

Title	Fission yeast and human blood metabolomic comparison with focus on age related compounds( Digest_要約 )
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Citation	Kyoto University (京都大学)
Issue Date	2014-09-24
URL	<a href="http://dx.doi.org/10.14989/doctor.k18626">http://dx.doi.org/10.14989/doctor.k18626</a>
Right	学位規則第9条第2項により要約公開; 許諾条件により要約は2015/07/15に公開; 許諾条件により要旨は2014/12/23に公開
Type	Thesis or Dissertation
Textversion	none

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

July 2014



## TABLE OF CONTENTS

<u>ABSTRACT .....</u>	<u>iii</u>
<u>INTRODUCTION.....</u>	<u>1</u>
1.1 Metabolomics .....	1
1.2 Fission yeast .....	4
1.3 Human blood .....	4
1.4 Comparison of fission yeast and human red blood cells .....	10
1.5 The aim of work.....	11
<u>RESULTS.....</u>	<u>12</u>
2.1 Isolation of metabolites from human blood.....	12
2.1.1 Methods for separation of blood components .....	12
2.1.2 Peak detection and identification of the compounds .....	17
2.2 Comparison of red blood cell and fission yeast metabolomes .....	19
2.2.1 Hundred thirty-three compounds identified in human blood.....	19
2.2.2 Thirty-two compounds identified in human blood were not detected in yeast.....	24
2.2.3 Eighteen <i>S. pombe</i> compounds not detected in human blood .....	25
2.2.4 Compounds detected in WBCs in comparison with those from RBCs and <i>S. pombe</i> .....	27
2.2.5 Fourteen newly identified blood compounds .....	27
2.2.6 Semi-quantification of blood metabolite peaks .....	28
2.2.7 Fifty-seven RBC-enriched compounds.....	33
2.2.8 Comparison of semi-quantified metabolites between blood and <i>S. pombe</i> .....	35
2.2.9 Summary and categorization of detected blood compounds .....	36
2.2.10 Energy and anti-oxidant metabolites abundant in human blood and fission yeast .....	38
<u>DISCUSSION AND PERSPECTIVES .....</u>	<u>42</u>
3.1 Comparison of human blood and fission yeast metabolomes.....	42
3.2 Conclusions.....	54
<u>MATERIALS AND METHODS .....</u>	<u>55</u>



4.1 Chemicals and reagents .....	55
4.2 Human subject characteristics .....	56
4.3 Ethics statement .....	56
4.4 Blood sample preparation for metabolomic analysis .....	56
4.5 Isolation of lymphocytes and RBC by ficoll gradient .....	57
4.6 Fission yeast growth conditions and preparation for metabolomics analysis .....	58
4.7 LC-MS analysis .....	58
4.8 Supplemental Material .....	59
<u>REFERENCES .....</u>	<u>60</u>
<u>ACKNOWLEDGMENTS .....</u>	<u>71</u>
<u>LIST OF TABLES .....</u>	<u>72</u>
<u>LIST OF FIGURES .....</u>	<u>73</u>

## ABSTRACT

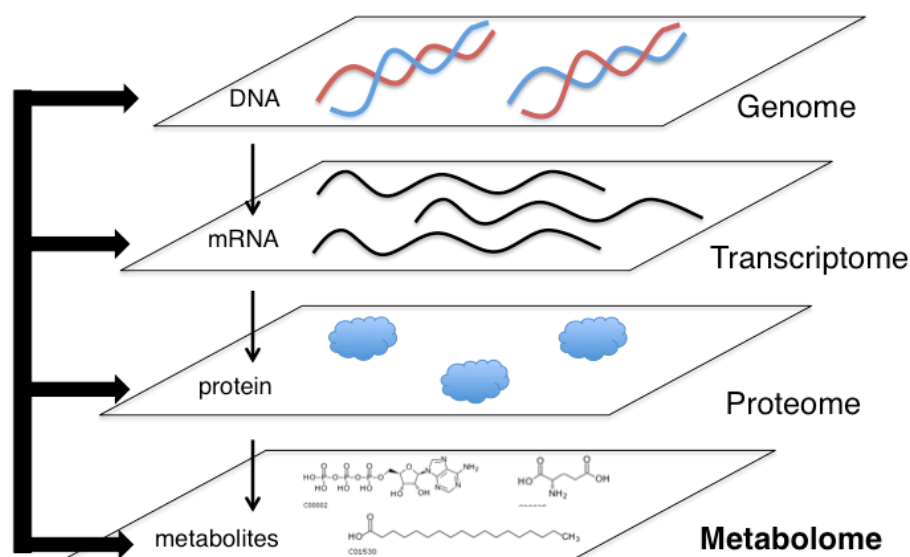
Metabolomics, a modern branch of chemical biology, provides qualitative and quantitative information about the metabolic states of organisms or cells at the molecular level. Here I report non-targeted, metabolomic analyses of human blood, using liquid chromatography-mass spectrometry (LC-MS). I compared 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*<sub>2</sub>-acetyl-lysine, and *N*<sub>6</sub>-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

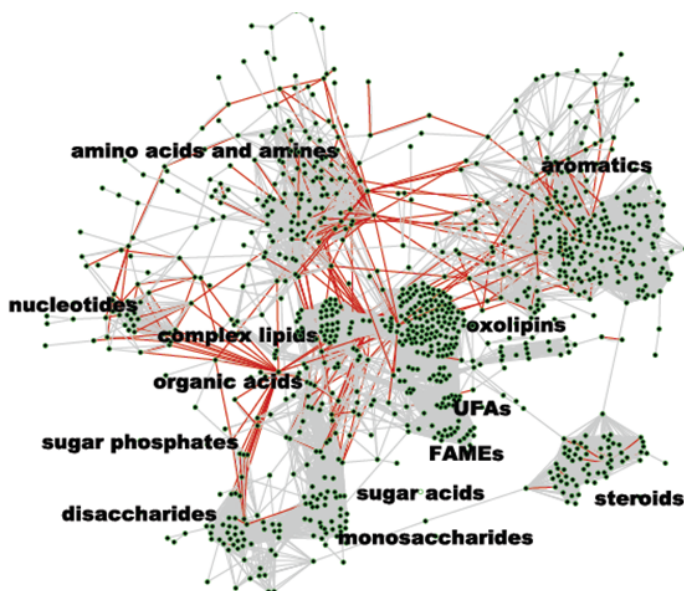
### 1.1 Metabolomics

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	<ul style="list-style-type: none"><li>• Relatively low-cost method</li><li>• No ion suppression</li><li>• Long-term stability and robustness</li><li>• Large spectral libraries available</li></ul>	<ul style="list-style-type: none"><li>• Requires derivatization</li><li>• Not suited for thermo labile compounds</li><li>• Impractical for identification of unknowns</li></ul>
LC-MS	<ul style="list-style-type: none"><li>• Compatible with a large number of compound classes, both charged and uncharged</li><li>• Utilizes organic solvents well-suited for MS</li><li>• Good sensitivity</li></ul>	<ul style="list-style-type: none"><li>• Poor reproducibility of retention times</li><li>• Different classes of compounds require different columns/solvents to achieve optimal separation</li></ul>
CE-MS	<ul style="list-style-type: none"><li>• Ideal for ionic or very polar metabolites</li><li>• High resolution separation</li><li>• High peak capacity</li><li>• Low cost of operation due to minimal consumption of solvents</li><li>• Low sample consumption</li></ul>	<ul style="list-style-type: none"><li>• Sensitive to sample ion strength</li><li>• Higher detection limits due to small injection volumes</li></ul>
NMR	<ul style="list-style-type: none"><li>• High analytical reproducibility</li><li>• Non-destructive method</li><li>• High speed of analysis</li><li>• Ability to study living systems</li></ul>	<ul style="list-style-type: none"><li>• Unable to identify individual metabolite signals in complex NMR spectra</li><li>• Low sensitivity compared to MS</li></ul>

(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

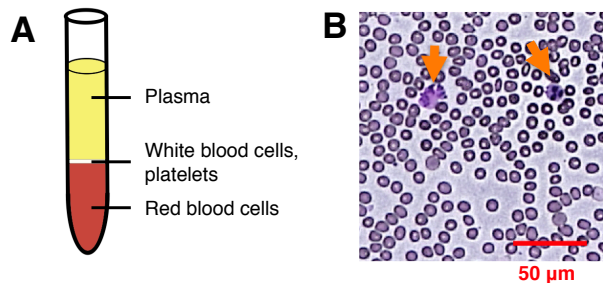
The fission yeast, *Schizosaccharomyces pombe*, a eukaryotic microbe with a genome encoding only ~5,000 genes (Wood et al. 2002; Wood et al. 2012), is an excellent model organism to study nutritional control of the transition between proliferation and quiescence (Yanagida et al. 2011).

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  $\mu\text{m}$  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**

	<b>Platelets</b>	<b>Red blood cells</b>	<b>White blood cells</b>
<b>Number of cells (cell/mL)</b>	$5 \times 10^8$	$5 \times 10^9$	$1 \times 10^7$
<b>Cell volume (fL)</b>	10	90	200 <sup>a</sup>

<sup>a</sup> 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  $\mu\text{m}$  (thickness, 2-2.5  $\mu\text{m}$ ). 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  $\mu\text{m}$  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 acids 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**

<b>Pathway</b>	<b>Function</b>
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<sup>+</sup>/K<sup>+</sup>-ATPase and Ca<sup>++</sup>-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, Heillier, 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 <i>P.falciparum</i> 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  $\mu\text{m}$  diameter (Mitchison 1957) and 13  $\mu\text{m}$  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
<b>Shape</b>	 Cylinder	 Disc
<b>Dimensions (<math>\mu\text{m}</math>)</b>	3.5 (Diameter) 12 (Length)	6-8 (Diameter) 2 (Height)
<b>Volume (fL)</b>	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*.

