calculated from four blood samples donated by the same person within 24 hours. Values and standard deviations rounded to 2 significant numbers. ND - not detected. Values >2 shown in bold. [d]Peak areas defined as H, high (>10^8 AU); M, medium (10^7-10^8 AU); L, low (<10^7 AU); ND - not detected. In case of blood, equivalent of ~5µl of blood was injected into the LC-MS system, for fission yeast ~1µl of internal cell volume (see Materials and Methods).

For comparison of blood metabolomics data with those of *S. pombe*, I performed analysis of the metabolites of *S. pombe* cells growing and dividing in the low glucose concentrations (0.1%). These *S. pombe* data obtained were basically the same as reported previously and quite significantly different from that cultured in the high glucose (111 mM) synthetic medium (Pluskal et al. 2011). In the following results sections, *S. pombe* data is shown for comparison which was obtained from cells cultured at 26° C in synthetic, minimal EMM2 medium with 0.1% glucose (5.6 mM; roughly the same glucose concentration found in blood).

2.2.2 Thirty-two compounds identified in human blood were not detected in yeast

Among 133 identified blood metabolites, 32 were not found in fission yeast (Table 6 p.22, Figure 18 p.37). They include three nucleoside bases and derivatives, one coenzyme precursor (4-aminobenzoate), one nucleotide-sugar derivative (UDP-glucuronate), one sugar derivative (1,5-anhydroglucitol), two organic acids (chenodeoxycholic acid, glyceric acid), six methylated amino acids including dimethyl-proline, eight other amino acid derivatives including creatine, creatinine, and taurine, and ten carnitines. Thus, 24 of 32 compounds are derived from three categories, namely, methylated amino acids, other amino acids, and carnitines. These three categories illustrate the major difference between human blood and *S. pombe* metabolomes.

Some compounds may play specific roles in higher eukaryotes. For example, creatine and phosphocreatine are stored in muscle as an energy source, and creatinine is the metabolic byproduct of creatine phosphate (Snow

& Murphy 2001). Carnitines are produced in liver and stored in muscle for consumption and transport of fatty acids (Evans & Fornasini 2003). Urate is a purine metabolic byproduct, high blood concentrations of which can cause gout (Ames et al. 1981), while caffeine is a xanthine alkaloid and of dietary origin (Barone & Roberts 1996; Benowitz 1990). *S. pombe* does not produce caffeine, but can become hypersensitive or resistant to caffeine by certain mutations (Kumada et al. 1996; Ohkura et al. 1988).

I examined whether caffeine and carnitine added to the culture medium of *S. pombe* could be metabolized. For 6 h I cultured *S. pombe* cells in the presence of 0.1 and 1 mM caffeine or carnitine. I could detect caffeine or carnitine peaks in the metabolome sample, but I could not find any metabolites of the two compounds nor other compounds affected by the addition of caffeine or carnitine.

2.2.3 Eighteen *S. pombe* compounds not detected in human blood

Eighteen compounds present in *S. pombe* were not detected in RBCs or in plasma (Table 7 p.26). Compounds that control fast cell division and growth, such as cyclic AMP (an activator of protein kinase A (Yamashita et al. 1996)), AICAR (5'-phosphoribosyl-5-amino-4-imidazolecarboxamide, an activator of AMP-dependent protein kinase, AMPK (Corton et al. 1995)), SAICAR (succinyl-aminoimidazolecarboxamide ribose-5'-phosphate, an activator of pyruvate kinase PKM2 (Keller et al. 2012)) and PRPP (phosphoribosyl pyrophosphate, involved in nucleotide metabolism (Murray 1971)) are present in *S. pombe*, but not in blood. Acetyl-CoA, biotin, Coenzyme A, FAD (flavine adenine dinucleotide) and HMG-CoA may be required for rapid cell division.

Ferrichrome is a cyclic hexapeptide present in lower eukaryotes, such as *Schizosaccharomyces* and *Ustilago*, which forms a complex with iron (Schrettl et al. 2004). Trehalose is a disaccharide having an α, α -1,1-glucosidic bond implicated in anhydrobiotic (anti-desiccant) and anti-oxidant mechanisms (Elbein et al. 2003). Trehalose is not synthesized in the human body. Trehalose-6-phosphate has been implicated in plant flowering (Wahl et al. 2013), but no function in fission yeast has been proposed. Saccharopine is an intermediate of lysine metabolism (Xu et al. 2006).

Table 7. List of 18 identified metabolites detected in fission yeast, but not in blood.

Compound	Status	Peak area Fission yeast	Detected in WBC
Nucleotides			
3',5'-cAMP	STD	L	O
CMP	STD	L	O
Nucleosides, nucleobases and derivatives			
AICAR	STD	L	
PRPP (5-Phospho-alpha-D-ribose 1-diphosphate)	STD	M	O
SAICAR	MS/MS	L	
Vitamins, Coenzymes			
Acetyl-CoA	STD	L	O
Biotin	STD	L	
CoA	STD	L	O
FAD	STD	L	O
HMG-CoA	STD	L	O
Sugar phosphates			
Trehalose-6-phosphate	STD	M	
Sugars and derivatives			
Trehalose	STD	H	
Methylated amino acids			
Dimethyl-histidine	MS/MS	M	
Other amino acid derivatives			
Deferrichrome	STD	L	
Ferrichrome	STD	H	
Histidinol	STD	M	
S-adenosyl-cysteine	STD	L	
Saccharopine	STD	L	

[a] Fission yeast cells were grown at 26°C in EMM2 medium with 0.1% glucose (for details see Materials and methods). Status of the compounds was either confirmed by comercially available standard standard (STD) or MS/MS analysis (MS/MS). Peak areas defined as H, high (>10^8 AU); M, middle (10^7-10^8AU); L, low (<10^7 AU). Compounds detected also in the WBC are marked by O.

2.2.4 Compounds detected in WBCs in comparison with those from RBCs and *S. pombe*

I then examined metabolites of WBCs isolated by Ficoll gradient centrifugation (Table 8 p.40). Seven compounds that seem to be mainly involved in cell growth and division in eukaryotes were found in both WBCs and *S. pombe* (Table 7 p.26). While the metabolic profile of WBCs was similar to that of RBCs, seven metabolites, 3'-5'-cAMP, acetyl-CoA, CMP, CoA, FAD (flavin adenine dinucleotide), HMG-CoA, and PRPP, were detected only in WBCs (Table 8 p.40). Differences between the RBCs and WBCs are mainly the types of vitamins and coenzymes. 4-Aminobenzoate was detected in blood and RBCs, but not in WBCs. Acetyl-CoA, biotin, FAD, and HMG-CoA, described above, belong to the coenzyme category.

2.2.5 Fourteen newly identified blood compounds

To my knowledge, 14 metabolites have not hitherto been reported in human blood, based on a recent report of detected blood metabolites (Rappaport et al. 2014) and literature database searches (Table 6 p.22 and Figure 21 p.47). These new blood metabolites include **citramalate**, dimethyl-lysine, **GDP-glucose**, glutamate methyl ester, *N*-acetyl-glutamate, *N*-acetyl-(iso)leucine, N_2-acetyl-lysine, N_6-acetyl-lysine, **trimethyl-histidine**, **trimethyl-phenylalanine**, **trimethyl-tryptophan**, **trimethyl-tyrosine**, **UDP-acetyl-glucosamine** and **UDP-glucuronate.** The eight compounds in boldface were enriched in RBCs, while ten underlined compounds were also found in *S. pombe*. Ten of the 14 novel blood metabolites are methylated or acetylated amino acids.

2.2.6 Semi-quantification of blood metabolite peaks

Each blood sample produced thousands of peaks in positive and negative ionization modes with a broad range (10^4~10^9 AU) of peak areas. Number of peaks and number of compounds detected in blood and fission yeast metabolome samples are compared in Figure 12 p.28.

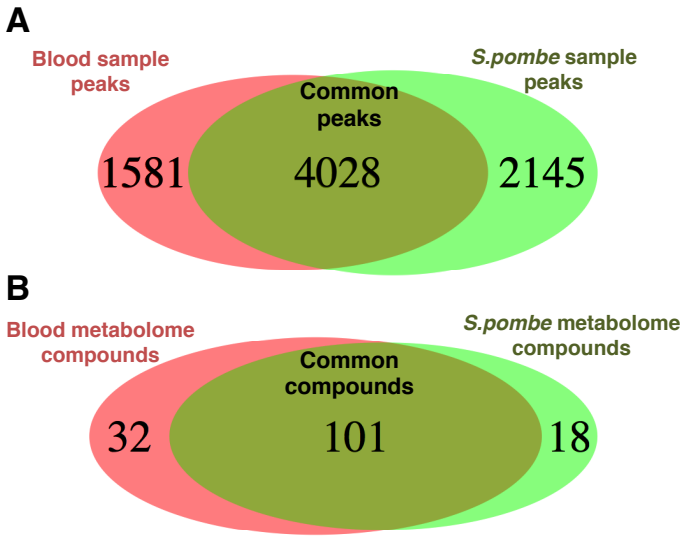


Figure 12. Number of peaks and compounds detected in human blood and fission yeast metabolomes.
(A) Number of peaks detected in representative blood and fission yeast metabolome sample (data acquired in same sequence, positive and negative ionization modes combined). **(B)** Number of identified compounds in blood and fission yeast metabolome samples.

I quantified compounds on the basis of their peak areas: High (H, over 10^8 AU), Medium (M, 10^7-10^8 AU) and Low (L, <10^7 AU). In blood samples, L, M, and H groups comprised 92, 7, and 1% of all peaks, respectively.

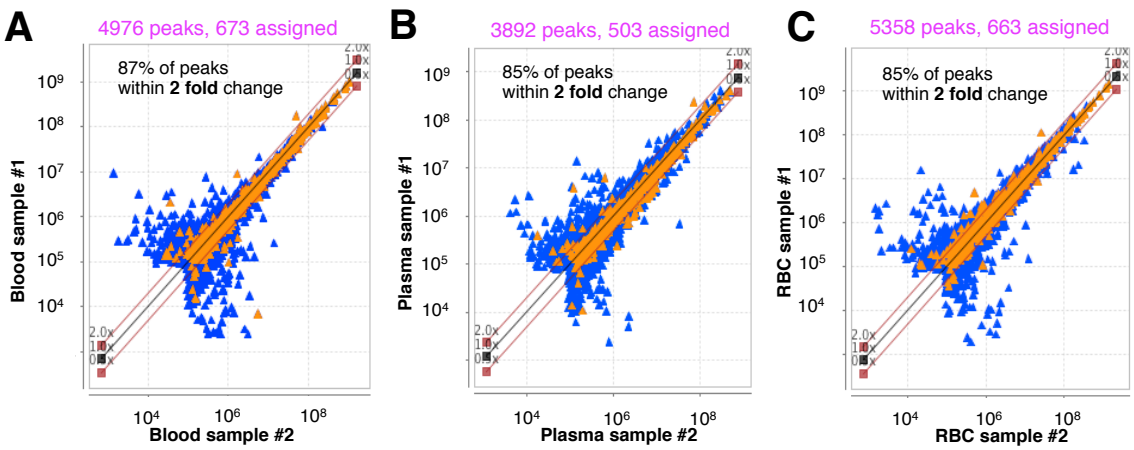


Figure 13. Scatter plots of all peaks detected in blood, plasma and RBC samples

Blood was donated twice by the same person in 1 h, and the two **(A)** blood, **(B)** plasma, **(C)** RBC samples were processed separately (samples #1 and #2). Scatter plot of all peaks detected in both blood samples (positive and negative ionization modes combined). 87 % of peaks differed less than 2-fold. Less than 15% of these peaks could be assigned to a known compound (assigned peaks marked yellow).

Quantitative reproducibility of peak areas was examined by collecting two blood samples independently from the same person at 1 h intervals. Each pair of samples (#1 and #2) of blood, plasma, and RBCs was compared in a scatter plot (Figure 13 p.29). In all cases, 85-87% of peak areas varied less than 2-fold (0.5 – 2.0x). Fission yeast samples obtained under identical conditions showed similar reproducibility (Pluskal, Nakamura, et al. 2010). Very small peaks (area $<10^6$ AU) showed larger deviations. For 133 compounds identified in blood, plasma, or RBCs, however, 97% of peaks in the compared samples changed less than 2-fold (Figure 14 p.30). Thus, in both, blood and fission yeast metabolomes, quantitative reproducibility was better for identified peaks (Pluskal, Nakamura, et al. 2010).

