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Metabolomics profiles of patients with Wilson disease reveal a distinct metabolic signature

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Abstract

Introduction—Wilson disease (WD) is characterized by excessive intracellular copper accumulation in liver and brain due to defective copper biliary excretion. With highly varied phenotypes and a lack of biomarkers for the different clinical manifestations, diagnosis and treatment can be difficult.

Objective—The aim of the present study was to analyze serum metabolomics profiles of patients with Wilson disease compared to healthy subjects, with the goal of identifying differentially abundant metabolites as potential biomarkers for this condition.

Methods—Hydrophilic interaction liquid chromatography-quadrupole time of flight mass spectrometry was used to evaluate the untargeted serum metabolome of 61 patients with WD (26 hepatic and 25 neurologic subtypes, 10 preclinical) compared to 15 healthy subjects. We conducted analysis of covariance with potential confounders (body mass index, age, sex) as covariates and partial least-squares analysis.

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Author contributions

All authors took part in the study design and contributed to the final draft of the paper. In addition, VM conceived and designed the study, and wrote the paper. GS analyzed and interpreted the data, and wrote the paper. AC and TL provided the samples for the studies. NS proofread, contributed to the discussion, and assisted with arranging the final draft. KK performed the data analysis. DK managed the human subject samples and database, and contributed to data interpretation. All authors read and approved the final manuscript.

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Compliance with ethical standards

This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board at the University of California, Davis.

Data Availability Statement

The metabolomics and metadata reported in this paper are available via Metabolomics Workbench http://www.metabolomicsworkbench.org/, and study can be found under ST001118.

Informed consent

Written informed consent was obtained from each patient and healthy subject in this study.

Results—After adjusting for clinical covariates and multiple testing, we identified 99 significantly different metabolites (FDR < 0.05) between WD and healthy subjects. Subtype comparisons also revealed significantly different metabolites compared to healthy subjects: WD hepatic subtype (67), WD neurologic subtype (57), WD hepatic-neurologic combined (77), and preclinical (36). Pathway analysis revealed these metabolites are involved in amino acid metabolism, the tricarboxylic acid cycle, choline metabolism, and oxidative stress.

Conclusions—Patients with WD are characterized by a distinct metabolomics profile providing new insights into WD pathogenesis and identifying new potential diagnostic biomarkers.

Keywords

copper; metabolomics; biomarkers; phenotype

1 Introduction

Wilson disease (WD) is an autosomal recessive disorder caused by mutations in the copper-transporting, P-type ATPase gene, *ATP7B* (Bull *et al.*, 1993). Disease-causing mutations lead to impaired intracellular copper trafficking and biliary excretion, deficient ceruloplasmin maturation, and consequent copper accumulation mainly in the liver and brain (Czlonkowska *et al.*, 2018). Clinical manifestations of WD are widely variable and include hepatic, neurologic, and psychiatric signs and symptoms. Hepatic manifestations range from mild (elevated liver enzymes) to severe (acute liver failure or cirrhosis). The neurologic signs and symptoms include Parkinson-like tremors, dysarthria, and ataxia (Ala *et al.*, 2007).

Numerous factors appear to influence the WD phenotype. Although many different *ATP7B* mutations have been identified, linking which variants specifically affect enzyme catalytic and transport activity has proven difficult (Huster *et al.*, 2012) Epigenetic, environment, age, and sex-related factors may also influence the WD phenotype (Ferenci *et al.*, 2018; Kieffer and Medici, 2017; Medici *et al.*, 2016; Medici *et al.*, 2014; Medici *et al.*, 2013) further complicating diagnosis. Currently, patients with WD are identified by a combination of clinical and laboratory findings, including low ceruloplasmin, increased urinary copper excretion, elevated hepatic copper levels, and the presence of copper deposits in the cornea (Kayser–Fleischer rings); however, these tests are neither highly specific (Cabras *et al.*, 2015) nor considered a gold standard for diagnostic purposes. Screening for *ATP7B* mutations is helpful though not routinely used in clinical practice due to confusion generated by the high number of potential disease-causing mutations. Also, a negative screening does not exclude a WD diagnosis. Due to these limitations, identifying new WD biomarkers is needed to improve diagnostic tools. High-throughput techniques used to identify biomarkers, such as metabolomic profiling, can deepen our understanding of disease pathogenesis and may eventually lead to new therapeutic targets (Aliasgharpour, 2015).

A small number of in vivo (Lee *et al.*, 2011; Simpson *et al.*, 2004; Wilmarth *et al.*, 2012; Xu *et al.*, 2015) and in vitro (Roelofsen *et al.*, 2004) studies have utilized high-throughput techniques to study copper accumulation. One study used comparative proteome analyses to study asymptomatic and early-stage patients with WD; the results revealed increased serum levels of oxidative stress and inflammation-related proteins in WD (Park *et al.*, 2009).

In the present study, untargeted metabolomics analyses were performed to characterize serum profiles of patients with hepatic, neurologic, or preclinical WD compared to healthy subjects, with the goal of identifying key metabolites and potential diagnostic biomarkers involved in the WD pathogenesis.

