



HAL
open science

ATP7B variant spectrum in a French pediatric Wilson disease cohort

Eduardo Couchonnal, Sophie Bouchard, Thomas Damgaard Sandahl, Cecile Pagan, Laurence Lion-Francois, Olivier Guillaud, Dalila Habes, Dominique Debray, Thierry Lamireau, Pierre Broue, et al.

► **To cite this version:**

Eduardo Couchonnal, Sophie Bouchard, Thomas Damgaard Sandahl, Cecile Pagan, Laurence Lion-Francois, et al.. ATP7B variant spectrum in a French pediatric Wilson disease cohort. *European Journal of Medical Genetics*, 2021, 64 (10), pp.104305. 10.1016/j.ejmg.2021.104305 . hal-03468275

HAL Id: hal-03468275

<https://hal.science/hal-03468275>

Submitted on 22 Aug 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

ATP7B variant spectrum in a French pediatric Wilson disease cohort

Eduardo Couchonnal^{1,2}, Sophie Bouchard³, Thomas Damgaard Sandahl^{2,4}, Cecile Pagan⁵, Laurence Lion-François^{1,2}, Olivier Guillaud^{1,2}, Dalila Habes^{2,6}, Dominique Debray^{2,7}, Thierry Lamireau⁸, Pierre Broué⁹, Alexandre Fabre^{10,11}, Claire Vanlemmens¹², Rodolphe Sobesky^{2,13}, Frederic Gottrand¹⁴, Laure Bridoux-Henno¹⁵, Abdelouahed Belmalih^{1,2}, Aurelia Poujois^{2,16}, Anne Sophie Brunet¹, Alain Lachaux^{1,2,3}, Muriel Bost^{1,2,5}.

¹ Hospices Civils de Lyon. National Center for Wilson's disease and Department of pediatric gastroenterology, hepatology and nutrition children's hospital of Lyon, France.

² European Reference Network on Hepatological Diseases (ERN RARE-LIVER)

³ Claude Bernard Lyon 1 university Lyon, France.

⁴ Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus N, Denmark.

⁵ Department of Biochemistry and Molecular Biology, LBMMS, Hospices Civils de Lyon, France

⁶ Pediatric Hepatology and Liver Transplantation Unit, National Reference Centre for Biliary Atresia and Genetic Cholestasis, FILFOIE, ERN RARE LIVER, Bicêtre Hospital, Assistance Publique – Hôpitaux de Paris, Le Kremlin-Bicêtre, France.

⁷ Inserm U1193, Hepatinov, University of Paris Saclay, Orsay, France.

⁸ Children's Hospital, Paediatric Gastroenterology Unit, Bordeaux, France.

⁹ Children University Hospital, Metabolic Disease Department, Toulouse, France.

¹⁰ APH, Timone Enfant, Service de pédiatrie multidisciplinaire, Marseille, France.

¹¹ Aix Marseille Univ, INSERM, MMG ; Marseille, France.

¹² University Hospital of Besancon, Paediatric Gastroenterology Unit, Besacon, France,

¹³ Paul Brousse Hospital, Hepatobiliary Centre, Hepatobiliary Centre, France,

¹⁴ Univ- Lille, CHU Lille, UMR1286 Department of Pediatric Gastroenterology Hepatology and Nutrition, Lille, France.

¹⁵ CHU Rennes, Department of Pediatric Gastroenterology, Rennes, France.

¹⁶ French National Rare Disease Reference Centre "Wilson's disease and other copper-related rare diseases", Rothschild Foundation Hospital, Neurology Department, Paris, France.

The authors report no conflicts of interest

Authors' contribution

EC wrote the manuscript and performed statistical calculations

SB, TS, CP, and MB helped in writing the manuscript

OG performed statistical calculations and revised the manuscript

DH, DD, TL, PB, AF, CV, RS, FG, LBH, AP: contributed to data and revision of the manuscript

AB: collected the data

ASB and AL conceived and developed the project.

MB: manuscript revision and approval.

Address correspondence and reprint requests to Eduardo Couchonnal.

Service de Gastroentérologie, Hépatologie et Nutrition. Hôpital Femme Mère Enfant.
59 boulevard Pinel 69677 Bron cedex, France (email : eduardo.couchonnal-
bedoya@chu-lyon.fr) Tel +33 4 27 35 70 50 ;

Keywords : Wilson's disease, *ATP7B*, p.His1069Gln, phenotype-genotype
correlation

Abbreviations

WD: Wilson disease

ALF acute liver failure

UCE: urinary copper excretion

H: hepatic manifestations

N: neurologic manifestations

AS: asymptomatic

KF ring: Kayser-Fleischer ring

MRI: magnetic resonance imaging.

ABSTRACT

Background/Aim: The spectrum of *ATP7B* variants varies significantly according to geographic distribution, and there is insufficient data on the variants observed in the French population.

Methods. Clinical data of 113 children included in the French WD national registry were gathered from 01/03/1995 to 01/07/2020. Data included epidemiological, clinical, laboratory, genetics.

Results: Diagnosis was made at a mean age of 11.0 ± 4.1 years (range 1-18 years). At diagnosis, 91 patients (79.8%) had hepatic manifestations, 18 (15.8%) presented neurological manifestations, and 4 patients (3.5%) were asymptomatic. Only 29 patients (25%) were homozygous for a variant. We have found a total of 102 different variants including 14 novel variants. Recurrent variant p.His1069Gln was the most prevalent, $n = 31$ alleles (14,2%), with only seven homozygous; in contrast 55% of variants are identified in only one family. 45% were truncating variants. In respect of mutated exon, the three most prevalent were exon 14 (16.5%), exon 8 (13.8%), and exon 3 (11.5%).

When considering patients with two Nonsense / Frameshift variants as a group and those with two Missense variants, we found significantly lower ceruloplasmin for the former: 2.8 ± 0.7 mg/dl vs 8.4 ± 5 mg/dl ($p < 0.05$).

Conclusion: p.His1069Gln is the most frequent variant (14,2%) and exons 14, 8, and 2 of the *ATP7B* gene account for 41.7% of total variants. However, there is significant heterogeneity in the French population concerning the other *ATP7B*

variants. Nonsense / Frameshift variants were associated with lower ceruloplasmin levels.