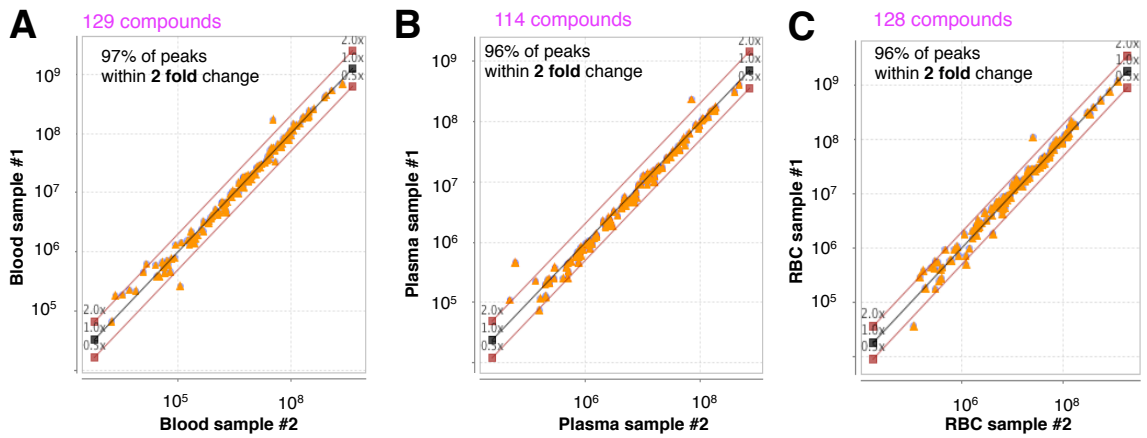
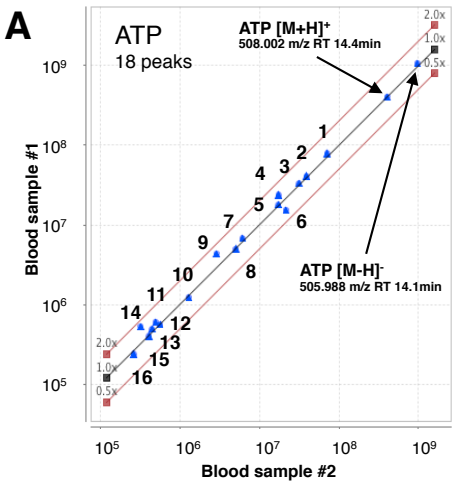
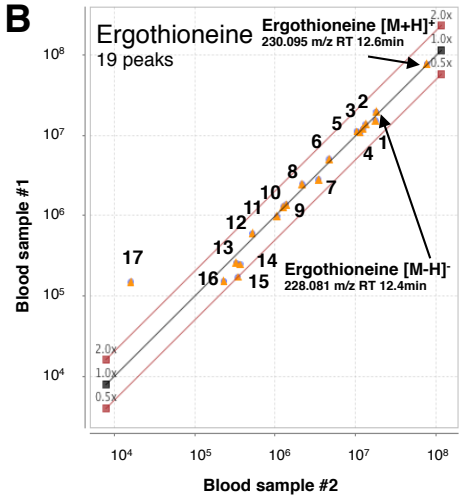


Figure 14. Scatter plots of identified compounds (representative single-charged peaks) in blood, plasma and RBC samples
Blood was donated twice by the same person in 1 h, and the two **(A)** blood, **(B)** plasma, **(C)** RBC samples were processed separately (samples #1 and #2). A scatter plot of 129 identified compounds detected in blood samples #1 and #2. Approximately 97% of these peaks were found within 2-fold change (see Table 8 p.40 for the peak details).

Highly abundant metabolites form various adducts or fragments, resulting in multiple MS peaks. For quantification, I used singly charged proton adducts in positive $[M+H]^+$ and negative modes $[M-H]^-$. ATP produced these two peaks as its highest signals (Figure 15 A p.31). ATP also produced 16 additional peaks (6 in positive and 10 in negative ionization mode). Since their retention time (RT) was basically identical to that of the corresponding primary peak, I suspect that these additional peaks were produced during ionization in the MS. For ergothioneine, 17 peaks were identified in addition to the primary peaks (Figure 15 B p.31).



Peak	m/z	RT (min)	Description
1	426.022	14.1	ATP [M-HPO_3]$^-$
2	525.028	14.4	ATP [M+NH_4]$^+$
3	603.965	14.1	ATP [M+H_2PO_4]$^-$
4	252.490	14.1	ATP [M-2H]$^{2-}$
5	136.061	14.5	ATP [M-$C_5H_{11}O_{13}P_3$]$^+$
6	134.048	14.2	ATP [M-$C_5H_{11}O_{13}P_3$]$^-$
7	529.983	14.4	ATP [M+Na]$^+$
8	408.011	14.1	ATP [M-H_3PO_4]$^-$
9	527.970	14.2	ATP [M+Na-2H]$^-$
10	428.036	14.4	ATP [M-HPO_3]$^+$
11	759.487	14.1	ATP [3M-2H]$^{2-}$
12	410.026	14.4	ATP [M-H_3PO_4]$^+$
13	487.979	14.1	ATP [M-H_2O-H]$^-$
14	527.961	14.1	ATP [M+Mg-3H]$^-$
15	348.070	14.4	ATP [M-$H_2P_2O_6$]$^+$
16	328.045	14.1	ATP [M-$H_4P_2O_7$]$^-$



Peak	m/z	RT (min)	Name
1	186.105	12.6	Ergothioneine [M-CO_2]$^+$
2	361.165	12.6	Ergothioneine, creatine complex [M+H]$^+$
3	457.168	12.7	Ergothioneine dimer [M+H]$^+$
4	374.150	12.5	Ergothioneine, glutamine complex [M-H]$^-$
5	408.145	12.4	Ergothioneine, glucose complex [M-H]$^-$
6	125.018	12.4	Ergothioneine [M-$C_4H_9O_2N$]$^-$
7	127.032	12.6	Ergothioneine [M-$C_4H_9O_2N$]$^+$
8	169.008	12.4	Ergothioneine [M-C_3H_9N]$^-$
9	376.164	12.7	Ergothioneine, glutamine complex [M+H]$^+$
10	459.183	12.6	Ergothioneine [2M+H]$^+$
11	264.058	12.4	Ergothioneine [M+Cl]$^-$
12	457.170	12.4	Ergothioneine [2M-H]$^-$
13	252.077	12.6	Ergothioneine [M+Na]$^+$
14	355.110	12.5	Ergothioneine, taurine complex [M+H]$^+$
15	171.022	12.6	Ergothioneine [M-C_3H_9N]$^+$
16	481.164	12.5	Ergothioneine [2M+Na]$^+$
17	487.196	12.7	Ergothioneine, glycerophosphocholine complex [M+H]$^+$

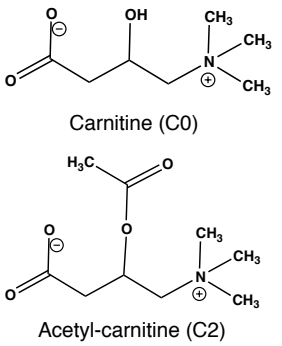
Figure 15. Multiple peaks produced by the same compound
Multiple peaks are produced by abundant compounds such as ATP **(A)** and ergothioneine **(B)**. In addition to the primary single-charged ions (indicated by arrows), compounds produce multiply charged ions, fragments of the molecule, adducts with salts, and complexes with other compounds eluting at similar retention times. I was able to identify 18 peaks related to ATP and 17 peaks related to ergothioneine, listed in the inset tables.

In a representative blood sample I was able to identify 37 (74%) peaks in group H, 118 (33%) peaks in group M, and 518 peaks (11%) in group L. The total number of assigned peaks (673) is much larger than that of actually identified compounds (133), due to the fact that many metabolites produced multiple peaks. A number of peaks were also produced by electrolytes such as NH_4Cl, originating from NaCl in blood samples. While several thousand peaks

were obtained by LC-MS (Figure 12 p.28), the actual number of compounds that can be detected in blood may be much less, possibly ~1,000.

The levels detected within chemically similar compound groups by our LC-MS system correlate quite well with reports in the literature. In Figure 16 p.32 the levels for the acyl-carnitines are shown. Acyl-carnitines are formed when the –OH in carnitine is linked to an acyl group (Figure 16 A p.32). Names of the acyl-carnitines are derived from the corresponding side chain (Figure 16 B p.32). Measured peak areas of the carnitines in the blood samples (Figure 16 C p.32) correlate quite well with concentration values reported in the literature (Figure 16 D p.32). Carnitines have a positively charged trimethylammonium group, and are thus easy to ionize and can be detected to low micromolar concentrations.

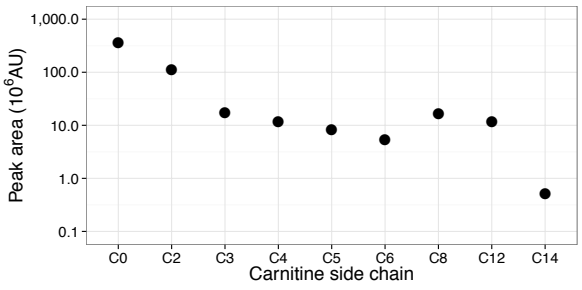
A



B

Side chain	Name
C0	Carnitine
C2	Acetyl-carnitine
C3	Propionyl-carnitine
C4	Butyryl-carnitine
C5	Isovaleryl-carnitine
C6	Hexanoyl-carnitine
C8	Octanoyl-carnitine
C12	Dodecanoyl-carnitine
C14	Tetradecanoyl-carnitine

C



D

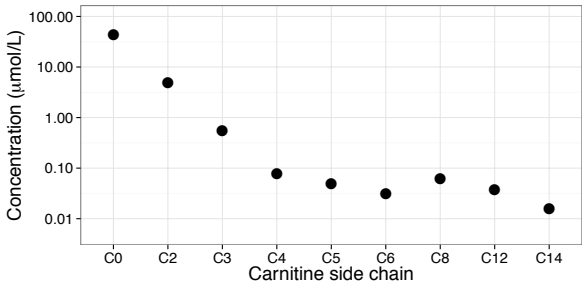


Figure 16. Semi-quantification of carnitines in blood
(A) Structural formula of carnitine and acetyl-carnitine. **(B)** Number of carbon atoms in the side chain and names of the carnitine acetyl esters. **(C)** Measured peak areas of the detected carnitine and carnitine derivatives in the blood sample. **(D)** Carnitine and acyl-carnitines concentrations in blood as reported in literature (Böhmer et al. 1974; Costa et al. 1997).

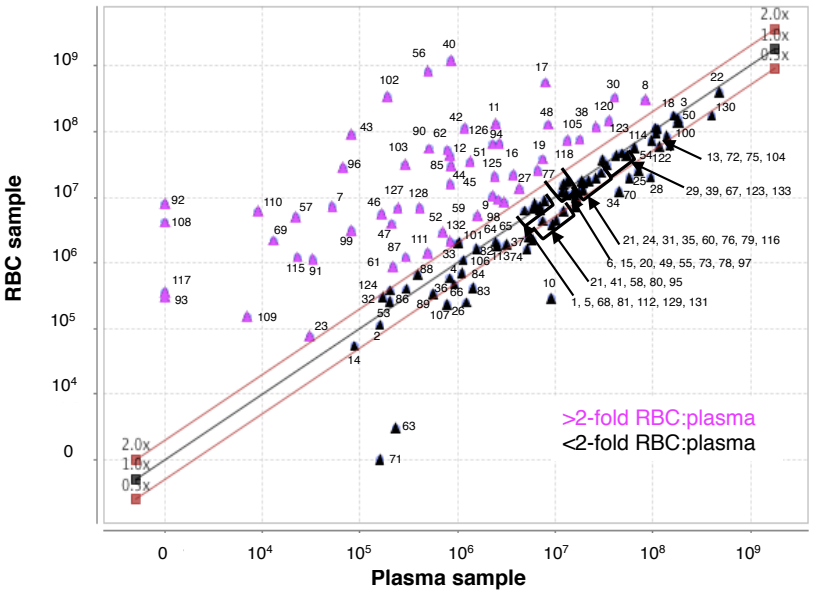
2.2.7 Fifty-seven RBC-enriched compounds

To determine the degree of compound enrichment in RBCs *vs.* plasma, samples of both were prepared from the same blood donor several times. I designated RBC-enriched compounds as those having an RBC:plasma ratio greater than 2.0 (Figure 17 p.34 and Table 6 p.22).

ATP and glutathione showed particularly large peak areas (>10^8 AU) in the RBC sample, but much smaller in the plasma sample (RBC:plasma ratios of 81 and 1900, respectively). In contrast, carnitine and urate showed RBC:plasma ratios of 0.85 and 0.69, respectively, even though their peak areas were large (>10^8 AU) in both samples.

Fifty-seven compounds were enriched in RBCs (Table 6 p.22). Most metabolites highly enriched in RBC-fractions (RBC:plasma ratio >30) were nucleotides (ADP, AMP, ATP, GDP, GTP, IMP), sugar phosphates (6-phosphogluconate, diphospho-glycerate, fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, pentose-phosphate, phosphoenolpyruvate, phosphoglycerate), vitamins (NAD^+, NADH, $NADP^+$, NADPH), antioxidants (ergothioneine, glutathione disulfide (GSSG), ophthalmic acid), methylated compounds (*S*-adenosyl-homocysteine, *S*-methyl-ergothioneine, tetradecanoyl-carnitine, trimethyl-histidine), and *N*-acetyl-D-glucosamine. There was no evidence of leakage of these highly enriched compounds from RBCs, confirming that cells were not damaged during handling. Metabolites moderately enriched in RBCs (RBC:plasma ratio between 2 and 30) contain compounds from the categories mentioned above, as well as nucleotide-sugar

derivatives and at least one or two compounds from other categories (except antioxidants and choline derivatives).



No	Compound
1	1-Methyl-adenosine
2	1-Methyl-guanosine
3	1,5-Anhydroglucitol
4	2-Aminoadipate
5	2-Oxoglutarate
6	4-Aminobenzoate
7	6-Phosphogluconate
8	Acetyl-carnitine
9	Adenine
10	Adenosine
11	ADP
12	AMP
13	Arginine
14	Arginino-succinate
15	Asparagine
16	Aspartate
17	ATP
18	Betaine
19	Butyro-betaine
20	Butyryl-carnitine
21	Caffeine
22	Carnitine
23	CDP
24	CDP-choline
25	Chenodeoxycholate
26	*cis*-Aconitate
27	Citramalate
28	Citrate
29	Citrulline
30	Creatine
31	Creatinine
32	CTP
33	Cytidine
34	Decanoyl-carnitine

No	Compound
35	Dimethyl-arginine
36	Dimethyl-guanosine
37	Dimethyl-lysine
38	Dimethyl-proline (stachydrine)
39	Dimethyl-xanthine
40	Diphospho-glycerate
41	Dodecanoyl-carnitine
42	Ergothioneine
43	Fructose-1,6-diphosphate
44	Fructose-6-phosphate
45	Fumarate
46	GDP
47	GDP-glucose
48	Gluconate
49	Glucosamine
50	Glucose
51	Glucose-6-phosphate
52	Glutamate
53	Glutamate methyl ester
54	Glutamine
55	Glutarate
56	Glutathione disulfide (GSSG)
57	Glyceraldehyde-3-phosphate
58	Glycerate
59	Glycerol-2-phosphate
60	Glycerophosphocholine
61	GMP
62	GTP
63	Guanosine
64	Hexanoyl-carnitine
65	Hippurate
66	Histamine
67	Histidine

No	Compound
68	Hypoxanthine
69	IMP
70	Indoxyl-sulfate
71	Inosine
72	Isoleucine
73	Isovaleryl-carnitine
74	Kynurenine
75	Leucine
76	Lysine
77	Malate
78	Methionine
79	Methyl-histidine
80	Methyl-lysine
81	*myo*-Inositol
82	*N*-Acetyl-(iso)leucine
83	*N*-Acetyl-arginine
84	*N*-Acetyl-aspartate
85	*N*-Acetyl-D-glucosamine
86	*N*-Acetyl-glutamate
87	*N*-Acetyl-ornithine
88	N_2-Acetyl-lysine
89	N_6-Acetyl-lysine
90	NAD^+
91	NADH
92	$NADP^+$
93	NADPH
94	Nicotinamide
95	Octanoyl-carnitine
96	Ophthalmic acid
97	Ornithine
98	Pantothenate
99	Pentose-phosphate
100	Phenylalanine

No	Compound
101	Phosphocreatine
102	Phosphoenolpyruvate
103	Phosphoglycerate
104	Proline
105	Propionyl-carnitine
106	Quinic acid
107	Quinolinic acid
108	*S*-Adenosyl-homocysteine
109	*S*-Adenosyl-methionine
110	*S*-Methyl-ergothioneine
111	Sedoheptulose-7-phosphate
112	Serine
113	Succinate
114	Taurine
115	Tetradecanoyl-carnitine
116	Threonine
117	Trimethyl-histidine (hercynine)
118	Trimethyl-lysine
119	Trimethyl-phenylalanine*
120	Trimethyl-tryptophan (hypaphorine)
121	Trimethyl-tyrosine*
122	Tryptophan
123	Tyrosine
124	UDP
125	UDP-acetyl-glucosamine
126	UDP-glucose
127	UDP-glucuronate
128	UMP
129	Uracil
130	Urate
131	Uridine
132	UTP
133	Valine

*Trimethyl-phenylalanine and -tyrosine not detected in these samples

Figure 17. Scatter plot comparison of the compounds detected in plasma and RBC samples

Peak areas of each compound identified in RBCs and plasma, plotted in a scatter plot. Each compound is indicated with the number with the compound name in the table inset. Purple numbers and names represent the compounds enriched in RBCs (the ratio RBC:plasma is more than 2-fold).