2 Subjects and Methods

Serum samples from patients with WD and healthy subjects were obtained from the Institute of Neurology and Psychiatry in Warsaw (Table 1). Subjects fasted for 8 hours prior to sampling. Whole venous blood was collected in 3 × 7.5 ml plastic Vacutainer tubes and allowed to clot for 45–60 min. Blood samples were centrifuged for 10 minutes at 1500 × g (4C), and serum aliquoted and stored at −80C until shipped. Serum samples were de-identified, shipped to the University of California, Davis and stored at −80°C until further analysis. Hemolytic samples were excluded. Informed written consent was obtained from each patient and healthy subject, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board at the University of California, Davis. Patients with WD were recruited at diagnosis [determined by Leipzig criteria (Ferenci *et al.*, 2003)] and hence were not on any anti-copper treatment. According to the clinician's experience, patients were categorized by hepatic or neurologic presentation and grouped as "symptomatic." A subgroup of asymptomatic patients was diagnosed based on a family member's diagnosis and defined as "preclinical."

Untargeted metabolomics profiling was performed at the UC Davis West Coast Metabolomics Center by hydrophilic interaction liquid chromatography-quadrupole time of flight mass spectrometry (HILIC-QTOF MS).

For data analysis methods, see Supplementary Data.

3 Results

A total of 363 distinct metabolites were identified between 15 healthy and 61 patients with WD. After adjusting for clinical covariates and multiple testing, 99 metabolites (including 36 annotated) were found to be significantly different in WD compared to healthy subjects with false discovery rate (FDR) <0.05 (Fig. 1a). A partial least-squares regression with linear discriminant analysis (PLS-LDA) was able to separate WD patients from healthy controls based on their metabolomics profiles (Fig. 1b), with 97.37% classification accuracy using only the first latent component for the covariate-adjusted analysis and 100% using the first three latent components for the covariate-unadjusted analysis.

Among the 36 annotated metabolites (Table S1), 9 were more abundant and 27 were less abundant in WD. These metabolites included amino acids associated with the tricarboxylic acid (TCA) cycle (glutamic acid and α-ketoglutarate) and choline metabolism (cysteine), and metabolites related to the gut microbiota (indole-2-propionic acid, glucose, and glutamine). Glutamic acid, sorbitol, pseudo-uridine, and threonic acid exhibited changes greater than 2-fold between WD and healthy subjects. Figure 2 shows differentially

abundant serum metabolites selected based on their link to copper metabolism and oxidative stress.

Further, we analyzed sex-specific metabolite profiles between WD and healthy subjects. Thirty-two metabolites were significantly different (FDR<0.05) in males while 56 metabolites were significant in females (Fig. S1, S2). Among them, 8 and 32 metabolites were found to be sex-specific in males and females, respectively. When comparing male and female patients with WD, no metabolites were found to differ significantly at FDR<0.05 (Fig. S3).

3.1 Metabolomics profiling analyses based on clinical manifestations

Metabolomics profiles for hepatic and neurologic presentations were analyzed in patients with WD compared to healthy subjects. Post hoc pairwise subtype comparisons, after adjusting for clinical covariates, identified 67 significantly different metabolites (FDR<0.05) between healthy and hepatic subtype, and 57 between healthy and neurologic subtype. Twenty-one of the 67 metabolites between healthy and hepatic were unique, including 2-hydroxybutanoic acid, 2-ketoisocaproic acid, α-ketoglutarate, glycerol-alpha-phosphate, oxamic acid, urea, and uridine. Eleven unique metabolites were identified between healthy and neurologic, including pyrophosphate and uric acid (Fig. 3a, Table S2). No significant metabolites were identified when directly comparing hepatic and neurologic subtypes after FDR correction. PLS-LDA analysis could not discriminate well between hepatic and neurologic subtypes by the first two latent components, although healthy and combined WD phenotypes (neurologic and hepatic combined) showed clear separation (Fig. 3b). Volcano plots for differential analysis, comparing healthy to hepatic and healthy to neurologic, are shown in Fig. S4.

3.2 Metabolomics profiling analyses based on diagnosis (preclinical and symptomatic)

Patients with WD were subdivided into either preclinical or symptomatic (HN, hepatic and neurologic combined) and compared to healthy subjects. Post hoc pairwise subtype comparisons, after adjusting for clinical covariates, resulted in 77 significant metabolites (FDR<0.05) between healthy and HN, and 36 between healthy and preclinical subtypes. Forty-three metabolites were specific to healthy vs. HN whereas only 2 metabolites were specific to healthy vs. preclinical (Table S3). No significant metabolites were found between HN and preclinical subtypes after FDR correction. Volcano plots for differential analysis, comparing healthy to HN and healthy to preclinical, are shown in Fig. 3c and 3d.

3.3 Pathways associated with differentially abundant metabolites in patients with WD compared to healthy subjects

Differentially abundant metabolites in WD compared to healthy subjects were used to identify pathways and biological functions potentially impacted by WD (Fig. 4). The metabolic pathway associated with the highest number of significant metabolites was aminoacyl-tRNA biosynthesis. The most affected pathways, in general, were related to amino acid metabolism, biosynthesis, and degradation. The metabolites associated with each pathway are listed in Table S4. For our discussion, we selected metabolites involved in major biophysiological pathways such as the TCA cycle, methionine metabolism, and

glutathione (GSH) biosynthesis. Importantly, these metabolites are also related to copper metabolism, oxidative stress, and WD.

4 Discussion

Uncertain phenotype-genotype correlations, variability in clinical manifestations, and lack of specific diagnostic biomarkers are some of the challenges faced in WD diagnosis and treatment. Understanding WD pathogenesis and enabling access to improved diagnostic methods is of great importance. Metabolomics studies provide a useful approach to do this by identifying key metabolites with potential diagnostic and innovative therapeutic significance (Kim *et al.*, 2016).