INTRODUCTION

Wilson's disease (WD) is a rare autosomal recessive genetic disorder associated with *ATP7B* gene variants and causing an impaired biliary copper elimination and consequently a copper accumulation, mainly in the liver and the brain. Clinical expressions are variable; the hepatic manifestations prevail during childhood, and the first neurological signs classically appear in the second and third decades of life as the copper accumulation progresses ^(1, 2). The prevalence of WD is considered as 1:30,000, although the existing data on WD epidemiology and genetics are somewhat contradictory ⁽³⁻⁶⁾. According to the recent data from the European population, WD's prevalence may account for 1:10,000 or 1:7,000, being much more frequent than the earlier estimations ⁽⁷⁾. Such a contradiction may occur due to poor diagnosis of WD, the presence of latent forms, various methodological issues and presence of consanguinity which increases disease frequency.

The diagnosis of WD is based on a pattern of clinical signs, laboratory results, and genetic analysis ^(2, 8). To establish a WD diagnosis can be particularly challenging in paediatric cases since young children often do not display clinical and laboratory hallmarks (elevated urinary copper excretion, Kayser-Fleischer ring, abnormal brain MRI) ⁽²⁾, thus enhancing the importance of concomitant genetic analysis.

To date, more than 900 WD-causing variants in the *ATP7B* gene have been identified in WD patients, and most of them have been identified in compound heterozygote patients. Despite considerable research efforts, this broad spectrum of

phenotypic and genotypic WD heterogeneities has hampered the study of phenotype-genotype correlation.

The spectrum of *ATP7B* variants varies significantly between different geographical populations. p.His1069Gln is considered the most frequent variant in the European population whereas p.Arg778Leu and c.del441-427 are the most common in the Asian population and Sardinia respectively⁽⁹⁾. In France, data on the observed variants are insufficient, with only one previous publication in 2012 reporting the distribution of *ATP7B* variants in French WD patients ⁽¹⁰⁾.

Therefore, in our study, we aim to provide a detailed analysis of the *ATP7B* variants from a paediatric cohort of the French WD registry and their possible correlation with clinical and laboratory data.

PATIENTS AND METHODS

Subjects and sample collection

Clinical data of children included in the French WD national registry were gathered from 01/03/1995 to 01/07/2020 (retrospective collection between 1995 and 2005 and prospective collection since 2006).

The patients were followed by paediatricians, hepatologists and neurologists in the two reference centers (Paris and Lyon) and the eight centers of competence.

The diagnosis of WD was based on clinical manifestations, biochemical parameters, and/or genetic analysis as previously published ^(1, 2, 11, 12). Collected data included: clinical data, presence of a Kayser-Fleischer ring (KF ring), liver function tests, immuno-nephelometric measurement of serum ceruloplasmin levels, serum copper level, determination of 24-hours urinary copper excretion (UCE), genetics. Patients without genetic information were excluded. In family cases, only the index case was included. All patients included in the registry had a clinical score ≥ 4 using the score proposed by Ferenci et al ⁽⁸⁾.

Depending on the primary clinical manifestations, cases were classified as hepatic (H), neurological (N), or asymptomatic (AS); we considered as asymptomatic only those with normal hepatic function tests and no neurological symptom.

For the genotype assessment, we hypothesized that the following variables would be associated with variant type: age at onset of first symptoms, neurological *versus* hepatic form of the illness, ceruloplasmin level, serum copper values, and 24h urine copper level, and the presence of KF rings. Many comparisons were made: first for the homozygous for the most common *ATP7B* variants (≥ 2), then between groups composed of homozygous and compound heterozygous for the most common

variants. We also compared patients with homozygous missense variants (MSM) *versus* patients with frameshift-nonsense variants.

Genetic analysis of *ATP7B* gene

Genetic analyses after 1994 were performed for patients and their families by bi-directional sequencing on the 21 exons and intron-exon boundary regions of *ATP7B* gene. For patients with only one *ATP7B* variant detected by sequencing, a Multiplex Ligation-dependent Probe Amplification (MLPA) assay was performed using the SALSA MLPA P098 for Wilson Disease kit (MRC-Holland, Amsterdam, Netherlands) to detect large deletions. Since 2017, next-generation sequencing (NGS) was used for *ATP7B* variant detection in WD patients with a bioinformatic pipeline allowing the detection of SNV and copy number variation.

Ethics and regulatory aspects

The study received approval from the INSERM (Institut National de la Santé et de la Recherche Médicale) Ethics evaluation committee (IRB 00003888, authorization No.19-550/January 24, 2019). Written informed consent was obtained from parents or adult patients.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Statistical analysis

Normally distributed quantitative variables were expressed as mean \pm SD; non-normally distributed quantitative variables were expressed as median [IQR], and were compared using the Student t-test or non-parametric tests (Mann-Whitney, Kruskal-Wallis) when appropriate. Qualitative variables were expressed as count (percentage), and compared using the χ^2 test or Fischer's exact test when appropriate. Statistical analysis was performed with SPSS version 23.0 (IBM, New York, USA); p-values lower than 0.05 were considered statistically significant.

RESULTS

Study population

A total of 113 children (aged 0 to 18 years) were included in the register analysis. Demographic and laboratory parameters are summarized in Table 1.

Table 1. Characteristics of the study population (n=113)

Gender was quite equally distributed M/F= 56/57. Diagnosis was made at a mean age of 11.0 ± 4.1 years (range 1-18 years). At diagnosis, 91 patients (79.8%) had hepatic manifestations (H), 18 (15.8%) presented neurological manifestations (N), and 4 patients (3.5%) were asymptomatic (AS).

Mean age at diagnosis was 10.4 ± 4.0 years (range 2-18) for H patients, 14.6 ± 1.9 years (range 11-18) for N patients ($p < 0.001$), and 8.3 ± 6.0 years (range 1-17) for AS patients.

Biochemical results at diagnosis

The mean serum ceruloplasmin level was 7.8 ± 5.5 mg/dL. A total of 8 patients (5.5%) had serum ceruloplasmin levels ≥ 20 mg/dl.

At diagnosis, the median urinary copper excretion (UCE) was $4.1 \mu\text{mol}/24$ hours (IRQ 5.2).

The median urinary copper excretion was significantly lower for AS patients $1.3 \mu\text{mol}/24$ hours (IRQ 2.1) compared to H patients $3.8 \mu\text{mol}/24$ hours (IRQ 5.6) and to N patients $5.0 \mu\text{mol}/24$ hours (IRQ 2.5), $p < 0.001$.