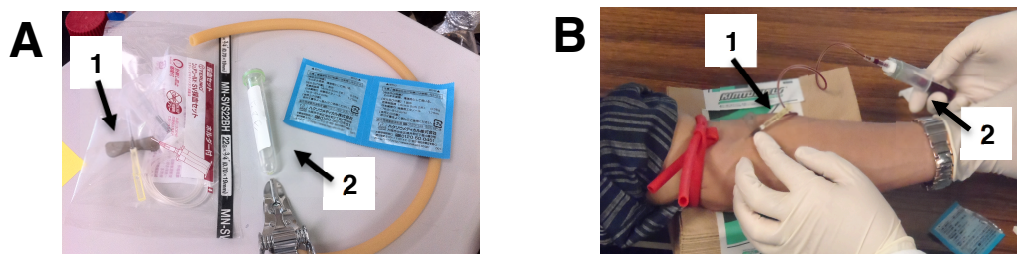
### **1.5 The aim of work**

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.

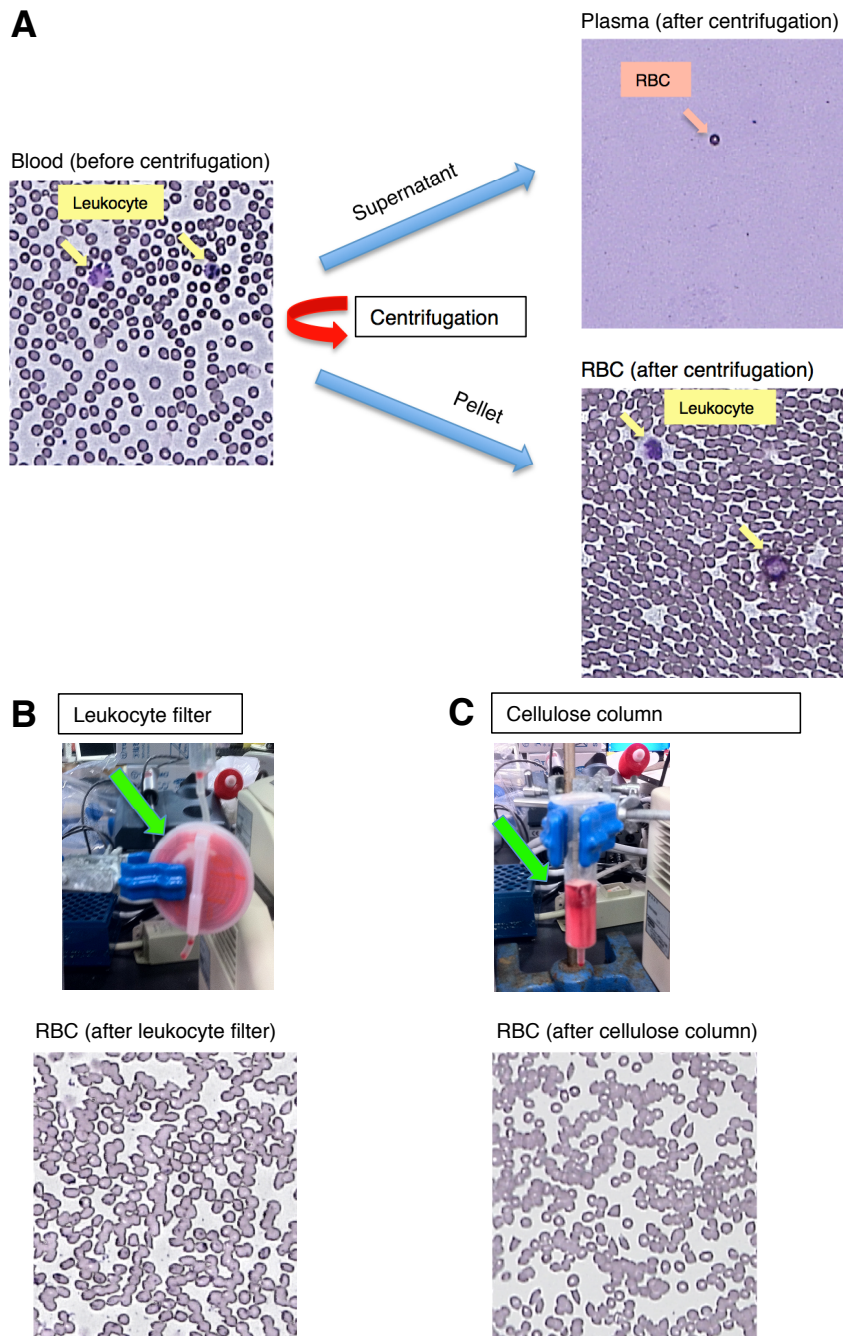
An easy and straightforward way to fractionate plasma and blood cells is centrifugation. After centrifugation of a whole blood sample, the plasma is on top, RBCs on the bottom, with a thin milky layer of WBCs (“buffy coat”) in between. To retrieve the RBCs without contamination with the “buffy coat” 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



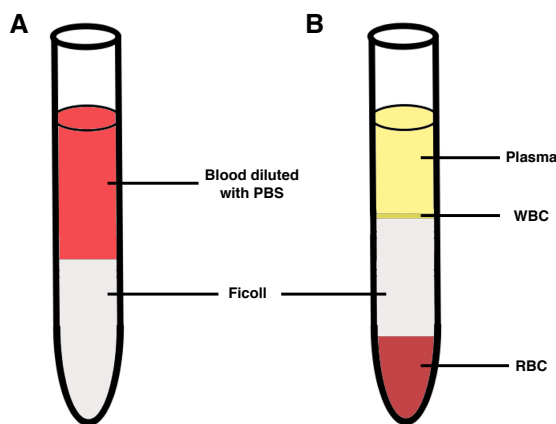
during processing through filters or columns it turns bright red, possibly due to binding oxygen. Furthermore, I frequently experienced column and filter clogging, which prolonged the procedure. Thus, I decided to work with the whole blood and RBC samples, containing leukocytes. To assess the contribution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

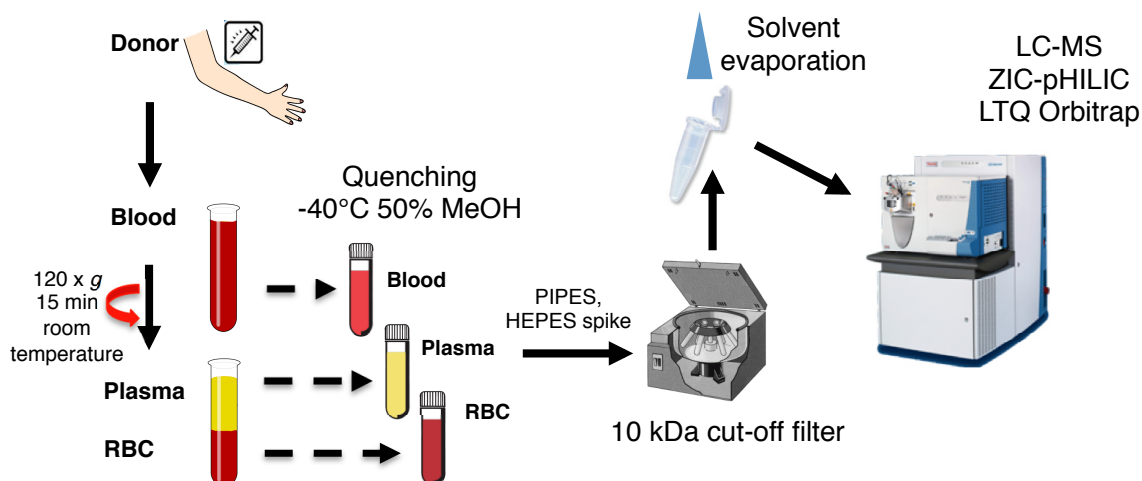
(A) Blood diluted with PBS is layered above Ficoll solution and (B) after centrifugation the blood components are separated into fractions (see Materials and Methods).

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  $-40^{\circ}\text{C}$  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  $4^{\circ}\text{C}$ , concentrated on a rotary evaporator, re-suspended in  $40\ \mu\text{L}$  50% acetonitrile, and stored at  $-80^{\circ}\text{C}$  until analysis. The whole blood, plasma (supernatant) and RBC (pellets), were employed for metabolomics analysis.