Many RBC-enriched compounds, such as nucleotides and sugar-phosphates, are involved in RBC metabolic pathways (Nishino et al. 2009). Others such as acetyl-carnitine (Cooper et al. 1988) and trimethyl-lysine (Mizobuchi et al. 1990) were previously reported to be enriched in RBCs, but their origins and roles in RBCs are unknown. Interestingly, eight of the fourteen newly discovered blood components (nucleotide-sugar derivatives and methylated amino acids) were also enriched in RBCs.

In contrast to RBC-enriched compounds, adenosine, guanosine, and inosine were scarcely detected in RBCs. Thus, brief centrifugation to sediment RBCs in combination with LC-MS quantification enabled us to classify metabolites into several groups based upon their RBC:plasma ratios (>30 highly RBC-enriched, <30, >2 RBC-enriched; <2 present in both plasma and RBC).

2.2.8 Comparison of semi-quantified metabolites between blood and *S. pombe*

Some compounds exhibited similar abundance in blood and *S. pombe* cells (Table 6 p.22). ATP (H), ADP (M), AMP (M) and GTP (M) were similar in both, whereas UTP was abundant in *S. pombe* (H), but not in blood (L). With the exception of UTP, these nucleotides are enriched in RBCs; thus RBCs evidently have a low requirement for UTP, since RNA synthesis does not occur in RBCs. 2,3-Diphospho-glycerate, an allosteric regulator of hemoglobin present at mM concentrations in RBCs (Joshi & Palsson 1990), was highly abundant (H) in blood, but not in *S. pombe* (L). Pentose-phosphate and sedoheptulose-7-phosphate were more abundant in *S. pombe* (M) than in blood

(L), indicating that growing and dividing yeast cells require greater amounts of pentose phosphate pathway intermediates.

Seventeen regular and seven acetylated amino acids were commonly present in blood and yeast. Fourteen methylated amino acids were significantly different between blood and *S. pombe*, while seven methylated amino acids (betaine, dimethyl-arginine, dimethyl-lysine, methyl-histidine, methyl-lysine, trimethyl-histidine, trimethyl-lysine) were commonly present in both, and seven others (butyro-betaine, dimethyl-proline, *S*-methyl-ergothioneine, trimethyl-phenylalanine, -tryptophan, -tyrosine) were detected only in human samples. Three anti-oxidants - glutathione, ergothioneine, and ophthalmic acid - were present both human and yeast samples.

2.2.9 Summary and categorization of detected blood compounds

Human blood metabolites identified in this study are summarized in Figure 18 p.37. Three nucleosides, adenosine, guanosine, and inosine, may be restricted to plasma, as RBC:plasma ratios are close to zero (Table 6 p.22). *cis*-Aconitate (0.3), citrate (0.3), indoxyl-sulfate (0.4), kynurenine (0.4), *N*-acetyl-arginine (0.3), and quinolinic acid (0.4) appear to be found primarily in plasma. In contrast, $NADP^+$, *S*-adenosyl-homocysteine, phosphoenolpyruvate, glutathione disulfide (GSSG), diphospho-glycerate, and fructose-1,6-diphosphate were highly enriched in RBC fractions. Their RBC:plasma ratios are quite large (>1,000). RBC compounds of intermediate abundance (ratio >50 - <1,000) included 6-phosphogluconate, ADP, AMP, ATP, ergothioneine, GTP, IMP, NAD^+, NADPH, ophthalmic acid, phosphoglycerate, *S*-methyl-

ergothioneine, tetradecanoyl-carnitine, and trimethyl-histidine. All other compounds are presumably present in both RBCs and plasma.

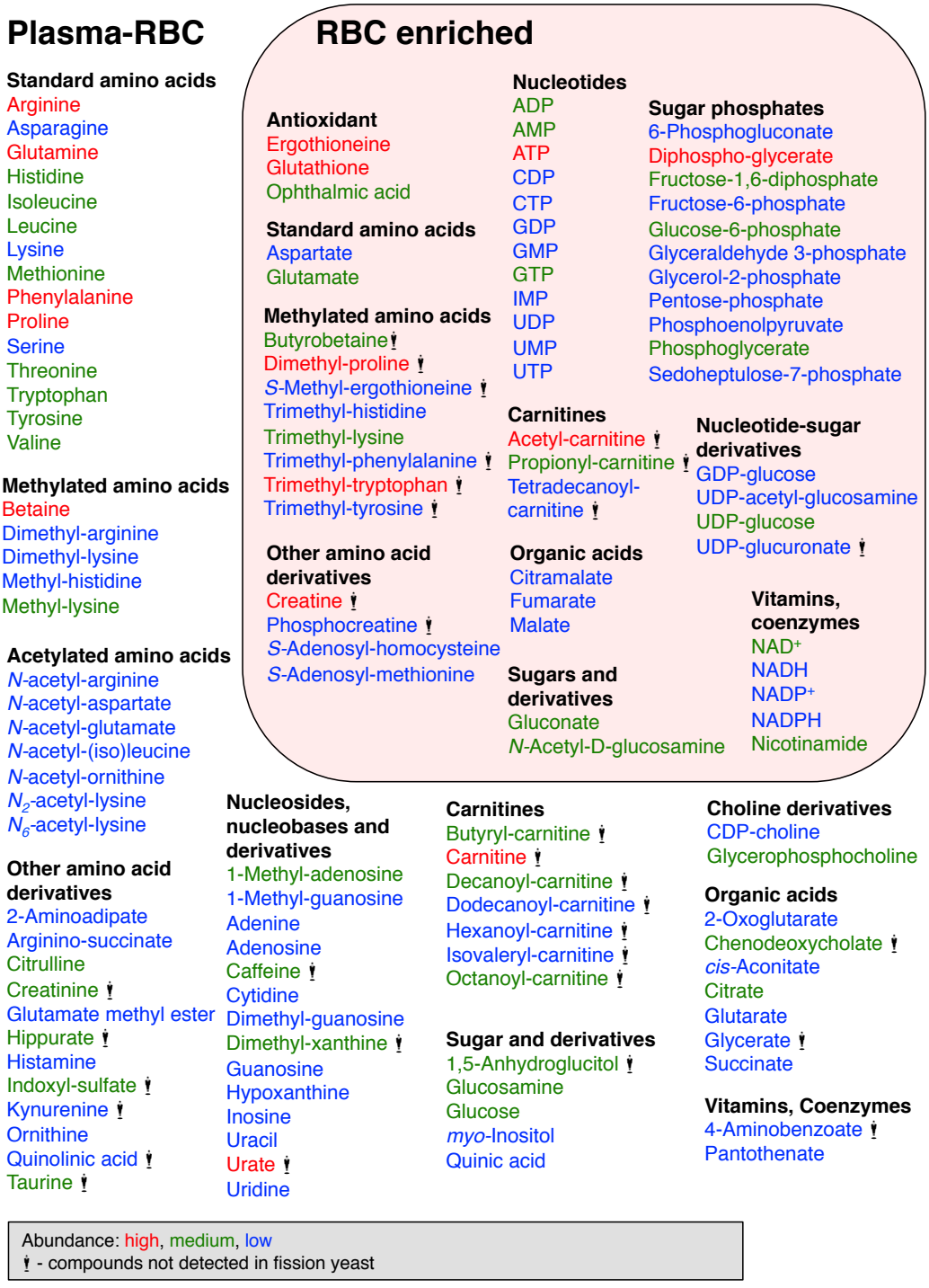


Figure 18. 133 compounds detected in human blood metabolome samples

Compounds that were either RBC-enriched (57) or not (76), based on whether the ratios of their RBC:plasma peak areas, were either >2 or <2, respectively (Table 6 p.22). Abundance of compounds classified by peak area size, indicated by color, red (high), green (medium) and blue (low). Compounds with the statue symbol are not present in *S. pombe*. See text for detail.

2.2.10 Energy and anti-oxidant metabolites abundant in human blood and fission yeast

Some compounds (ATP, glutathione and glutamine) were highly abundant in both RBCs and *S. pombe* (Figure 19 p.39). Most compounds in the High and Medium categories are implicated in energy metabolism, anti-oxidation, or amino acid metabolism. In the WBC metabolome (Table 8 p.40), ATP, glutathione, and glycerophosphocholine (instead of glutamine) were abundant. Some medium-level compounds (e.g. NAD^+ and UDP-glucose) are required for production of high-energy compounds such as ATP, GTP, and UTP. Ergothioneine and ophthalmic acid are thought to be anti-oxidants. It thus appears that energy metabolites, anti-oxidants, and amino acid metabolites may be the most highly conserved in eukaryotes.

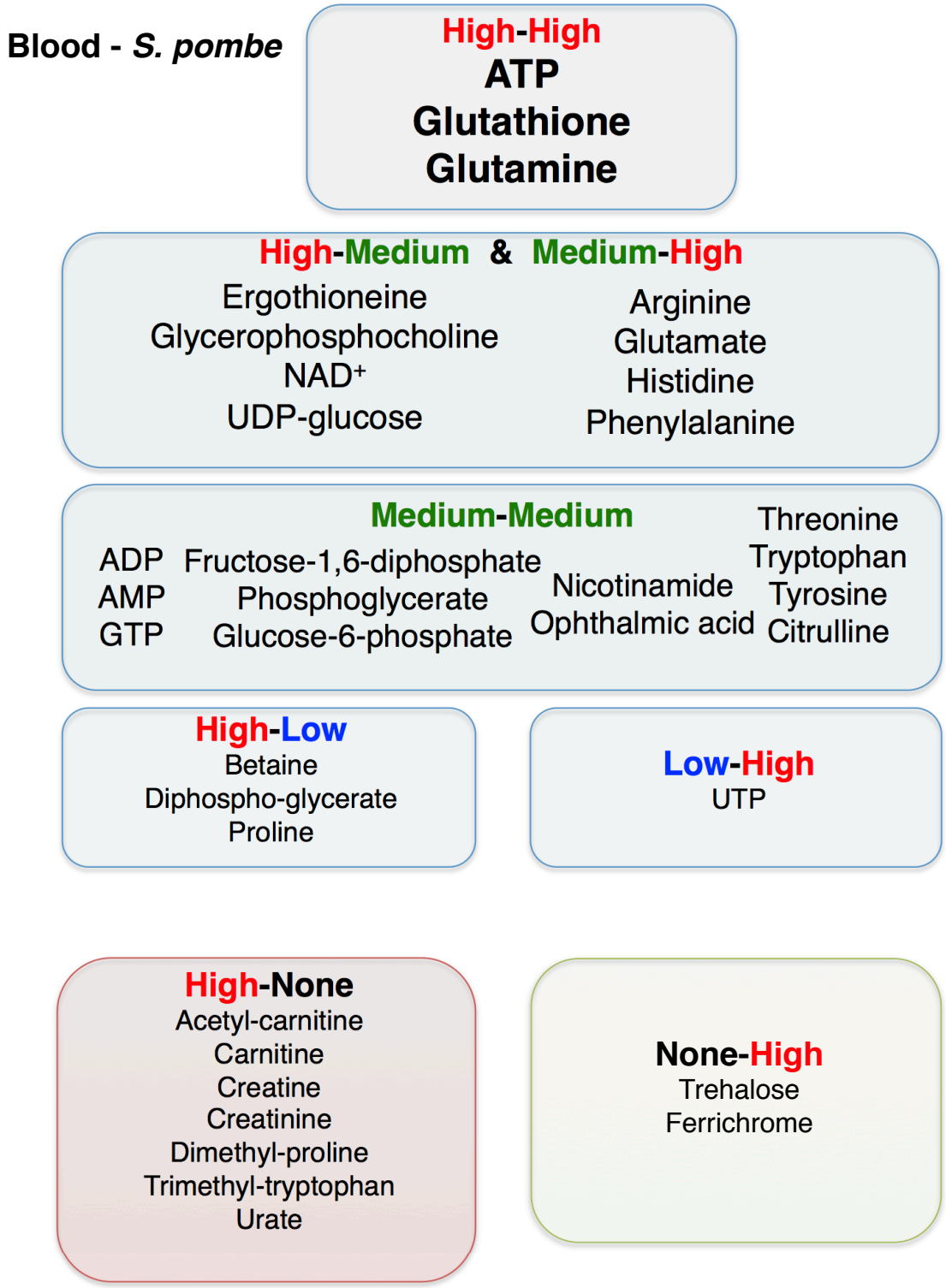


Figure 19. Highly abundant compounds in blood and *S. pombe*
Compound abundance in human blood and *S. pombe*. For example, "High-High" indicates that ATP, glutathione, and glutamine are highly abundant in both blood and *S. pombe*. See text