Variant analysis

ATP7B gene analysis was performed in 113 WD patients. Two variants within the *ATP7B* gene were identified in 98 patients, just one variant in 13, and 2 patients had three variants (patient 1: variants p.Asn41Ser and variant p.Ile1021Val in *cis* and variant p.Arg1319* in *trans*; patient 2: variants p.Lys175Serfs*28 and variant p.Gln260Profs*10 in *cis* and variant p.Gly1266Arg in *trans*). MLPA allowed to detect large exon deletions in 4 patients. Only 29 patients (25%) were homozygous for a variant. We have found a total of 102 different variants; details about variants are listed in Table 2. p.His1069Gln was the most prevalent, n = 31 alleles (14,2%), with only seven homozygous. The frequency of the three other most prevalent variants were: p.Gln111* (3.7%), c.1708-1G>A, p.Arg1319*, p.Met769HisFs*26 and p.Val890Met (2.3% each). The allele frequency of other variants was $\leq 2\%$; 55% of variants were present in only one family, confirming genetic heterogeneity of WD in France. 14 novel variants were identified and submitted to Clinvar. Data used to classified novel missense variants and variants previously classified as variants of unknown significance are detailed in table 3.

Table 2. Variants detected in children with Wilson disease in this study

Table 3. Data used to classified novel missense variants and variants previously classified as variants of unknown significance

In respect of mutated exon, the 3 most prevalent were exon 14 (16.5%), exon 8 (13.8%), and exon 3 (11.5%). Very few variants were identified in exons 7, 3, 12, 9 and 21. 12.4% of variants were located in intron-exon boundary regions of *ATP7B*

gene; introns 4, 5, 8, and 12 were the most frequently involved (Figure 1). This point is important according epidemiological level.

Figure 1: Variant frequency of exons

Concerning the type of variant, the repartition was as follow (n alleles - %): Missense (127 – 58.2%), Splice site (30 – 13,8%), Frameshift (30 – 13.8%), Nonsense (24 – 11%), Deletion (7 – 3.2%). Overall, 45% of variants identified were truncating variants.

Variant distribution in the ATP7B protein domains was as follow: ATP binding domain (29.4%), transmembrane domain (23.9%), copper-binding domain (22.9%), phosphatase domain, and phosphorylation domain (4.1% and 1.8%, respectively), combined domains accounted for the rest of variants (17,9%).

Genotype-phenotype.

Missense variants (p.His1069Gln, p.Val890Met, p.Lys1020Arg) – Splice site (c.1708-1G>A)

The 7 homozygous for p.His1069Gln were compared with 3 other homozygous variants with a frequency of at least 2 patients (p.Val890Met, p.Lys1020Ar, c.1708-1G>A). We didn't observe any statistically significant difference for clinical phenotype, age at diagnosis, presence of KF ring, ceruloplasmin levels, serum copper values and 24h urine copper excretion. When adding compound heterozygous for these variants, only p.His1069Gln was enlarged with 13 patients and only one for c.1708-1G>A. Comparison between p.His1069Gln homozygous with compound heterozygous did not find significant statistical differences for any of the variables previously mentioned. Details are shown in table 3.

Nonsense / Frameshift variants

For Nonsense / Frameshift variants, there were only compound heterozygotes and no homozygous. When considering patients with two Nonsense / Frameshift variants as a group and compared with those with two Missense variants, we found significant lower ceruloplasmin for the former: 2.8 ± 0.7 mg/dl vs 8.4 ± 5 mg/dl ($p < 0.05$). No differences were found in age at diagnosis, serum copper values or 24h urine copper excretion. Details are shown in table 3.

Acute liver failure: 8 patients had acute liver failure at diagnosis of which only 3 were homozygous, each one of a different type hence no comparisons were made.

Table 4. Clinical and biochemical features of Patients Homozygous or Compound Heterozygous for Common Variants

DISCUSSION

The p.His1069Gln variant was the most frequent variant in our cohort and the prevalence was very similar to the reported by Bost et al 2012⁽¹⁰⁾, this prevalence is one of the lowest ones reported in Central and East Europe where ranges of prevalence are between 15% to 72% ⁽⁹⁾.

The distribution of mutated exons could give the spectrum of *ATP7B* variants in the French population since exons 14, 8 and 2 account for 42.6% of total variants. However, 80% of the variants in this population span at least 14 exons contrary to just eight exons in the UK population according to Coffey et al ⁽⁷⁾.

The considerable diversity of variants and the low percentages of homozygous patients, make it difficult to have more conclusive results on a genotype-phenotype correlation in our cohort. This correlation has been extensively studied over the years in WD with no definitive results.

We followed similar approaches as those used by Panagiotakaki et al.⁽¹³⁾, Nicastro et al.⁽¹⁴⁾ and Merle et al. ⁽¹⁵⁾, but we could only verify lower ceruloplasmin levels associated with Frameshift / Nonsense variants. Those studies founded an association of p.His1069Gln homozygous variant with neurologic and later presentation⁽¹³⁾; early onset of the disease, lower ceruloplasmin levels, higher urinary copper excretion, and lower serum copper associated with Nonsense / Frameshift variants ^(14–16). Type of variants have been associated with clinical phenotype and disease severity, but often other studies have opposite or not confirmatory results. A recent publication from Kluska et al⁽¹⁷⁾ found an association with frameshift variant and a risk for neurological presentation. Neurological forms have been associated

with p.His1069Gln homozygous variant according to some studies ^(13, 18, 19) but one study showed a more frequent hepatic presentation in patients with this variant ⁽²⁰⁾.

Many reasons could explain some of our differences with those studies. First of all, our population's extreme heterogeneity doesn't allow to perform very meaningful statistical calculations despite the important population since each of the multiple groups comprises only a few subjects. Being our cohort exclusively pediatric, the differences between the two main clinical forms might not yet be evident since hepatic forms are known to be predominant in this age group. Nevertheless, Nicastro et al⁽¹⁴⁾ reported a pediatric population with differences in serum copper and ceruloplasmin levels related with genotype.

An impressive report of 1357 patients from Ferenci et al⁽²¹⁾ found that clinical phenotype was more influenced by age and sex rather than by genotype, being that neurologic forms start later and are more common in men than the hepatic presentations. Other reports describe the association of age and sex ^(17, 18, 22–24) with WD's clinical forms. In our study, we've also found a later onset of neurologic forms comparing to asymptomatic and hepatic patients but no clear gender predominance. It has been speculated that the gender difference could be hormone-related. Following this line of thinking, this could also explain the lack of difference in our young cohort since estrogen exposure is much limited than of the women from adults cohorts.

Other factors that affect disease severity might include the copper levels in the diet, the amount of the metal in drinking water or other genetic modifying factors at nutritional and environmental levels.