In this study, HILIC-QTOF MS-based serum metabolomics revealed patients with WD can be distinguished from healthy subjects based on metabolites associated with aminoacyl-tRNA biosynthesis, the TCA cycle, and choline metabolism, and metabolites related to gut microbiota. Metabolite profile comparisons could also distinguish between clinical manifestations (hepatic from neurologic) as well as healthy from preclinical subtypes. Although most of the identified metabolites were concordant between the hepatic, neurologic, and preclinical groups, we postulate a subset of differentially abundant metabolites can serve as potential WD biomarkers, providing new insights into its pathogenesis.

Copper-induced oxidative stress is known to contribute to the pathogenesis of WD (Kalita *et al.*, 2014; Nagasaka *et al.*, 2006). Our data reveal a significant alteration in metabolite levels which might be linked to copper-induced oxidative stress (Fig. 5). Among the increased annotated metabolites, sorbitol was augmented more than 3-fold in WD. Sorbitol is a sugar alcohol and plays an important role in the polyol pathway. Underlying mechanisms for the higher sorbitol are unknown, but it might be associated with glucose metabolism. Excess copper accumulation has long been reported to inhibit glycolysis and some of its enzymes (Lai and Blass, 1984), theoretically resulting in cytoplasmic accumulation of excess glucose. Removal of glucose from the cell, mediated by the polyol pathway, could cause an increased release of sorbitol into the bloodstream. Previous studies in diabetes have also indicated that impaired glucocorticoid receptor (GR) signaling induces sorbitol accumulation via polyol pathways (Eriksson *et al.*, 1986; Gaynes and Watkins, 1989; Poulsom and Heath, 1983) and there is evidence showing a positive correlation between blood copper level and risk for developing diabetes mellitus, possibly due to augmented copper-induced oxidative stress (Zhang *et al.*, 2017). Our analysis confirmed a previous finding in $Atp7b^{-/-}$ mice, a mouse model of WD, of higher plasma sorbitol levels (Wilmarth *et al.*, 2012) . These mice also exhibited significantly reduced GR levels, which might indicate attenuated GR signaling. Furthermore, glucose is converted to pyruvate during glycolysis. Pyruvate enters the mitochondrion where it is converted into acetyl-CoA by pyruvate dehydrogenase (PDH). Since excess copper leads to copper-induced oxidative stress, which produces reactive oxygen species (ROS) known to inhibit PDH (Sheline and Choi, 2004), excess pyruvate may accumulate and be converted to sorbitol. In diabetes, sorbitol can also lead to increased production of mitochondrial superoxide anion radicals causing poly(ADP-ribose) polymerase activation which has been reported to exacerbate oxidative stress further through

generation of ROS and reactive nitrogen species (Naik and Kokil, 2013; Obrosova, 2005). Sorbitol has also been shown to have a strong copper-chelating action (Briggs *et al.*, 1981), thus high sorbitol levels in WD patients could help maintain cellular homeostasis by attempting chelation of excess copper.

We observed significant changes in metabolites related to the TCA cycle in patients with WD compared to healthy subjects. In particular, glutamic acid and α-ketoglutarate were both found to be increased in WD. α-ketoglutarate is an intermediate in the TCA cycle interconvertible with glutamic acid by transamination; therefore, glutamic acid can directly impact energy metabolism via the TCA cycle (Maus and Peters, 2017; Zheng *et al.*, 2014). Copper-induced ROS was shown to block the TCA cycle by inhibiting α-ketoglutarate dehydrogenase, which converts α-ketoglutarate to succinyl CoA (Sheline and Choi, 2004), with a consequent increase in α-ketoglutarate levels. The elevated glutamic acid levels observed in WD patients are likely due to conversion from the increased α-ketoglutarate. Our data showing high glutamic acid levels is consistent with previously published data indicating that copper-induced oxidative stress might be responsible for elevated glutamic acid and cytokines (Kalita *et al.*, 2014). Copper, then, may have duo impact on the TCA cycle through oxidative stress affecting both α-ketoglutarate dehydrogenase and glutamic acid.

Metabolites in GSH biosynthesis and methionine metabolism e.g., cysteine, 2-hydroxybutanoic acid, and oxoproline, were significantly different in WD. Cysteine was reduced more than 2-fold in patients with WD. Cysteine functions as an antioxidant; clinical studies have shown cysteine supplementation improves skeletal muscle and immune system functions by reducing plasma levels of tumor necrosis factor α, decreasing body fat vs. lean body mass, and increasing plasma albumin levels (Droge, 2005). N-acetyl-cysteine, a derivative of cysteine, is used in the treatment of alcoholic hepatitis and acetaminophen poisoning (Whyte *et al.*, 2007). Cysteine is also a primary component in GSH production, a critical antioxidant against oxidative stress, particularly in the liver. In a mouse model with a hepatocyte-specific deletion in the glutamate-cysteine ligase catalytic subunit gene, causing impaired hepatic GSH production, mice became moribund by about 4 weeks of age (Chen *et al.*, 2007). They exhibited highly elevated liver enzymes, steatosis, abnormal rough endoplasmic reticulum, and multiple abnormal hepatic mitochondria morphologies leading to compromised membrane potential and respiration. Previous studies by us and others showed copper accumulation is associated with reduced S-adenosyl homocysteine hydrolase expression and activity (Li *et al.*, 2007; Medici *et al.*, 2013), possibly leading to reduced cysteine and GSH levels with consequent compromised antioxidant defense. This may explain the reduced plasma cysteine observed in this study and previous reports of significantly low hepatic GSH in WD patients (Summer and Eisenburg, 1985). Orally administered cysteine can also act as a copper chelator by forming a copper-cysteine complex, thereby inhibiting copper absorption in the gut (Baker and Czarnecki-Maulden, 1987). In untreated patients with WD, high plasma copper levels were shown to inhibit L-cysteine/L-cystine uptake in erythrocytes (Mandal *et al.*, 2016) and GSH concentration was found to be reduced and significantly dependent on the L-cysteine/L-cystine uptake. Excess copper, then, can reduce cysteine bioavailability which may lead to increased cysteine demand (Robbins and Baker, 1980).

