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# Genetics and epigenetic factors of Wilson disease

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**Abstract:** Wilson disease (WD) is a complex condition due to copper accumulation mainly in the liver and brain. The genetic base of WD is represented by pathogenic mutations of the copper-transporting gene *ATP7B* with consequent lack of copper excretion through the biliary tract. *ATP7B* is the only gene so far identified and known to be responsible for the development of the disease. Our understanding of the disease has been evolving as functional studies have associated specific disease-causing mutations with specific copper-transporter impairments. The most frequent variant in patients of European descent is the H1069Q missense mutation and it has been associated with protein misfolding, aberrant phosphorylation of the P-domain, and altered ATP binding orientation and affinity. Conversely, there is much less understanding of the relation between the genotype and the clinical manifestations of WD. WD is characterized by a highly varied and unpredictable presentation with different combined hepatic, neurological, and psychiatric symptoms. Several studies have attempted to correlate genotype and phenotype but the most recent evidences on larger populations failed to identify a relation between genotype and clinical presentations. Given that so far also modifier genes have not shown convincing association with WD, there is growing interest to identify epigenetic mechanisms of gene expression regulation as underlying the onset and progression of WD phenotype. Evidence from animal models indicated changes in methionine metabolism regulation with possible effects on DNA methylation. Mouse models of WD have indicated transcript level changes of genes related to DNA methylation in fetal and adult livers. And finally, evidence is accumulating regarding DNA methylation changes in patients with WD. It is unexplored how *ATP7B* genetic mutations combine with epigenetic changes to affect the phenotype. In conclusion, WD is a genetic disease with a complex regulation of its phenotype that includes molecular genetics and epigenetic mechanisms.

**Keywords:** Copper; gene; methylation

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## ***ATP7B* gene, protein structure and function**

Wilson disease (WD) is considered a monogenic autosomal recessive disease and *ATP7B* is the only gene that has been identified as causative of this condition. Disease-causing mutations affecting the *ATP7B* gene are associated with lack of biliary copper excretion and its consequent accumulation in the hepatocytes. The *ATP7B* gene encodes

for a transmembrane P-type cation copper transporter ATPase and can be affected by homozygous or compound heterozygous mutations, meaning that each gene allele presents a different mutation. The gene is located on human chromosome 13q14, it contains 20 introns and 21 exons, and almost 800 mutations have been described according to the Human Gene Mutation Database (accessed January 2019) (1-3). Not all mutations are causative of

copper accumulation. The clinical phenotype and the relation between *ATP7B* gene mutations with the extent of the copper transporter function impairment are not predictable (4). Therefore, even the most advanced technologies for genetic diagnosis, including next generation sequencing, still require evidence of abnormal copper metabolism parameters for the definite clinical diagnosis of WD (5). There have been reports of pseudo-dominant inheritance where the disease presents in 2 or more consecutive generations within one family (6,7). Rarely, one individual can present with three mutations. Cases of segmental uniparental disomy, occurring when both copies of a single chromosome are inherited from one parent, have been described (8).

The *ATP7B* gene is highly expressed in the liver but the protein is also detected in the brain, kidney, lung, intestinal epithelial cells, placenta, and mammary glands (9). The gene encodes for a 1465-aminoacid protein which is synthesized in the endoplasmic reticulum and is expressed in the trans-Golgi network (10). The ATP7B protein belongs to class 1B (PIB) P-type ATPase superfamily, responsible for actively exporting copper and other heavy metal ions from the cytoplasm of cells. These highly conserved structure proteins present a phosphatase domain (A-domain), a phosphorylation domain (P-domain), a nucleotide-binding domain (N-domain), and M-domains, which includes eight transmembrane ion channels. The copper transport through the membrane is an active complex mechanism which is ATP-dependent and functionally affected by the gene mutations. Many of the described mutations map at the N-domain, potentially impairing the transporter function by interfering with the ATP binding (11,12).