Table 8. All compounds detected in blood and fission yeast, and their properties

Category	Name	Status	m/z	Ionisation	RT	In fission yeast	In blood	RBC enriched	New blood component	Blood	RBC	WBC	Fission yeast
Nucleotides	*3′,5′*-cAMP	STD	328.045	NEG	7.1	O				ND	ND	L	L
	ADP	STD	428.037	POS	12.4	O	O	R		M	M	H	M
	AMP	STD	348.070	POS	11.5	O	O	R		M	M	H	M
	ATP	STD	508.003	POS	13.8	O	O	R		H	H	H	H
	CDP	STD	402.011	NEG	14.7	O	O	R		L	L	L	L
	CMP	STD	322.045	NEG	13.2	O				ND	ND	L	L
	CTP	STD	481.977	NEG	15.5	O	O	R		L	L	M	M
	GDP	STD	442.017	NEG	15.5	O	O	R		L	L	M	M
	GMP	STD	362.051	NEG	14.1	O	O	R		L	L	M	L
	GTP	STD	521.983	NEG	16.4	O	O	R		M	M	H	M
	IMP	STD	349.054	POS	13.1	O	O	R		L	L	M	L
	UDP	STD	402.995	NEG	14.0	O	O	R		L	L	M	M
	UMP	STD	323.029	NEG	12.6	O	O	R		L	L	M	L
	UTP	STD	482.961	NEG	15.1	O	O	R		L	L	M	H
Nucleosides, nucleobases and derivatives	1-Methyl-adenosine	STD	282.120	POS	11.1	O	O			M	L	L	M
	1-Methyl-guanosine	STD	298.115	POS	7.1	O	O			L	L	L	L
	Adenine	STD	136.062	POS	6.6	O	O			L	L	L	M
	Adenosine	STD	268.104	POS	6.2	O	O			L	L	M	L
	AICAR	STD	337.055	NEG	12.7	O				ND	ND	ND	L
	Caffeine	STD	195.088	POS	3.8		O			M	M	L	ND
	Cytidine	STD	244.093	POS	9.1	O	O			L	L	L	L
	Dimethyl-guanosine	STD	312.130	POS	6.0	O	O			L	L	L	L
	Dimethyl-xanthine	STD	181.072	POS	4.5		O			M	M	L	ND
	Guanosine	STD	282.084	NEG	9.8	O	O			L	L	L	L
	Hypoxanthine	STD	135.031	NEG	7.6	O	O			L	L	M	L
	Inosine	STD	269.088	POS	8.2	O	O			L	L	L	L
	PRPP (5-Phospho-alpha-D-ribose 1-dip	STD	388.945	NEG	16.5	O				ND	ND	L	M
	SAICAR	MS/MS	455.081	POS	17.1	O				ND	ND	ND	L
	Uracil	STD	111.020	NEG	7.2	O	O			L	L	L	L
	Urate	STD	167.021	NEG	10.7		O			M	M	L	ND
	Uridine	STD	243.062	NEG	7.1	O	O			L	L	L	L
Vitamins, coenzymes	4-Aminobenzoate	STD	138.055	POS	7.2		O			L	L	ND	ND
	Acetyl-CoA	STD	810.133	POS	10.5	O				ND	ND	L	L
	Biotin	STD	245.095	POS	6.2	O				ND	ND	ND	L
	CoA	STD	768.122	POS	11.7	O				ND	ND	L	L
	FAD	STD	786.164	POS	9.4	O				ND	ND	L	L
	HMG-CoA	STD	912.165	POS	13.2	O				ND	ND	L	L
	NAD^+	STD	664.116	POS	11.9	O	O	R		M	M	M	H
	NADH	STD	666.132	POS	11.1	O	O	R		L	L	L	M
	$NADP^+$	STD	744.083	POS	14.4	O	O	R		L	L	L	L
	NADPH	STD	746.098	POS	14.8	O	O	R		L	L	L	L
	Nicotinamide	STD	123.055	POS	4.7	O	O	R		M	M	M	M
	Pantothenate	STD	220.118	POS	6.1	O	O			L	L	L	M
Nucleotide-sugar derivatvies	GDP-glucose	STD	606.084	POS	15.5	O	O	R	O	L	L	L	M
	UDP-acetyl-glucosamine	STD	606.074	NEG	12.8	O	O	R	O	L	L	M	M
	UDP-glucose	STD	565.048	NEG	13.9	O	O	R		M	M	M	H
	UDP-glucuronate	STD	579.027	NEG	16.5		O	R	O	L	L	M	ND
Sugar phosphates	6-Phosphogluconate	STD	275.017	NEG	15.0	O	O	R		L	L	L	L
	Diphospho-glycerate	STD	264.952	NEG	15.6	O	O	R		H	H	L	L
	Fructose-1,6-diphosphate	STD	338.989	NEG	15.5	O	O	R		M	M	M	M
	Fructose-6-phosphate	STD	259.022	NEG	13.3	O	O	R		L	L	L	M
	Glucose-6-phosphate	STD	259.022	NEG	14.2	O	O	R		M	M	M	M
	Glyceraldehyde-3-phosphate	STD	168.991	NEG	13.2	O	O	R		L	L	L	L
	Glycerol-2-phosphate	STD	171.006	NEG	12.2	O	O	R		L	L	L	M
	Pentose-phosphate	STD	229.012	NEG	13.0	O	O	R		L	L	L	M
	Phosphoenolpyruvate	STD	166.975	NEG	15.1	O	O	R		L	L	L	M
	Phosphoglycerate	STD	184.986	NEG	14.4	O	O	R		M	M	L	M
	Sedoheptulose-7-phosphate	STD	289.033	NEG	13.7	O	O	R		L	L	L	M
	Trehalose-6-phosphate	STD	421.075	NEG	14.7	O				ND	ND	ND	M
Sugar and derivatives	1,5-Anhydroglucitol	STD	163.061	NEG	9.2		O			M	M	L	ND
	Gluconate	STD	195.051	NEG	11.4	O	O	R		M	M	L	L
	Glucosamine	STD	180.087	POS	11.6	O	O			M	M	ND	L
	Glucose	STD	179.056	NEG	12.0	O	O			M	M	ND	L
	myo-Inositol	STD	179.056	NEG	14.4	O	O			L	L	M	L
	N-Acetyl-D-glucosamine	STD	222.097	POS	9.3	O	O	R		M	M	L	L
	Quinic acid	STD	191.056	NEG	10.4	O	O			L	L	L	M
	Trehalose	STD	341.109	NEG	13.6	O				ND	ND	ND	H
Organic acids	2-Oxoglutarate	STD	145.014	NEG	12.9	O	O			L	L	L	L
	Chenodeoxycholate	STD	391.285	NEG	3.9		O			M	M	L	ND
	cis-Aconitate	STD	175.024	POS	15.5	O	O			L	L	L	L
	Citramalate	STD	147.030	NEG	12.6	O	O	R	O	L	L	M	M
	Citrate	STD	191.020	NEG	15.5	O	O			M	L	M	M
	Fumarate	STD	115.004	NEG	13.1	O	O	R		L	L	L	L
	Glutarate	STD	131.035	NEG	12.0	O	O			L	L	L	L
	Glyceric acid	STD	105.019	NEG	10.1		O			L	L	L	ND
	Malate	STD	133.014	NEG	13.3	O	O	R		L	L	L	L
	Succinate	STD	117.019	NEG	12.6	O	O			L	L	L	L

Table 8. (continued)

Category	Name	Status	m/z	Ionisation	RT	In fission yeast	In blood	RBC enriched	New blood component	Blood	RBC	WBC	Fission yeast
Standard amino acids	Arginine	STD	175.119	POS	24.8	O	O			H	M	M	H
	Asparagine	STD	131.046	NEG	12.4	O	O			L	L	L	L
	Aspartate	STD	132.030	NEG	12.5	O	O	R		L	L	M	M
	Glutamate	STD	146.046	NEG	12.1	O	O	R		M	M	M	H
	Glutamine	STD	147.076	POS	12.2	O	O			H	H	M	H
	Histidine	STD	156.077	POS	12.0	O	O			M	M	L	H
	Isoleucine	STD	130.087	NEG	8.6	O	O			M	M	L	L
	Leucine	STD	130.087	NEG	7.6	O	O			M	M	L	L
	Lysine	STD	145.098	NEG	23.3	O	O			L	L	L	L
	Methionine	STD	150.058	POS	8.7	O	O			M	M	M	L
	Phenylalanine	STD	166.086	POS	7.3	O	O			H	H	M	M
	Proline	STD	116.071	POS	10.2	O	O			H	H	H	L
	Serine	STD	104.035	NEG	13.1	O	O			L	L	L	L
	Threonine	STD	120.066	POS	11.7	O	O			M	M	M	M
	Tryptophan	STD	205.097	POS	8.7	O	O			M	M	M	M
	Tyrosine	STD	182.081	POS	10.3	O	O			M	M	L	M
	Valine	STD	118.086	POS	9.8	O	O			M	M	M	L
Methylated amino acids	Betaine	STD	118.086	POS	8.6	O	O			H	H	H	L
	Butyro-betaine	STD	146.118	POS	10.9		O	R		M	M	M	ND
	Dimethyl-arginine	STD	203.150	POS	20.0	O	O			L	L	L	L
	Dimethyl-histidine	STD	184.108	POS	9.7	O				ND	ND	ND	M
	Dimethyl-lysine	STD	175.144	POS	19.2	O	O		O	L	L	L	L
	Dimethyl-proline (stachydrine)	STD	144.102	POS	7.9		O	R		H	H	M	ND
	Methyl-histidine	STD	170.092	POS	10.2	O	O			L	L	L	M
	Methyl-lysine	MS/MS	161.128	POS	22.1	O	O			M	L	L	L
	S-Methyl-ergothioneine	STD	244.111	POS	7.9		O	R		L	M	L	ND
	Trimethyl-histidine (hercynine)	MS/MS	198.124	POS	9.7	O	O	R	O	L	L	L	M
	Trimethyl-lysine	STD	189.160	POS	20.7	O	O	R		M	M	L	L
	Trimethyl-phenylalanine	MS/MS	208.133	POS	5.4		O	R	O	L	L	ND	ND
	Trimethyl-tryptophan (hypaphorine)	STD	247.144	POS	6.0		O	R	O	H	H	M	ND
	Trimethyl-tyrosine	MS/MS	224.128	POS	7.5		O	R	O	L	L	L	ND
Acetylated amino acids	*N*-Acetyl-(iso)leucine	STD	174.112	POS	4.4	O	O		O	L	L	L	L
	N-Acetyl-arginine	STD	217.130	POS	12.3	O	O			L	L	L	M
	N-Acetyl-aspartate	STD	174.041	NEG	12.5	O	O			L	L	L	L
	N-Acetyl-glutamate	STD	190.071	POS	11.6	O	O		O	L	L	L	M
	N-Acetyl-ornithine	STD	175.108	POS	11.1	O	O			L	L	L	L
	N_{ε}-Acetyl-lysine	STD	189.123	POS	12.7	O	O		O	L	L	ND	L
	N_{6}-Acetyl-lysine	STD	189.123	POS	10.5	O	O		O	L	L	L	M
Other amino acid derivatives	2-Aminoadipate	STD	162.076	POS	12.2	O	O			L	L	L	L
	Arginino-succinate	STD	291.130	POS	14.2	O	O			L	L	L	M
	Citrulline	STD	176.103	POS	13.0	O	O			M	M	M	M
	Creatine	STD	132.077	POS	12.1		O	R		H	H	H	ND
	Creatinine	STD	114.066	POS	6.9		O			H	H	M	ND
	Deferrichrome	STD	688.326	POS	5.9	O				ND	ND	ND	L
	Ferrichrome	STD	741.238	POS	4.7	O				ND	ND	ND	H
	Glutamate methyl ester	STD	162.076	POS	7.1	O	O		O	L	L	L	L
	Hippurate	STD	180.066	POS	4.4		O			M	L	L	ND
	Histamine	STD	112.087	POS	24.8	O	O			L	L	M	L
	Histidinol	STD	142.097	POS	16.3	O				ND	ND	ND	M
	Indoxyl-sulfate	STD	212.002	NEG	4.9		O			M	M	L	ND
	Kynurenine	STD	209.092	POS	8.0		O			L	L	L	ND
	Ornithine	STD	131.083	NEG	20.8	O	O			L	L	L	M
	Phosphocreatine	STD	212.043	POS	12.7		O	R		L	L	L	ND
	Quinolinic acid	STD	166.015	NEG	12.7		O			L	L	L	ND
	S-adenosyl-cysteine	STD	371.113	POS	10.6	O				ND	ND	ND	L
	S-Adenosyl-homocysteine	STD	385.129	POS	11.2	O	O	R		L	L	L	M
	S-Adenosyl-methionine	STD	399.145	POS	14.2	O	O	R		L	L	L	L
	Saccharopine	STD	275.125	NEG	13.3	O				ND	ND	ND	L
	Taurine	STD	124.007	NEG	12.1		O			M	M	H	ND
Carnitines	Acetyl-carnitine	STD	204.123	POS	8.5		O	R		H	H	H	ND
	Butyryl-carnitine	STD	232.154	POS	6.2		O			M	M	M	ND
	Carnitine	STD	162.112	POS	10.9		O			H	H	H	ND
	Decanoyl-carnitine	STD	316.248	POS	4.2		O			M	M	L	ND
	Dodecanoyl-carnitine	STD	344.280	POS	3.8		O			L	L	L	ND
	Hexanoyl-carnitine	STD	260.186	POS	4.8		O			L	L	L	ND
	Isovaleryl-carnitine	STD	246.170	POS	5.4		O			L	L	L	ND
	Octanoyl-carnitine	STD	288.217	POS	4.1		O			M	L	L	ND
	Propionyl-carnitine	STD	218.139	POS	7.2		O	R		M	M	M	ND
	Tetradecanoyl-carnitine	STD	372.311	POS	3.8		O	R		L	L	L	ND
Choline derivatives	CDP-choline	STD	489.115	POS	13.0	O	O			L	L	H	L
	Glycerophosphocholine	STD	258.110	POS	12.1	O	O			M	M	H	H
Antioxidant	Ergothioneine	STD	230.096	POS	12.1	O	O	R		H	H	M	M
	Glutathione disulfide (GSSG)	STD	613.159	POS	15.0	O	O	R		H	H	H	H
	Ophthalmic acid	STD	290.135	POS	11.2	O	O	R		L	M	L	M

[a] From total 151 compounds 133 were detected in blood and 119 were detected in fission yeast.