Even considering the French population's extreme genetic heterogeneity, it would be useful to include adults in the analysis of the WD's population to have a complete picture of phenotype evolution through age and its possible association with a genotype.

CONCLUSION

p.His1069Gln is the most frequent variant (14,2%) and exons 14, 8, and 2 of the *ATP7B* gene account for 41.7% of total variants. However, there is significant heterogeneity in the French population concerning the other *ATP7B* variants. Nonsense / Frameshift variants were associated with lower ceruloplasmin levels.

Acknowledgments

The authors would like to thank Micheline Misrahi et Bruno Francou from Bicetre Hospital, Corine Collet from Lariboisiere Hospital and all teams from other hospitals that helped with the genetic and copper analysis .

Table 1. Characteristics of the study population (n=113)

	Asymptomatic	Hepatic	Neurologic	All
Total patients n (%)	4 (3.5%)	91 (79.8%)	18 (15.8%)	113
Male / Female	2/2	45/46	9/9	56/57
Age at diagnosis (mean +/- sd)	8.3 ± 6.0	10.4 ± 4.0	14.6 ± 1.9	11.0 ± 4.1
Kayser-Fleischer ring (n,%)	0	13 (14.2%)	15 (83.3%)	28 (24.8%)
Serum ceruloplasmin mg/dl (mean +/- sd)	8.0 ± 5.5	8.5 ± 5.6	4.6 ± 2.7	7.8 ± 5.5
Urinary copper excretion, µmol/24 hours - median (IQR)	1.3 (2.1)	3.8 (5.6)	5.0 (2.5)	4.1 (5.2)

Serum copper $\mu\text{mol/L}$ - median (IQR)	3.94 (3.31)	4.88 (6.14)	6.30 (2.52)	5.04 (5.2)
---	-------------	-------------	-------------	-------------------

Reference ranges: ceruloplasmin (20-35 mg/dL) - serum copper (12.6– 31.5 $\mu\text{mol/L}$) -
urinary copper excretion <0.6 $\mu\text{mol}/24\text{h}$

Table 2. Variants detected in children with Wilson disease in this study

Protein change	cDNA change	ClinVar accession	Variant classification (source)	Exon or Intron	Variant type	Protein domain	Variant allele frequency (%)	Age of onset mean(range)	Clinical form (n)
p.His1069Gln	c.3207C>A	VCV000003848	5 (CV, HGMD)	E 14	MS	Ph	14,22%	12,3 (4-18)	H=21 N=9 AS=1
p.Gln111*	c.331C>T	VCV000188899	5 (CV, HGMD)	E 2	NS	Cu1	3,67%	12,2 (5-17)	H=8
	c.1708-1G>A	VCV000370195	5 (CV, HGMD)	I 4	Spl	Cu6	2,29%	13,8 (9-17)	H=5
p.Met769HisFs*26	c.2304dupC	VCV000456552	5 (CV, HGMD)	E 8	FS	TM4	2,29%	10,4 (1-15)	H=3 AS=2
p.Val890Met	c.2668G>A	VCV000189121	5 (CV, HGMD)	E 11	MS	Td/TM5	2,29%	8,2 (7-13)	H=5
p.Arg1319*	c.3955C>T	VCV000035728	5 (CV, HGMD)	E 19	NS	TM7	2,29%	6,4 (4-11)	H=5
p.Arg226Trp	c.676C>T	VCV000312397	4 (This study)	E 2	MS	Cu2/Cu3	1,83%	10,7 (8-12)	H=2 N=2
p.Met645Arg	c.1934T>G	VCV000003862	5 (CV, HGMD)	E 6	MS	Cu6/TM1	1,83%	9 (6-13)	H=4
p.Arg778Trp	c.2332C>G	VCV000456553	5 (CV, HGMD)	E 8	MS	TM4	1,83%	7,75 (4-11)	H=4
	c.2865+1G>A	VCV000157942	5 (CV, HGMD)	I 12	Spl	TM5/TM6	1,83%	12,75 (11-15)	H=3 N=1
p.Lys1020Arg	c.3059A>G	pending	5 (HGMD)	E 13	MS	Ph/ATP Loop	1,83%	11 (6-16)	H=2 N=2
p.Asn1270Ser	c.3809A>G	VCV000003859	5 (CV, HGMD)	E 18	MS	ATP Hinge	1,83%	9,5 (3-18)	H=3 N=1
p.Ser1365CysFs*12	c.4092_4093del	VCV000371438	5 (CV, HGMD)	E 20	FS	TM8	1,83%	12 (7-16)	H=2 N=2
	c.51+4A>T	VCV000312401	5 (CV, HGMD)	I 1	Spl	before Cu1	1,38%	10 (5-14)	H=3
	c.1869+2T>C	pending	5 (HGMD)	I 5	Spl	Cu6	1,38%	12,33 (11-15)	H=2 AS=1
p.Asp765Asn	c.2293G>A	VCV000003855	5 (CV, HGMD)	E 8	MS	TM4	1,38%	8 (6-9)	H=3
p.Val845SerFs*28	c.2532delA	VCV000188883	5 (CV, HGMD)	E 10	FS	Td	1,38%	10 (8-14)	H=2 N=1
p.Thr977Met	c.2930C>T	VCV000035710	5 (CV)	E 13	MS	Ch/TM6	1,38%	8,66 (4-11)	H=3
p.Ile1148Thr	c.3443T>C	VCV000037122	5 (CV, HGMD)	E 16	MS	ATP Loop	1,38%	10,3 (4-16)	H=2 N=1
p.Asp1267Ala	c.3800A>C	VCV000552417	5 (CV, HGMD)	E 18	MS	ATP Hinge	1,38%	12,6 (7-17)	H=3
	del_E4	NA	5 (This study)	E 4	Del	Cu5/Cu6	1,38%	11,6 (11-12)	H=3
p.Leu168Pro	c.503T>C	VCV000967323	5 (CV, HGMD)	E 2	MS	Cu2	0,92%	3	H=2
p.Gln260ProFs*10	c.778dupC	VCV000188938	5 (CV, HGMD)	E 2	FS	Cu2/Cu3	0,92%	8 (7-9)	H=2
p.Gln289*	c.865C>T	VCV000003864	5 (CV, HGMD)	E 2	NS	Cu3	0,92%	14 (12-16)	N=2
p.Ala553Glu	c.1658C>A	pending	5 (HGMD)	E 4	MS	Cu5/6	0,92%	9	H=2