### **ATP7B gene mutations and population prevalence**

More than 50% of the described mutations are single-nucleotide missense and nonsense, whereas the remaining are insertions/deletions and splice site mutations. The missense mutation His1069Gln on exon 14 is the most prevalent in patients of Central and Eastern European descent (13). However, its prevalence varies greatly according to the geographic area. The allele frequency is the highest in Poland, Latvia, Former East Germany, Bulgaria, and Czech Republic ranging from 72% to 57% (14-18). The mutation is also the one with highest prevalence in North America with about 40% allele frequency (19). A study on a large group of Polish patients with WD using

whole-exome sequencing, confirmed the high prevalence of the H1069Q mutation and identified other low-prevalence mutations (20). Another described mutation in Central and Eastern Europe is the G710S on exon 8, having a prevalence of 6.4% in Austria (21). In United Kingdom, the most frequent mutation is the H1069Q but with a prevalence of 19%, much lower compared to other European countries (8). The second most common identified mutation in UK is the Met769Val with a frequency of 6% (8). Patients from continental Italy present a lower prevalence of the H1069Q mutation (17.5%) and other mutations (p.Val845fs and p.Met769fs) are described with a prevalence lower than 10% (22). Spain represents an exception compared to other European countries with a high frequency of the missense mutation M645R on exon 6 (23). A study on French patients with WD also identified the M645R as highly prevalent with a carrier frequency of 1.8% (5). Regions where consanguinity is highly prevalent, present different patterns of mutations distribution. Sardinia has a high prevalence (60%) of the deletion c.-441\_-427del15 in the *ATP7B* gene promoter region (24) and Canary Island has a 64% allele frequency of the p.Leu708Pro (25). In Costa Rica, patients present with a high prevalence (61%) of the N1270S mutation (19), also described in Italian and Turkish populations (26,27). In China, Japan, and Korea the prevalent mutation is the missense R778L on exon 8 with an allele frequency ranging from 45% to 12% (28-31). In Japan, the 2871delC and 2874delC are also described (32). This variant is associated with replacement of arginine with leucine in the transmembrane domain. India is a vast country with high ethnic diversity and different prevalence of ATP7B mutations has been described according to the geographic localization. As pointed out by Gomes et al, the p.Cys271 mutation appears to be the most frequent mutation in the Indian sub-continent (33,34) even though it has not been described in the north of the country.

### **ATP7B mutations, copper transporter function, and genotype-phenotype correlation**

Whereas the pathogenesis of WD is known to be rooted in copper accumulation and ATP7B copper transporter impaired function, it is much less clear if and how *ATP7B* mutations influence the phenotype. At the molecular level, previous studies have shown a relationship between the type of gene mutation and the extent of copper transporter functional impairment. A study has characterized *in vitro*

and *in silico* the activity changes induced by the H1069Q mutation showing that its main consequences are protein misfolding, aberrant phosphorylation of the P-domain, and altered ATP binding orientation and affinity (35). Another study using immunogold electron microscopy to explore the intracellular localization of ATP7B in liver tissue from WD patients homozygous for the H1069Q mutation, identified an aberrant localization of the mutated protein in the endoplasmic reticulum whereas the non-mutated ATP7B was in the trans-Golgi network (36). An extensive *in vitro* study assessed the catalytic and transport activity as well as the intracellular localization of 28 ATP7B variants. The selected variants, including the H1069Q, were all known to be associated with clinical manifestations. Various mutations were associated with different functional impairments but notably variants domain-localization was not the cause of the same type or extent of dysfunction (37). Common missense mutations tend to impair ATP7B stability and are associated with its reduced levels (38,39). In addition, several mutations impair only partially ATP7B function with some residual ATP binding and copper transporter activity (36). There is evidence that certain ATP7B polymorphisms may interact with metabolic factors, specifically copper availability, with effects on the copper transporter activity itself. In particular, the Arg875 causes lack of copper transporter activity but in response to elevated copper, the trafficking activity was restored along with the copper delivery (40). A study on 59 patients with WD found that protein-truncating mutations were associated with lower serum ceruloplasmin oxidase activity compared to missense mutations (41). A study from India identified 28 *ATP7B* variants in 50 patients with WD and conducted *in vitro* functional analysis describing 5 new variants associated with protein misfolding (34). When transitioning from the molecular to the disease level, several studies embarked in the difficult task to identify a correlation between genotype and phenotype but the results have been non-conclusive and affected by several challenges. First, most patients are compound heterozygous. Second, the clinical practice (for example, hepatology versus neurology) could have influenced the initial selection of cases and in some cases, liver or neurological signs or symptoms may have not been properly investigated or diagnosed. Third, most studies include a overall small number of cases, assessed and diagnosed with non-standardized criteria, often uniquely based on the single clinician experience. The most recent and relevant evidence of a limited role of the genotype on the phenotypic manifestations is provided by a European