DISCUSSION AND PERSPECTIVES

3.1 Comparison of human blood and fission yeast metabolomes

During my graduate studies at Kyoto University I performed LC-MS-based metabolomic analysis of human blood, plasma, and RBCs in comparison with previously published metabolomic results from the fission yeast, *S. pombe* (Pluskal, Nakamura, et al. 2010; Pluskal et al. 2011). Analysis of the *S. pombe* metabolome was performed simultaneously, and results were consistent with those of previous reports. This comparative study enabled me to learn which metabolomic features are conserved between these distantly related cellular systems. The LC column employed in this study can separate hydrophilic compounds with high resolution, but is not appropriate for separating hydrophobic compounds; therefore, my data contain limited information on lipids, except for two choline derivatives, CDP-choline and glycerophosphocholine. Also the detection range of the mass spectrometer was set to the range between 100 and 1000 *m/z*, thus compounds below 100 Da (e.g. glycine, alanine) were not detected. For this reason, conclusions regarding metabolite conservation should be restricted to hydrophilic compounds between 100 and 1000 Da.

Thirty-two compounds were found in blood, but not in *S. pombe* (Figure 11 p.21). Metabolite compositions of blood and fission yeast are unexpectedly similar, with 76% of identified compounds present in both. The WBC metabolome is also highly similar to those of RBCs and yeast. However, metabolites mostly belonging to three categories (ten carnitines, six methylated amino acids, eight other amino acid derivatives) were not detected in *S. pombe*

(Table 6 p.22). If I compare metabolites in the remaining 11 categories, only 8 of 89 compounds were not present in *S. pombe* (caffeine, dimethyl-xanthine, urate, 4-aminobenzoate and UDP-glucuronate, 1,5-anhydroglucitol, chenodeoxycholic acid, and glyceric acid), therefore the overall similarity between the two metabolomes is over 90% (81/89×100). Differences are mostly restricted to carnitines and amino acid derivatives.

Overlap of the compounds detected in this study with that of the other RBC studies is rather large. I could detect all metabolites reported in two recent metabolomics studies on RBC storage (Nishino et al. 2009; D'Alessandro et al. 2013) as well as 69 of 91 metabolites reported in a study on sickle RBCs (Darghouth, Koehl, Madalinski, et al. 2011). Darghouth et al. used a C18 column enabling them to detect more hydrophobic compounds, while our method by using hydrophilic interaction chromatography enabled us to separate the polar compounds.

Methyl-histidine is detected in plasma, RBCs, and fission yeast. Human and fission yeast peaks are slightly offset by the retention time. In humans methyl-histidine comes most likely from diet, anserine or balenine (methylated on imidazole ring, 1- or 3-methyl-histidine). 3-Methyl-histidine has been reported at 8 µM in plasma (Dohm et al. 1982). In fission yeast samples it could be the *N*-Methyl-histidine, which is involved in the ergothioneine biosynthesis pathway. In fission yeast methyl-histidine increases with lower glucose concentrations together with other ergothioneine biosynthesis pathway metabolites (Pluskal et al. 2011).

Many of the compounds detected in fission yeast, but not in blood in this study are known to be present at low concentrations in blood or in other tissues. For example, CoA has been reported to be involved in RBC membrane metabolism and at low concentrations (~1 μM) (Arduini et al. 1992). CTP, a compound involved in various metabolic pathways such as phosphatidylcholine or DNA synthesis, is absent in non-dividing RBC cells as reported (Traut 1994).

Several metabolites are specific to fission yeast. Fission yeast contains metabolic pathways absent in the human genome. For example, histidinol is an intermediate in the histidine pathway and it was detected in fission yeast samples, but not in human samples. Also fission yeast is capable of synthesizing ergothioneine (Pluskal et al. 2014), synthesis of which goes through the intermediate of dimethyl-histidine, detected in fission yeast samples only.

Trehalose is a non-reducing disaccharide formed from two glucose units joined by a 1-1 alpha bond and can be synthesized by fission yeast. In fission yeast the levels of trehalose increase in low glucose conditions (Pluskal et al. 2011). While humans do intake disaccharides with food, disaccharides are cleaved in the small intestine by disaccharidases to monosacharides. Concentrations in blood of disaccharides are less than a few μg/mL in adult blood. Following a sucrose tolerance test blood glucose markedly increases, while blood sucrose levels are not substantially increased (Nakamura & Tamura 1972).

Among the compounds not detected in the fission yeast are carnitine and its derivatives. Carnitine is a trimethylbetaine of γ -amino-β- hydroxybutyric

acid with various functions in humans. One of the functions is the transport of the fatty acids from cytosol compartment into mitochondria. It also modulates the acetyl coenzyme A(CoA)/CoA ratio by acting as a reservoir for activated acetyl units (Rebouche & Seim 1998). It has been shown that acyl-carnitines can be synthetized inside the RBC and used for the membrane phospholipids turnover (Arduini et al. 1992). Budding yeast can not synthesize carnitine, but are capable of utilizing it for acetyl group transfer into mitochondria (Swiegers et al. 2001). In fission yeast, after addition of carnitine I could not detect any carnitine acyl derivatives consistent with the absence of the carnitine utilizing genes (Sohn et al 2012).

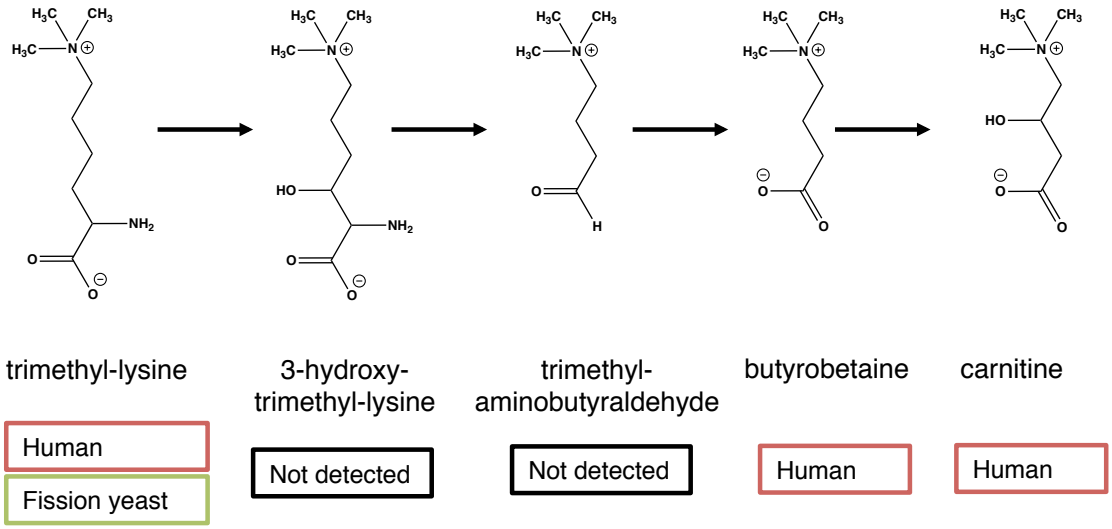


Figure 20. Carnitine biosynthesis pathway
Adapted from (Strijbis et al. 2010). Boxes indicate in my data where metabolites were detected.

Many bacteria and mammalian species can synthetize carnitine (Hulse et al. 1978; Strijbis et al. 2009). The carnitine synthesis pathway can be found in Figure 20 p.45. The precursor for carnitine synthesis is trimethyl-lysine and humans are known to be capable of synthesizing it or obtaining it from foods (Rebouche & Seim 1998). While in human blood samples I could find in

addition to the trimethyl-lysine, butyrobetaine and carnitine itself, in fission yeast I could detect only trimethyl-lysine (Figure 20 p.45).

In addition to carnitines, another group of metabolites not detected in fission yeast and detected only in human samples are creatine related compounds. Both carnitines and creatine related compounds are related to energy production. While high-energy demands in human tissues such as the brain and muscle require the presence of such specialized “energy” compounds, fission yeast apparently does not require them.

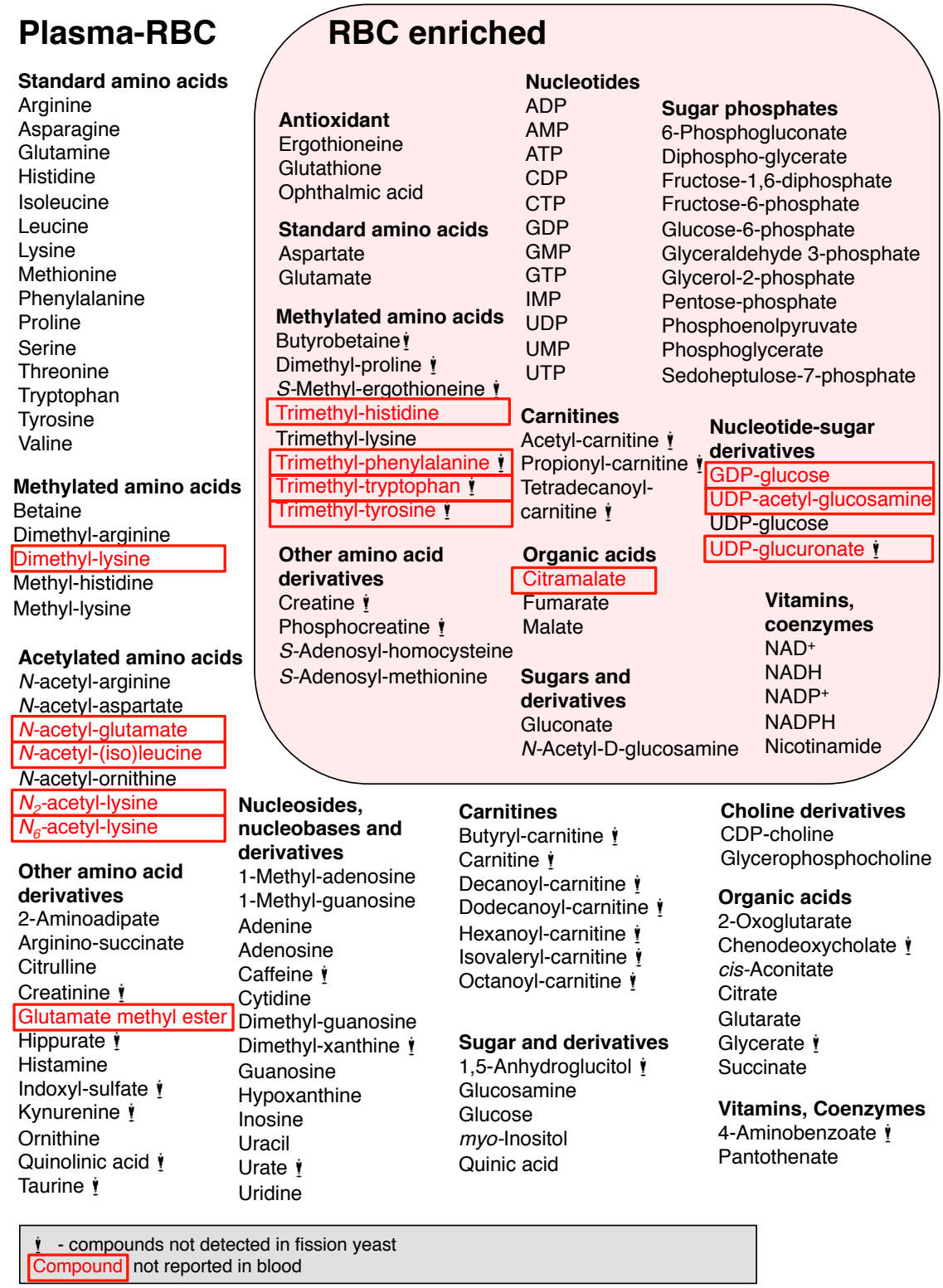


Figure 21. Fourteen compounds not yet reported in blood

I found 14 novel human blood compounds (Figure 21 p.47), ten of which were methylated or *N*-acetylated amino acids. To my knowledge, there has been no report describing these as blood components. Blood data presented in this report came from four healthy volunteers. We further extended our analysis to more than 30 people (Chaleckis et al., manuscript in preparation), and these compounds were universally present, suggesting that their occurrence is neither accidental nor specific to certain dietary customs. Interestingly, ten of these compounds (GDP-glucose, UDP-acetyl-glucosamine, citramalate, dimethyl-lysine, trimethyl-histidine, *N*-acetyl-(iso)leucine, *N*-acetyl-glutamate, N_2-acetyl-lysine, N_6-aceltyl-glutamate and glutamate methyl ester) were also present in *S. pombe*. Their physiological roles can thus be further investigated using *S. pombe* as a model. Eight compounds are RBC-enriched. RBCs may require UDP-acetyl-glucosamine, a nucleotide sugar and a coenzyme, as a signaling molecule of sugar metabolism, as in other eukaryotic cells, including *S. pombe* (Wellen & Thompson 2012). UDP-glucuronic acid is synthesized in liver, binds to hormones or toxic compounds, and is also used for the synthesis of glucuronic acid-containing polysaccharides (Mulloy & Forster 2000; Tukey & Strassburg 2000). It is not present in lower eukaryotes. Citramalate is an intermediate in bacterial glutamate degradation (Barker 1981). Citramalate and glutamate methyl ester are also present in *S. pombe*, but their physiological role is not understood. Two acetylated amino acids N_2-acetyl and N_6-acetyl-lysine, are present in plasma and RBCs, as well as in *S. pombe*, but their biological role is little understood. *N*-acetyl-glutamate is involved in the removal of waste from the body in the urine as it is an allosteric cofactor of

carbamyl phosphate synthetase I, the first enzyme in the urea cycle (Caldovic & Tuchman 2003).

