Spectrum and classification of *ATP7B* variants with clinical correlation in children with Wilson disease

Ruqayah GY. Al-Obaidi, MBChB, MSc, *Bassam MS. Al-Musawi,* MBChB, PhD.

ABSTRACT

الأهداف: دراسة طيف وتصنيف متغيرات ATP7B في الأطفال العراقيين المصابين بمرض ويلسون عن طريق التسلسل الجيني المباشر مع الارتباط السريري.

المنهجية: اشتملت الدراسة على خمسة وخمسين طفلاً لا علاقة لهم بالتشخيص السريري لمرض ويلسون (WD). وتم استخلاص حمض الديوكسي ريبونوكلييك من عينات الدم المحيطية، وتم تحديد المتغيرات في جين ATP7B باستخدام تسلسل الجيل التالي.

النتائج: تم الكشف عن ستة وسبعين متغيرًا ضارًا في 97 من أصل 110 أليل لجين ATP7B. كان لدى ثلاثين (54.5%) من المرضى نوعان مختلفان مسببان للمرض (15 متماثل الزيجوت و15 مركب متغاير الزيجوت). كان لدى اثني عشر (21.8%) من المرضى متغير واحد مسبب للمرض ومتغير واحد ذو أهمية غير مؤكدة (VUS) مع إمكانية الإمراضية. ثلاثة عشر (23.6%) من المرضى كانوا حاملين لمتغير واحد مسبب للمرض. تم اكتشاف المتغيرات الأكثر شيوعًا، c.3305T>C وc.956delC، في 4 أليلات لكل منهما، تليها c.3741_3742dupCA وc.3694A>C، والتي تم اكتشافها في 3 أليلات لكل منهما. من بين 76 متغيرًا، كان 42 منها عبارة عن خطأ، و13 كانت عبارة عن توقف، و9 كانت عبارة عن تغيير إطار، وكان 1 عبارة عن حذف داخل الإطار، و11 كانت متغيرات intronic. والجدير بالذكر أنه لم يتم اكتشاف المتغير الشائع عالميًا H1069Q في هذه الدراسة.

الخلاصة: إن الطيف الطفري لـ ATP7B في السكان العراقيين متنوع، على الرغم من ارتفاع معدلات زواج الأقارب. وهي تختلف عن الدول المجاورة. لقد قدمنا أدلة على إعادة تصنيف عشرة VUS على أنها ضارة، مما يثير تساؤلات حول معايير التشخيص للمرضى الذين لديهم درجات أعلى في لايبزيغ ومتغير واحد ضار.

Objectives: To study the spectrum and classification of *ATP7B* variants in Iraqi children with Wilson disease by direct gene sequencing with clinical correlation.

Methods: Fifty-five unrelated children with a clinical diagnosis of Wilson disease (WD) were recruited. Deoxyribonucleic acid was extracted from peripheral blood samples, and variants in the *ATP7B* gene were identified using next-generation sequencing.

Results: Seventy-six deleterious variants were detected in 97 out of 110 alleles of the *ATP7B* gene. Thirty (54.5%) patients had 2 disease-causing variants (15 homozygous and 15 compound heterozygous). Twelve (21.8%) patients had one disease-causing variant and one variant of uncertain significance (VUS) with potential pathogenicity. Thirteen (23.6%) patients were carriers of a single disease-causing variant. The most frequent variants, c.3305T>C and c.956delC, were detected in 4 alleles each, followed by c.3741-3742dupCA and c.3694A>C, which were detected in 3 alleles each. Among the 76 variants, 42 were missense, 13 were stop-gain, 9 were frameshift, 1 was an in-frame deletion, and 11 were intronic variants. Notably, the globally common variant H1069Q was not detected in this study.

Conclusion: The mutational spectrum of *ATP7B* in the Iraqi population is diverse, despite the high rates of consanguinity. It differs from that of neighboring countries. We provided evidence for ten VUS to be reclassified as deleterious, raising questions about the diagnostic criteria for patients with higher Leipzig scores and a single deleterious variant.

Keywords: *ATP7B*, Next-Generation Sequencing, Wilson disease, children, Iraq, c.3305T>C, c.956delC

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From the Department of Medical Genetics (Al-Obaidi), National Center for Educational Laboratories, Medical City Campus; and from Department of Pathology and Forensic Medicine (Al-Musawi), College of Medicine, University of Baghdad, Baghdad, Iraq.

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Address correspondence and reprint request to: Dr. Bassam Musa Sadik Al-Musawi, Department of Pathology and Forensic Medicine, College of Medicine, University of Baghdad, Baghdad, Iraq. E-mail: bm.al-musawi@comed.uobaghdad.edu.iq
ORCID ID: https://orcid.org/0000-0002-4404-377X

Wilson disease (WD) (hepatolenticular degeneration) (MIM #277900) is a rare autosomal recessive disease caused by a defect in copper metabolism, which primarily accumulates in the liver and brain.[1] Wilson disease is a well-established single-gene disease caused by biallelic mutations of the *ATP7B* gene (MIM number 606882), located at 13q14.3, which encodes a copper transporter responsible for biliary excretion of excess copper and its incorporation into ceruloplasmin.[2] The *ATP7B* gene spans 80 kb. The longest transcript (NM_000053), detected in the liver, ranges from 7.5–8.5 kb and comprises 21 exons (encoding an approximately 7.5 kb transcript) and 20 introns.[3]

Wilson disease is a multisystem disorder with variable symptoms characterized by the accumulation of intracellular hepatic copper, which then spills into the blood and begins to accumulate in other organs and tissues, such as the putamen of the brain, subthalamus, kidneys, and cornea.[4] The clinical manifestations depend on the organs in which copper accumulates during the pathological process. Therefore, liver and neurological involvement predominates. Wilson disease can have a pure hepatic, neurological, or a mixed presentation. Most pediatric patients present with liver disease, whereas patients diagnosed in adulthood predominantly have mixed presentations. Diagnosis is typically made in childhood, adolescence, or early adulthood (between 5 and 35 years of age); however, late presentation has also been described.[5]