2-hydroxybutanoic acid (2-HBA) is derived from α-ketobutyrate as a byproduct of GSH anabolism when cystathionine is converted to cysteine. Elevated levels of 2-HBA serves as an early indicator for insulin resistance and compromised glucose regulation, and seems to be derived via increased lipid oxidation and oxidative stress (Gall *et al.*, 2010). 2-HBA is primarily produced in the liver upon excessive demand for GSH under increased oxidative stress. In the WD hepatic phenotype, therefore, copper-induced oxidative stress and reduced cysteine levels might be possible underlying mechanisms involved in up-regulation of 2-HBA.

Oxoproline, reduced 1.5-fold in WD, is another metabolite involved with GSH biosynthesis and is converted to glutamate by 5-oxoprolinase. Previous studies have suggested 5-oxoproline levels indicate the availability of glycine and/or cysteine (Metges, 2000). Low levels of oxoproline may be a consequence of reduced cysteine levels in WD.

An intermediate likely contributing to many of the above altered metabolites is the iron sulfur cluster (ISC). ISCs play key roles in biochemical processes such as energy production, metabolic conversions, DNA maintenance, gene expression regulation, protein translation, and the anti-viral response (Braymer and Lill, 2017). They are required for functional enzymes involved in the TCA cycle e.g., succinate dehydrogenase and aconitase, and respiratory chain complexes I–III (Rouault, 2012) as well as for amino acid biosynthesis, including cysteine and glutamate (Rocha and Dancis, 2016).

In eukaryotic cells, mitochondrial ISC assembly proteins function in an environment known to contain a copper(I) pool. With a stronger binding affinity, it has been shown in vitro that copper can preferentially displace iron in the ISC assembly (Brancaccio *et al.*, 2017). It is also established that ISCs are highly vulnerable to ROS damage (Vernis et al., 2017). Moreover, previous studies in E. coli revealed copper overload inactivated ISC enzymes such as isopropyl malate dehydratase, fumarase A, and 6-phospho-gluconate dehydratase (Macomber and Imlay, 2009). ISC assembly processes are highly conserved among yeast, bacteria, and humans (Rouault, 2012); therefore, within the scope of our data, it is possible the impact of excess copper on ISCs is two-fold – directly inhibiting ISC synthesis or damaging existing ISCs by iron displacement and indirectly damaging existing ISCs by ROS generation with subsequent ROS-induced damage. This could lead to a deleterious cascade in which cysteine and glutamic acid levels and subsequent glutathione levels in WD patients are decreased. Since cysteine is also a required component of assembly proteins that form ISCs (Vallieres et al., 2017), reduced cysteine levels can further compound ISC damage as glutathione is required for maturation of cytosolic ISC proteins (Sipos et al., 2002) thus creating a continuously detrimental cycle of ISC/amino acid/glutathione damage and reduction.

Our study revealed threonic acid was reduced more than 3-fold in serum of patients with WD. Threonic acid is a breakdown product of ascorbic acid (AA) metabolism. This metabolite is linked with the insufficiency or deprivation of AA which scavenges free radicals such as oxygen, superoxide, and hydroxyl radicals (Gao *et al.*, 2012; Thomas and Hughes, 1983). Previous reports described significant AA reduction and compromised antioxidant defense in untreated WD patients (Attri *et al.*, 2006; Ogihara *et al.*, 1995), and

linked copper-mediated oxidative stress to mitochondrial dysfunction (Rossi *et al.*, 2004). AA accumulates in mitochondria as a protective agent where most free radicals are produced. If increased oxidative stress in WD mitochondria is a valid inference, increased AA utilization should initially elevate threonic acid until AA levels are depleted, at which point the threonic acid level would decrease.

Uric acid levels were reduced in WD neurologic vs. healthy subjects. Low plasma uric acid levels at presentation with hepatic or neurologic manifestations in patients with WD have been previously described and may be due to renal tubular failure and reduced uric acid synthesis in the liver (Nussinson *et al.*, 2013; Roberts *et al.*, 2008; Umeki *et al.*, 1986; Wang *et al.*, 2015). Reduced levels of uric acid were also associated with neurodegenerative diseases like Parkinson's disease (Winquist *et al.*, 2010). Uric acid acts as a natural antioxidant (Ames *et al.*, 1981) and low serum uric acid likely indicates, again, reduced antioxidant defense in WD.

We found reduced uridine in WD hepatic compared to healthy subjects. Uridine plays many important roles in hepatic metabolism, including liver detoxification, immune homeostasis (Chong *et al.*, 1999), enhancement of mitochondrial function (Banasch *et al.*, 2006), and suppression of fat accumulation (Le *et al.*, 2013); however, the exact mechanisms regulating its protective roles are unknown. As liver controls the formation and degradation of uridine (Gasser *et al.*, 1981), hepatic copper-induced liver damage in WD patients might be causatively associated with low uridine levels.