Five novel blood compounds are methylated amino acids, four of which are trimethylated and enriched in RBCs (histidine, phenylalanine, tryptophan, tyrosine). While the presence in blood of some methylated amino acids such as trimethyl-lysine (Mizobuchi et al. 1990), dimethyl-proline (Lever et al. 1994) and *S*-methyl-ergothioneine (Klein et al. 2011) has been previously documented, their role is not well understood.

Trimethyl-histidine (hercynine) is a precursor of ergothioneine, but humans do not synthesize ergothioneine; therefore this compound might be of dietary origin or possibly a degradation product of ergothioneine (Cheah & Halliwell 2012). Trimethyl-histidine has been reported to be present in *Boletus* mushrooms (Barger & Ewins 1913). RBCs, as well as other tissues, have been reported to have ergothioneine transporter (Gründemann et al. 2005; Gründemann 2012). As revealed through genetic and metabolomic studies *S. pombe* can produce ergothioneine and as an intermediate trimethyl-histidine (Pluskal et al. 2014). Further ergothioneine metabolite, *S*-methyl-ergothioneine, was detected in human as well as in fission yeast samples in klf1 mutant (Shimanuki et al. 2013). *S*-methyl-ergothioneine was also shown to be present in deep sea sponge (Gross et al. 2004).

Ergothioneine was discovered more than 100 years ago (Tanret 1909) and has been extensively researched ever since (Melville 1959; Cheah & Halliwell 2012). First it was thought that ergothioneine is incorporated into RBC only during erythrogenesis (Melville 1959), but later it was shown that mature

erythrocytes also incorporate ergothioneine (Mitsuyama & May 1999). Ergothioneine is among the highly abundant compounds accumulated in RBCs, as detected by our method, but differences among individuals in the levels ranged more than 10 fold. Ergothioneine concentrations in the RBC have been reported to be 10-960 mg/l (44-4200 μM) (Cheah & Halliwell 2012).

In low glucose conditions, levels of ergothioneine and other methylated compounds increase in fission yeast (Pluskal et al. 2011). Over 100 years of research on ergothioneine has not provided a clear answer on the role of this compound. Ergothioneine and its metabolites are present in such distantly related organisms as human and fission yeast. Also the fact that in fission yeast ergothioneine levels under stress conditions increase, suggest that this compound might also play a similar role in humans.

Trimethyl-tryptophan (hypaphorine) was variable, and highly abundant in some RBC samples. Trimethyl-tryptophan was originally discovered in the seeds of the *Erythryna Hypaphorus* (van Romburgh & Barger 1911). Structurally similar to indole acetic acid, it has been detected in fungi and shown to inhibit root hair elongation (Ditengou et al. 2000). Trimethyl-tryptophan has been reported to have sleep-inducing and sugar-lowering effects in mice (Ozawa et al. 2008; Chand et al. 2010). In humans, it was reported to be present in milk and associated with legume consumption (Keller et al. 2013), but not previously detected in blood. I could confirm the trimethyl-tryptophan by obtaining a standard compound. For trimethyl-tyrosine, trimethyl-phenylalanine the standard compounds were not available, but I could confirm them by MS/MS analysis. I could not find any biological reports on *N*-

trimethylated tyrosine and phenylalanine. Compounds enriched in the RBC could have a yet undiscovered influence on the RBC metabolism. Metabolic changes in RBCs, as an oxygen supplier, could affect the whole organism.

The high similarity of fission yeast and human blood metabolomes raises the possibility that *S. pombe* genetics might be useful to understand the role of certain metabolites, such as small anti-oxidants (ophthalmic acid, ergothioneine, and glutathione), which are enzymatically synthesized in *S. pombe*. *S*-adenosyl-homocysteine (SAH) and *S*-adenosyl-methionine (SAM), coenzymes involved in the methionine cycle, were also enriched in RBCs. *S*-adenosyl-methionine synthase has been reported in RBCs (Oden & Clarke 1983). In RBCs, SAM has been shown to act as a coenzyme for protein carboxyl methylation (Perna et al. 1993), synthesis of phosphatidylcholine (Hirata & Axelrod 1978), and *N*-, *O*- and *S*-methyltransferase activities (Weinshilboum et al. 1999). It remains to be determined how SAH and SAM in RBCs are involved in the production of methylated compounds. We are particularly interested in determining whether levels of free methylated amino acids are controlled by SAM and SAH. To this end, in the OIST G0 unit, a number of *S. pombe* mutants of methionine cycle enzymes were isolated and their metabolic profiles are currently being examined (T. Hayashi et al., unpublished results).

Gluconate, which is not included in the current RBC models, was detected at quite high levels enriched in the RBCs. It has been used in delivery of iron (Jaber et al. 2010), blood storage solutions (Burger et al. 2012), in vitro osmotic stress studies e.g. (Floride et al. 2008). Gluconate has been reported

as tentatively identified in a RBC metabolomics study (Darghouth, Koehl, Madalinski, et al. 2011) and has been reported in plasma at a concentration less than 5 μM (Psychogios et al. 2011). In RBC the source of glucuronate is not clear. It has been shown that the rate of spontaneous hydrolysis of 6-phosphogluconate increases sharply at high pH values (Bauer et al. 1983). As HILLIC column solvent is pH 9.3, the gluconate could be a result of the 6-phosphogluconate hydrolysis.

ATP and glutathione were selectively enriched in RBCs (scarcely present in plasma), while glutamine was found in both plasma and RBCs in roughly equal amounts. Eleven sugar phosphate compounds required for sugar and energy metabolism were all found in RBC-enriched fractions and also in *S. pombe*. Similarly, all twelve nucleotides, four nucleotide-sugar derivatives, and five coenzyme NAD-related compounds were selectively enriched in RBCs. Fifty-six percent of RBC-enriched compounds are energy-related; these compounds are also found in *S. pombe*. Three anti-oxidant compounds, glutathione, ergothioneine, and ophthalmic acid, were enriched in RBCs and abundant in *S. pombe*. Glutathione and ophthalmic acid may be synthesized in RBCs, as the synthetic enzymes encoded by the human genes are present in RBCs (Hirono et al. 1996). Aspartate and glutamate were selectively enriched in RBCs. Both are excitatory neurotransmitters. Inhibitory transmitters, GABA and glycine, are difficult to measure using our method. Glutamate may be partly utilized for the synthesis of glutathione (Whillier et al. 2011). These energy and anti-oxidant compounds are most likely essential for

maintaining RBCs during their relatively long lifespan of 120 days, and these compounds are also common to *S. pombe.*

Among the fifteen high abundance blood compounds, however, *S. pombe* lacks six (40%) metabolites (acetyl-carnitine, carnitine, creatine, dimethyl-proline, trimethyl-tryptophan and urate), indicating that the most abundant compounds in blood are quite different from those of *S. pombe*. Conversely, among the 14 high abundance *S. pombe* compounds, only 2 (trehalose, ferrichrome) were not present in human blood, indicating that 86% are also found in human blood. I presume that the high abundance metabolites of *S. pombe* may be more 'fundamental' than those of blood, since *S. pombe* is a single-celled eukaryote. Blood contains a multitude of specialized metabolites. Carnitine is not strictly a metazoan compound, as it is also synthesized from lysine in *Neurospora* (Rebouche & Broquist 1976). However, a great variety of carnitine derivatives (9) carrying different fatty acids might be a higher eukaryotic feature.

3.2 Conclusions

Analysis of the RBC metabolome on the LC-MS platform optimized for polar metabolites revealed that 75% of the 133 metabolites detected in blood were also detected in fission yeast, yet at different levels. From the 133 metabolites detected in blood, 57 were more abundant in the RBC than in plasma. Fourteen of these compounds were previously unreported such as trimethylated amino acids trimethyl-tryptophan, -phenylalanine, -tyrosine, histidine. Highly abundant metabolites conserved between RBC and *S. pombe* exist, which seem to be implicated in structural maintenance, energy production and protection against stresses.

MATERIALS AND METHODS

4.1 Chemicals and reagents

Over 40 pure standards were obtained (listed in Table 9 p.55) in addition to the pure standards obtained for previous studies (Pluskal, Nakamura, et al. 2010; Pluskal et al. 2011; Sajiki et al. 2013; Shimanuki et al. 2013).

Table 9. List of analyzed standard compounds

Compound	Vendor	Name	Catalog #
1,5-Anhydroglucitol	Wako	1,5-Anhydro-D-glucitol	016-13531
4-Aminobenzoate	Sigma-Aldrich	4-aminobenzoic acid	A9878
Acetyl-carnitine	Sigma-Aldrich	Acetyl-L-Carnitine Chloride	17-0200-7
Arginino-succinate	Sigma-Aldrich	Argininosuccinic acid disodium salt hydrate	A5707
Butyro-betaine	Sigma-Aldrich	(3-Carboxypropyl)trimethylammonium chloride	403245
Butyryl-carnitine	Wako	Butyryl-L-Carnitine Chloride	17-0400-7
Caffeine	Sigma-Aldrich	Caffeine	C0750
Carnitine	Sigma-Aldrich	L-Carnitine	C0283
CDP-choline	Sigma-Aldrich	Cytidine 5′-diphosphocholine sodium salt dihydrate	C0256
Chenodeoxycholate	Sigma-Aldrich	Chenodeoxycholic acid	C9377
cis-Aconitate	Sigma-Aldrich	cis-Aconitic acid	A3412
Creatine	Wako	Creatine	A17477
Creatinine	Wako	Creatinine	033-04591
Decanoyl-carnitine	Wako	Decanoyl-L-Carnitine Chloride	17-1000-7
Dimethyl-proline (stachydrine)	TCI	Stachydrine Hydrochloride	S0358
Dimethyl-xanthine	Sigma-Aldrich	1,7-Dimethylxanthine	D5385
Diphospho-glycerate	Sigma-Aldrich	2,3-Diphospho-D-glyceric acid pentasodium salt	D5764
Dodecanoyl-carnitine	Wako	Lauroyl-L-Carnitine Chloride	17-1200-7
Glyceraldehyde-3-phosphate	Sigma-Aldrich	DL-Glyceraldehyde 3-phosphate solution	G5251
Glycerate	Sigma-Aldrich	DL-Glyceric acid hemicalcium salt hydrate	G5000
Glycerophosphocholine	Santa Cruz Bio	sn-Glycero-3-phosphocholine	sc-301813
Hexanoyl-carnitine	Wako	Hexanoyl-L-Carnitine Chloride	17-0600-7
Hippurate	Sigma-Aldrich	Hippuric acid	112003
Histamine	Sigma-Aldrich	Histamine	H7125
Hypoxanthine	Sigma-Aldrich	Hypoxanthine	H9377
Indoxyl-sulfate	Sigma-Aldrich	Indoxyl sulfate potassium salt	I3875
Isovaleryl-carnitine	Wako	Isovaleryl-L-Carnitine Chloride	17-0550-7
Kynurenine	Sigma-Aldrich	L-kynurenine	K8625
Methyl-histidine	Sigma-Aldrich	1-Methyl-L-histidine	67520
myo-Inositol	Wako	myo- Inositol	092-00282
N-acetyl-aspartate	Fluka	N-Acetyl-L-aspartic acid	920
N-acetyl-D-glucosamine	Sigma-Aldrich	N-Acetyl-D-glucosamine	A8625
N-acetyl-isoleucine	Sigma-Aldrich	N-ACETYL-L-ISOLEUCINE	S783501
Octanoyl-carnitine	Wako	Octanoyl-L-Carnitine Chloride	17-0800-7
Phosphocreatine	Wako	Disodium Creatinephosphate Tetrahydrate	922-32-7
Propionyl-carnitine	Wako	Propionyl-L-Carnitine Chloride	17-0300-7
Quinolinic acid	Wako	2, 3-pyridinedicarboxylic acid (Quinolinic acid)	174-00271
S-Methyl-ergothioneine	Tetrahedron	S-Methyl-L-ergothioneine	THD-300
Sedoheptulose-7-phosphate	Sigma-Aldrich	D-Sedoheptulose 7-phosphate lithium salt	78832
Succinate	Sigma-Aldrich	Succinic acid	S3674
Taurine	Sigma-Aldrich	Taurine	T0625
Tetradecanoyl-carnitine	Wako	Myristoyl-L-Carnitine Chloride	17-1400-7
Trimethyl-tryptophan (hypaphorine)	Research Plus	Hypaphorine HCL	08-8016-17
UDP-glucuronate	Sigma-Aldrich	Uridine 5′-diphosphoglucuronic acid trisodium salt	U6751
Urate	Sigma-Aldrich	Uric acid	U0881
Uridine	Sigma-Aldrich	Uridine	U3750

[a] Previously reported standard compounds vendors can be found in Pluskal et al. 2010

4.2 Human subject characteristics

Three healthy male and one female volunteer (23-33 years old) provided blood samples for the fission yeast and blood metabolome comparison study. Blood samples for metabolomic analysis were taken in the morning and subjects were asked not to eat breakfast to ensure at least 12 h of fasting prior to sampling.

4.3 Ethics statement

Written informed consent was obtained from all donors in accordance with the Declaration of Helsinki. All experiments were performed in compliance with relevant Japanese laws and institutional guidelines. All protocols were approved by the Ethical Committee on Human Research of Kyoto University Hospital and by the Human Subjects Research Review Committee of the Okinawa Institute of Science and Technology Graduate University (OIST).

4.4 Blood sample preparation for metabolomic analysis

The workflow of the blood metabolome samples preparation is shown in Figure 8 p.17. Blood samples for metabolomic analysis were drawn by venipuncture into 5 mL heparinized tubes (Terumo). Immediately, 0.2 mL blood (8-12×10^8 RBC) were quenched in 1.8 mL –40° C 55% methanol. The remainder of each blood sample was centrifuged at 120 x *g* for 15 min at room temperature to separate plasma and RBCs. After centrifugation, 0.2 mL of separated plasma and RBCs (14-20×10^8 RBC), respectively, were quenched in 1.8 mL –40°C 55% methanol. Ten nmol each of HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and PIPES, piperazine-N,N′-bis(2-

ethanesulfonic acid) were added to each sample to serve as standards. After brief vortexing, samples were transferred to Amicon Ultra 10 kDa cut-off filters (Millipore, Billerica, MA, USA) to remove proteins and cell debris. Following sample concentration by vacuum evaporation, each sample was re-suspended in 40 µL of 50% acetonitrile and 1 µL (equivalent to 5 µL of initial sample) was used for each injection into the LC-MS system.