An estimated disease prevalence is 1:30,000–1:50,000 in Europe, USA, and Asia. It is surprisingly close to the 1984 proposals of Scheinberg and Sternlieb.[6] Other populations have a higher prevalence, such as the Middle East, Pakistan, and India.[6]

There is significant variability in WD in terms of the age at onset, impact on the liver or brain, severity, and response to treatment. Analysis of hundreds of different deleterious variants in WD has failed to find convincing evidence of genotype-phenotype correlations.[7]

The diagnosis of WD is currently based on a scoring system developed at the 8th International Meeting on WD in Leipzig,[8] which includes clinical signs, histopathological studies, biochemical tests, and genetic analyses. A score of 4 or more confirms WD. This is achievable if 2 deleterious mutations in *ATP7B* are detected segregating with the disease. With a score of three, the diagnosis is possible, and more tests are indicated; while a score of 2 or less, the diagnosis is unlikely.[2]

In the modified Leipzig score, other points are assigned for example a family history of WD and a serum ceruloplasmin value of <5 mg/dL versus a value of serum ceruloplasmin ≥5.[9]

Molecular analysis of *ATP7B* is an essential step for diagnosing WD to initiate proper treatment and prevent or delay the development of fulminant hepatic failure, liver cirrhosis, or neurological manifestations, as neither clinical nor laboratory results are sufficient to confirm the diagnosis in many patients.

The study aims to detect variants of the *ATP7B* gene by long-read next-generation sequencing (NGS) among a group of clinically diagnosed or highly suspected children with WD in Iraq and to analyze the detected variants to determine the disease-causing variants in the *ATP7B* gene.

Methods. This cross-sectional study was carried out between October 2022 and October 2023. The patients were recruited from the Children's Welfare Teaching Hospital, Medical City, Baghdad, Iraq. In total, 55 unrelated children with a diagnosis or high clinical suspicion of WD were recruited to this study. A thorough history was obtained, and physical examination and appropriate investigations were performed. Patients aged <18 years with clinical features suggestive of Wilson disease supported by laboratory evidence at presentation (such as: elevated 24-hour urinary copper level, reduced serum ceruloplasmin, and elevated liver enzymes), the presence of Kayser-Fleischer (KF) ring, and a family history of Wilson disease were included in this study. The data obtained were converted into numbers using a modified Leipzig scoring system.[10] As a rule, any patient with a modified Leipzig score of ≥2 was recruited into this study. Patients with chronic viral hepatitis, autoimmune hepatitis, or other metabolic liver diseases were also excluded.

Two milliliter of peripheral blood samples were withdrawn from each patient, collected in K2EDTA tubes, and kept at 4°C for deoxyribonucleic acid (DNA) extraction and downstream molecular workup. DNA was extracted using a Reliaprep Blood gDNA Miniprep System (Promega, USA). DNA concentration was measured by the Qubit™ dsDNA HS Assay Kit from Invitrogen-Thermo Fisher®, USA.

Polymerase chain reaction (PCR), performed using the KAPA HiFi PCR Kit from Roche®, Switzerland,

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was used to amplify the whole *ATP7B* gene using 10 specific primers from Macrogen®, Korea in 14 different combinations (forward and reverse), which were put in 2 separate tubes for each combination. Polymerase chain reaction products were purified using Agencourt AMPure XP PCR Purification (Beckman Coulter, USA). Sequencing was performed using MinION mk1C Oxford Nanopore (Oxford, UK). Sequencing results were analyzed using MinKNOW, which acquires raw signals from the device and sends them to the analysis pipeline (basecaller) in chunks of defined size, according to the manufacturer's instructions.

The entire coding, non-coding, promoter, 3'-UTR, and 5'-UTR regions of the *ATP7B* gene were sequenced using long-read NGS. This test can detect SNPs as well as small Integrative Genomics Viewer (IGV), Mutation Taster, and other software were used for data analysis. Bidirectional sequence reads were assembled and aligned to reference sequences based on the NCBI Reference Sequence Transcripts and the human genome build GRCh37/UCSC hg19.

Following gene-specific filtering, the data were analyzed to identify sequence variants as well as most deletions and duplications involving the entire gene. Alternative sequencing or copy number detection methods were used to analyze regions with inadequate sequences or copy number data. Reportable variants included pathogenic variants, likely pathogenic variants, and variants of uncertain significance.In silico analysis of all the variants was performed to assess their predicted effects on protein function.

This study was performed according to the Declaration of Helsinki and approved by the Research Ethics Committee in the Department of Pathology & Forensic Medicine, College of Medicine, University of Baghdad (issue no. 18 dated January 19th, 2023). Informed consent for the publication of medical information was obtained from parents/legal guardians of the enrolled patients.

Statistical analysis. Statistical data description and analysis were performed by Microsoft Excel version 2016 and by the IBM SPSS Statistics for Windows version 21 (IBMCorp, Armonk, NY, USA). Qualitative data were described by frequency and percentage. Fisher Exact Test was used to determine non-random associations between 2 categorical variables. A *p*-value <0.05 was considered statistically significant.

Results. For this study, 55 unrelated children from Iraq were recruited. There were 34 (61.8%) males and 21 (38.2%) females, with a male-to-female ratio of 1.62:1. Their ages ranged between 6 and 17 years (12.07±3.36).The detailed demographic and clinical data are presented in **Table 1**.

The asymptomatic patient showed elevated urinary copper levels. The patient was recruited because he had a deceased sister with WD and a cousin with liver cirrhosis.

Results of genetic analysis. Direct *ATP7B* gene sequencing using long-read NGS detected a wide range of disease-causing variants and a group of VUS with conflicting interpretations of their probable deleterious or damaging effects on protein function.