An additional novel finding is reduced indole-3-proprionic acid (IPA) levels in our patients with WD. In the human gastrointestinal microbiome, IPA is endogenously produced and detected in vivo only in the presence of *Clostridium sporogenes* (Wikoff *et al.*, 2009). By using tryptophan, *C. sporogenes* synthesizes indole and, subsequently, IPA at detectable levels in the host plasma. IPA plays a role in fibrosis and inflammatory-related gene suppression in proximal tubular cells (Yisireyili *et al.*, 2017) and protects hepatic microsomal membranes from iron-induced oxidative damage in cancer (Karbownik *et al.*, 2001). Human hepatocytes cultured with IPA also demonstrated attenuated copper- and arsenic-mediated mitochondrial and cellular DNA damage (Chinnasamy *et al.*, 2016). Considering its multiple protective roles, low IPA levels indicate yet another facet of compromised cellular defense in WD.

Microbiomics has become a very dynamic field with recently published data supporting the importance of microbiome-associated bacteria and their products for liver homeostasis (Mazagova *et al.*, 2015) and copper chelation in hepatocyte mitochondria (Lichtmannegger *et al.*, 2016), and dietary copper impact on microbial activity (Song *et al.*, 2018). Peripherally, the copper-regulatory role of ATP7B in intestinal epithelial cells has also been reported (Pierson *et al.*, 2018). Other metabolites from our analyses are also associated with microbial activity, but merit a separate space for adequate discussion. As such, we suspect microbiome status has a significant impact on WD copper metabolism, warranting further microbiomic analyses.

Metabolomics profiling has been studied in conditions that are included in the differential diagnosis of WD. One of the most extensive studies on metabolomics in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis (NASH) included the serum and liver metabolomics profiles in more than 500 patients (Alonso *et al.*, 2017). The profile of different subtypes of NASH patients included different levels of amino acids (methionine, glutamic acid, taurine, aspartic acid, and glutamine), bile acids, fatty acids, triglycerides, glycerophospholipids, and sphingomyelins. The metabolic profile of autoimmune hepatitis was explored in 2 main studies. One of the studies described two bile acids, three long-chain acylcarnitines, seven glycerophospholipids, a bilirubin, and a retinyl ester as being associated with early autoimmune hepatitis (Zhou *et al.*, 2016). The second study showed that autoimmune hepatitis was associated with changes in metabolites typically associated with changes in energy metabolism, including pyruvate, lactate, acetate, acetoacetate, and glucose (Wang *et al.*, 2014). Therefore, even though we cannot make a direct comparison, it appears a WD metabolomics profile has distinct features that could be implemented in the diagnostic process of this condition.

In summary, we investigated the serum metabolomics profiles of a group of patients with WD compared to healthy subjects, identifying differentially abundant metabolites that may contribute to understanding WD pathogenesis and could lead to novel diagnostic biomarker development. Study limitations include the lack of significant findings in subgroup comparisons after correction for multiple testing at FDR<0.05 due to the relatively small number of subjects in subgroups. Untargeted metabolomics profiling also has the disadvantage of detecting high numbers of unannotated compounds, which was the case in our analysis. Many of the unannotated metabolites could have biological implications relevant to WD but could not be studied. Future validation studies in gene/gene expression analysis should be conducted to confirm the present findings and determine the impact of any altered enzymatic activity, providing more mechanistic explanations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations:

WD	Wilson disease
HILIC-QTOF MS	hydrophilic interaction liquid chromatography-quadrupole time of flight mass spectrometry
PLS-LDA	partial least-squares regression with linear discriminant analysis

FDR	false discovery rate
TCA	tricarboxylic acid
HN	hepatic-neurologic manifestations combined
GSH	glutathione
GR	glucocorticoid receptor
PDH	pyruvate dehydrogenase
ROS	reactive oxygen species
2-HBA	2-hydroxybutanoic acid
AA	ascorbic acid
IPA	indole-3-proprionic acid

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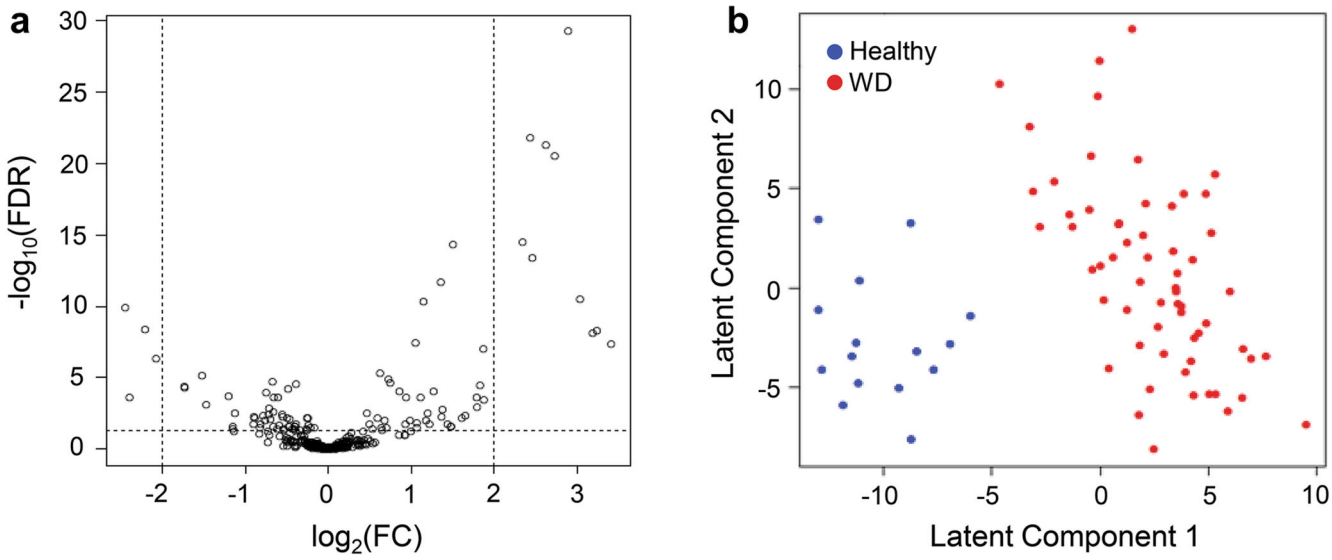

Fig. 1.