study on more than 1300 patients with WD, including 702 children and 655 adults. Patients presented either hepatic or neurological manifestations and more than 50% of them had a liver biopsy-confirmed diagnosis. The described population included both acute liver failure and cirrhosis patients carrying a high allele frequency of the H1069Q mutation (46.9%) (42). The major finding of the study was the lack of correlation between *ATP7B* gene mutations and the phenotype. It should be mentioned that this study was preceded by multiple other smaller studies attempting to identify a genotype-phenotype correlation. In particular, the H1069Q mutation was associated more frequently with neurological phenotype in some studies (16,43). A Dutch study showed an association between homozygous H1069Q and neurological phenotype (44). A study on 58 pediatric patients with WD affected by 34 different *ATP7B* mutations showed that nonsense and frameshift mutations were associated with lower serum ceruloplasmin and copper levels (45). Genotype-phenotype correlation studies in 126 Bulgarian patients presenting a H1069Q allele frequency in 78% of cases indicated an association between the mutation and hepatic presentation (46). Tarnacka *et al.* reported on 148 Polish patients with a high H1069Q frequency and did not find any genotype-phenotype correlation (47).

Other interesting information can be derived from studies describing siblings and homozygous twins. Chabik *et al.* studied 73 unrelated Polish families including 73 index cases and 95 siblings, presenting with a H1069Q allelic frequency of 77% (48). They found a high concordance rate of the initial clinical presentations, being 86% concordance for hepatic symptoms and 66% for neurological symptoms. However, there are also case reports of siblings and homozygous twins presenting discordant clinical presentations or different post liver transplant outcomes (49-51). A Japanese study in 11 families including 23 sibilings diagnosed with WD described that 5 families had identical phenotypes and 6 families different phenotypes (52). Interestingly, there is evidence of geographical clustering within regions with smaller or isolated communities with various levels of consanguinity and presenting phenotype homogeneity. Two large families in small mountain community in the region of Rucar in Romania were studied given the high prevalence of WD cases (53). Of the 50 screened living members, 5 individuals with clinical diagnosis of WD and 2 asymptomatic subjects presented the H1069Q/M769H mutations. Of note, there was a significant phenotypic concordance between all WD patients presenting neurological and psychiatric phenotype.

Patients presented mostly dysarthria and dysphagia as initial symptoms and had similar age of onset (17–20 years old). The authors concluded that their findings demonstrate the influence of both genetic and environmental factors on the phenotype (53). More than 70 members of a single Lebanese family were investigated for the c.2299insC and p.Ala1003Thr mutations. The clinical diagnosis of WD was confirmed in 9 subjects (54). The c-2299insC mutation was associated hepatic and the Ala1003Thr was associated with neurological phenotype. A study on 4 generations of an Italian family, showed a possible association between the homozygotic mutation T1288R and the hepatic phenotype (55). Different evidence comes from Gran Canaria, where the Leu708Pro mutation was highly prevalent, affecting 18 out of 24 patients with WD, including 12 homozygous patients, 4 compound heterozygous, and 2 with only one identified mutation (25). However, the phenotype was variable and included prevalently neurological manifestations but also hepatic involvement with no obvious association between the genotype and phenotype. Therefore, despite the methodological challenges, the evidence points to the presence of additional factors, other than gene mutations, affecting the clinical presentation.

### Modifier genes

A modifier gene has been defined as a gene that alters the expression of a gene at another locus or the phenotypic expression of another gene (56). It is plausible to hypothesize the presence of modifier genes influencing the varied WD phenotype. Several genes have been proposed as possible candidates for this role. Kluska *et al.* conducted whole-exome sequencing analysis on 248 patients with WD (20). The study identified two new possible variants in esterase D (*ESD*) and *INO80* genes being associated with increased and reduced risk of neurological presentation, respectively. *ESD*, encoding for a polymorphic red cell enzyme, was previously linked to WD (57). *INO80* has important transcription regulation functions through chromatin remodeling action (58). In addition, rare *APOE* and *MBD6* variants were associated with lower risk of early onset WD (20). Other studies have previously associated *APOE* variants with WD (59), in one case with delayed neurological symptoms (60). However, other studies could not confirm the association (61,62). Interestingly, *MBD6* encodes for a protein belonging to the methyl-CpG-binding domain family. These proteins have central regulating

function in epigenetic mechanisms, including the readout of DNA methylation (63). *MBD6* interacts with the human deubiquitinase complex and reported to be a target of Oct4 in stem cells derived from adipose tissue (64). Conversely, the analysis could not confirm any association between WD phenotypes and other previously proposed modifier genes allelic variants (20). Even though the study results may have been affected by the relatively small population or by the analysis methodologies, the results question the validity and clinical significance of previous findings on candidate modifier genes.

