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Original article

Phenotype and molecular characterization of Wilson's disease in Morocco

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ABSTRACT

Background and study aims: In Morocco the prevalence of Wilson disease (WD) and the spectrum of mutations are not known. The aim of the present study was to estimate the prevalence of WD in Morocco, to evaluate the phenotype among a large cohort of WD patients, and to characterize *ATP7B* variants in a subgroup of WD patients.

Patients and methods: We collected data from 226 patients admitted to five university hospital centers in Morocco between 2008 and 2020. The diagnosis was based on clinical manifestations, function tests and biochemical parameters. The genotype was characterized in 18 families diagnosed at the University Hospital Center of Marrakesh, by next generation sequencing.

Results: The mean annual prevalence in Morocco was 3.88 per 100,000 and the allele frequency was 0.15 %. Among the 226 patients included (121 males and 105 females), 196 were referred for a hepatic or neurological involvement and 30 were asymptomatic. The mean age at diagnosis was 13 ± 5.1 years (range: 5 - 42 years). Consanguinity was found in 63.3 % of patients. The mean duration of illness was 2.8 ± 1.9 years. Kayser-Fleischer rings were found in 131 (67.9 %) of 193 patients. Among the 196 symptomatic patients, 141/159 (88.7 %) had low serum ceruloplasmin (<0.2 g/L) and a high 24-hours urinary copper (>100 µg/day) was found in 173/182 (95.1 %) patients. The initial treatment was D-penicillamine in 207 patients, zinc acetate in five, zinc sulfate in five, and nine patients were not treated; 60/207 (29 %) patients have stopped treatment. A total of 72 patients died; the mortality rate was 31.9 %. Eight different *ATP7B* variants were identified among the 18

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patients studied, of which two were novel (p.Cys1104Arg and p.Gln1277Hisfs*52), and six previously published (p.Gln289Ter, p.Cys305Ter, p.Thr1232Pro, p.Lys1020Arg, p.Glu583ArgfsTer25 and c.51+4A>*T*). All informative patients were homozygous for the disease-causing mutation.
Conclusion: In Morocco, a high prevalence due to consanguinity and a high mortality rate due to the difficulty of diagnosis and lack of treatment were observed in WD patients. NGS sequencing identified new *ATP7B* variants in WD patients from Morocco.

1. Introduction

Wilson disease (WD) is a rare autosomal recessive genetic disease, caused by a large number of different mutations in the *ATP7B* gene [1] that leads to excessive copper accumulation in the liver and then in the brain and other tissues and produces protean clinical manifestations, including variable combinations of hepatic, neurological, psychiatric, ophthalmological, and other disorders [2]. WD typically begins with a presymptomatic period, during which copper accumulation in the liver causes subclinical hepatitis, and progresses to liver cirrhosis and development of neuropsychiatric symptoms [3]. The diagnosis of WD is based on the combination of clinical, biochemical, and genetic analyses [4]. However, the molecular genetic spectrum of WD is wide and heterogeneous. To date, more than 900 variants have been identified in *ATP7B* gene. The frequency of common variants varies greatly among different ethnic groups [5–8]. The disease prevalence varies with the geographic area and usually ranges from 1 case per 30,000 to 100,000 live births in most populations [9]. In Morocco, there are great diagnostic difficulties such as: genetic analysis is not performed and the prevalence of WD is not known [10], but we expect to have a high prevalence of the disease as a result of frequent consanguineous marriages [11]. In addition, only a few studies investigating WD, which included very few patients, have been reported [12–17]. For these reasons, we therefore conducted a study to estimate the prevalence of WD in Morocco and to investigate the clinical presentations, diagnostic modalities and genetic features of WD patients in Morocco.

2. Patients and methods

2.1. Patients, clinical, and biochemical data

A retrospective study of 226 patients with WD was conducted between 2008 and 2020. The age at diagnosis ranged from 5 to 42 years (median age 13 years). These patients were referred to the departments of Neurology, Paediatrics, Child Neurology, and Internal Medicine of the five University Hospital Centers (CHU) in Morocco: CHU Mohammed VI Marrakesh, CHU Ibn Rochd Casablanca, CHU ibn Sina Rabat, CHU Hassan II Fez and CHU Mohammed VI Oujda.

The diagnosis of WD was based on clinical manifestations, function tests and biochemical parameters. The collected data included: clinical data, the presence of a Kayser-Fleischer ring (K-F ring), liver function test, measurement of serum ceruloplasmin levels, determination of 24-h urinary copper excretion, age at symptom onset and at diagnosis, age at death, treatment, consanguinity, geographic origin. All patients included in the present study had to have a Leipzig score ≥ 4 [18].