**Figure 8. Preparation and analysis of blood metabolome samples**

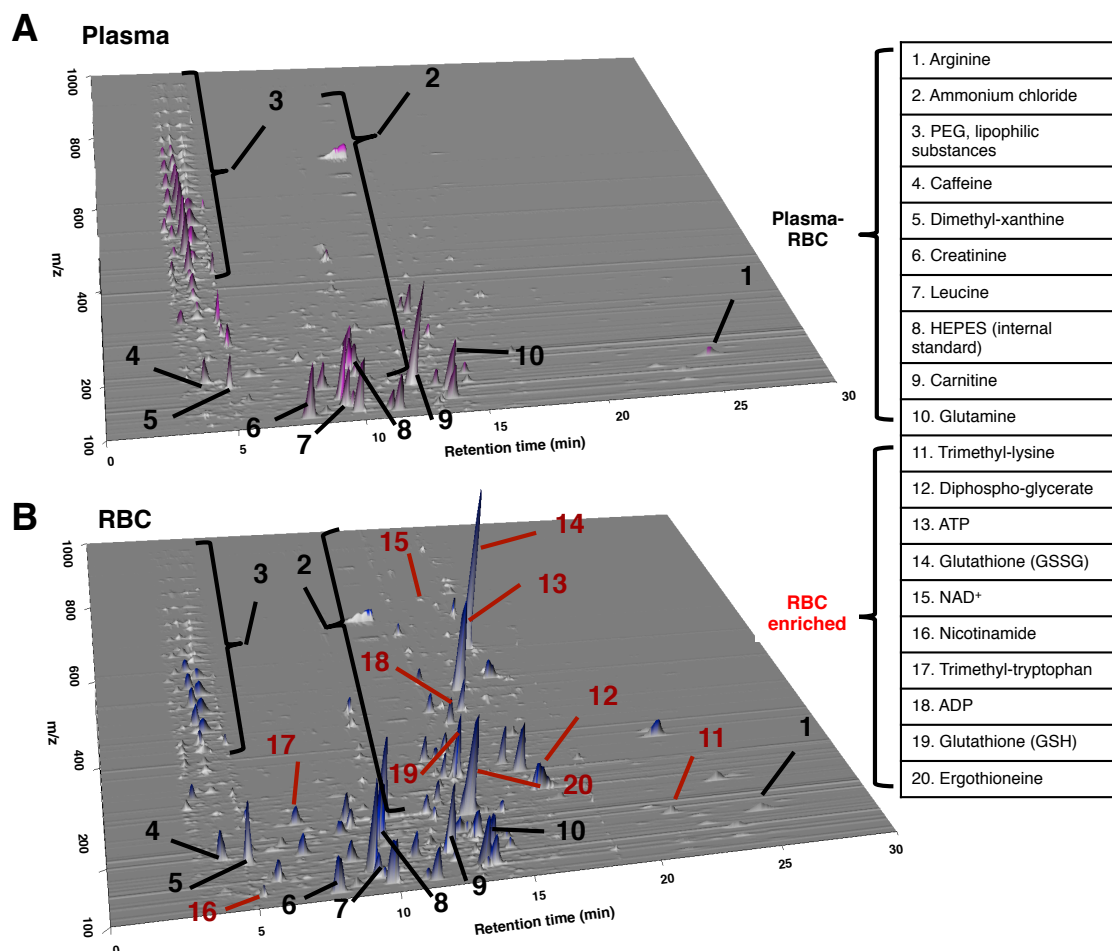
Metabolic compounds were extracted in 50% MeOH at -40° C from whole blood, plasma, and RBCs (Materials and Methods). Extracted metabolites were isolated with a 10-kDa cut-off filter, concentrated by a rotary evaporator, and analyzed on an LC-MS system, as illustrated.

### 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**

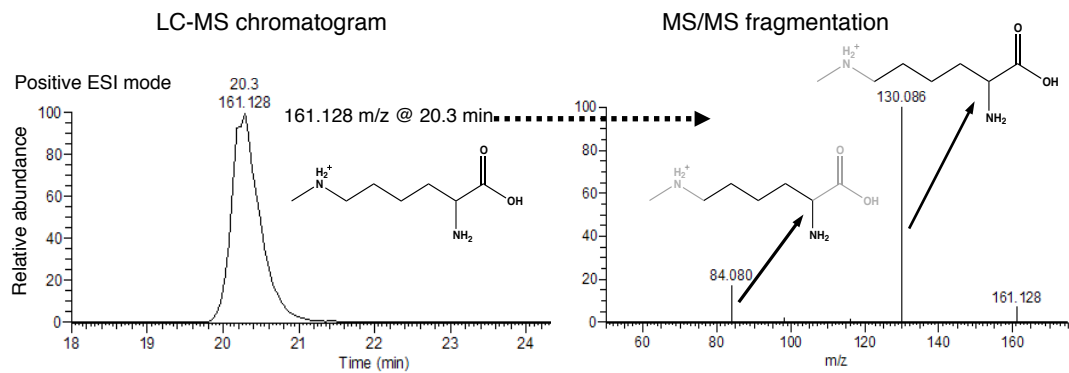
Raw LC-MS data 3D plots of plasma fraction (top) and RBC fraction (bottom) obtained in positive ionization mode are shown: X-axis, retention time (RT, min); Y-axis,  $m/z$ ; Z-axis, signal intensity. Twenty identified peaks are shown as examples. Peaks 1-10 are detected in both plasma and RBCs. These are amino acids, creatine, carnitine, dietary metabolites (caffeine, dimethyl-xanthine), and compounds introduced during sample preparation (HEPES as internal standard,  $\text{NH}_4\text{Cl}$  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

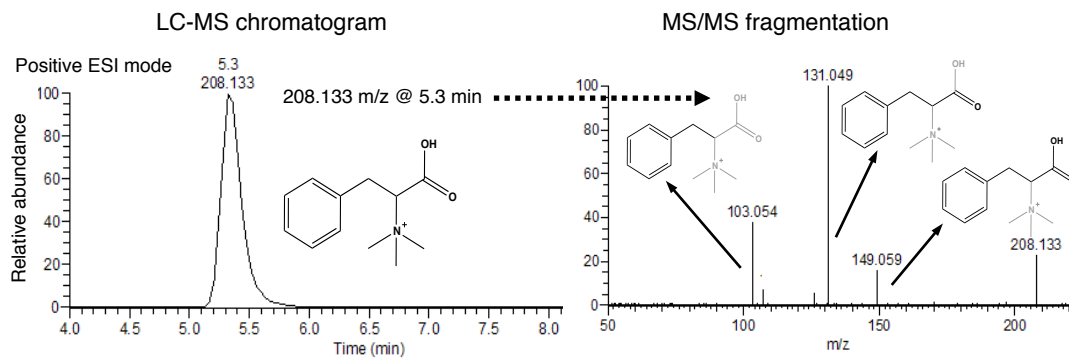
### 2.2.1 Hundred thirty-three compounds identified in human blood

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).