4.5 Isolation of lymphocytes and RBC by ficoll gradient

For isolation of leucocytes I employed Ficoll gradient centrifugation. Blood of two donors, 20 mL each, was collected into heparinized tubes (Terumo) and subsequently diluted with the same volume of PBS buffer. Diluted blood was carefully layered onto the same volume of Lympholite-H (Cosmo Bio, Tokyo, Japan) and centrifuged at room temperature for 20 min at 800 x *g*. Separated leucocytes were washed 3x in PBS (1 min, 350 x *g*, 4° C). After dilution, 0.2 mL of leucocytes (~5×10^7 cells) were quenched in 1.8 mL –40° C 55% methanol. After addition HEPES and PIPES, samples were processed as described above. Metabolites were isolated from 10 µL of leukocyte cell volume (assuming an individual leukocyte cell volume of 200 fL (Segel et al. 1981) and an estimated 5×10^7 cells per sample). One µL of the metabolome sample was injected into the LC-MS system, corresponding to ~0.25 µL leukocyte intracellular volume.

4.6 Fission yeast growth conditions and preparation for metabolomics analysis

The wild-type heterothallic haploid 972 h^- *S. pombe* strain (Gutz et al. 1974) was used for metabolomic experiments. Cells were cultivated at 26° C in minimal synthetic medium EMM2 (Mitchison 1970; Nurse 1975) with 0.1% glucose (5.6 mM) content. Limited glucose media were prepared by mixing regular EMM2 (2% glucose) medium with EMM2-G (0% glucose) in an appropriate ratio. Preparation of fission yeast samples was done as described previously (Pluskal, Nakamura, et al. 2010). Metabolites were isolated from 30 µL of total *S. pombe* cell volume (*S. pombe* cell diameter 3.5 µm (Mitchison 1957); cells grown at 5.6 mM glucose have lengths of 13 µm (Pluskal et al. 2011), thus using a formula (Mitchison 1957), mean cell volume is 120 fL; total number of cells per sample 2.5×10^8). LC-MS sample injections (1 µL) corresponded to ~0.75 µL *S. pombe* intracellular volume.

4.7 LC-MS analysis

LC-MS data were obtained using a Paradigm MS4 HPLC system (Michrom Bioresources, Auburn, CA, USA) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Pluskal, Nakamura, et al. 2010). Briefly, LC separation was performed on a ZIC-pHILIC column (Merck SeQuant, Umeå , Sweden; 150 mm × 2.1 mm, 5 µm particle size). Acetonitrile (A) and 10 mM ammonium carbonate buffer, pH 9.3 (B) were used as the mobile phase, with gradient elution from 80% A to 20% A in 30 min, at a flow rate of 100 µL mL^{-1}. Peak areas for metabolites of interest were integrated using MZmine 2 software

version 2.10 (Pluskal, Castillo, et al. 2010). Detailed data analytical procedures and parameters were described previously (Pluskal, Nakamura, et al. 2010). Metabolites were initially identified by searching their *m/z* values in on-line databases (see text) or by predicting chemical formulae from mass spectra (Pluskal et al. 2012). Identified peaks were verified by analyzing pure standards (STD category) to confirm their retention times, or in cases where pure standards were not available, by analyzing their fragmentation patterns (MS/MS category).

4.8 Supplemental Material

Raw LC-MS data in mzML format were submitted to the MetaboLights repository (URL: http://www.ebi.ac.uk/metabolights). The accession number for the fission yeast and human blood metabolome comparison is MTBLS87, while that for metabolomic samples of a single individual's blood donated 4 times within 24 h to determine RBC:plasma metabolite distribution is MTBLS88.

REFERENCES

A, J. et al., 2005. Extraction and GC/MS analysis of the human blood plasma metabolome. *Analytical Chemistry*, 77(24), pp.8086–94.

Alberts, B. et al., 2002. *Molecular Biology of the Cell, Fourth Edition*, Garland Science.

Allison, A.C., 1960. Turnovers of erythrocytes and plasma proteins in mammals. *Nature*, 188(4744), pp.37–40.

Ames, B.N. et al., 1981. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proceedings of the National Academy of Sciences of the United States of America*, 78(11), pp.6858–62.

Arduini, A. et al., 1992. Role of carnitine and carnitine palmitoyltransferase as integral components of the pathway for membrane phospholipid fatty acid turnover in intact human erythrocytes. *The Journal of Biological Chemistry*, 267(18), pp.12673–81.

Barger, G. & Ewins, A.J., 1913. The identity of trimethylhistidine (histidine-betaine) from various sources. *The Biochemical Journal*, 7(2), pp.204–6.

Barker, H.A., 1981. Amino acid degradation by anaerobic bacteria. *Annual Review of Biochemistry*, 50, pp.23–40.

Barone, J.J. & Roberts, H.R., 1996. Caffeine consumption. *Food and Chemical Toxicology*, 34(1), pp.119–29.

Bauer, H.P. et al., 1983. 6-phosphogluconolactonase. Purification, properties and activities in various tissues. *European Journal of Biochemistry*, 133(1), pp.163–8.

Benowitz, N.L., 1990. Clinical pharmacology of caffeine. *Annual Review of Medicine*, 41, pp.277–88.

Böhmer, T., Rydning, A. & Solberg, H.E., 1974. Carnitine levels in human serum in health and disease. *Clinica Chimica Acta*, 57(1), pp.55–61.

Brauer, M.J. et al., 2006. Conservation of the metabolomic response to starvation across two divergent microbes. *Proceedings of the National Academy of Sciences of the United States of America*, 103(51), pp.19302–7.

Bruce, S.J. et al., 2009. Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance

liquid chromatography/mass spectrometry. *Analytical Chemistry*, 81(9), pp.3285–96.

Burger, P. et al., 2012. Collection and storage of red blood cells with anticoagulant and additive solution with a physiologic pH. *Transfusion*, 52(6), pp.1245–52.

Caldovic, L. & Tuchman, M., 2003. N-acetylglutamate and its changing role through evolution. *The Biochemical Journal*, 372(Pt 2), pp.279–90.

Catalán, Ú. et al., 2013. Biomarkers of food intake and metabolite differences between plasma and red blood cell matrices; a human metabolomic profile approach. *Molecular BioSystems*, 9(6), pp.1411–22.

Chaleckis, R. et al., 2013. Metabolomic analysis of human blood, plasma and red blood cells in comparison with eukaryotic microbe. *[Rinshō ketsueki] The Japanese Journal of Clinical Hematology*, 54(10), pp.1603–14.

Chand, K. et al., 2010. Major alkaloidal constituent from Impatiens niamniamensis seeds as antihyperglycemic agent. *Medicinal Chemistry Research*, 20(9), pp.1505–1508.

Cheah, I.K. & Halliwell, B., 2012. Ergothioneine; antioxidant potential, physiological function and role in disease. *Biochimica et Biophysica Acta*, 1822(5), pp.784–93.

Cooper, M.B., Forte, C.A. & Jones, D.A., 1988. Carnitine and acetylcarnitine in red blood cells. *Biochimica et Biophysica Acta*, 959(2), pp.100–5.

Corton, J.M. et al., 1995. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *European Journal of Biochemistry*, 229(2), pp.558–65.

Costa, C.G. et al., 1997. Quantitative analysis of plasma acylcarnitines using gas chromatography chemical ionization mass fragmentography. *Journal of Lipid Research*, 38(1), pp.173–82.

D'Alessandro, A. et al., 2012. Time-course investigation of SAGM-stored leukocyte-filtered red bood cell concentrates: from metabolism to proteomics. *Haematologica*, 97(1), pp.107–15.

D'Alessandro, A., Gevi, F. & Zolla, L., 2011. A robust high resolution reversed-phase HPLC strategy to investigate various metabolic species in different biological models. *Molecular BioSystems*, 7(4), pp.1024–32.

D'Alessandro, A., Gevi, F. & Zolla, L., 2013. Red blood cell metabolism under prolonged anaerobic storage. *Molecular BioSystems*, 9(6), pp.1196–209.

Darghouth, D., Koehl, B., Heilier, J.F., et al., 2011. Alterations of red blood cell metabolome in overhydrated hereditary stomatocytosis. *Haematologica*, 96(12), pp.1861–5.

Darghouth, D. et al., 2010. Metabolomic analysis of normal and sickle cell erythrocytes. *Transfusion Clinique et Biologique*, 17(3), pp.148–50.

Darghouth, D., Koehl, B., Madalinski, G., et al., 2011. Pathophysiology of sickle cell disease is mirrored by the red blood cell metabolome. *Blood*, 117(6), pp.e57–66.

Ditengou, F. a, Béguiristain, T. & Lapeyrie, F., 2000. Root hair elongation is inhibited by hypaphorine, the indole alkaloid from the ectomycorrhizal fungus Pisolithus tinctorius, and restored by indole-3-acetic acid. *Planta*, 211(5), pp.722–8.

Dohm, G.L. et al., 1982. Increased excretion of urea and N tau-methylhistidine by rats and humans after a bout of exercise. *Journal of Applied Physiology*, 52(1), pp.27–33.

Dzik, S., 1993. Leukodepletion blood filters: filter design and mechanisms of leukocyte removal. *Transfusion Medicine Reviews*, 7(2), pp.65–77.

Elbein, A.D. et al., 2003. New insights on trehalose: a multifunctional molecule. *Glycobiology*, 13(4), p.17R–27R.

Evans, A.M. & Fornasini, G., 2003. Pharmacokinetics of L-carnitine. *Clinical Pharmacokinetics*, 42(11), pp.941–67.

Fernie, A.R. et al., 2004. Metabolite profiling: from diagnostics to systems biology. *Nature Reviews Molecular Cell Biology*, 5(9), pp.763–9.

Fiehn, O., 2002. Metabolomics - the link between genotypes and phenotypes. *Plant Molecular Biology*, 48(1-2), pp.155–71.

Floride, E. et al., 2008. Caffeine inhibits suicidal erythrocyte death. *Cellular Physiology and Biochemistry*, 22(1-4), pp.253–60.

Franco, R.S., 2009. The measurement and importance of red cell survival. *American Journal of Hematology*, 84(2), pp.109–14.

Goodacre, R. et al., 2004. Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends in Biotechnology*, 22(5), pp.245–52.

Gross, H., Reitner, J. & König, G.M., 2004. Isolation and structure elucidation of azoricasterol, a new sterol of the deepwater sponge Macandrewia azorica. *Die Naturwissenschaften*, 91(9), pp.441–6.

Gründemann, D. et al., 2005. Discovery of the ergothioneine transporter. *Proceedings of the National Academy of Sciences of the United States of America*, 102(14), pp.5256–61.

Gründemann, D., 2012. The ergothioneine transporter controls and indicates ergothioneine activity-a review. *Preventive Medicine*, 54 Suppl, pp.S71–4.

Guo, Y. & Gaiki, S., 2005. Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography. *Journal of Chromatography A*, 1074(1-2), pp.71–80.

Gutz, H. et al., 1974. Schizosaccharomyces pombe. In R. C. King, ed. *Handbook of Genetics*. New York: Plenum Press, pp. 395–446.

Hirai, M.Y. et al., 2004. Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America*, 101(27), pp.10205–10.

Hirata, F. & Axelrod, J., 1978. Enzymatic synthesis and rapid translocation of phosphatidylcholine by two methyltransferases in erythrocyte membranes. *Proceedings of the National Academy of Sciences of the United States of America*, 75(5), pp.2348–52.

Hirono, a et al., 1996. Three cases of hereditary nonspherocytic hemolytic anemia associated with red blood cell glutathione deficiency. *Blood*, 87(5), pp.2071–4.

Hulse, J.D., Ellis, S.R. & Henderson, L.M., 1978. Carnitine biosynthesis. beta-Hydroxylation of trimethyllysine by an alpha-ketoglutarate-dependent mitochondrial dioxygenase. *The Journal of Biological Chemistry*, 253(5), pp.1654–9.

Jaber, L. et al., 2010. Iron polymaltose versus ferrous gluconate in the prevention of iron deficiency anemia of infancy. *Journal of Pediatric Hematology/Oncology*, 32(8), pp.585–8.

Janse, C.J. et al., 1994. Removal of leucocytes from Plasmodium vivax infected blood. *Annals of Tropical Medicine and Parasitology*, 88(2), pp.213–216.

Joshi, A. & Palsson, B.O., 1990. Metabolic dynamics in the human red cell. Part IV—Data prediction and some model computations. *Journal of Theoretical Biology*, 142(1), pp.69–85.

Kanehisa, M. & Goto, S., 2000. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, 28(1), pp.27–30.

Kell, D.B., 2004. Metabolomics and systems biology: making sense of the soup. *Current Opinion in Microbiology*, 7(3), pp.296–307.

Keller, B.O. et al., 2013. Hypaphorine is present in human milk in association with consumption of legumes. *Journal of Agricultural and Food Chemistry*, 61(31), pp.7654–60.

Keller, K.E., Tan, I.S. & Lee, Y.-S., 2012. SAICAR stimulates pyruvate kinase isoform M2 and promotes cancer cell survival in glucose-limited conditions. *Science*, 338(6110), pp.1069–72.

Kimura, T. et al., 2009. Plasma amino acid analysis for diagnosis and amino acid-based metabolic networks. *Current Opinion in Clinical Nutrition and Metabolic Care*, 12(1), pp.49–53.

Kinoshita, A. et al., 2007. Simulation study of methemoglobin reduction in erythrocytes. Differential contributions of two pathways to tolerance to oxidative stress. *The FEBS Journal*, 274(6), pp.1449–58.

Klein, M. et al., 2011. Identification in human urine and blood of a novel selenium metabolite, Se-methylselenoneine, a potential biomarker of metabolization in mammals of the naturally occurring selenoneine, by HPLC coupled to electrospray hybrid linear ion trap-orbital MS. *Metallomics*, 3(5), pp.513–20.