p.Thr569del	c.1705_1707+10del	pending	5 (HGMD)	E 4	Del	Cu5/6	0,92%	17	N=2
p.Gly710Ser	c.2128G>A	VCV000156281	5 (CV, HGMD)	E 8	MS	TM2	0,92%	11 (9-13)	H=2
p.Gly711Arg	c.2131G>A	VCV000495405	5 (CV, HGMD)	E 8	MS	TM2	0,92%	10 (7-13)	H=2
p.Thr766Arg	c.2297C>G	VCV000003861	5 (CV, HGMD)	E 8	MS	TM4	0,92%	5,5 (3-8)	H=2
p.Met769Val	c.2305A>G	VCV000035706	5 (CV)	E 8	MS	TM4	0,92%	10 (8-12)	H=2
p.Arg778Gly	c.2332C>G	VCV000156283	5 (CV, HGMD)	E 8	MS	TM4	0,92%	14,5 (12-17)	H=1 N=1
p.Trp779*	c.2336G>A	VCV000156284	5 (CV, HGMD)	E 8	NS	TM4	0,92%	8 (6-10)	H=2
	c.2356-2A>G	pending	5 (HGMD)	I 9	Spl	TM4/Td	0,92%	11	N=2
p.Gly836Glu	c.2507G>A	pending	5 (HGMD)	E 10	MS	Td	0,92%	8	H=2
p.Ile857Thr	c.2570T>C	VCV000377538	5 (CV, HGMD)	E 10	MS	Td	0,92%	6	H=2
p.Asp918Asn	c.2752G>A	VCV000552229	5 (HGMD)	E 12	MS	Td/TM5	0,92%	15	H=2
p.Ala1018Val	c.3053C>T	VCV000188722	5 (CV, HGMD)	E 13	MS	Ph/ATP Loop	0,92%	5 (1-9)	H=1 AS=1
p.Lys1028SerFs*40	c.3083_3085delinsG	VCV000633073	5 (CV, HGMD)	E 14	FS	Ph	0,92%	12,5 (10-15)	H=2
p.Ser1067Arg ^a	c.3201T>G ^a	pending	4 (This study)	E 14	MS	ATP Loop	0,46%	18	H=1
p.Ala1135GlnFs*13	c.3402delC	VCV000088958	5 (CV, HGMD)	E 15	FS	ATP Loop	0,92%	8 (6-10)	H=2
p.Ala1183Thr	c.3547G>A	pending	5 (HGMD)	E 16	MS	ATP Loop	0,92%	13 (10-16)	H=2
p.Gly1186Ser	c.3556G>A	VCV000188900	5 (CV, HGMD)	E 16	MS	TM6	0,92%	13,5 (9-18)	H=2
p.Gly1266Arg	c.3796G>A	VCV000003849	5 (CV, HGMD)	E 18	MS	ATP Hinge	0,92%	9	H=2
	c.3904-2A>G	VCV000371387	5 (CV, HGMD)	I 19	Spl	ATP Loop	0,92%	3	H=2
	c.4125-1G>T ^a	pending	5 (This study)	I 20	Spl	TM8	0,92%	8,5 (4-13)	H=2
p.Glu1382* ^a	c.4144G>T ^a	pending	5 (This study)	E 21	NS	after TM8	0,92%	15 (15-15)	N=2
p.Asn41MetFs*26 ^a	c.122delA ^a	pending	5 (This study)	E 2	FS	Cu1	0,46%	10	H=1
p.Asn41Ser	c.122A>G	VCV000157928	<u>5 (CV, HGMD)</u>	E 2	MS	Cu1	0,46%	3	AS=1
p.Ser105*	c.314C>A	VCV000189193	5 (CV, HGMD)	E 2	NS	Cu1	0,46%	14	H=1
p.Glu127LysFs*26	c.379del	pending	5 (HGMD)	E 2	FS	Cu1	0,46%	18	H=1
p.Lys175SerFs*28	c.524_525delAA	VCV000188995	5 (CV, HGMD)	E 2	FS	Cu2	0,46%	9	H=1
p.Tyr187SerFs*16 ^a	c.560_561del ^a	pending	5 (This study)	E 2	FS	Cu2/3	0,46%	11	H=1
p.Gly379AlaFs*2	c.1136delG	pending	5 (HGMD)	E 2	FS	Cu4	0,46%	8	H=1
p.Arg414SerFs*9 ^a	c.1242_1243del ^a	pending	5 (This study)	E 2	FS	Cu4	0,46%	12	H=1
	c.1286-2A>G	pending	5 (HGMD)	I 2	Spl	Cu4	0,46%	10	H=1