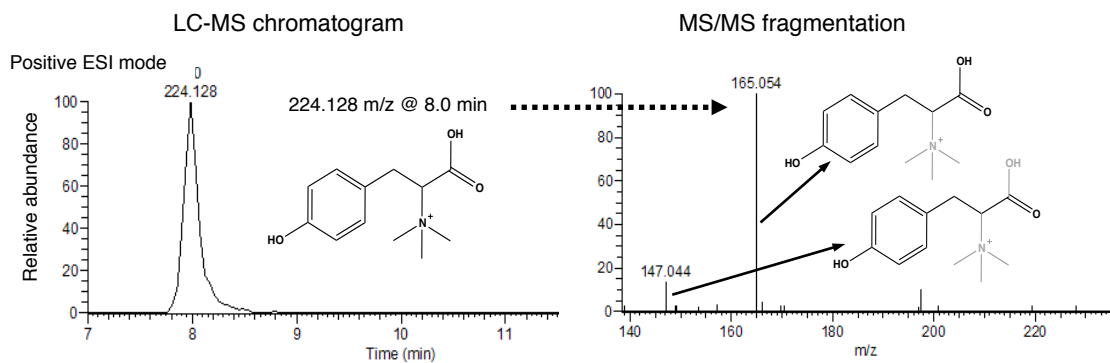
### A Methyl-lysine MS/MS analysis



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

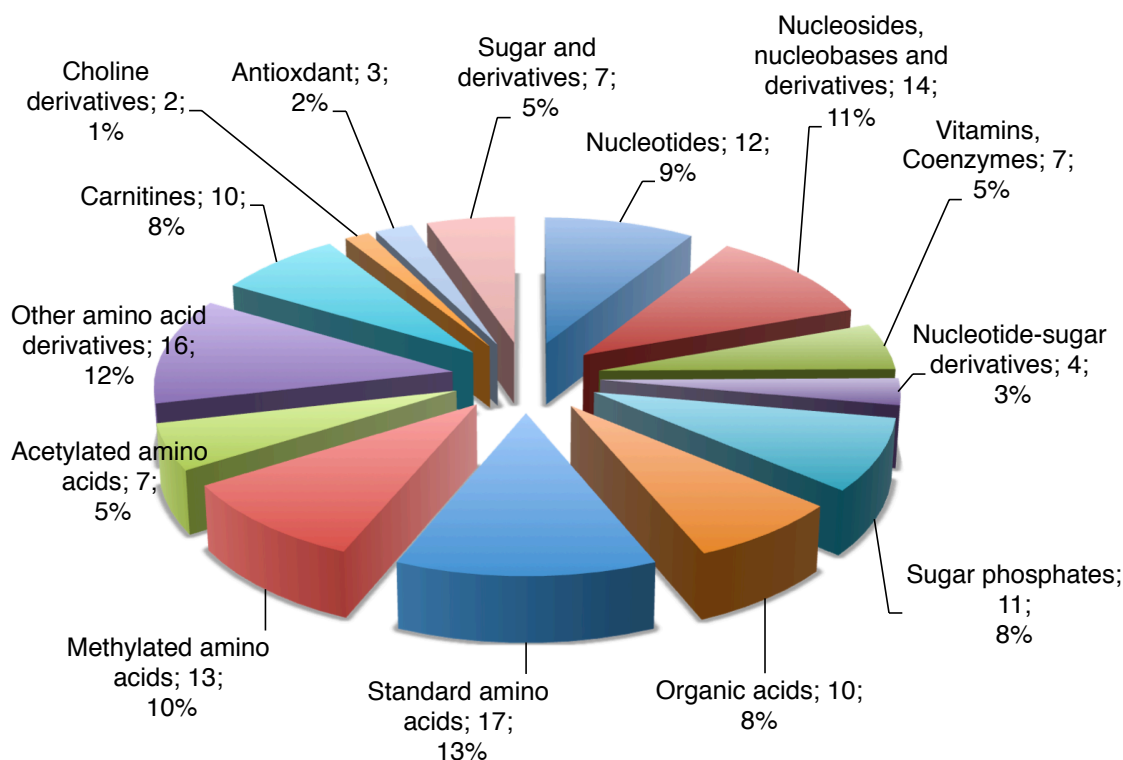


### C N-Trimethyl-tyrosine MS/MS analysis



**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  $\text{NH}_2(\text{CH}_3)$  (31.043 Da), the 84.080 Da fragment to loss of  $\text{NH}_2(\text{CH}_3)$  (31.043) and  $\text{CO}_2\text{H}_2$  (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  $\text{N}(\text{CH}_3)_3$  (59.074 Da), the 131.049 Da fragment to loss of  $\text{N}(\text{CH}_3)_3$  (59.074 Da) and  $\text{H}_2\text{O}$  (18.010 Da), and the 103.054 Da fragment to loss of  $\text{N}(\text{CH}_3)_3$  (59.074 Da) and  $\text{CO}_2\text{H}_2$  (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  $\text{N}(\text{CH}_3)_3$  (59.074 Da), the 147.044 Da fragment to loss of  $\text{N}(\text{CH}_3)_3$  (59.074 Da) and  $\text{H}_2\text{O}$  (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 <sup>b</sup>	Ratio RBC:plasma ± standard deviation <sup>c</sup>	Peak area <sup>d</sup> Blood	Peak area <sup>d</sup> Fission yeast
<b>Nucleotides</b>							
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
<b>Nucleosides, nucleobases and derivatives</b>							
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	○			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	○			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	○			0.69 ± 0.22	M	ND
Uridine	STD				1.0 ± 0.027	L	L
<b>Vitamins, Coenzymes</b>							
4-Aminobenzoate	STD	○			2.5 ± 0.86	L	ND
NAD <sup>+</sup>	STD			R	130.0 ± 30.0	M	H
NADH	STD			R	80.0 ± 68.0	L	M
NADP <sup>+</sup>	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
<b>Nucleotide-sugar derivatives</b>							
GDP-glucose	STD		○	R	20.0 ± 3.6	L	M
UDP-acetyl-glucosamine	STD		○	R	11.0 ± 1.4	L	M
UDP-glucose	STD			R	35.0 ± 6.6	M	H
UDP-glucuronate	STD	○	○	R	37.0 ± 6.2	L	ND
<b>Sugar phosphates</b>							
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
<b>Sugars and derivatives</b>							
1,5-Anhydroglucitol	STD	○			0.95 ± 0.14	M	ND
Glucuronate	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
<b>Organic acids</b>							
2-Oxoglutarate	STD				0.94 ± 0.16	L	L
Chenodeoxycholate	STD	○			0.49 ± 0.14	M	ND
cis-Aconitate	STD				0.33 ± 0.11	L	L
Citramalate	STD		○	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	○			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 <sup>b</sup>	Ratio RBC:plasma ± standard deviation <sup>c</sup>	Peak area <sup>d</sup> Blood	Peak area <sup>d</sup> Fission yeast
<b>Standard amino acids</b>							
Arginine	STD				0.57 ± 0.072	H	H
Asparagine	STD				1.6 ± 0.18	L	L
Aspartate	STD			R	<b>6.3</b> ± 0.91	L	M
Glutamate	STD			R	<b>3.4</b> ± 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
<b>Methylated amino acids</b>							
Betaine	STD				1.2 ± 0.11	H	L
Butyro-betaine	STD	○		R	<b>5.3</b> ± 0.52	M	ND
Dimethyl-arginine	STD				0.95 ± 0.12	L	L
Dimethyl-lysine	STD		○		0.51 ± 0.09	L	L
Dimethyl-proline (stachydrine)	STD	○		R	<b>4.9</b> ± 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	○		R	<b>1500.0</b> ± <b>2300.0</b>	L	ND
Trimethyl-histidine (hercynine)	MS/MS		○	R	<b>110.0</b> ± <b>140.0</b>	L	M
Trimethyl-lysine	STD			R	<b>6.3</b> ± 1.5	M	L
Trimethyl-phenylalanine	MS/MS	○	○	R	<b>14.0</b> ± <b>9.0</b>	L	ND
Trimethyl-tryptophan (hypaphorine)	STD	○	○	R	<b>6.1</b> ± 1.7	H	ND
Trimethyl-tyrosine	MS/MS	○	○	R	ND <sup>e</sup>	L	ND
<b>Acetylated amino acids</b>							
N-Acetyl-(iso)leucine	STD		○		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		○		0.82 ± 0.18	L	M
N-Acetyl-ornithine	STD				<b>2.6</b> ± 1.2	L	L
N <sub>ε</sub> -Acetyl-lysine	STD		○		<b>2.1</b> ± 0.3	L	L
N <sub>ε</sub> -Acetyl-lysine	STD		○		0.88 ± 0.26	L	M
<b>Other amino acid derivatives</b>							
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	○		R	<b>7.0</b> ± 1.6	H	ND
Creatinine	STD	○			0.97 ± 0.079	H	ND
Glutamate methyl ester	STD		○		1.5 ± 0.28	L	L
Hippurate	STD	○			0.54 ± 0.083	M	ND
Histamine	STD				1.0 ± 0.5	L	L
Indoxyl-sulfate	STD	○			0.43 ± 0.092	M	ND
Kynurenine	STD	○			0.41 ± 0.081	L	ND
Ornithine	STD				1.2 ± 0.16	L	M
Phosphocreatine	STD	○		R	<b>3.0</b> ± 0.95	L	ND
Quinolinic acid	STD	○			0.35 ± 0.04	L	ND
S-Adenosyl-homocysteine	STD			R	<b>2100.0</b> ± <b>1600.0</b>	L	M
S-Adenosyl-methionine	STD			R	<b>57.0</b> ± <b>62.0</b>	L	L
Taurine	STD	○			0.92 ± 0.18	M	ND
<b>Carnitines</b>							
Acetyl-carnitine	STD	○		R	<b>4.0</b> ± 0.58	H	ND
Butyryl-carnitine	STD	○			0.95 ± 0.2	M	ND
Carnitine	STD	○			0.85 ± 0.1	H	ND
Decanoyl-carnitine	STD	○			0.42 ± 0.11	M	ND
Dodecanoyl-carnitine	STD	○			0.51 ± 0.072	L	ND
Hexanoyl-carnitine	STD	○			0.68 ± 0.087	L	ND
Isovaleryl-carnitine	STD	○			0.94 ± 0.25	L	ND
Octanoyl-carnitine	STD	○			0.45 ± 0.086	M	ND
Propionyl-carnitine	STD	○		R	<b>5.3</b> ± 0.82	M	ND
Tetradecanoyl-carnitine	STD	○		R	<b>11.0</b> ± <b>15.0</b>	L	ND
<b>Choline derivatives</b>							
CDP-choline	STD				0.98 ± 0.37	L	L
Glycerophosphocholine	STD				1.5 ± 0.24	M	H
<b>Antioxidant</b>							
Ergothioneine	STD			R	<b>100.0</b> ± <b>4.8</b>	H	M
Glutathione disulfide (GSSG)	STD			R	<b>1900.0</b> ± <b>430.0</b>	H	H
Ophthalmic acid	STD			R	<b>310.0</b> ± <b>150.0</b>	L	M

<sup>a</sup> One hundred thirty-three identified metabolic compounds detected in human blood metabolome samples by LC-MS. Status of the compounds was either confirmed by commercially 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 ○ in respective columns. <sup>b</sup>on average in multiple 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. <sup>c</sup>Peak areas defined as H, high (>10<sup>4</sup> AU); M, medium (10<sup>3</sup>-10<sup>4</sup> AU); L, low (<10<sup>3</sup> 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  $\alpha, \alpha$ -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
<b>Nucleotides</b>			
3',5'-cAMP	STD	L	○
CMP	STD	L	○
<b>Nucleosides, nucleobases and derivatives</b>			
AICAR	STD	L	
PRPP (5-Phospho-alpha-D-ribose 1-diphosphate)	STD	M	○
SAICAR	MS/MS	L	
<b>Vitamins, Coenzymes</b>			
Acetyl-CoA	STD	L	○
Biotin	STD	L	
CoA	STD	L	○
FAD	STD	L	○
HMG-CoA	STD	L	○
<b>Sugar phosphates</b>			
Trehalose-6-phosphate	STD	M	
<b>Sugars and derivatives</b>			
Trehalose	STD	H	
<b>Methylated amino acids</b>			
Dimethyl-histidine	MS/MS	M	
<b>Other amino acid derivatives</b>			
Deferrichrome	STD	L	
Ferrichrome	STD	H	
Histidinol	STD	M	
S-adenosyl-cysteine	STD	L	
Saccharopine	STD	L	

<sup>a</sup> 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 commercially available standard (STD) or MS/MS analysis (MS/MS). Peak areas defined as H, high (>10<sup>8</sup> AU); M, middle (10<sup>7</sup>-10<sup>8</sup> AU); L, low (<10<sup>7</sup> AU). Compounds detected also in the WBC are marked by ○.