Kumada, K., Yanagida, M. & Toda, T., 1996. Caffeine-resistance in fission yeast is caused by mutations in a single essential gene, crm1+. *Molecular and General Genetics : MGG*, 250(1), pp.59–68.

Lawton, K.A. et al., 2008. Analysis of the adult human plasma metabolome. *Pharmacogenomics*, 9(4), pp.383–397.

Lee, R. & Britz-McKibbin, P., 2009. Differential rates of glutathione oxidation for assessment of cellular redox status and antioxidant capacity by capillary electrophoresis-mass spectrometry: an elusive biomarker of oxidative stress. *Analytical Chemistry*, 81(16), pp.7047–56.

Lever, M. et al., 1994. Glycine betaine and proline betaine in human blood and urine. *Biochimica et Biophysica Acta*, 1200(3), pp.259–64.

Melville, D.B., 1959. Ergothioneine. In R. S. Harris, G. F. Marrian, & K. V. Thimann, eds. *Vitamins & Hormones Volume 17*. Academic Press, pp. 155–204.

Mitchison, J.M., 1970. Physiological and cytological methods for Schizosaccharomyces pombe. *Methods in Cell Biology*, 4, pp.131–165.

Mitchison, J.M., 1957. The growth of single cells. I. Schizosaccharomyces pombe. *Experimental Cell Research*, 13(2), pp.244–62.

Mitsuyama, H. & May, J.M., 1999. Uptake and antioxidant effects of ergothioneine in human erythrocytes. *Clinical Science*, 97(4), pp.407–11.

Mizobuchi, M. et al., 1990. High concentration of free trimethyllysine in red blood cells. *Biochimica et Biophysica Acta*, 1033(2), pp.119–23.

Mohandas, N. & Gallagher, P.G., 2008. Red cell membrane: past, present, and future. *Blood*, 112(10), pp.3939–48.

Mulloy, B. & Forster, M.J., 2000. Conformation and dynamics of heparin and heparan sulfate. *Glycobiology*, 10(11), pp.1147–56.

Murray, A.W., 1971. The biological significance of purine salvage. *Annual Review of Biochemistry*, 40, pp.811–26.

Nakamura, H. & Tamura, Z., 1972. Gas chromatographic analysis of mono- and disaccharides in human blood and urine after oral administration of disaccharides. *Clinica Chimica Acta*, 39(2), pp.367–381.

Nakamura, T. et al., 2012. Impaired coenzyme A synthesis in fission yeast causes defective mitosis, quiescence-exit failure, histone hypoacetylation and fragile DNA. *Open Biology*, 2(9), p.120117.

Nakayama, Y., Kinoshita, A. & Tomita, M., 2005. Dynamic simulation of red blood cell metabolism and its application to the analysis of a pathological condition. *Theoretical Biology and Medical Modelling*, 2, p.18.

Nicholson, J.K., Holmes, E., Kinross, J., et al., 2012. Host-gut microbiota metabolic interactions. *Science*, 336(6086), pp.1262–7.

Nicholson, J.K., Holmes, E., Kinross, J.M., et al., 2012. Metabolic phenotyping in clinical and surgical environments. *Nature*, 491(7424), pp.384–92.

Nicholson, J.K. & Lindon, J.C., 2008. Systems biology: Metabonomics. *Nature*, 455(7216), pp.1054–6.

Nicholson, J.K. & Wilson, I.D., 2003. Opinion: understanding “global” systems biology: metabonomics and the continuum of metabolism. *Nature Reviews Drug Discovery*, 2(8), pp.668–76.

Nishino, T. et al., 2009. In silico modeling and metabolome analysis of long-stored erythrocytes to improve blood storage methods. *Journal of Biotechnology*, 144(3), pp.212–23.

Nurse, P., 1975. Genetic control of cell size at cell division in yeast. *Nature*, 256(5518), pp.547–551.

Oden, K.L. & Clarke, S., 1983. S-adenosyl-L-methionine synthetase from human erythrocytes: role in the regulation of cellular S-adenosylmethionine levels. *Biochemistry*, 22(12), pp.2978–86.

Ohkura, H. et al., 1988. Cold-sensitive and caffeine-supersensitive mutants of the Schizosaccharomyces pombe dis genes implicated in sister chromatid separation during mitosis. *The EMBO Journal*, 7(5), pp.1465–73.

Olszewski, K.L. & Llinás, M., 2013. Extraction of hydrophilic metabolites from Plasmodium falciparum-infected erythrocytes for metabolomic analysis. In R. Ménard, ed. *Malaria*. Methods in Molecular Biology. pp. 259–66.

Ozawa, M. et al., 2008. Hypaphorine, an indole alkaloid from Erythrina velutina, induced sleep on normal mice. *Bioorganic & Medicinal Chemistry Letters*, 18(14), pp.3992–4.

Paglia, G. et al., 2012. Intracellular metabolite profiling of platelets: evaluation of extraction processes and chromatographic strategies. *Journal of Chromatography B*, 898, pp.111–20.

Pasini, E.M. et al., 2006. In-depth analysis of the membrane and cytosolic proteome of red blood cells. *Blood*, 108(3), pp.791–801.

Patti, G.J., Yanes, O. & Siuzdak, G., 2012. Innovation: Metabolomics: the apogee of the omics trilogy. *Nature Reviews Molecular Cell Biology*, 13(4), pp.263–9.

Pence, H.E. & Williams, A., 2010. ChemSpider: an online chemical information resource. *Journal of Chemical Education*, 87(11), pp.1123–1124.

Perna, A.F. et al., 1993. Enzymatic methyl esterification of erythrocyte membrane proteins is impaired in chronic renal failure. Evidence for high levels of the natural inhibitor S-adenosylhomocysteine. *The Journal of Clinical Investigation*, 91(6), pp.2497–503.

Pluskal, T., Nakamura, T., et al., 2010. Metabolic profiling of the fission yeast S. pombe: quantification of compounds under different temperatures and genetic perturbation. *Molecular BioSystems*, 6(1), pp.182–98.

Pluskal, T., Castillo, S., et al., 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics*, 11, p.395.

Pluskal, T. et al., 2011. Specific biomarkers for stochastic division patterns and starvation-induced quiescence under limited glucose levels in fission yeast. *The FEBS Journal*, 278(8), pp.1299–315.

Pluskal, T., Uehara, T. & Yanagida, M., 2012. Highly accurate chemical formula prediction tool utilizing high-resolution mass spectra, MS/MS fragmentation, heuristic rules, and isotope pattern matching. *Analytical Chemistry*, 84(10), pp.4396–403.

Pluskal, T., Ueno, M. & Yanagida, M., 2014. Genetic and metabolomic dissection of the ergothioneine and selenoneine biosynthetic pathway in the fission yeast, S. pombe, and construction of an overproduction system. *PLOS ONE*, 9(5), p.e97774.

Psychogios, N. et al., 2011. The human serum metabolome. D. Flower, ed. *PLOS ONE*, 6(2), p.e16957.

Qin, J. et al., 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*, 464(7285), pp.59–65.

Rapoport, S.M., Schewe, T. & Thiele, B.-J., 1990. Maturational breakdown of mitochondria and other organelles in reticulocytes. In J. R. Harris, ed. *Erythroid Cells*. Springer US, pp. 151–194.

Rappaport, S.M. et al., 2014. The blood exposome and its role in discovering causes of disease. *Environmental Health Perspectives*, 122(8), pp.769–74.

Rappaport, S.M. & Smith, M.T., 2010. Epidemiology. Environment and disease risks. *Science*, 330(6003), pp.460–1.

Rebouche, C.J. & Broquist, H.P., 1976. Carnitine biosynthesis in Neurospora crassa: enzymatic conversion of lysine to epsilon-N-trimethyllysine. *Journal of Bacteriology*, 126(3), pp.1207–14.

Rebouche, C.J. & Seim, H., 1998. Carnitine metabolism and its regulation in microorganisms and mammals. *Annual Review of Nutrition*, 18, pp.39–61.

Van Romburgh, P. & Barger, G., 1911. Preparation of the betaine of tryptophan and its identity with the alkaloid hypaphorine. *Journal of the Chemical Society, Transactions*, 99, p.2068.

Roux-Dalvai, F. et al., 2008. Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry. *Molecular & Cellular Proteomics*, 7(11), pp.2254–69.

Ruud, J.T., 1954. Vertebrates without erythrocytes and blood pigment. *Nature*, 173(4410), pp.848–50.

Sajiki, K. et al., 2013. Metabolomic analysis of fission yeast at the onset of nitrogen starvation. *Metabolites*, 3(4), pp.1118–1129.

Saltiel, A.R. & Kahn, C.R., 2001. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*, 414(6865), pp.799–806.

Savage, D.C., 1977. Microbial ecology of the gastrointestinal tract. *Annual Review of Microbiology*, 31(70), pp.107–33.

Schrettl, M., Winkelmann, G. & Haas, H., 2004. Ferrichrome in Schizosaccharomyces pombe-an iron transport and iron storage compound. *Biometals*, 17(6), pp.647–54.

Segel, G.B., Cokelet, G.R. & Lichtman, M.A., 1981. The measurement of lymphocyte volume: importance of reference particle deformability and counting solution tonicity. *Blood*, 57(5), pp.894–9.

Serkova, N.J., Standiford, T.J. & Stringer, K.A., 2011. The emerging field of quantitative blood metabolomics for biomarker discovery in critical illnesses. *American Journal of Respiratory and Critical Care Medicine*, 184(6), pp.647–55.

Shimanuki, M. et al., 2013. Klf1, a C2H2 zinc finger-transcription factor, is required for cell wall maintenance during long-term quiescence in differentiated G0 phase. *PLOS ONE*, 8(10), p.e78545.

Singh, S. & Kumar, A., 2009. Leukocyte depletion for safe blood transfusion. *Biotechnology Journal*, 4(8), pp.1140–51.

Snow, R.J. & Murphy, R.M., 2001. Creatine and the creatine transporter: a review. *Molecular and Cellular Biochemistry*, 224(1-2), pp.169–81.

Soga, T. et al., 2003. Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. *Journal of Proteome Research*, 2(5), pp.488–94.

Sriprawat, K. et al., 2009. Effective and cheap removal of leukocytes and platelets from Plasmodium vivax infected blood. *Malaria Journal*, 8, p.115.

Steck, T.L., 1974. The organization of proteins in the human red blood cell membrane. *The Journal of Cell Biology*, 62(1), pp.1–19.

Strijbis, K. et al., 2009. Identification and characterization of a complete carnitine biosynthesis pathway in Candida albicans. *The FASEB Journal*, 23(8), pp.2349–59.

Strijbis, K., Vaz, F.M. & Distel, B., 2010. Enzymology of the carnitine biosynthesis pathway. *IUBMB life*, 62(5), pp.357–62.

Swiegers, J.H. et al., 2001. Carnitine-dependent metabolic activities in Saccharomyces cerevisiae: three carnitine acetyltransferases are essential in a carnitine-dependent strain. *Yeast*, 18(7), pp.585–95.

Sze, D.Y. & Jardetzky, O., 1990. Determination of metabolite and nucleotide concentrations in proliferating lymphocytes by 1H-NMR of acid extracts. *BBA - Molecular Cell Research*, 1054(2), pp.181–197.

Takeda, K. et al., 2010. Synergistic roles of the proteasome and autophagy for mitochondrial maintenance and chronological lifespan in fission yeast. *Proceedings of the National Academy of Sciences of the United States of America*, 107(8), pp.3540–5.

Tanret, C., 1909. Sur une base nouvelle retiree du seigle ergote, l'ergothioneine. *Rend. Acad. Sci*, 149, pp.222–224.

Traut, T.W., 1994. Physiological concentrations of purines and pyrimidines. *Molecular and Cellular Biochemistry*, 140(1), pp.1–22.

Tukey, R.H. & Strassburg, C.P., 2000. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annual Review of Pharmacology and Toxicology*, 40, pp.581–616.

Wahl, V. et al., 2013. Regulation of flowering by trehalose-6-phosphate signaling in Arabidopsis thaliana. *Science*, 339(6120), pp.704–7.

Weinshilboum, R.M., Otterness, D.M. & Szumlanski, C.L., 1999. Methylation pharmacogenetics: catechol O-methyltransferase, thiopurine methyltransferase, and histamine N-methyltransferase. *Annual Review of Pharmacology and Toxicology*, 39, pp.19–52.

Wellen, K.E. & Thompson, C.B., 2012. A two-way street: reciprocal regulation of metabolism and signalling. *Nature Reviews Molecular Cell Biology*, 13(4), pp.270–276.

Whillier, S. et al., 2011. Glutamine and α-ketoglutarate as glutamate sources for glutathione synthesis in human erythrocytes. *The FEBS Journal*, 278(17), pp.3152–63.

Van Wijk, R. & van Solinge, W.W., 2005. The energy-less red blood cell is lost: erythrocyte enzyme abnormalities of glycolysis. *Blood*, 106(13), pp.4034–42.

Wishart, D.S. et al., 2013. HMDB 3.0 - The Human Metabolome Database in 2013. *Nucleic Acids Research*, 41(Database issue), pp.D801–7.

Wood, V. et al., 2012. PomBase: a comprehensive online resource for fission yeast. *Nucleic Acids Research*, 40(Database issue), pp.D695–9.

Wood, V. et al., 2002. The genome sequence of Schizosaccharomyces pombe. *Nature*, 415(6874), pp.871–80.

Xu, H. et al., 2006. The alpha-aminoadipate pathway for lysine biosynthesis in fungi. *Cell Biochemistry and Biophysics*, 46(1), pp.43–64.

Yamashita, Y.M. et al., 1996. 20S cyclosome complex formation and proteolytic activity inhibited by the cAMP/PKA pathway. *Nature*, 384(6606), pp.276–9.

Yanagida, M. et al., 2011. Nutrient limitations alter cell division control and chromosome segregation through growth-related kinases and phosphatases. *Philosophical Transactions of the Royal Society Series B*, 366(1584), pp.3508–20.

Zhang, Y. et al., 2009. Organic solvent extraction and metabonomic profiling of the metabolites in erythrocytes. *Journal of Chromatography B*, 877(18-19), pp.1751–7.

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