p.Gln457*	c.1369C>T	pending	5 (HGMD)	E 3	NS	Cu4/5	0,46%	10	H=1
p.Asn505*	c.1512dupT	VCV000370348	5 (CV, HGMD)	E 3	NS	Cu5	0,46%	7	H=1
	c.1543+1G>T	VCV000590806	5 (CV, HGMD)	I 3	Spl	Cu5	0,46%	14	H=1
	c.1708-34G>A ^a	pending	4 (This study)	I 5	Spl	Cu6	0,46%	14	H=1
p.His580GlnFs*3 ^a	c.1740del ^a	pending	5 (This study)	E 5	FS	Cu6	0,46%	7	H=1
p.Ile582ArgFs*25	c.1745_1746del	VCV000189112	5 (CV, HGMD)	E 5	MS	Cu6	0,46%	8	H=1
p.Gly591Asp	c.1772G>A	VCV000210482	5 (CV, HGMD)	E 5	MS	Cu6	0,46%	4	H=1
p.Ala604Asp	c.1811C>A	pending	4 (This study)	E 5	MS	Cu6	0,46%	12	H=1
	c.1869+5_1869+8del	pending	5 (HGMD)	I 6	Spl	Cu6	0,46%	7	H=1
p.Gly626Ala	c.1877G>C	VCV000157930	5 (HGMD)	E 6	MS	Cu6/TM1	0,46%	18	H=1
p.Leu641Ser	c.1922T>C	VCV000420002	5 (HGMD)	E 6	MS	Cu6/TM1	0,46%	16	H=1
p.Asp642His	c.1924G>C	VCV000189109	5 (CV, HGMD)	E 6	MS	Cu6/TM1	0,46%	7	H=1
	c.1946+6T>C	VCV000495403	5 (HGMD)	I 6	Spl	Cu6/TM1	0,46%	14	N=1
p.Gly691Arg	c.2071G>A	VCV000003866	5 (CV, HGMD)	E 7	MS	TM2	0,46%	18	H=1
	c.2122-8T>G	VCV000035705	5 (CV, HGMD)	I 8	Spl	TM2	0,46%	15	N=1
p.Leu708Pro	c.2123T>C	VCV000003865	5 (CV, HGMD)	E 8	MS	TM2	0,46%	6	AS=1
p.Pro768Leu	c.2303C>T	VCV000370887	4 (CV)	E 8	MS	TM4	0,46%	14	N=1
p.Met769ThrFs*26	c.2304dup	VCV000456552	5 (CV, HGMD)	E 8	FS	TM4	0,46%	15	N=1
	c.2333G>A	VCV000550914	5 (CV, HGMD)	E 8	MS	TM4	0,46%	16	H=1
p.Arg778Gln	c.2337G>C	pending	4 (This study)	E 8	MS	TM4	0,46%	5	H=1
p.Leu795Phe	c.2383C>T	VCV000188814	5 (CV, HGMD)	E 9	MS	TM4/Td	0,46%	7	H=1
p.Gly836Arg	c.2506G>A	pending	5 (HGMD)	E 10	MS	Td	0,46%	4	H=1
p.Lys838Glu	c.2512A>G	VCV000860738	4 (This study)	E 10	MS	Td	0,46%	14	H=1
p.Gly881*	c.2637_2650del	pending	5 (This study)	E 11	NS	Td/TM5	0,46%	15	N=1
p.Thr991Ala	c.2971A>G	pending	4 (This study)	E 13	MS	TM6/Ph	0,46%	12	H=1
p.Ala1003Pro	c.3007G>C	pending	4 (This study)	E 13	MS	TM6/Ph	0,46%	16	H=1
p.Ala1003Val	c.3008C>T	VCV000188781	5 (CV, HGMD)	E 13	MS	TM6/Ph	0,46%	10	H=1
	c.3061-12T>A	VCV000557116	5 (CV, HGMD)	I 13	Spl	Ph	0,46%	16	H=1
p.Ile1021Val	c.3061A>G	VCV000430276	5 (HGMD)	E 14	MS	Ph	0,46%	3	AS=1
p.Met1025Lys	c.3074T>A	pending	5 (HGMD)	E 14	MS	Ph	0,46%	10	H=1

p.Arg1041Trp	c.3121C>T	VCV000312384	5 (CV, HGMD)	E 14	MS	ATP Loop	0,46%	8	H=1
p.Gly1099Ser	c.3295G>A	VCV000370820	5 (CV, HGMD)	E 15	MS	ATP Loop	0,46%	6	AS=1
p.Ile1102Thr	c.3305T>C	VCV000430725	5 (CV, HGMD)	E 15	MS	ATP Loop	0,46%	18	H=1
p.Val1106Leu	c.3316G>C	pending	5 (HGMD)	E 15	MS	ATP Loop	0,46%	15	H=1
p.Thr1178Ala	c.3532A>G	VCV000959197	5 (CV, HGMD)	E 16	MS	ATP loop	0,46%	17	H=1
	c.3556+1G>T	VCV000495416	5 (CV, HGMD)	I 16	Spl	ATP Loop	0,46%	12	H=1
p.Gln1210_Ser1211del	c.3627_3632del	pending	5 (HGMD)	E 7	Del	ATP Bind	0,46%	11	H=1
p.Val1217_Leu1218del	c.3649_3654del	VCV000189015	5 (CV, HGMD)	E 17	Del	ATP Bind	0,46%	14	N=1
p.Thr1220Met	c.3659C>T	VCV000035725	5 (CV, HGMD)	E 17	MS	ATP Bind	0,46%	10	H=1
p.Thr1232Pro	c.3694A>C	VCV000555144	5 (CV, HGMD)	E 17	MS	ATP Bind	0,46%	15	N=1
p.Ala1274Thr	c.3820G>A	pending	5 (HGMD)	E 18	MS	ATP Hinge	0,46%	12	H=1
p.Gly1281Asp	c.3842G>A	VCV000550301	5 (HGMD)	E 18	MS	ATP Hinge	0,46%	18	H=1
p.Val1282LysFs*22	c.3843dup	VCV000958649	5 (CV, HGMD)	E 18	FS	ATP Hinge	0,46%	12	H=1
p.Ala1283GlyFs*21	c.3845dupT	pending	5 (This study)	E 18	FS	ATP Hinge	0,46%	16	N=1
p.Gln1351*	c.4051C>T	VCV000188947	5 (CV, HGMD)	E 20	NS	TM7	0,46%	12	H=1
p.Leu1373Pro	c.4118T>C	VCV000552788	5 (HGMD)	E 20	MS	TM8	0,46%	11	H=1
	del_E1	NA	5 (This study)	E 1	Del	before Cu1	0,46%	3	H=1

Variant nomenclature based on Refseq NM_000053.3

Variant classification according to ACMG guidelines. 5: pathogenic. 4: likely pathogenic. 3: unknown significance. 2: likely benign. 1: benign.

Variant classification source. CV: ClinVar database (searched on 21/04/21). HGMD: Human Gene Mutation Database Professional (searched on 21/04/21). This study: for variants not referenced in ClinVar nor HGMD Pro, or referenced with uncertain significance or conflicting interpretation, authors interpretation based on ACMG recommendations.

Variant location and type. E: exon. I: Intron. MS: missense. NS: nonsense. FS: frameshift. Spl: splice site. Del: deletion.

Protein domain. Cu: copper-binding domain. TM: transmembrane domain. Td: Transduction domain ATP Bind: ATP binding domain. Ph: Phosphorylation domain.

Clinical form. H: hepatic. N: neurologic. AS: asymptomatic.

Table 3. Data used to classified novel missense variants and variants previously classified as variants of unknown significance