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

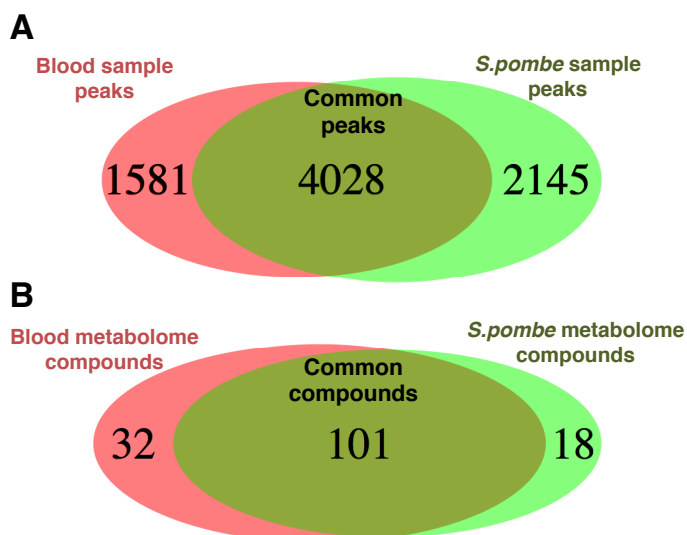
I then examined metabolites of WBCs isolated by Ficoll gradient centrifugation (Table 8 p.40). Seven compounds that seem to be mainly involved in cell growth and division in eukaryotes were found in both WBCs and *S. pombe* (Table 7 p.26). While the metabolic profile of WBCs was similar to that of RBCs, 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<sub>2</sub>-acetyl-lysine, N<sub>6</sub>-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

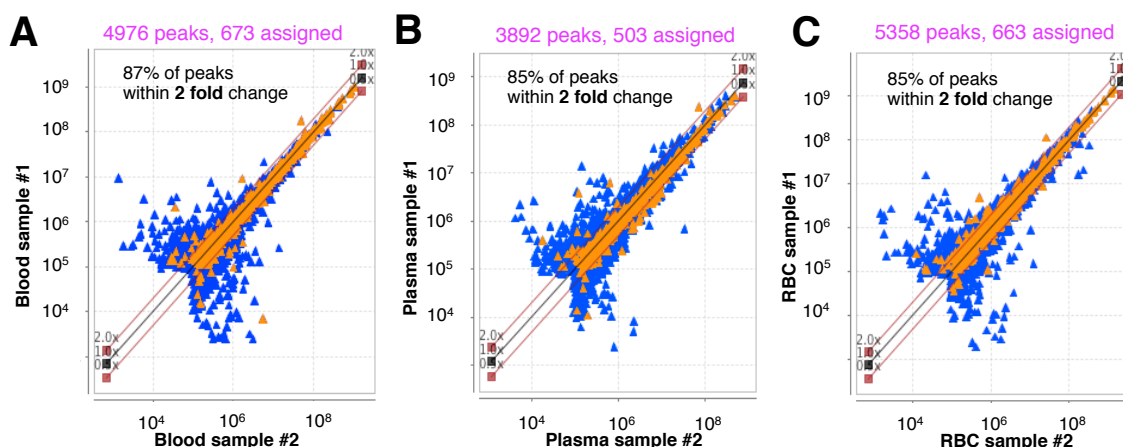
Each blood sample produced thousands of peaks in positive and negative ionization modes with a broad range ( $10^4 \sim 10^9$  AU) of peak areas. Number of peaks and number of compounds detected in blood and fission yeast metabolome samples are compared in Figure 12 p.28.



**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.

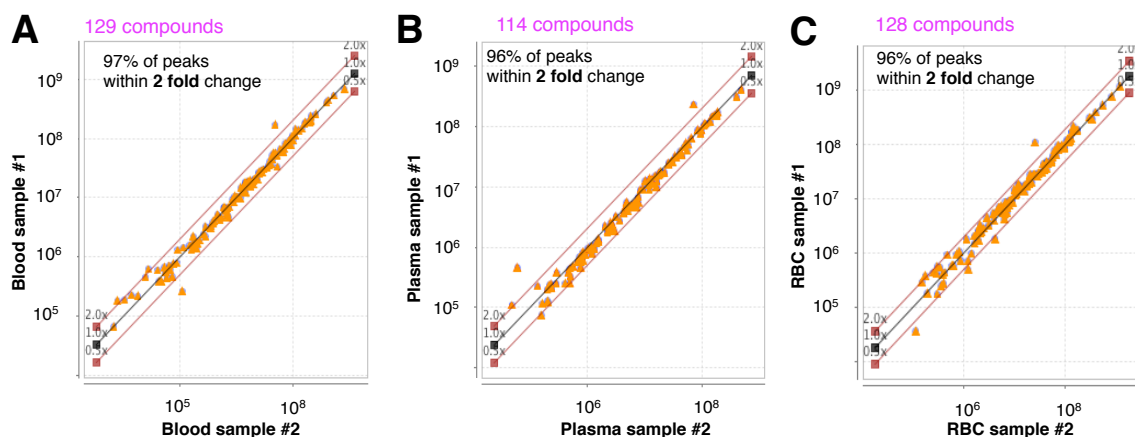


**Figure 13. Scatter plots of all peaks detected in blood, plasma and RBC samples**

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).

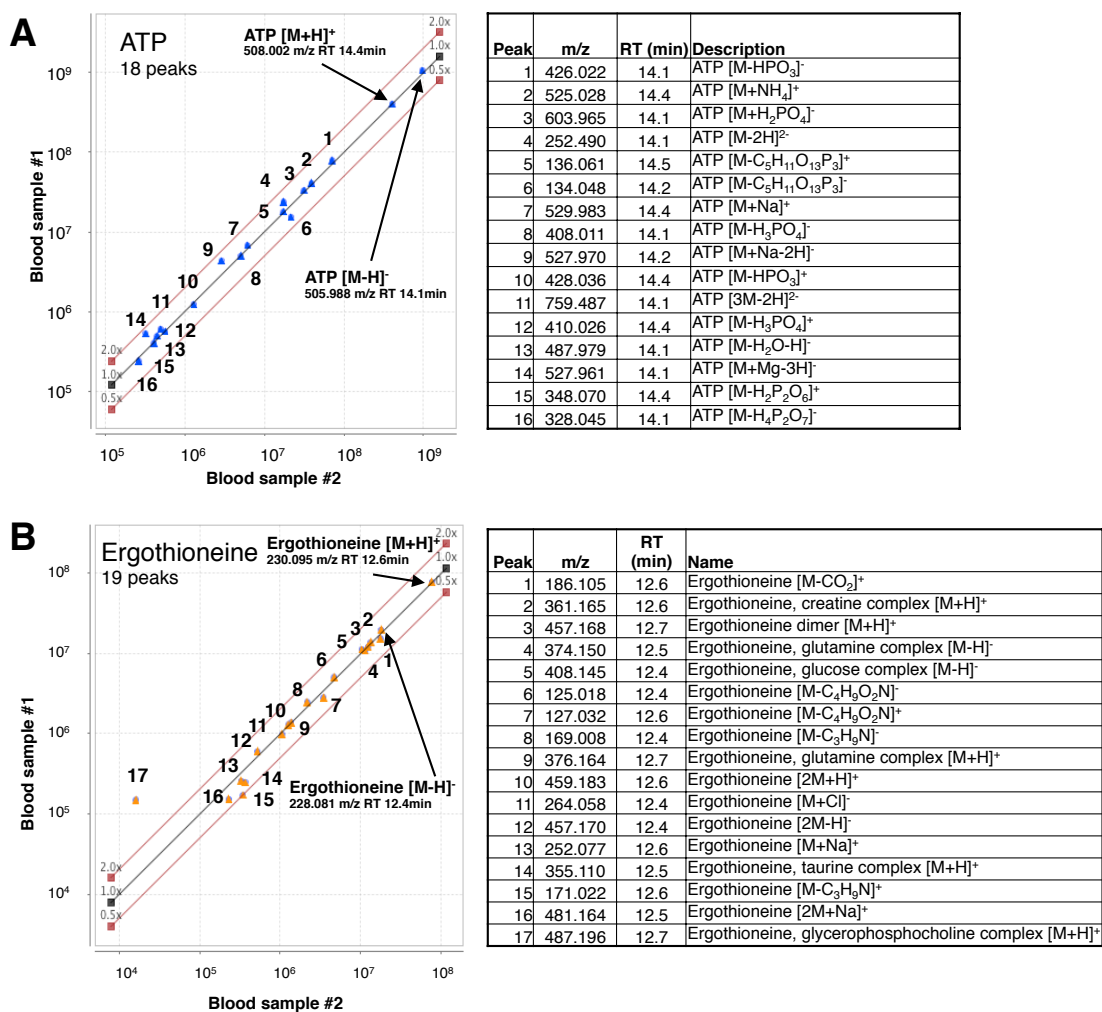




**Figure 14. Scatter plots of identified compounds (representative single-charged peaks) in blood, plasma and RBC samples**

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).