Overall findings. Molecular confirmation of the diagnosis of WD was obtained in 33 of the 55 patients, yielding a WD diagnostic rate of 60% among the recruited children. Thirty-four patients had a score of >4 prior to NGS, and the diagnosis was molecularly confirmed in 30 patients; the remaining four were heterozygous carriers (have one deleterious variant). Thirteen patients had a score of 3 prior to NGS, and the diagnosis was confirmed in 6 patients (4/13 were heterozygous carriers). The remaining 3 had one variant

Table 1 - Demographic and clinical characteristics with age of onset of the enrolled children

Parameters	n	%
Gender		
Male	34	61.8%
Female	21	38.2%
Parental consanguinity		
Positive	47	85.5%
Negative	8	14.5%
Family history of WD or a similar condition		
Positive	27	49.1%
Negative	28	50.9%
Ethnicity		
Arabs	52	94.5%
Kurds	3	5.5%
Kayser-Fleischer ring		
Positive	18	32.7%
Negative	37	67.3%
Clinical presentation		
Hepatic	40	72.7%
Neurologic	2	3.6%
Mixed	12	21.8%
Asymptomatic	1	1.8%
Main clinical presentation		
Age (years)		
Hepatic	Mixed	Neurologic
N=40	N= 12	N= 2
Mean±Standard deviation	Mean±SD	Mean±SD
At presentation		
9.4±3.1	8.6±3.3	8.5±7.7*
At diagnosis		
12.05±3.2	12.4±3.7	13.35±3.7**

*p=0.814; **p=0.742, N: number, WD: Wilson disease

Table 2 - Variants identified by targeted ATP7B gene sequencing by next-generation sequencing arranged by frequency.

#	Nucleotide change	Amino acid change	Variant effect	Variant classification by ClinVar	Region Exon/ intron	Location on chromosome 13	dbSNP code	No. of alleles
1	c.3305T>C	p.Ile1102Thr	Missense	Pathogenic	15	52516629	560952220	4
2	c.956delC	p.Pro319HisfsTer44	Frameshift	Pathogenic	2	52548399	753674382	4
3	c.3741-3742 dupCA	p.Lys1248ThrfsTer83	Frameshift	Pathogenic	18	52511772	1462451206	3
4	c.3694A>C	p.Thr1232Pro	Missense	Likely pathogenic	17	52513192	568009639	3
5	c.4309A>T	p.Lys1437Ter	Stop-gain	Pathogenic	21	52508981	768833241	2
6	c.2575+1G>C	-	Splice-donor	Pathogenic	Intron 10	52524407	766149114	2
7	c.4021G>A	p.Gly1341Ser	Missense	Likely pathogenic	19	52511412	587783317	2
8	c.2336G>A	p.Trp779Ter	Stop-gain	Pathogenic	8	52532466	137853283	2
9	c.2866-2A>C	-	Splice-acceptor	Pathogenic	Intron 12	52520616	1377418826	2
10	c.2905C>T	p.Arg969Trp	Missense	Likely pathogenic	13	52520575	774028495	2
11	c.3061-12T>A	-	Intronic	Pathogenic	Intron 13	52518439	1045194246	2
12	c.2987T>C	p.Met996Thr	Missense	Pathogenic	13	52520493	770782111	2
13	c.2304dupC	p.Met769HisfsTer26	Frameshift	Pathogenic	8	52532497	193922103	2
14	c.4092-4093 delGT	p.Ser1365CysfsTer12	Frameshift	Likely pathogenic	20	52509759	747301758	2
15	c.3443T>C	p.Ile1148Thr	Missense	Pathogenic	16	52515330	60431989	2
16	c.3547-3548 delGC	p.Ala1183TyrfsTer2	Frameshift	Pathogenic	16	52515224	765139243	2
17	c.4022G>A	p.Gly1341Asp	Missense	Pathogenic	20	52509831	779494870	2
18	c.3263T>A	p.Leu1088Ter	Stop-gain	Pathogenic	15	52516671	753250853	2
19	c.2297C>G	p.Thr766Arg	Missense	Pathogenic	8	52532505	121907997	2
20	c.2827G>A	P.Gly943Ser	Missense	Pathogenic	12	52523836	28942076	2
21	c.2447+11delG	-	Intronic	Likely pathogenic	Intron 9, 11bp from exon 9	52531640	758601871	1
22	c.4051C>T	p.Gln1351Ter	Stop gain	Pathogenic	20	52509802	786204578	1
23	c.3517G>A	p.Glu1173Lys	Missense	Pathogenic	16	52515256	756029120	1
24	c.1924G>C	p.Asp642His	Missense	Pathogenic	6	52535995	72552285	1
25	c.1870-39T>C	-	Intronic	Likely pathogenic	Intron 5, 38 bp from exon 6	52536088	747432408	1
26	c.2663C>T	p.Thr888Ile	Missense	Likely pathogenic	11	52524210	935426164	1
27	c.1745-1746delTA	p.Ile582ArgfsTer25	Frameshift	Pathogenic	5	52539130	753962912	1
28	c.2332C>G	p.Arg778Gly	Missense	Pathogenic	8	52532470	137853284	1
29	c.562C>T	p.Gln188Ter	Stop-gain	Pathogenic	2	52548794	1412593296	1
30	c.2426G>A	p.Gly809Asp	Missense	VUS	9	52531673	762578415	1
31	c.1870-39T>G	-	Intronic	Not reported in ClinVar. Found in low % in ExAC	Intron 5, 39bp from exon 6	52536088	747432408	1
32	c.3556G>A	p.Gly1186Ser	Missense	Pathogenic	16	52515217	786204547	1
33	c.2002A>G	P.Met668Val	Missense	Conflicting (VUS / Likely pathogenic)	7	52534403	587783301	1
34	c.3955C>T	p.Arg1319Ter	Stop gain	Pathogenic	19	52511478	193922109	1
35	c.3472-3482delGGTTTAACCAT	p.Gly1158phefsTer	Frameshift	Pathogenic	Gross deletion	52515290	-	1
36	c.3538A>G	p.Ile1180Val	Missense	Likely pathogenic	16	52515235	1324203873	1
37	c.2730+39-2730+41delGTT	-	Intronic deletion	Likely pathogenic	Intron 11, 41bp from exon 11	52524101	751433161	1
38	c.2513delA	Lys838SerfsTer35	Frameshift	Pathogenic	10	52524469	777362050	1
39	c.314C>A	p.Ser105Ter	Stop gain	Pathogenic	2	52549042	753236073	1
40	c.3895C>T	p.Leu1299Phe	Missense	Pathogenic	18	52511620	749472361	1
41	c.2715G>C	p.Glu905Asp	Missense	VUS	11	52524158	923227127	1
42	c.3649-3654delGTTCTG	p.Val1217-Leu1218del	Inframe-deletion	Pathogenic	17	52513231	781266802	1
43	c.2804C>T	p.Thr935Met	Missense	Pathogenic	12	52523859	750019452	1
44	c.3960G>C	p.Arg1320Ser	Missense	Pathogenic	19	52511473	778732681	1
45	c.3121C>T	p.Arg1041Trp	Missense	Likely pathogenic	14	52518367	746485916	1
46	c.3836A>G	p.Asp1279Gly	Missense	Likely pathogenic	18	52511679	778914828	1
47	c.4114C>T	p.Gln1372Ter	Stop gain	Pathogenic	20	52509739	755584106	1