A candidate modifier gene with mechanistic relevance is the patatin-like phospholipase domain-containing 3 gene (*PNPLA3*). *PNPLA3* rs738409 polymorphism has been associated with increased risk of nonalcoholic fatty liver disease development (65) and with hepatic steatosis in hepatitis B (66) and C (67). The protein presents various functions in lipids metabolism as it can both synthesize intracellular triglycerides and also has hydrolyzing activity against triglycerides (68,69). Therefore, mutations affecting its function could favor steatosis. A study on 98 male patients with WD showed that, on multivariate logistic regression, *PNPLA3* was an independent variable associated with moderate/severe steatosis (70). *MTHFR* mutations are also potential genetic modifiers of WD. *MTHFR* encodes 5,10-methylenetetrahydrofolate reductase, an enzyme in methionine metabolism that affect homocysteine levels. Two polymorphisms in *MTHFR* were associated with WD expressivity. The *MTHFR* 667T allele was associated with hepatic phenotype and the *MTHFR* 1298C allele was associated with earlier WD presentation (71). This is interesting as it provides evidence that aberrant homocysteine and methionine metabolism may interact with copper accumulation in the pathogenesis of WD and provides a possible explanation for the variable expressivity of this monogenetic disease.

Other previously proposed modifier genes for WD include antioxidant 1 copper chaperone (*ATOX*) (72), copper metabolism domain-containing 1 (*COMMD*) (73–75), X-linked inhibitor of apoptosis (*XIAP*) (76), hemochromatosis gene (*HFE*) (77–79), human prion gene (*PrP*) (80), *ATP7A* (81), and divalent metal transporter1 (*DMT1*) (81) but none was confirmed in additional or larger studies (Table 1).

### Introduction to epigenetics

The field of epigenetics, literally meaning “on top of

**Table 1** Proposed modifier genes for Wilson disease

Proposed modifier gene	Studied population(s)	Publication year	Associated phenotype	Results validation
<i>APOE</i>	121 WD patients (60)	2000	Epsilon3/3 was associated with delayed onset	Results not confirmed by Gu <i>et al.</i> (61) and Kocabay <i>et al.</i> (62);
	383 WD patients (59)	2012	Women with Epsilon4 have earlier onset of symptoms	Kluska <i>et al.</i> reduced risk of early onset WD (20)
<i>COMMD1</i>	63 WD patients (73)	2004	GAT/GAC heterozygous: 19% with hepatic presentation; 25% with neurological presentation	Results not confirmed by Weiss <i>et al.</i> (75) and Bost <i>et al.</i> (74)
<i>PRNP</i>	134 WD patients (80)	2006	M129V: similar prevalence compared to control; PRNP codon 129: later disease onset and later neurological presentation	No additional studies
<i>ATOX1</i>	63 WD patients (72)	2008	5'UTR-99 T>C: earlier onset of disease	Results not confirmed by Bost <i>et al.</i> (74)
<i>XIAP</i>	98 WD patients (76)	2010	p.T470S and p.N340S: early disease onset	No additional studies
<i>HFE</i>	32 WD patients (77)	2010	H63D mutation in 20% of cases, increased hepatic iron concentration	Results not confirmed by Erhardt <i>et al.</i> (78), and Pfeifferberger <i>et al.</i> (79)
<i>MTHFR</i>	245 WD patients (71)	2011	C677T allele: hepatic onset; A1298: younger age at presentation	No additional studies
<i>ATP7A</i>	108 WD patients (81)	2014	No association with WD	No additional studies
<i>DMT1</i>	108 WD patients (81)	2014	IVS4 C(+): in 83% of patients with WD; no association with phenotype	No additional studies
<i>PNPLA3</i>	98 WD patients (70)	2015	G allele associated with moderate/severe steatosis	No additional studies
<i>ESD</i>	248 WD patients (20)	2018	Increased risk of neurological phenotype	No additional studies
<i>INO80</i>	248 WD patients (20)	2018	Reduced risk of neurological phenotype	No additional studies