Variant		previous classification	authors classification	AF in GnomAD	In silico predictions	Variant associated in <i>trans</i>	Biochemical phenotype	Clinical phenotype
p.Arg226Trp	c.676C>T	Unknown significance (CV, HGMD)	Likely pathogenic	max 0.039% (all 0.0040%)	prob. damaging (Polyphen), deleterious (SIFT)	c.2332C>T (p.Arg778Trp)	mild cytolysis	compensated cirrhosis
						c.2297C>G (p.Thr766Arg)	cytolysis	fulminant hepatitis, liver transplantation
						c.676C>T (p.Arg226Trp) homoz.	NA	neurologic form, KF ring. Liver transplantation
/	c.1708-34G>A	ND	Likely pathogenic	absent	creation of alternative consensus acceptor splice site	c.3800A>C (p.Asp1267Ala)	mild cytolysis	compensated cirrhosis, hepatomegaly
p.Ala604Asp	c.1811C>A	ND	Likely pathogenic	absent	prob. damaging (Polyphen), deleterious (SIFT)	c.3843dup (p.Val1282LysFs*22)	Cp<0,03	hepatitis, haemolytic anemia
p.Trp779Cys	c.2337G>C	ND	Likely pathogenic	absent	prob. damaging (Polyphen), deleterious (SIFT)	not identified	Persistent cytolysis, very low Cp, increased hepatic Cu	chronic hepatitis
p.Lys838Glu	c.2512A>G	Unknown significance (CV)	Likely pathogenic	max 0.017% (all 0.0024%)	prob. damaging (Polyphen), deleterious (SIFT)	c.51+4A>T	mild cytolysis, Cp=0,12 g/L CuS=5,75 µmol/L CuEx=0,69 µmol/L CuU = 14,4 µmol/24H	compensated cirrhosis, hepatomegaly
p.Thr991Ala	c.2971A>G	ND	Likely pathogenic	absent	prob. damaging (Polyphen), tolerated (SIFT)	c.1242_1243del (p.Arg414SerFs*9)	Cytolysis - Cp=0,14g/L - Cuu=1,86 µmol/L - Cus=4,40 µmol/L - CuEXC=0,65 µmol/L- REC=14,6%	hepato-splenomegaly
p.Ala1003Pro	c.3007G>C	ND ^a	Likely pathogenic	absent	prob. damaging (Polyphen), deleterious (SIFT)	not identified ^b	Cp>0,03 g/L CuS=1,10 µmol/L CuEx=0,38 µmol/L REC=34,1%	neurologic form, KF ring
p.Ser1067Arg	c.3201T>G	ND	Likely pathogenic	absent	prob. damaging (Polyphen), deleterious (SIFT)	c.3305T>C (p.Ile1102Thr)	Cp=0,05g/L - Cus=6,9µmol/L - CuEXC=1,86µmol/L - REC=27% - Cuu=27,8µmol/L	decompensated cirrhosis

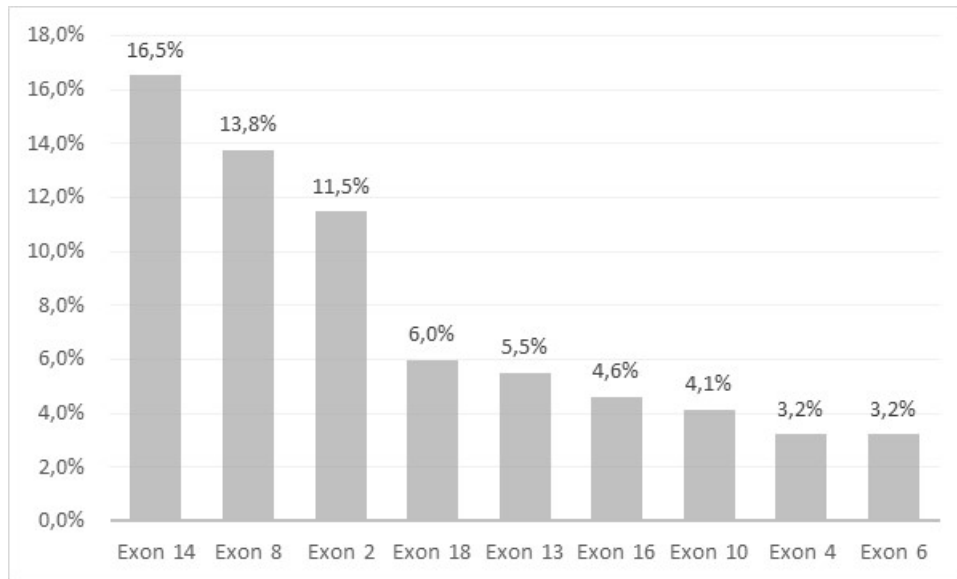
Reference ranges for copper status explorations: Ceruloplasmin (Cp) >0.2 g/L. Serum copper (CuS): 13-19 µmol/L. Exchangeable copper (CuEx): 0,38-1 µmol/L. Relative Exchangeable Copper (REC): 3-9%. Urinary copper (CuU) <0.8 µmol/24h. ^a c.3007G>A (p.Ala1003Thr) reported pathogenic in CV and HGDM ^b Same genotype in affected sibling.

Table 4. Clinical and biochemical features of Patients Homozygous or Compound Heterozygous for Common Variants

Genotype	n	Age (mean ± sd)	Phenotype	KF ring	Ceruloplasmin (mg/dL) (mean ± sd)	Serum copper (µmol/L) (mean ± sd)	Urinary copper (µmol/24 hours) (mean ± sd)
p.His1069Gln/p.His1069Gln	7	12,7 ± 3,5	H = 4 N = 1 AS = 2	29%	12,0 ± 5,0	0,58 ± 0,2	4,6 ± 2,4
c.1708-1G>A/c.1708-1G>A	2	15,0 ± 2,8	H = 2 N = 0 AS = 0	0%	15,5 ± 3,5	0,73 ± 0,5	48,7 ± 65,3
p.Val890Met/p.Val890Met	2	7,0 ± 0,0	H = 2 N = 0 AS = 0	0%	11,5 ± 3,5	0,63 ± 0,1	4,2 ± 0,6
p.Lys1020Arg/p.Lys1020Arg	2	11,0 ± 7,1	H = 2 N = 0 AS = 0	0%	3,0 ± 0,0	0,89 ± 1,0	8,9 ± 6,1
p.His1069Gln HMZ + compound HTZ	20	11,7 ± 3,5	H = 16 N = 2 AS = 2	15%	12,1 ± 5,0	0,50 ± 0,3	4,8 ± 3,2
c.1708-1G>A HMZ + compound HTZ	3	13,0 ± 2,8	H = 3 N = 0 AS = 0	0%	11,0 ± 8,2	0,73 ± 0,4	34,2 ± 52,6
p.Val890Met HMZ + compound HTZ	2	7,0 ± 0,0	H = 2 N = 0 AS = 0	0%	11,5 ± 3,5	0,63 ± 0,1	4,2 ± 0,6
p.Lys1020Arg HMZ + compound HTZ	2	11,0 ± 7,1	H = 2 N = 0 AS = 0	0%	3,0 ± 0,0	0,89 ± 1,0	8,9 ± 6,1
Missense	39	10,7 ± 4,7	H = 33 N = 4 AS = 2	18%	8,2 ± 5,1	0,48 ± 0,4	4,9 ± 4,7
Frameshift/Nonsense	8	10,8 ± 4,0	H = 7 N = 1 AS = 0	38%	2,8 ± 0,7	0,33 ± 0,2	4,4 ± 3,6

H: hepatic phenotype, N: neurological, AS: asymptomatic. KF: Kayser–Fleischer. HTZ: heterozygotes. HMZ: homozygotes.