VUS: variant of uncertain significance, SIFT: Sorting Tolerant From Intolerant, #: number

Table 2 - Variants identified by targeted ATP7B gene sequencing by next-generation sequencing arranged by frequency (continuation).

#	Nucleotide change	Amino acid change	Variant effect	Variant classification by ClinVar	Region Exon/ intron	Location on chromosome 13	dbSNP code	No. of alleles
48	c.623C>T	p.Ala208Val	Missense	VUS but polyphen-2 possibly damaging; align-GVGD: Class C0)	2	52548733	754738204	1
49	c.3317T>A	p.Val1106Asp	Missense	Likely pathogenic	15	52516617	775541743	1
50	c.2606G>A	p.Gly869Glu	Missense	Likely pathogenic	11	52524267	775553302	1
51	c.2924C>A	p.Ser975Tyr	Missense	Pathogenic	13	52520556	778163447	1
52	c.2972C>T	p.Thr991Met	Missense	Pathogenic	13	52520508	41292782	1
53	c.2897T>G	p.Val966Gly	Missense	VUS by ACMG, SIFT: Damaging	13	52520583	761430052	1
54	c.1630C>T	p.Gln544Ter	Stop gain	Pathogenic	4	52542657	766906034	1
55	c.2576-44G>T	-	Intronic	VUS, previous analysis from invitae labs showed it is related to disease	Intron 10, 66bp from exon 10, 43bp from exon 11	52524341	746739918	1
56	c.3892G>A	p.V.1298Ile	Missense	VUS / SIFT: affect protein function	18	52511623	753044473	1
57	c.2507G>A	p.Gly836Glu	Missense	Likely pathogenic	10	52524476	773809011	1
58	c.915T>A	p.Cys305Ter	Stop gain	Pathogenic	2	52548441	398123137	1
59	c.347T>C	p.Ile116Thr	Missense	VUS Conflicting (Pathogenic by invitae)	2	52549009	199773340	1
60	c.352G>A	p.Asp118Asn	Missense	VUS by ACMG/ SIFT is deleterious	2	52549004	769655497	1
61	c.1318A>G	p.Ser440Gly	Missense	VUS	3	52544853	759000301	1
62	c.1616C>T	p.pro539Leu	Missense	VUS / Likely pathogenic by ACMG	4	52542671	572122562	1
63	c.3646G>A	p.Val1216Met	Missense	Likely pathogenic	17	52513240	776280797	1
64	c.2807T>A	p.Leu936Ter	Stop gain	Pathogenic	12	52523856	776002066	1
65	c.4125-1G>A	-	Splice-acceptor	VUS / Suspicious for pathogenicity	Intron 20	52509166	1293549383	1
66	c.3182G>A	p.Gly1061Glu	Missense	Pathogenic	14	52513806	764131178	1
67	c.1924G>T	p.Asp642Tyr	Missense	Pathogenic	6	52535995	72552285	1
68	c.1934T>G	p.Met645Arg	Missense	Likely pathogenic	6	52535985	121907998	1
69	c.2530A>T	p.Lys844Ter	Stop gain	Pathogenic	10	52524453	780292767	1
70	c.2532delA	p.Val845SerfsTer28	Frameshift	Pathogenic	10	52524450	755709270	1
71	c.1708-1G>A	-	Splice acceptor	Pathogenic	Intron4	52539170	137853280	1
72	c.1543+1G>C	-	Splice donor	Pathogenic	intron3	52544627	1360279134	1
73	c.2333G>T	p.Arg778Leu	Missense	Pathogenic	8	52532469	28942074	1
74	c.2930C>T	p.Thr977Met	Missense	Pathogenic	13	52520550	72552255	1
75	c.2549C>T	p.Thr850Ile	Missense	Pathogenic	10	52524434	777629392	1
76	c.2000T>A	p.Leu667Ter	Stop gain	Likely pathogenic	7	52534405	1474837260	1

VUS: variant of uncertain significance, SIFT: Sorting Tolerant From Intolerant, #: number

of uncertain significance (VUS) in addition to the disease-causing variant. Eight patients had a score of 2 prior to NGS, and the diagnosis was confirmed in 3 patients; while 5 patients were heterozygous.

Variants' characteristics. Seventy-six distinct variants were identified in 97 out of 110 alleles of the *ATP7B* gene, with a detection rate of 88.18%; detailed data are presented in **Table 2**. Of the 76 variants, 42 (55.3%) were missense variants, 13 (17.1%) were stop-gains, 9 (11.8%) were frameshifts, 11 (14.5%) were intronic variants, and 1 (1.3%) was an in-frame deletion. The common worldwide variant (p.H1069Q) was not detected in any of the enrolled patients. Of the 76 detected variants, 2 were detected in four alleles each, 2 were detected in three alleles each, 16 were detected in 2 alleles each, and 56 were detected in one allele.

Exon 2 harbors 7 (9.2%); exon 13 harbors 6 (7.9%); exons 8, 10, and 16 harbor 5 (6.6%) each; exons 18 and 20 harbor 4 (5.3%) each; exons 6, 11, 12, 15, 17, and 19 harbor 3 (3.95%) each; exons 4, 7, and 14 harbor 2 (2.6%) each; exons 3, 5, 9, and 21 harbor one (1.3%) each, while introns harbor 11 (14.5%) of the detected variants. No variants were detected in exon 1.