Metabolomics profiling analyses for healthy subjects vs. WD patients

1a – Volcano plot of metabolite abundance changes (covariate-adjusted). The x-axis specifies the $\log_2$ fold changes and the y-axis specifies the negative logarithm to the base 10 of the FDR values. Dotted vertical and horizontal lines reflect the filtering criteria ($\log_2$ FC= ±2.0 and FDR=0.05). 1b – Score plots of the PLS-LDA analysis distinguishing WD patients (n=61) from healthy subjects (n=15) based on their metabolomics pattern. WD, Wilson disease; FC, fold change; FDR, false discovery rate, PLS-LDA, partial least-squares regression with linear discriminant analysis

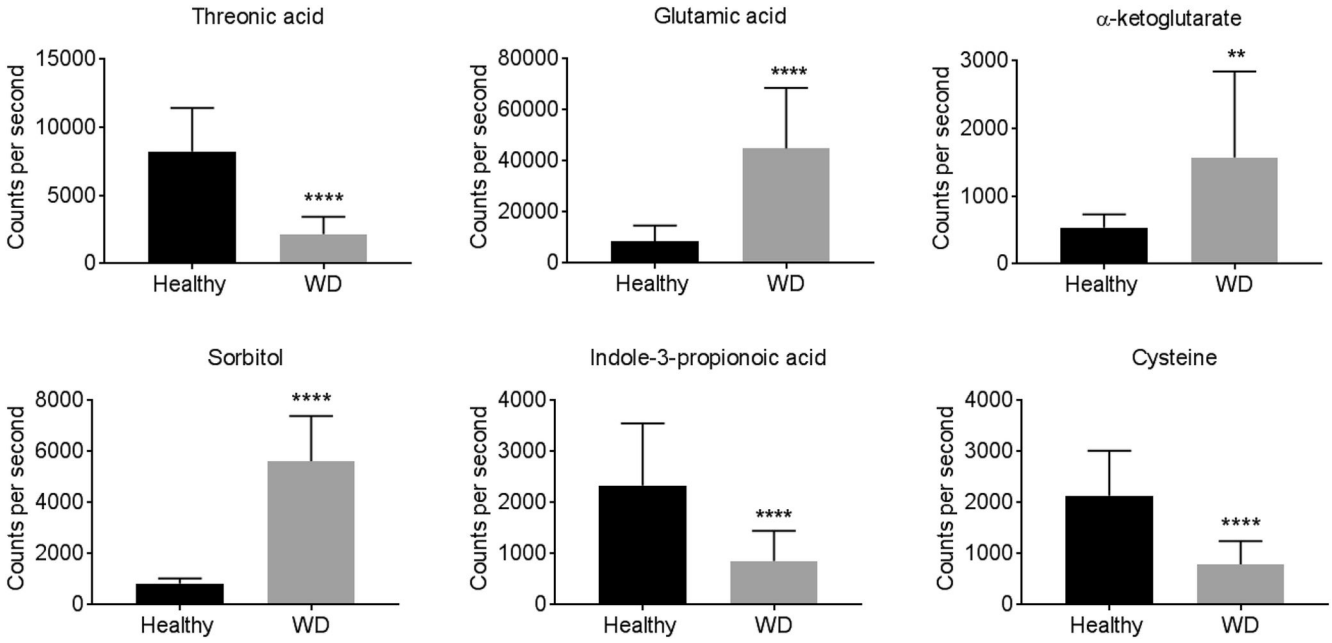

Fig. 2.

Comparison of select serum metabolites between healthy subjects and WD patients

Results are presented as mean ± SD; healthy subjects n=15, WD patients n=61. **p< 0.01, ****p< 0.0001 compared to healthy subjects. WD, Wilson disease