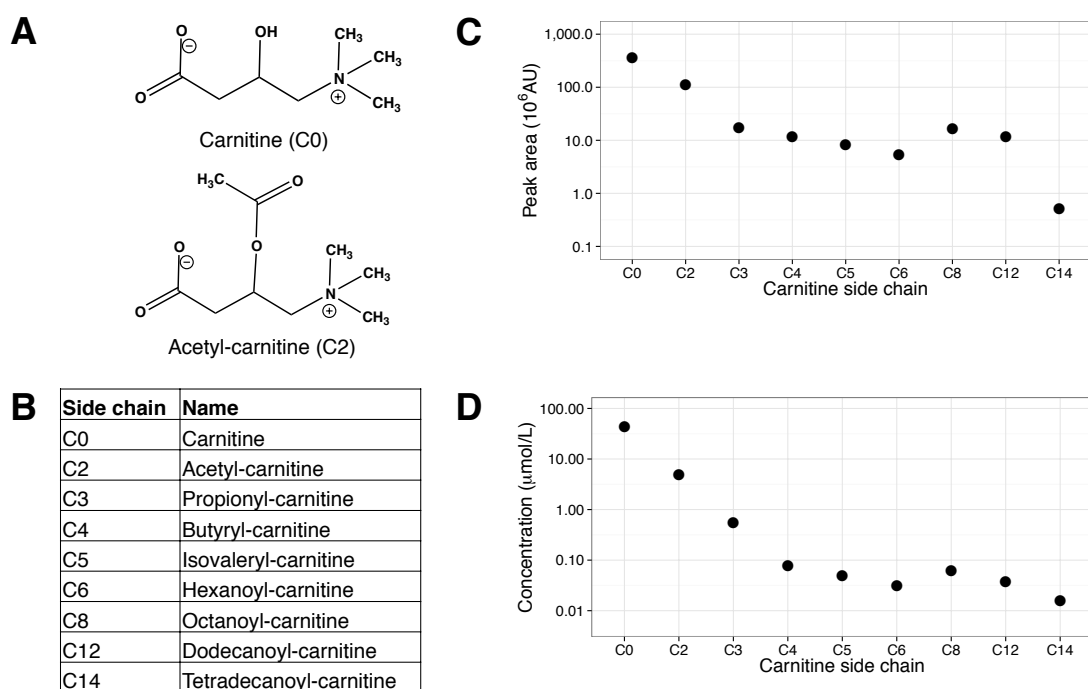
### Figure 15. Multiple peaks produced by the same compound

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<sub>4</sub>Cl, 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.



**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).

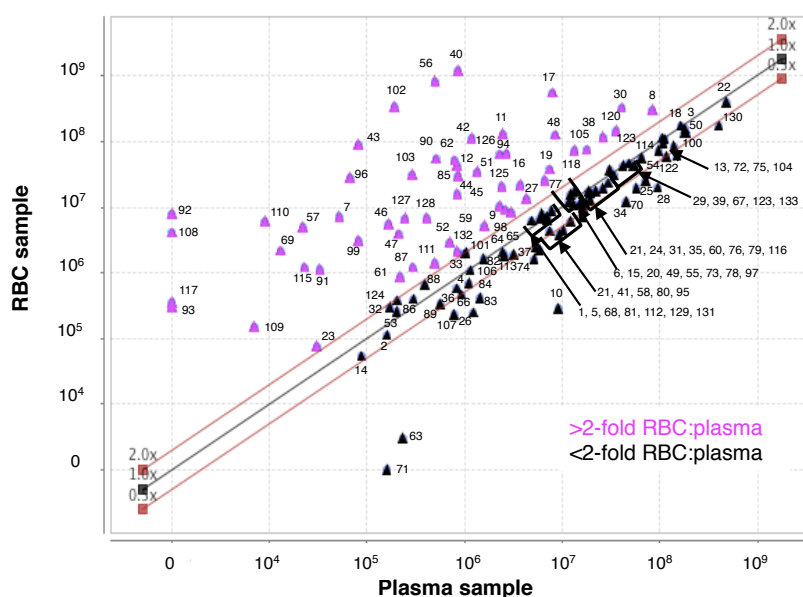
### 2.2.7 Fifty-seven RBC-enriched compounds

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 ( $\text{NAD}^+$ , NADH,  $\text{NADP}^+$ , 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	No	Compound	No	Compound	No	Compound
1	1-Methyl-adenosine	35	Dimethyl-arginine	68	Hypoxanthine	101	Phosphocreatine
2	1-Methyl-guanosine	36	Dimethyl-guanosine	69	IMP	102	Phosphoenolpyruvate
3	1,5-Anhydroglucitol	37	Dimethyl-lysine	70	Indoxyl-sulfate	103	Phosphoglycerate
4	2-Amino adipate	38	Dimethyl-proline (stachydrine)	71	Inosine	104	Proline
5	2-Oxoglutarate	39	Dimethyl-xanthine	72	Isoleucine	105	Propionyl-carnitine
6	4-Aminobenzoate	40	Diphospho-glycerate	73	Isovaleryl-carnitine	106	Quinic acid
7	6-Phosphogluconate	41	Dodecanoyl-carnitine	74	Kynurenine	107	Quinolinic acid
8	Acetyl-carnitine	42	Ergothioneine	75	Leucine	108	S-Adenosyl-homocysteine
9	Adenine	43	Fructose-1,6-diphosphate	76	Lysine	109	S-Adenosyl-methionine
10	Adenosine	44	Fructose-6-phosphate	77	Malate	110	S-Methyl-ergothioneine
11	ADP	45	Fumarate	78	Methionine	111	Sedoheptulose-7-phosphate
12	AMP	46	GDP	79	Methyl-histidine	112	Serine
13	Arginine	47	GDP-glucose	80	Methyl-lysine	113	Succinate
14	Arginino-succinate	48	Gluconate	81	myo-Inositol	114	Taurine
15	Asparagine	49	Glucosamine	82	N-Acetyl-(iso)leucine	115	Tetradecanoyl-carnitine
16	Aspartate	50	Glucose	83	N-Acetyl-arginine	116	Threonine
17	ATP	51	Glucose-6-phosphate	84	N-Acetyl-aspartate	117	Trimethyl-histidine (hercynine)
18	Betaine	52	Glutamate	85	N-Acetyl-D-glucosamine	118	Trimethyl-lysine
19	Butyro-betaine	53	Glutamate methyl ester	86	N-Acetyl-glutamate	119	Trimethyl-phenylalanine*
20	Butyryl-carnitine	54	Glutamine	87	N-Acetyl-ornithine	120	Trimethyl-tryptophan (hypaphorine)
21	Caffeine	55	Glutarate	88	N <sub>2</sub> -Acetyl-lysine	121	Trimethyl-tyrosine*
22	Carnitine	56	Glutathione disulfide (GSSG)	89	N <sub>6</sub> -Acetyl-lysine	122	Tryptophan
23	CDP	57	Glyceraldehyde-3-phosphate	90	NAD <sup>+</sup>	123	Tyrosine
24	CDP-choline	58	Glycerate	91	NADH	124	UDP
25	Chenodeoxycholate	59	Glycerol-2-phosphate	92	NADP <sup>+</sup>	125	UDP-acetyl-glucosamine
26	cis-Aconitate	60	Glycerophosphocholine	93	NADPH	126	UDP-glucose
27	Citramalate	61	GMP	94	Nicotinamide	127	UDP-glucuronate
28	Citrate	62	GTP	95	Octanoyl-carnitine	128	UMP
29	Citrulline	63	Guanosine	96	Ophthalmic acid	129	Uracil
30	Creatine	64	Hexanoyl-carnitine	97	Ornithine	130	Urate
31	Creatinine	65	Hippurate	98	Pantothenate	131	Uridine
32	CTP	66	Histamine	99	Pentose-phosphate	132	UTP
33	Cytidine	67	Histidine	100	Phenylalanine	133	Valine
34	Decanoyl-carnitine						