The molecular data of those patients are summarized

as follows. Group 1: 30 patients with definitive diagnosis of WD: a) 15 Homozygous: 3 of them had additional deleterious variant; 13 variants were detected in those 15 patients, example were 2 variants were detected in 2 patients each; Table 3. b) 15 compound heterozygous: 1 of them had additional intronic pathogenic variant; Table 4.

Group 2: 12 heterozygous – 6 had additional VUS that affect protein function by SIFT; 4 had additional variant with conflicting interpretation by ClinVar and shown to affect protein function by SIFT; and 2 had additional VUS that is shown to be tolerated by SIFT; Table 5. Group 3: 13 heterozygous; 12 symptomatic, and 1 was asymptomatic carrier; Table 6.

In this study, 12 (21%) patients had 2 heterozygous variants, one of which was disease-causing, whereas the second was either a VUS or had a conflicting interpretation. Six of them were VUS but showed affected protein function evidenced by Sorting Tolerant From Intolerant (SIFT) tool. Four of them had conflicting interpretations but showed evidence of affecting protein function by SIFT. The remaining 2 were VUS, which showed no evidence of an effect on protein function (tolerated) by SIFT (Table 5).

The 13 (23.63%) patients were carriers of a single pathogenic or likely pathogenic variant. One of them was asymptomatic and was included because of a positive family history and elevated urinary copper excretion (Table 6).

Additional findings. Two patients had neuropsychiatric manifestations. One with pure neurological manifestations (ID: 47) was found to be compound heterozygote for a missense pathogenic variants (p.Arg778Leu/p.Ile1148Thr), whereas the other patient (ID: 27) had neuropsychiatric manifestations and a single pathogenic stop-gain variant (p.Leu1088Ter).

Three patients harbored more than one disease-causing variant in the same allele, with a homozygous variant and one additional pathogenic variant.

Discussion. In this study, the target patients were children aged <18 years. The number of males was slightly higher than females, giving an M:F ratio of 1.62:1. This finding is similar to other local and regional studies.[10-12] The vast majority (84%) of patients were offsprings of consanguineous unions. The overall consanguinity rate reported earlier in Iraq ranged from 47–60% in various studies.[13] A high rate of consanguinity has also been reported in other clinical studies from Iraq[10,14] and other regional countries, such as Turkey[12] and the Arab Gulf.[15] In this study, a family history of WD was found in 49.1% of patients, which is less than that reported by Hameed et al,[16] who reported it in 64% of patients. Therefore, screening of first-degree relatives of the proband is highly recommended by the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL).[8,17]

The age of onset and diagnosis has been reported to

Table 3 - Variants detected in homozygous state in fifteen patients with Wilson disease.

Patients' IDs	No. of alleles	Nucleotide change	Codon change	Variant effect	Variant classification	Presentation	MLS score after NGS
14, 23	4	c.956delC	p.Pro319HisfsTer44	Frameshift	Pathogenic	Mixed, Hepatic	10, 11
26, 50	4	c.3305T>C	p.Ile1102Thr	Missense	Pathogenic	Hepatic	8, 9
13	3	c.3741-3742dupCA*	p.Lys1248ThrfsTer83	Frameshift	Pathogenic	Hepatic	10
51	3	c.3694A>C&	p.Thr1232Pro	Missense	Likely pathogenic	Hepatic	8
1	2	c.4309A>T	p.Lys1437Ter	Stop-gain	pathogenic	Mixed	7
4#	2	c.2575+1G>C	-	Splice-donor	pathogenic	Hepatic	9
30#	2	c.4021G>A	p.Gly1341Ser	Missense	Likely pathogenic	Hepatic	10
33	2	c.2336G>A	p.Trp779Ter	Stop-gain	pathogenic	Mixed	8
43#	2	c.2866-2A>C	-	Splice acceptor	pathogenic	Hepatic	7
45	2	c.2905C>T	p.Arg969Trp	Missense	Likely pathogenic	Hepatic	8
48	2	c.3061-12T>A	-	Intronic variant	pathogenic	Hepatic	6
53	2	c.2987T>C	p.Met996Thr	Missense	pathogenic	Hepatic	7
54	2	c.2304dupC	p.Met769HisfsTer26	Frameshift	pathogenic	Mixed	10
Total: 15	32	No. of variants = 13					

#Those patients have one additional variant in heterozygous state, namely: (c.2663C>T, p.Thr888Ile), (c.2924C>A, p.Ser975Tyr), and (c.1934T>G, p.Met645Arg). *This variant is also detected in patient no. 5 but in heterozygous state, so total no. of alleles carrying this variant were 3. &: This variant is also detected in patient no. 38 but in heterozygous state, so total no of alleles carrying this variant were 3. MLS: modified Leipzig score

Table 4 - Variants detected in compound heterozygous state in 15 patients with Wilson disease.