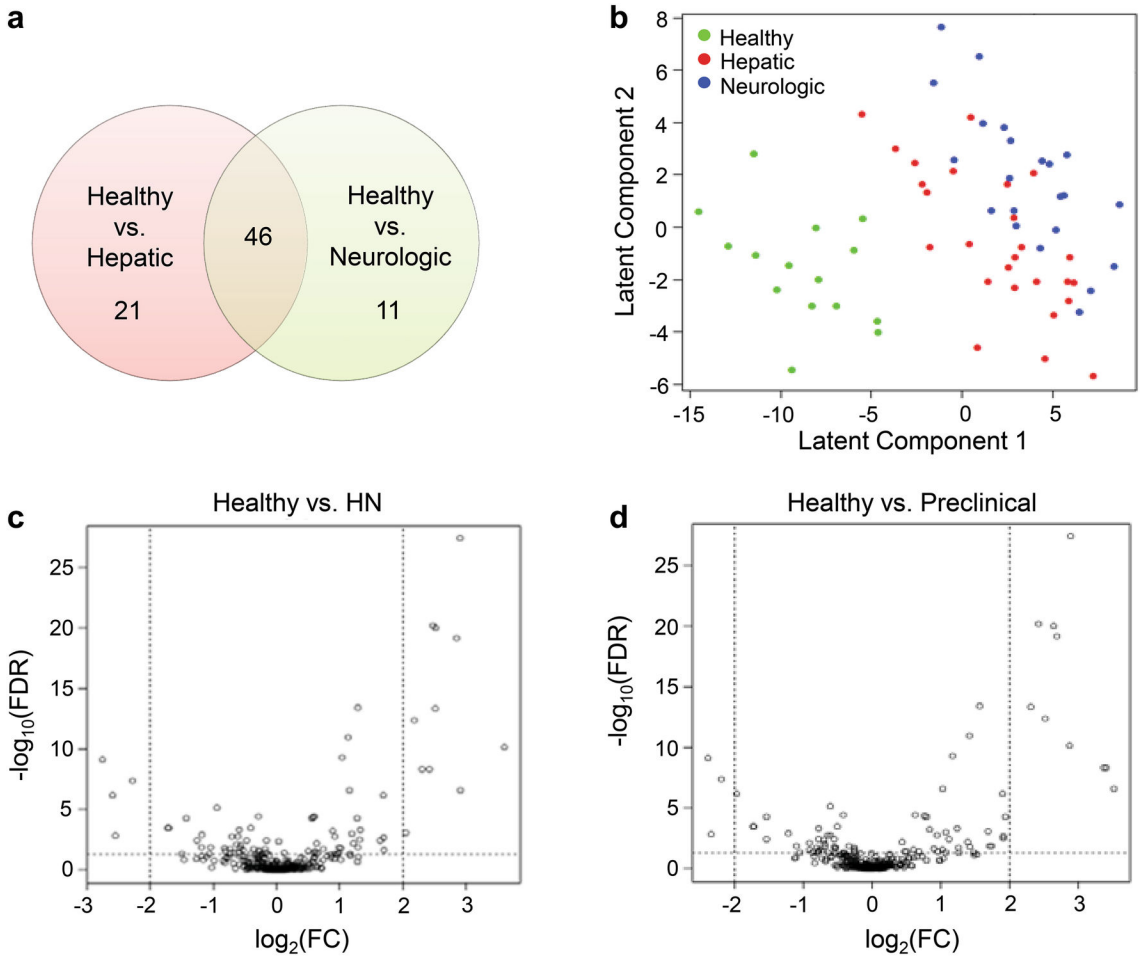

Fig. 3.

Metabolomics profiling analyses based on clinical manifestations

3a – Number of unique and concordant metabolites in healthy vs. hepatic and healthy vs. neurologic. 3b – Score plots of the PLS-LDA analysis for healthy subjects, hepatic subtype, and neurologic subtype. 3c, 3d – Volcano plot of metabolite abundance changes (covariate-adjusted), comparing comparing healthy vs. HN (3c) and healthy vs. preclinical (3d). The x-axis specifies the $\log_2$ fold changes and the y-axis specifies the negative logarithm to the base 10 of the FDR values. Dotted vertical and horizontal lines reflect the filtering criteria ($\log_2$ FC=± 2.0 and FDR=0.05). FC, fold change; FDR, false discovery rate; PLS-LDA, partial least-squares regression with linear discriminant analysis

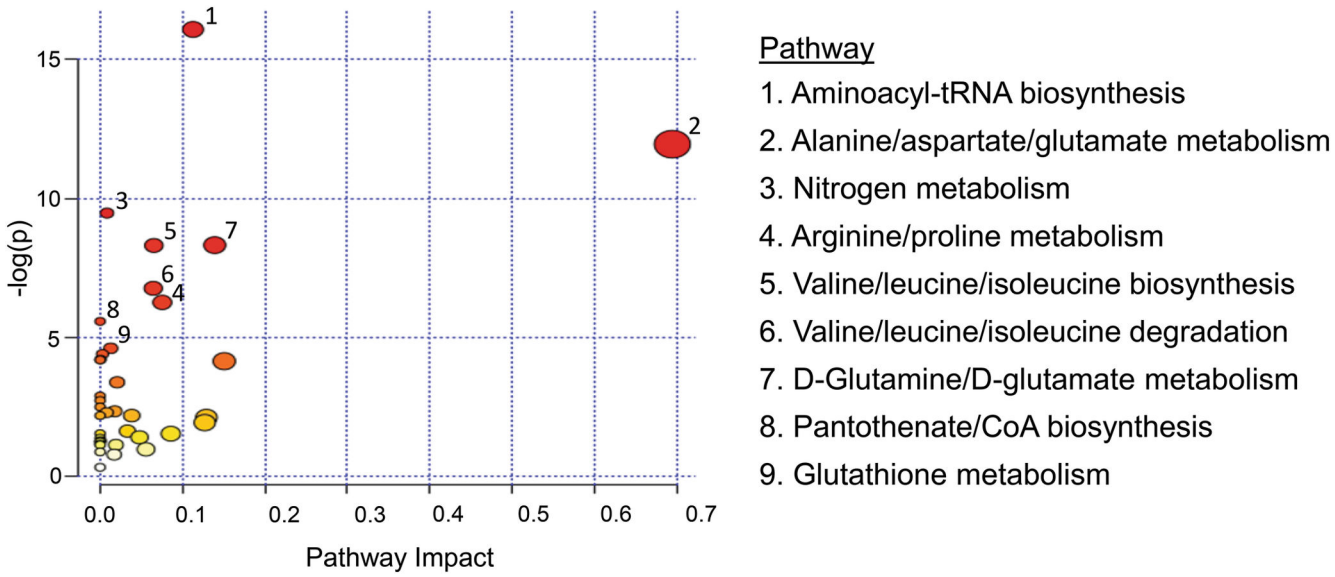

Fig. 4.