*APOE*, Apolipoprotein E; *COMMD1*, copper metabolism domain-containing 1; *PRPN*, Prion Protein; *ATOX1*, human homologue antioxidant 1 copper chaperone; *XIAP*, X-linked inhibitor of apoptosis protein; *HFE*, hemochromatosis; *MTHFR*, 5, 10-methylenetetrahydrofolate reductase; *ATP7A*, ATPase copper transporting alpha; *DMT1*, divalent metal transporter 1; *PNPLA3*, patatin-like phospholipase domain-containing 3 gene; *ESD*, esterase D; *INO80*, INO80 complex subunit; WD, Wilson disease.

genetics”, lies at the critical intersection where nature meets nurture, allowing a static genome to adapt to a dynamic environment. Epigenetics can explain long-lived effects from in utero and early life environments on human disease susceptibility and resilience. The “Developmental Origins of Adult Disease” hypothesis (82) relies heavily of epigenetic mechanisms to explain how maternal in utero effects can influence risk for common diseases in adulthood, including obesity, cancer, cardiovascular disease, addiction, and other mental health disorders (83,84).

Epigenetic information is layered on top of the DNA sequence through covalent modifications to nucleotides or nucleosomes (85,86). The first layer of epigenetic information is DNA methylation. The majority of CpG

(meaning cytosine preceding a guanine) dinucleotides in the human genome are methylated at the 5' position of cytosine. In contrast, CpGs are predominantly unmethylated when they are located in “CpG islands” at the promoters of approximately 70% of mammalian gene promoters. DNA methylation patterns are dynamically erased and reestablished each generation during the formation of the gametes, which occurs in utero in mammals (87). Implantation of the embryo is another critical time for genome-wide changes in DNA methylation patterns that can be modified by maternal environment. Adding to the complexity of DNA methylation dynamics, there are variations on 5-methylcytidine (mC), including 5-hydroxymethylation (hmC), which is correlated with gene



activity and demethylation (88). Furthermore, while CpG dinucleotides are the most methylated, the more abundant CpH (C preceding an A, T, or C) dinucleotides are methylated at low frequency in mature oocytes, embryonic stem cells, and postmitotic neurons.

While DNA methylation has historically been considered to be a mechanism for silencing gene expression, the genome-wide view has demonstrated multiple complexities to that simple interpretation. 5-methylcytosine at CpG sites at gene promoters is associated with gene repression. In contrast, a positive association with expression and CpG methylation is observed over gene bodies, particularly in pre-implantation embryonic tissues and placenta (87,89,90). Interpretation of CpH methylation is less clear, although it appears to mark genes that were active in earlier development that were later silenced (91,92). In these ways, the layer of DNA methylation can provide important clues about past, present, and future gene expression patterns.

In addition to DNA methylation, there are several other layers of epigenetic information. DNA in the nucleus is organized and assembled through the action of nucleosomes, which are octamers of pairs of four histone core subunits (H2A, H2B, H3, H4) and the H1 linker histone. All of the core histones have N- and C-terminal tails that are post-translationally modified by methylation, phosphorylation, ubiquitylation, acetylation, to name a few (93). A large number of nuclear proteins in mammalian genomes are dedicated to the writing, reading, or erasing of these histone modifications collectively known as the “histone code”. Genome-wide, histone codes have been mapped for a large variety of human tissues, developmental stages, and cell types in order to functionally annotate the genome into promoters versus enhancers, active versus repressed versus “poised” genes, as well as inactive heterochromatin. Chromatin loops between enhancers-promoter pairs, or groups of genes with a single “super-enhancer” have also been mapped in specific cell types and tissues genome-wide (94). Lastly, noncoding RNAs are emerging as having a variety of functional roles in defining chromatin states and chromatin loop interactions between promoters and enhancers (95).