Pt's ID	First variant	Effect / Classification	Second variant	Effect / Classification	Presentation	Score after NGS
2#	c.3517G>A (p.Glu1173Lys)	Missense/pathogenic	c.4051C>T (p.Gln1351Ter)	Stop-gain / pathogenic	Hepatic	10
3	c.1924G>C (p.Asp642His)	Missense/pathogenic	c.1870-39T>C (Intronic variant)	Intronic / Likely pathogenic	Mixed	11
7	c.2332C>G (p.Arg778Gly)	Missense/pathogenic	c.562C>T (p.Gln188Ter)	Stop-gain / pathogenic	Hepatic	9
9	c.4092-4093delGT (p.Ser1365CysfsTer12)	Frameshift/pathogenic	c.3556G>A (p.Gly1186Ser)	Missense / pathogenic	Hepatic	8
11	c.3955C>T (p.Arg1319Ter)	Stop-gain / pathogenic	c.3472-3482del GGTTTAACCAT (p.Gly1158PhefsTer2)	Frameshift / pathogenic	Mixed	8
12	c.3538A>G (p.Ile1180Val)	Missense/likely pathogenic	c.2730+39-2730+41delGTT (Intronic deletion)	Intronic deletion / Likely pathogenic	Mixed	10
19	c.3649-3654del GTTCTG (p.Val1217-Leu1218del)	Inframe-deletion / pathogenic	c.2804C>T (p.Thr935Met)	Missense / pathogenic	Mixed	10
20	c.3960G>C (p.Arg1320Ser)	Missense/ pathogenic	c.3121C>T (p.Arg1041Trp)	Missense / likely pathogenic	Hepatic	7
28	c.3317T>A (p.Val1106Asp)	Missense / likely pathogenic	c.3263T>A (p.Leu1088Ter)	Stop-gain / pathogenic	Mixed	10
32	c.2972C>T (p.Thr991Met)	Missense/ pathogenic	c.2297C>G (p.Thr766Arg)	Missense / pathogenic	Hepatic	7
42	c.3182G>A (p.Gly1061Glu)	Missense/ pathogenic	c.1924G>T (p.Asp642Tyr)	Missense / pathogenic	Hepatic	8
44	c.2827G>A (p.Gly943Ser)	Missense/ pathogenic	c.2530A>T (p.Lys844Ter)	Stop-gain / Pathogenic	Hepatic	6
46	c.1708-1G>A (Splice acceptor variant)	Splice acceptor/ pathogenic	c.1543+1G>C (Splice donor variant)	Splice donor / pathogenic	Hepatic	9
47	c.3443T>C (p.Ile1148Thr)	Missense/ pathogenic	c.2333G>T (p.Arg778Leu)	Missense / pathogenic	Neuro	8
49	c.2930C>T (p.Thr977Met)	Missense/ pathogenic	c.2549C>T (p.Thr850Ile)	Missense / pathogenic	Hepatic	7

#This patient had additional third variant, c.2447+11delG (intronic variant, classified as likely pathogenic). NGS: next-generation sequencing

coincide with clinical presentation, where neurological symptoms are typically preceded by liver involvement.[2] However, this was not observed in the present study. The clinical manifestations of WD may vary from asymptomatic patients with only biochemical abnormalities, to those with advanced disease such as liver cirrhosis, neurological deficits, and neuropsychiatric disorders. Hepatic manifestations are the predominant presenting features in pediatric patients.[18] In the current study, the highest percentage of patients with WD presented with pure hepatic manifestations (72.7%), followed by mixed presentations (21.8%), similar to the pattern observed worldwide. The factors that determine whether a WD patient will develop hepatic or neurological diseases, or both, remains unclear.[9]

The diagnosis of WD, based on clinical and biochemical abnormalities alone, is often difficult. Similar non-specific chronic liver diseases with elevated urinary copper, reduced serum ceruloplasmin levels, and liver cirrhosis can be found in other inherited conditions, such as glycosylation disorders and manganese storage disease.[2,19] Therefore, genetic testing using microarray-based methods or next-generation sequencing (NGS) is essential. Sequencing the entire *ATP7B* gene using NGS is an essential tool for diagnosing Wilson disease.[9]

In the present study, direct NGS analysis of the entire *ATP7B* gene in Iraqi patients with WD was performed. Previous Iraqi articles have studied the clinical and epidemiological characteristics of patients with WD.[10,16] To date, 1,275 unique variants of the *ATP7B* gene have been identified.[20] In this study, 76 variants were found in 97 of the 110 alleles; 56/76 were detected in only one allele, whereas only 2 variants were detected in four alleles of 2 homozygous patients. This demonstrated the tremendous heterogeneity of the detected variants, even in consanguineous marriages. This was also observed in other studies from India[9] and China[21] but not in Turkey.[22] This heterogeneity among the Iraqi population has also been observed for other diseases.[23]

Single-nucleotide missense and nonsense variants are the most common types of the *ATP7B* gene worldwide,

Table 5 - Variants characteristics detected in 12 children with 2 variants: a disease-causing variant plus a VUS or a variant with conflicting pathogenicity.

Pt's ID	First variant (Disease causing)	Variant effect / Classification	Second variant :(Variant effect/ classification (VUS/ conflicting)	SIFT Score for the VUS *	Predictions of functional effect with SIFT	Score after NGS
8#	c.4092-4093delGT (p.Ser1365CysfsTer12)	Frameshift/ likely pathogenic	c.2426G>A (p.Gly809Asp): missense/VUS	0.2	Tolerated	3
10	c.3443T>C (p.Ileu1148Thr)	Missense/ pathogenic	c.2002A>G (p.Met668Val): missense/ conflicting (VUS/Likely pathogenic)	0.04	Affect protein function	8
18	c.3547-3548delGC (p.Ala1183TyrfsTer2)	Frameshift/ pathogenic	c.2715G>C (p.Glu905Asp): missense/VUS	0.04	Affect protein function	9
24	c.4114C>T (p.Gln1372Ter)	Stop-gain/ pathogenic	c.623C>T (p.Ala208Val) missense/ VUS	0.4	Tolerated	5
34	c.2297C>G (p.Thr766Arg)	Missense/ pathogenic	c.2897T>G (p.Val966Gly): missense/VUS	0.06	Affect protein function	6
35	c.1630C>T (p.Gln544Ter)	Stop-gain/ pathogenic	c.2576-44G>T: Intronic variant/ VUS	0.05	Affect protein function	5
36	c.2507G>A (p.Gly836Glu)	Missense/ likely pathogenic	c.3892G>A (p.Val1298Ile): missense/ VUS	0.03	Affect protein function	4
37	c.915T>A (p.Cys305Ter)	Stop-gain/ pathogenic	c.347T>C (p.Ile116Thr): missense/ Conflicting (VUS/Likely pathogenic)	0.03	Affect protein function	8
38	c.3694A>C (p.Thr1232Pro)	Missense/ likely pathogenic	c.352G>A (p.Asp118Asn): missense/ conflicting (VUS/Likely benign)	0.04	Affect protein function	4
39	c.3547-3548delGC (P.Ala1183TyrfsTer2)	Frameshift/ pathogenic	c.1318A>G (p.Ser440Gly): missense/ VUS	0.02	Affect protein function	6
40	c.2827G>A (p.Gly943Ser)	Missense/ pathogenic	c.1616C>T (p.Pro539Leu): missesnse/ conflicting (VUS/Likely pathogenic)	0.02	Affect protein function	6
41	c.2807T>A (p.Leu936Ter)	Stop-gain/ pathogenic	c.4125-1G>A (Splice acceptor variant)/ VUS	0.04	Affect protein function	7

*Scores less than 0.05 are considered deleterious. #This patient (ID 8) has additional heterozygous variant (13:52536088, rs747432408, c.1870-39T>G, intronic variant, has not been reported before and found in low penetrance in ExAC). Pt' ID: Patient's identification, NGS: next-generation sequencing

Table 6 - Variant characteristics, clinical presentation, and MLS scores of 13 highly suspected children to have Wilson disease with a single heterozygous deleterious variant.