Figure 1: Variant frequency of exons



References

1. Schilsky ML. Wilson Disease: Diagnosis, Treatment, and Follow-up. *Clin Liver Dis* 2017; 21:755–767. <https://doi.org/10.1016/j.cld.2017.06.011>
2. Socha P, Janczyk W, Dhawan A, et al. Wilson's Disease in Children: A Position Paper by the Hepatology Committee of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2018; 66:334–344. <https://doi.org/10.1097/MPG.0000000000001787>
3. O'Brien M, Reilly M, Sweeney B, et al. Epidemiology of Wilson's disease in Ireland. *Mov Disord* 2014; 29:1567–1568. <https://doi.org/10.1002/mds.25983>
4. Collet C, Laplanche J-L, Page J, et al. High genetic carrier frequency of Wilson's disease in France: discrepancies with clinical prevalence. *BMC Med Genet* 2018; 19:143. <https://doi.org/10.1186/s12881-018-0660-3>
5. Poujois A, Woimant F, Samson S, et al. Characteristics and prevalence of Wilson's disease: A 2013 observational population-based study in France. *Clin Res Hepatol Gastroenterol* 2018; 42:57–63. <https://doi.org/10.1016/j.clinre.2017.05.011>
6. Sandahl TD, Laursen TL, Munk DE, et al. The Prevalence of Wilson's Disease: An Update. *Hepatology* 2020; 71:722–732. <https://doi.org/10.1002/hep.30911>
7. Coffey AJ, Durkie M, Hague S, et al. A genetic study of Wilson's disease in the United Kingdom. *Brain* 2013; 136:1476–1487. <https://doi.org/10.1093/brain/awt035>
8. Ferenci P, Caca K, Loudianos G, et al. Diagnosis and phenotypic classification of Wilson disease. *Liver Int* 2003; 23:139–142.
9. A G, Gv D. Geographic distribution of ATP7B mutations in Wilson disease. *Annals of human biology*. <https://pubmed.ncbi.nlm.nih.gov/26207595/>. Published 2016. Accessed December 3, 2020
10. Bost M, Piguet-Lacroix G, Parant F, et al. Molecular analysis of Wilson patients: direct sequencing and MLPA analysis in the ATP7B gene and Atox1 and COMMD1 gene analysis. *J Trace Elem Med Biol* 2012; 26:97–101. <https://doi.org/10.1016/j.jtemb.2012.04.024>
11. Ferenci P. Diagnosis of Wilson disease. *Handb Clin Neurol* 2017; 142:171–180. <https://doi.org/10.1016/B978-0-444-63625-6.00014-8>
12. European Association for Study of Liver. EASL Clinical Practice Guidelines: Wilson's disease. *J Hepatol* 2012; 56:671–685. <https://doi.org/10.1016/j.jhep.2011.11.007>
13. Panagiotakaki E, Tzetis M, Manolaki N, et al. Genotype-phenotype correlations for a wide spectrum of mutations in the Wilson disease gene (ATP7B). *Am J Med Genet A* 2004; 131:168–173. <https://doi.org/10.1002/ajmg.a.30345>

14. E N, G L, L Z, et al. Genotype-phenotype correlation in Italian children with Wilson's disease. *Journal of hepatology*. <https://pubmed.ncbi.nlm.nih.gov/19118915/>. Published March 2009. Accessed December 2, 2020
15. Merle U, Weiss KH, Eisenbach C, et al. Truncating mutations in the Wilson disease gene ATP7B are associated with very low serum ceruloplasmin oxidase activity and an early onset of Wilson disease. *BMC Gastroenterol* 2010; 10:8. <https://doi.org/10.1186/1471-230X-10-8>
16. Gromadzka G, Schmidt HH-J, Genschel J, et al. Frameshift and nonsense mutations in the gene for ATPase7B are associated with severe impairment of copper metabolism and with an early clinical manifestation of Wilson's disease. *Clin Genet* 2005; 68:524–532. <https://doi.org/10.1111/j.1399-0004.2005.00528.x>
17. Kluska A, Kulecka M, Litwin T, et al. Whole-exome sequencing identifies novel pathogenic variants across the ATP7B gene and some modifiers of Wilson's disease phenotype. *Liver Int* 2019; 39:177–186. <https://doi.org/10.1111/liv.13967>
18. Stapelbroek JM, Bollen CW, van Amstel JKP, et al. The H1069Q mutation in ATP7B is associated with late and neurologic presentation in Wilson disease: results of a meta-analysis. *J Hepatol* 2004; 41:758–763. <https://doi.org/10.1016/j.jhep.2004.07.017>
19. Maier-Dobersberger T, Ferenci P, Polli C, et al. Detection of the His1069Gln mutation in Wilson disease by rapid polymerase chain reaction. *Ann Intern Med* 1997; 127:21–26. <https://doi.org/10.7326/0003-4819-127-1-199707010-00004>
20. Mihaylova V, Todorov T, Jelev H, et al. Neurological symptoms, genotype-phenotype correlations and ethnic-specific differences in Bulgarian patients with Wilson disease. *Neurologist* 2012; 18:184–189. <https://doi.org/10.1097/NRL.0b013e31825cf3b7>
21. Ferenci P, Stremmel W, Członkowska A, et al. Age and Sex but Not ATP7B Genotype Effectively Influence the Clinical Phenotype of Wilson Disease. *Hepatology* 2019; 69:1464–1476. <https://doi.org/10.1002/hep.30280>
22. Beinhardt S, Leiss W, Stättermayer AF, et al. Long-term outcomes of patients with Wilson disease in a large Austrian cohort. *Clin Gastroenterol Hepatol* 2014; 12:683–689. <https://doi.org/10.1016/j.cgh.2013.09.025>
23. Litwin T, Gromadzka G, Członkowska A. Gender differences in Wilson's disease. *J Neurol Sci* 2012; 312:31–35. <https://doi.org/10.1016/j.jns.2011.08.028>
24. N C, H W, W W, et al. Spectrum of ATP7B mutations and genotype-phenotype correlation in large-scale Chinese patients with Wilson Disease. *Clinical genetics*. <https://pubmed.ncbi.nlm.nih.gov/27982432/>. Published July 2017. Accessed December 2, 2020