### Integration of methionine metabolism with epigenetics and copper

There is a large variability in the expressivity of WD in terms of the age of onset, severity, response to treatment, and impacts to the liver versus brain. Analysis of hundreds

different disease-causing mutations in WD have failed to find convincing evidence of genotype-phenotype correlations. Therefore, WD appears to be a genetic disease with clear environmental and epigenetic modifications that are likely due to the complex relationship between copper accumulation and methionine metabolism pathways (96). Methionine metabolism in liver and brain is an important pathway that regulates the supply of methyl groups required for modifications of proteins, DNA, and RNA through the availability of the universal methyl donor S-adenosylmethionine (SAM). Methionine adenosyltransferase utilizes ATP to convert methionine into SAM. S-adenosylhomocysteine hydrolase (AHCY) is a key enzyme regulating the amount of SAM available for methylation reactions, since it bidirectionally catalyzes the conversion of SAM to S-adenosylhomocysteine (SAH). Bethin *et al.* initially demonstrated that in toxic milk mouse livers AHCY has copper binding properties and the enzymes levels were reduced by 42% compared to mice with normal copper metabolism (97). Subsequent studies demonstrated that excess copper inhibits the activity of AHCY (98). AHCY inhibition leads to reduced SAM/SAH ratios, inhibition of MAT, and impaired methylation reactions. Therefore, some of the variability of WD symptoms is likely to be due to wide-scale alterations in methyl donor supplies.

Studies on the toxic milk (tx-j) mouse model of WD are consistent with methionine metabolism alterations due to copper accumulation. Specifically, tx-j mice show reduced AHCY transcript and protein levels and elevated SAH and SAH/SAM ratios compared to wild-type mice (99). The methyl donor nutrient betaine counteracted the genetic effect, demonstrating further evidence that the tx-j phenotype could be modified by dietary methyl donors (99). Similar data in Long-Evans Cinnamon rats demonstrated a correlation between hepatic copper accumulation and reduced transcript levels of MAT and changes in methionine metabolism (100).

### Integration of copper with mitochondrial oxidative stress and epigenetics

Another way that excess copper levels in WD may impact epigenetic layers is through the mitochondrial oxidative stress pathways. The intracellular levels of reactive oxygen species (ROS) increase as a byproduct from mitochondrial activity during normal cellular metabolism. While ROS are signal transduction molecules that are part of normal

metabolism, high levels of ROS can cause detrimental effects on multiple cell components, including DNA, lipids, proteins, membranes, and mitochondria. ROS and additional mitochondrial metabolites have a detrimental impact on epigenetic layers, including DNA methylation and histone modifications such as methylation and acetylation (101,102).

Exposure to non-physiological levels of trace elements such as copper contributes to mitochondrial dysfunction, including oxidative stress. Other trace metals with an observed epigenetic impact in mammalian tissues, include nickel and iron. Interestingly, iron acts as a cofactor for the TET family proteins that catalyze the oxidation of DNA methylation from methylated to hydroxymethylated cytosine, while nickel acts as an inhibitor of the same reaction (103). Furthermore, nickel, chromium, and arsenic appear to be mechanistically involved in alterations to histone post-translational modifications by altering actions of the enzymes or “writers” that modify them (104). Therefore, the impact on the epigenome from even mild alterations to metals may have both direct effects on epigenetic enzymes or indirect effects through the mitochondrial and oxidative stress pathways.

### **Evidence for epigenetic and methionine alterations in animal models of WD**

Since alterations in methionine metabolism can directly affect methylation reactions to DNA and histone tails, the copper-induced alterations to methionine pathways can also adversely impact many downstream gene targets through epigenetics. The tx-j mouse model of WD supports epigenetic alterations in WD. An initial time-course study on tx-j mice livers from fetal life to adulthood, showed global DNA methylation starting at 20 weeks of age with a significant negative correlation with *Dnmt3a* and *Dnmt3b* transcript levels (105). Pooling data from 20 and 28 week old mice, global DNA methylation correlated negatively with hepatic copper concentration and with SAH levels and correlate positively with SAM/SAH ratio. Tx-j liver samples show reduced *Dnmt3b* transcript levels and global DNA methylation levels compared to wild-type that were able to be restored through betaine supplementation (105). In addition to methionine, mitochondrial pathways directly impacted by excess copper accumulation result in ROS and other metabolites that influence epigenetic control of gene expression throughout the genome (106).