Patients' IDs	Nucleotide change	Codon change	Variant effect	Variant classification	Presentation	MLS	
						Before NGS	After NGS
5	c.3741-3742dupCA	p.Lys1248ThrfsTer83	Frameshift	Pathogenic	Hepatic	4	5
6	c.1745-1746delTA	p.Ile582ArgfsTer25	Frameshift	Pathogenic	Mixed	3	4
15	c.2513delA	p.Lys838SerfsTer35	Frameshift	Pathogenic	Hepatic	2	3
16	c.314C>A	p.Ser105Ter	Stop-gain	Pathogenic	Hepatic	5	6
17	c.3895C>T	p.Leu1299phe	Missense	Pathogenic	Hepatic	4	5
22	c.3836A>G	p.Asp1279Gly	Missense	Pathogenic/likely pathogenic	Hepatic	2	3
25	c.4022G>A	p.Gly1341Asp	Missense	Pathogenic	Mixed	3	4
27	c.3263T>A	p.Leu1088Ter	Stop-gain	Pathogenic	Neuropsychiatric	2	3
29	c.2606G>A	p.Gly869Glu	Missense	VUS/Likely pathogenic	Hepatic	3	4
52	c.2000T>A	p.Leu667Ter	Stop-gain	Pathogenic/likely pathogenic	Hepatic	4	5
55	c.3646G>A	p.Val1216Met	Missense	Pathogenic/Likely pathogenic	Asymptomatic	3	4
56	c.2532delA	p.Val845SerfsTer28	Frameshift	Pathogenic	Hepatic	2	3
57	c.4022G>A	p.Gly1341Asp	Missense	Pathogenic	Hepatic	2	3

ID: identification, MLS: modified Leipzig score, NGS: next-generation sequencing

followed by insertions/deletions (indels) and splice-site mutations.[25] Rare genetic mechanisms include whole-exon deletions, promoter region mutations, three concurrent pathogenic variants, and uniparental disomy.[25,26] In the current study, the predominant variant type was missense, followed by nonsense, intronic, frameshift, and in-frame deletions, similar to what has been reported in other studies worldwide.[20]

The frequency of pathogenic variants associated with WD has regional differences. For example, the H1069Q variant is among the commonest mutations. It has a population allelic frequency of 10–40% (30–70% among Caucasians) and is detected most frequently in Central and Eastern Europe.[27] However, its prevalence greatly varies based on geographic area. The most common variants identified in this study were p.Ile1102Thr and p.Pro319HisfsTer44, followed by p.Lys1248ThrfsTer83 and p.Thr1232Pro.

Genetically, there were 4 groups of patients in this study: homozygotes, compound heterozygotes, heterozygotes without additional variants, and heterozygotes with another variant (VUS).

Homozygous and compound heterozygous patients. The diagnosis was straightforward with no clinical problems in this group of patients, as the variants were either reported in other studies or classified according to the ACMG guidelines as disease-causing (pathogenic or likely pathogenic variants).

One patient (ID=47) in this study had pure neurological manifestations. The patient was a 16-year-old boy with a positive consanguinity and a negative family history; his MLS score was 8. A compound heterozygous state of the 2 pathogenic variants, p.Ile1148Thr and p.Arg778Leu, was observed.

The (p.Arg778Leu) variant is the most prevalent in East Asia and is considered the first hotspot mutation in the Chinese population.[22] Recently published articles have reported that patients with WD with the p.Arg778Leu variant present with earlier disease onset and predominantly hepatic symptoms.[28,29]

Heterozygous patients with no additional variant. Twelve symptomatic patients (and one asymptomatic child) carried only one disease-causing variant without any additional variants on *ATP7B*. *ATP7B* is the only gene known to be responsible for WD.[30] The frequency of heterozygote *ATP7B* mutations was considerably higher than the previously reported.[31,32] The findings of heterozygous manifesting WD patients were also found in published articles, though they were not epidemiological studies, such as Paris[33] and China.[34]

Early evidence suggests that among carriers of autosomal recessive conditions, intermediate phenotypes are plausible.[35] The presence of symptoms suggestive of WD in patients with one deleterious variant can be explained by the inability of the test to detect a second variant. However, this was not the case in this study, as long-read NGS covered all regions of the *ATP7B* gene and all possible variant types, including gross deletions.

Another possibility is the presence of a modifier gene that can either alter gene function (mutation of a regulatory region) or presence of a coexisting gene mutation (in heterozygous state) causing hepatic manifestation. Such genes are *ATP7A*, *PNPLA3*, *MTHFR, ESD, INO80, HTT*, and others.[7] These genetic modifiers can alter disease onset and phenotype, and some can even result in similar biochemical changes. In fact, 3 of the reported patients in this study had a clinical diagnosis of Gilbert disease, Alagille syndrome, and beta thalassemia major. They were found to have a single variant in *ATP7B* gene. This cannot be excluded as we tested only for *ATP7B*, whole genome sequencing can detect such possibility.