Pathway analysis by MetaboAnalyst: metabolome view X-axis – pathway impact values from pathway topology analysis; y-axis – matched pathways from pathway enrichment analysis arranged by -log(*p*-value). Red indicates the most significant effects according to *p*-value and the node size is determined by pathway impact value

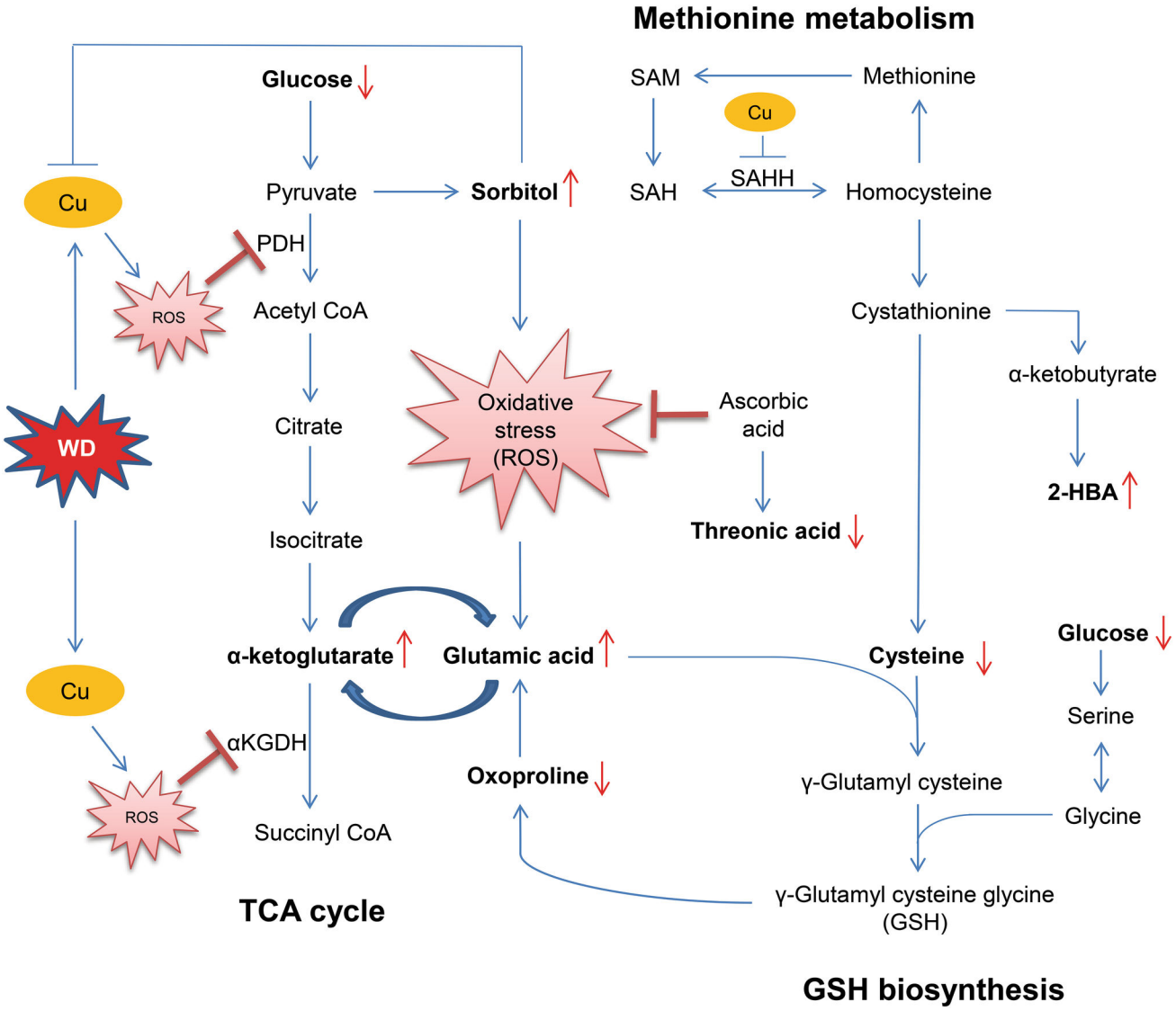

Fig. 5.

Network of key metabolites and their associated pathways relevant to copper and oxidative stress in WD Differential metabolites are indicated in bold text. Red arrows represent decreased or increased levels in the serum of WD patients. 2-HBA, 2-hydroxybutanoic acid; αKGDH, α-ketoglutarate dehydrogenase; Cu, copper; GSH, glutathione; PDH, pyruvate dehydrogenase; ROS, reactive oxygen species; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine

Table 1

Patient characteristics

	Healthy (n=15)		Wilson Disease (n=61)		*p*-value
	Males (n=5)	Females (n=10)	Males[a] (n=31)	Females (n=30)	
Age (years), mean ± SD	34.4 ± 11.4	37.7 ± 10.0	35.7 ± 10.5	32.3 ± 13.2	0.5926[b] 0.2408[c]
BMI (kg/m^2), mean ± SD	26.6 ± 3.9	22.0 ± 3.0	26.4 ± 6.2	25.0 ± 3.3	0.9351[b] 0.0139[c]
Hepatic phenotype, n (% total WD)	N/A	N/A	12 (20.0%)	14 (23.3%)	N/A
Neurologic phenotype, n (% total WD)	N/A	N/A	13 (21.7%)	9 (15.0%)	N/A
Preclinical, n (% total WD)	N/A	N/A	5 (8.3%)	7 (11.7%)	N/A

[a] Phenotype not available for one patient.

[b] males

[c] females.

BMI, body mass index.