\*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

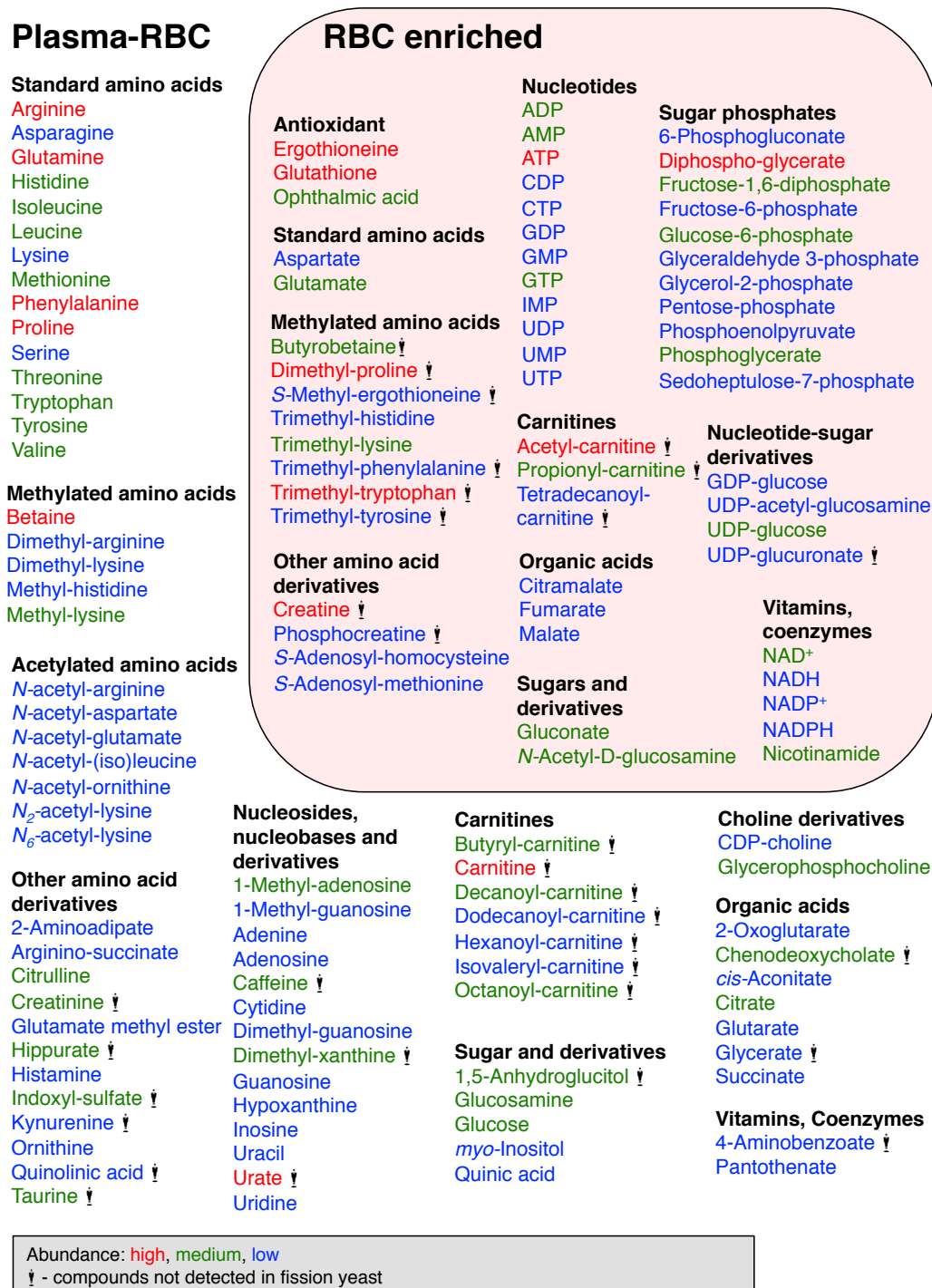
(L), indicating that growing and dividing yeast cells require greater amounts of pentose phosphate pathway intermediates.

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<sup>+</sup>, *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<sup>+</sup>, NADPH, ophthalmic acid, phosphoglycerate, *S*-methyl-

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



Abundance: high, medium, low  
 † - compounds not detected in fission yeast

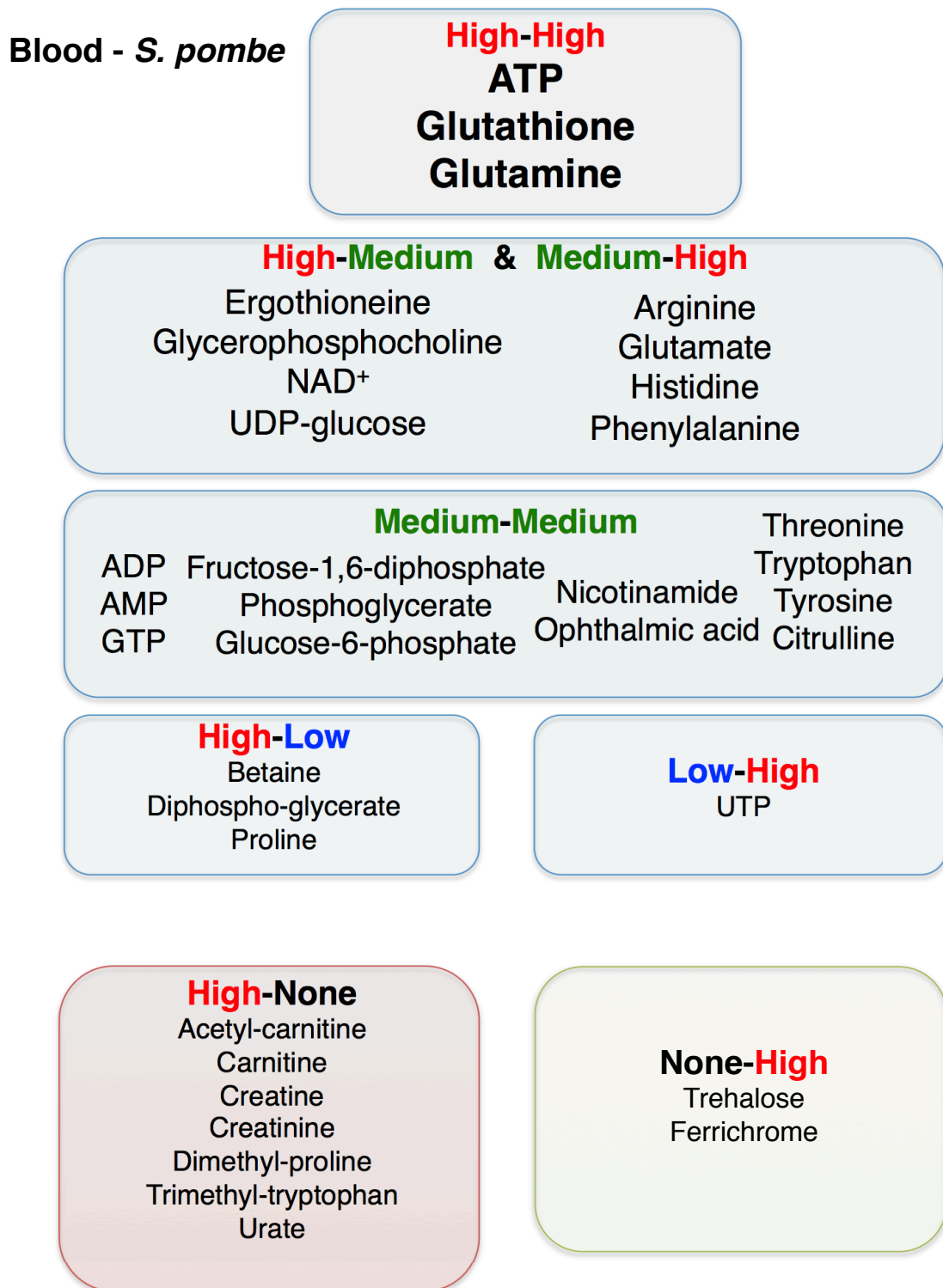
**Figure 18. 133 compounds detected in human blood metabolome samples**

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

<sup>a</sup> 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% ( $81/89 \times 100$ ). 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  $\mu\text{M}$  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).

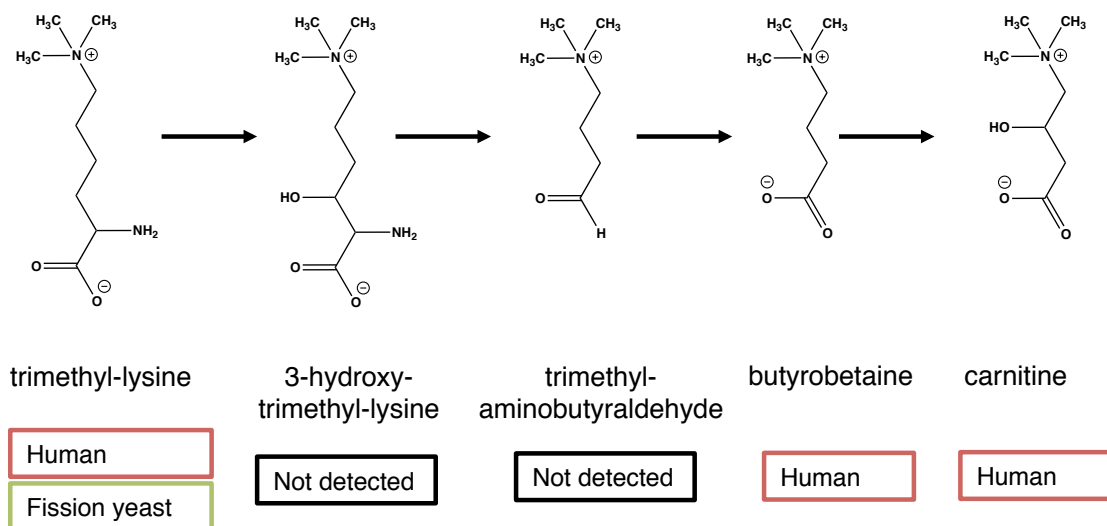
Many of the compounds detected in fission yeast, but not in blood in this study are known to be present at low concentrations in blood or in other tissues. For example, CoA has been reported to be involved in RBC membrane metabolism and at low concentrations (~1  $\mu\text{M}$ ) (Arduini et al. 1992). 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 monosaccharides. Concentrations in blood of disaccharides are less than a few  $\mu\text{g/mL}$  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  $\gamma$ -amino- $\beta$ -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 synthesized 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 synthesize 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.