A third possibility is uniparental segmental isodisomy, which was documented as a novel disease-causing mechanism of WD in one study.[25] This mechanism cannot be excluded from the present study because parental testing was not performed. A search for other causes of the WD phenotype for possible misdiagnosis, as it occurs in approximately 1% of cases, is necessary. The list of differential diagnosis is long, where similar clinical and/or biochemical changes are observed. This includes progressive familial intrahepatic cholestasis, congenital disorders of glycosylation, and manganese storage disease (referred to as the new Wilson disease).[19]

Urinary copper levels following a D-penicillamine challenge and symptomatic responses to chelation therapy can aid clinicians to manage these patients. Knowing that 7/12 of the carriers had an MLS score of 4 or more favored the diagnosis of WD. For patients with a score of 2, WD is unlikely.

Heterozygous patients with one additional variant. In this study, 12 patients carried a VUS in combination with a pathogenic or likely pathogenic variant. According to the ACMG, the most important criteria for establishing the causality of putative disease-causing mutations are minor allele frequency, co-segregation, and in silico pathogenicity scores (36).

In this study, an in-silico analysis of the variants was performed using the SIFT tool. According to the SIFT scores, 10 variants (p.Met668Val, p.Glu905Asp, p.Val966Gly, c.2576-44G>T, p.Val1298Ile, p.Ile116Thr, p.Asp118Asn, p.Ser440Gly, p.Pro539Leu, and c.4125-1G>A) were predicted to affect protein

function, and 2 (p.Gly809Asp, p.Ala208Val) were predicted to be tolerated (**Table 5**).

Being detected in symptomatic patients with biochemical changes suggesting WD, their MLS score of ≥4, and the presence of an additional single deleterious variant, these 10 VUS that show an effect on protein function can be reclassified as disease-causing.

Only one case of an intronic VUS affecting a splice site has been reported. All other VUS occurred in the coding regions. Given that the in-silico analysis of intronic variants can reveal their impact on the splicing process, the consequences of a given substitution are generally unpredictable. Functional in-vitro analysis of the effects of potential splicing mutations can confirm the putative pathogenicity of non-coding mutations and thus help guide the patient's clinical management and improve genetic counseling in affected families.[36]

The following cases showed evidence on the rationale for reclassification: i) An 8-year-old girl (ID=38) died from liver failure. Her MLS score was 4. She was born to consanguineous parents and had no relevant family history. The patient presented with hepatic manifestations at 5 years. She had 2 variants, p.Thr1232Pro (a likely pathogenic missense variant, also detected in patient 51 in the homozygous state and previously reported in Morocco.[37] The second variant was p.Asp118Asn, a missense variant classified as VUS according to the ACMG guidelines. In silico analysis using SIFT revealed that it was deleterious. This variant has been identified on 79/280,864 chromosomes in the general population, using the Genome Aggregation Database (gnomAD). The disease was severe enough to cause liver cirrhosis and death, with one LP variant and one VUS shown by SIFT to be deleterious, making it more likely to be the second deleterious variant.

ii) The patient (ID=34) was a 10-year-old Kurdish female with a negative family history of WD and positive consanguinity who presented with hepatic manifestations at 6 years of age, leading to liver cirrhosis, for which liver transplantation was performed. Her MLS score was 6. Two variants were detected in the heterozygous state: a missense pathogenic variant (p.Thr766Arg) and a missense VUS variant (p.Val966Gly), according to ACMG. In silico analysis using SIFT revealed a damaging effect.

iii) The patient (ID=39) was an 8-year-old male with a positive consanguinity and a negative family history of WD. The patient presented with liver cirrhosis for which liver transplantation was performed. His MLS score was 6. Two heterozygous variants were detected: a frameshift pathogenic variant (p.Ala1183TyrfsTer2) (which was also detected in a heterozygous state in another patient) and a missense variant classified according to the ACMG guidelines as VUS (p.Ser440Gly). This variant has not been reported in individuals with WD in the literature and has been identified on 2/248,776 chromosomes in the general population with gnomAD.

iv) A 10-year-old boy (ID=35), who was a child of a consanguineous union with a negative family history, presented with hepatic manifestations and a score of 5. The patient also had a congenital heart defect and clubfoot. Brain MRI revealed abnormal signal intensity in white matter and the basal ganglia with mild brain atrophy. A pathogenic (p.Gln544Ter) stop-gain variant was detected along with another intronic variant (c.2576-44G>T) located at 13:52524342, intron 10, approximately 66 bp from exon 10 and 43 bp from exon 11, which was classified as VUS. In silico analysis revealed that it affects proteins. A previous analysis by Invitrogen Laboratories indicated that it is related to disease. The presence of congenital heart defects and clubfoot raised the suspicion of concomitant Alagille syndrome. Amson et al[38] reported 2 siblings presented with liver disease and were carriers of 2 deleterious genetic variants.

v) A 17-year-old male (ID=36), who was a child of a consanguineous union and negative family history, presented with acute abdominal pain, shrunken lobulated liver, and signs of portal hypertension. His MLS was 4. Two variants were detected: a rare missense (p.Gly836Glu) likely pathogenic variant [also reported in 3 cases: one in a Moroccan patient,[37] a second in an Italic patient of Moroccan origin,[39] and a third in a French patient[40]]. The second variant was a missense VUS (p.Val1298Ile). Computational prediction in ClinVar suggests it may have a deleterious impact on protein structure and function. In-silico study by SIFT showed a score of 0.03, indicating that it affects protein function.

In summary, in a clinically relevant scenario, when a biallelic mutation is detected, no additional work is required. However, when a heterozygous variant is detected, with or without an additional VUS, a search for other variants in regulatory regions or in other related genes may be necessary. In addition, a comprehensive functional study is required to provide evidence of pathogenicity of the detected VUS with regular checkups for any update or reclassification.

Study limitation. Further molecular workup is needed for patients having disease manifestations and one deleterious variant, with or without additional VUS (variant functional study). This was not possible for this group of patients.

In conclusion, the *ATP7B* mutational spectrum in the Iraqi population is diverse, despite high rates of consanguinity. Evidence for ten VUS to be reclassified as deleterious was provided. Whether patients with a Leipzig score greater than 4 who carry a single deleterious variant can be definitively diagnosed as having WD or classified as manifesting carriers remains uncertain. Whole-exome or genome sequencing is recommended for patients with only one detected variant. Additionally, functional in-vitro or in-vivo studies are advised to ascertain the pathogenicity of the detected VUS.

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