Epigenetic mechanisms are especially important in

utero, because this is a critical time when the instructions of the genome are being followed within the context of the maternal environment, diet, and metabolism. In WD, this is particularly evident in the tx-j mouse model, as choline supplementation to the diet of dams from 2 weeks during pregnancy to gestational day 17 resulted in a 17% increase in global DNA methylation levels in the liver (107). Maternal choline supplementation also prevented the transcriptional deficits in fetal tx-j liver for multiple genes related to cell growth and rescued reduced body weight phenotypes of tx-j mice (107). Interestingly, copper levels in fetal livers of tx-j mice are actually lower than wild-type, which is the opposite pattern of the copper accumulation in postnatal livers of tx-j mice. What the two stages have in common is deficits in copper transport due to *Atp7b* mutation, as well as alterations to DNA methylation observed in liver.

Recently, we have applied genome-wide approaches to identifying the genes impacted by epigenetic changes in fetal livers of tx-j mice, as well as the effect of maternal choline supplementation (108). Using a whole methylome approach called whole genome bisulfite sequencing, we identified six differentially methylated genes with genome-wide significance, including *Atp7b* itself, as well as genes involved in oxidative stress and thioredoxin, including *Gpx4*, *Prdx2*, and *Hif1a*. Importantly, we also demonstrated that maternal choline supplementation corrected the tx-j differential methylation patterns observed. Secondly, by performing genome-wide analyses of transcriptional differences in the tx-j liver, we were able to demonstrate several associations between methylation and transcriptional changes in tx-j liver with and without choline supplementation (108). Together these results provided genome-wide evidence for epigenetic alterations in the tx-j model of WD that could be corrected through maternal choline supplementation.

### **Evidence for epigenetic alterations in samples from patients with WD**

Unlike mouse model studies of WD in which the genetics and dietary methyl donors can be controlled, human studies have been more challenging to assess the role of epigenetics. However, genome-wide methylome sequencing approaches have been recently utilized in order to gain important insights into epigenetic changes in human WD liver and blood. We utilized whole-genome bisulfite sequencing (WGBS) to identify differentially methylated regions that distinguished liver biopsies from



WD patients compared to both healthy and disease control subjects with other liver diseases. 18 regions at genome-wide significance were identified, as well as thousands of others at lower confidence, that were able to discriminate a WD-specific epigenomic signature. WD-specific differentially methylated regions were enriched for liver-specific enhancers and genes with functions in folate and lipid metabolism and acute inflammatory response.

We then sought to determine if blood samples from WD patients with hepatic versus neurologic symptoms could be distinguished by WGBS epigenomic signatures. While the differentially methylated regions identified were fewer than those identified in blood, there was a significant overlap (31% also in liver) with the >200 regions that distinguished WD blood DNA from healthy control or disease control samples. More importantly, differentially methylated regions in WD blood were identified that clearly discriminated those patients with hepatic versus neurologic symptoms in two different cohorts. These preliminary results suggest that epigenetic biomarkers in blood could be useful clinically in predicting the symptom and treatment types for early stage WD patients. To explore this possibility, we developed a classifier algorithm that predicted neurologic versus hepatic phenotypes in a separate cohort with 90% accuracy. Together these results suggest the potential clinical utility of mapping epigenetic alterations in WD human patients (109).

### Sex and epigenetic differences

WD clinical presentations can be different between males and females (110,111) and the potential explanation could be related to epigenetics mechanisms. Acute liver failure presents as WD onset more frequently in females whereas the neurological phenotype tends to manifest more in male patients. The explanation for these differences has not been completely clarified but in general has been attributed to hormonal influences or differences in iron metabolism (112). Hormonal factors are most likely involved but it is also likely that epigenetics factors are contributing to sex differences. Methylome and transcriptome differences have been described in human liver and brains and are associated with metabolic differences (113). Our studies in tx-j mice showed that female mice presented higher hepatic copper concentration compared to male mice after treatment with copper chelator penicillamine and global hepatic DNA methylation was different between male and female mice after treatment with choline (106). Moreover, female mice

showed changes of hepatic transcript levels of genes related to oxidative phosphorylation in response to choline and penicillamine treatments (106).

### Conclusions and questions for future research

Disease-causing mutations affecting the *ATP7B* gene cause a wide variety of functional alterations of the copper transporter. Genetic mutations are ultimately responsible for copper accumulation but other factors affect the clinical presentation. The interactions between genetic mutations and epigenetic factors may be the explanation for the heterogeneous phenotypic presentation of WD.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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