## Plasma-RBC

### Standard amino acids

Arginine  
Asparagine  
Glutamine  
Histidine  
Isoleucine  
Leucine  
Lysine  
Methionine  
Phenylalanine  
Proline  
Serine  
Threonine  
Tryptophan  
Tyrosine  
Valine

### Methylated amino acids

Betaine  
Dimethyl-arginine  
**Dimethyl-lysine**  
Methyl-histidine  
Methyl-lysine

### Acetylated amino acids

*N*-acetyl-arginine  
*N*-acetyl-aspartate  
***N*-acetyl-glutamate**  
***N*-acetyl-(iso)leucine**  
*N*-acetyl-ornithine  
***N*<sub>2</sub>-acetyl-lysine**  
***N*<sub>6</sub>-acetyl-lysine**

### Other amino acid derivatives

2-Amino adipate  
Arginino-succinate  
Citrulline  
Creatinine †  
**Glutamate methyl ester**  
Hippurate †  
Histamine  
Indoxyl-sulfate †  
Kynurenine †  
Ornithine  
Quinolinic acid †  
Taurine †

### Nucleosides, nucleobases and derivatives

1-Methyl-adenosine  
1-Methyl-guanosine  
Adenine  
Adenosine  
Caffeine †  
Cytidine  
Dimethyl-guanosine  
Dimethyl-xanthine †  
Guanosine  
Hypoxanthine  
Inosine  
Uracil  
Urate †  
Uridine

## RBC enriched

### Antioxidant

Ergothioneine  
Glutathione  
Ophthalmic acid

### Standard amino acids

Aspartate  
Glutamate

### Methylated amino acids

Butyrobetaine †  
Dimethyl-proline †  
S-Methyl-ergothioneine †

### **Trimethyl-histidine**

Trimethyl-lysine  
**Trimethyl-phenylalanine †**  
**Trimethyl-tryptophan †**  
**Trimethyl-tyrosine †**

### Other amino acid derivatives

Creatine †  
Phosphocreatine †  
S-Adenosyl-homocysteine  
S-Adenosyl-methionine

### Nucleotides

ADP  
AMP  
ATP  
CDP  
CTP  
GDP  
GMP  
GTP  
IMP  
UDP  
UMP  
UTP

### Carnitines

Acetyl-carnitine †  
Propionyl-carnitine †  
Tetradecanoyl-carnitine †

### Organic acids

**Citramalate**  
Fumarate  
Malate

### Sugars and derivatives

Gluconate  
*N*-Acetyl-D-glucosamine

### Sugar phosphates

6-Phosphogluconate  
Diphospho-glycerate  
Fructose-1,6-diphosphate  
Fructose-6-phosphate  
Glucose-6-phosphate  
Glyceraldehyde 3-phosphate  
Glycerol-2-phosphate  
Pentose-phosphate  
Phosphoenolpyruvate  
Phosphoglycerate  
Sedoheptulose-7-phosphate

### Nucleotide-sugar derivatives

**GDP-glucose**  
**UDP-acetyl-glucosamine**  
UDP-glucose  
**UDP-glucuronate †**

### Vitamins, coenzymes

NAD<sup>+</sup>  
NADH  
NADP<sup>+</sup>  
NADPH  
Nicotinamide

### Choline derivatives

CDP-choline  
Glycerophosphocholine

### Organic acids

2-Oxoglutarate  
Chenodeoxycholate †  
*cis*-Aconitate  
Citrate  
Glutarate  
Glycerate †  
Succinate

### Vitamins, Coenzymes

4-Aminobenzoate †  
Pantothenate

† - compounds not detected in fission yeast  
**Compound** not reported in blood

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*<sub>2</sub>-acetyl-lysine, *N*<sub>6</sub>-acetyl-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*<sub>2</sub>-acetyl and *N*<sub>6</sub>-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 erythropoiesis (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  $\mu$ M) (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 *Erythrina 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  $\mu$ M (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 HILIC 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	Tetradron	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

<sup>a</sup> 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 ( $8-12 \times 10^8$  RBC) were quenched in 1.8 mL  $-40^\circ$  C 55% methanol. The remainder of each blood sample was centrifuged at  $120 \times g$  for 15 min at room temperature to separate plasma and RBCs. After centrifugation, 0.2 mL of separated plasma and RBCs ( $14-20 \times 10^8$  RBC), respectively, were quenched in 1.8 mL  $-40^\circ$ 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  $\mu\text{L}$  of 50% acetonitrile and 1  $\mu\text{L}$  (equivalent to 5  $\mu\text{L}$  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 ( $\sim 5 \times 10^7$  cells) were quenched in 1.8 mL  $-40^\circ\text{C}$  55% methanol. After addition HEPES and PIPES, samples were processed as described above. Metabolites were isolated from 10  $\mu\text{L}$  of leukocyte cell volume (assuming an individual leukocyte cell volume of 200 fL (Segel et al. 1981) and an estimated  $5 \times 10^7$  cells per sample). One  $\mu\text{L}$  of the metabolome sample was injected into the LC-MS system, corresponding to  $\sim 0.25$   $\mu\text{L}$  leukocyte intracellular volume.

#### **4.6 Fission yeast growth conditions and preparation for metabolomics analysis**

The wild-type heterothallic haploid 972 h<sup>-</sup> *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<sup>8</sup>). 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<sup>-1</sup>. 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.

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## **ACKNOWLEDGMENTS**

This thesis was carried out under the supervision of Prof. Mitsuhiro Yanagida.

I thank Prof. Mitsuhiro Yanagida not only for his excellent supervision during my graduation, but also for my overall training as a member of scientific community. From the very beginning he has been a persistent and responsible teacher. All the way through my studies he has been extremely helpful and motivating.

I am also very grateful to Prof. Tadashi Uemura and Dr. Hiroshi Kondoh for their support, advice and helpful discussions. In addition, I am thankful to the members of the G0 unit in Okinawa and Kondoh lab in Kyoto University hospital for their co-operation and providing pleasant working environment. In particular, I would like to thank Tomáš Pluskal for an introduction into metabolomics, his critical and helpful advice.

In all, I am thankful to everybody for making my graduate studies in Kyoto and Okinawa a nice experience.

I would like to thank my family for support and motivation.

My graduate studies were supported by Japanese government MEXT scholarship.

This thesis is based on material contained in the following scholarly papers.

Chaleckis R, Ebe M, Pluskal T, Murakami I, Kondoh H & Yanagida M

“Unexpected similarities between the Schizosaccharomyces and human blood metabolomes, and novel human metabolites”

Molecular bioSystems, in press, 2014

DOI: 10.1039/c4mb00346b

## LIST OF TABLES

<b>Table</b>	<b>Page</b>
Table 1. Advantages and disadvantages of analytical techniques used in metabolomics research .....	3
Table 2. Comparison of blood cells in healthy individual.....	6
Table 3. Major metabolic pathways in RBC .....	7
Table 4. Recent reports covering RBC metabolome.....	9
Table 5 Comparison of RBC isolation methods .....	16
Table 6. 133 compounds detected in blood metabolome samples .....	22
Table 7. List of 18 identified metabolites detected in fission yeast, but not in blood. ....	26
Table 8. All compounds detected in blood and fission yeast, and their properties .....	40
Table 9. List of analyzed standard compounds.....	55

## LIST OF FIGURES

Figure	Page
Figure 1. “Omics” Sciences: Genomics, Transcriptomics, Proteomics, and Metabolomics .....	1
Figure 2. Biological molecules detectable in metabolomics experiments .....	2
Figure 3. Blood components .....	5
Figure 4. Fission yeast and RBC .....	10
Figure 5. Blood donation .....	12
Figure 6. Methods for isolation of RBCs .....	14
Figure 7. Isolation of blood cells by Ficoll gradient .....	15
Figure 8. Preparation and analysis of blood metabolome samples .....	17
Figure 9. Raw LC-MS data 3D plots of plasma and RBC fractions .....	18
Figure 10. MS/MS analysis of peaks identified as methyl-lysine, <i>N</i> -trimethyl-phenylalanine and <i>N</i> -trimethyl-tyrosine .....	20
Figure 11. Overview of 133 compounds detected in blood by HILIC LC/MS .....	21
Figure 12. Number of peaks and compounds detected in human blood and fission yeast metabolomes. ....	28
Figure 13. Scatter plots of all peaks detected in blood, plasma and RBC samples .....	29
Figure 14. Scatter plots of identified compounds (representative single-charged peaks) in blood, plasma and RBC samples .....	30
Figure 15. Multiple peaks produced by the same compound .....	31
Figure 16. Semi-quantification of carnitines in blood .....	32
Figure 17. Scatter plot comparison of the compounds detected in plasma and RBC samples .....	34
Figure 18. 133 compounds detected in human blood metabolome samples .....	37

Figure 19. Highly abundant compounds in blood and <i>S. pombe</i> .....	39
Figure 20. Carnitine biosynthesis pathway .....	45
Figure 21. Fourteen compounds not yet reported in blood .....	47