According to the primary clinical manifestations, patients were classified as follows: hepatic form, characterized by the presence of clinical and biological signs, indicating an hepatopathy, neurological/psychiatric form, characterized by neurological disturbances and/or psychiatric symptoms with or without hepatic symptoms, and asymptomatic form, characterized by an absence of symptoms but with laboratory findings confirming WD.

2.2. Genetic analysis

Genetic analysis was performed in the 18 WD patients and their families diagnosed in the university hospital center of Marrakech (CHU Mohammed VI). Written informed consent was obtained from all patients or patients' legal guardians, and the study was approved by the local ethics committee under protocol number 032/2020.

The *ATP7B* (NM_000053.4) full gene (including exons, introns, and promoter region from c.−1200) was sequenced, as well as coding sequences (exons ± 25 nucleotides of four genes reported to be associated with copper workup abnormalities, for differential diagnosis: *CP* (NM_NM_000096.4), *SLC33A1* (NM_004733), *NPC1* (NM_000271.5), and *NPC2* (NM_006432.5).

Genomic DNA was extracted from the whole venous blood collected in ethylenediaminetetraacetic acid (EDTA) tubes employing the salt precipitation method [19]. The targeted next generation sequencing (NGS) was performed after target capture, enrichment, and elution according to the manufacturer's protocols (Twist Bioscience) without modification except for library preparation performed using the NEBNext® Ultra II kit (New England Biolabs®). For the library preparation, 150 ng of each genomic DNA were fragmented by sonication and purified to yield fragments of 150–200 bp. Paired-end adaptor oligonucleotides from the NEBNext® Ultra II kit were ligated on repaired, a-tailed fragments then purified and enriched by 7 PCR cycles; 500 ng of these purified libraries were then hybridized to the Twist oligo probe capture library for 16 h in a singleplex reaction. After hybridization, washing, and elution, the eluted fraction is PCR-amplified with 8 cycles, purified and quantified by QPCR to obtain sufficient DNA template for downstream applications. Each eluted-enriched DNA sample was then sequenced on an Illumina NovaSeq as Paired End 100 reads. Image analysis and base calling were performed using Illumina Real-Time Analysis (3.4.4) with default parameters [20] (IntegraGen, Evry, France). All coding sequences (exons and intron-exon junctions) were covered above 25X. The overall design coverage was 81 %.

Single nucleotide variations were analyzed using an in-house bioinformatics pipeline developed by IntegraGen (Sirius platform). The copy Number Variations (CNV) were analysed using DeCovA tool [21]. The Genome Aggregation Database (gnomAD), Human Gene Mutation Database (HGMD), and ClinVar (CV) database were searched for previously to identify WD variants. Variants interpretation followed ACMG guidelines [22].

2.3. Statistical methods

The SPSS 20.0 (IBM SPSS statistics) was used for data entry and analysis. All numeric variables were expressed as mean ± standard deviation (SD). Comparison of variables in the various groups was made using Student's test. The Chi-squared test was used to compare frequency of qualitative variables among the different groups. For all the tests a probability (p), of less than 0.05 was considered significant.

The prevalence of WD was evaluated in accordance with the data obtained from the census population published by the Moroccan institute for statistics (*Haut-Commissariat au Plan*) [23]:

$$\text{Disease prevalence} = \frac{\text{Number of persons with Wilson disease}}{\text{Number of individuals in the population}}$$

3. Results

3.1. Prevalence and epidemiological profile of WD in Morocco

A total of 226 patients were examined including 121 males and 105

females. Most were identified from the medical files of the pediatrics department ($n = 188$), followed by the department of neurology ($n = 27$), and gastroenterology-hepatology ($n = 11$). The mean age at diagnosis was 13 ± 5.1 years (range, 5–42 years). Among them, 140 patients (61.9 %) were < 14 years of age, and three patients (1.3 %) were over 30 years of age. The oldest initial onset of WD was at the age of 42 years. Men were diagnosed with WD at a similar age as women (12.7 ± 5.4 years *vs* 13.3 ± 4.9 years; $p = 0.601$). Consanguinity was found in 63.3 % of patients. The 226 patients studied were drawn from 175 unrelated families. Ninety-four (53.7 %) parents were first degree cousin. The mean interval between first symptoms and diagnosis was noted among 196 patients, and it was 2.8 ± 1.9 years. The median duration of follow-up duration was 5 years.

Between 2008 and 2020, the mean annual prevalence was 3.88 per 100,000; the prevalence increased from 1.34 in 2008 to 6.61 per 100,000 in 2020 ($p < 0.001$; Table 1). Allele frequency was 0.15 %.

The geographical distribution of Wilsonian families is represented in Fig. 1; three of high prevalence were observed. They correspond to the Fez-Meknes, Draa-Tafilalet, and the Oriental region.

3.2. Clinical manifestations

A total of 114 patients (50.4 %; 53 males and 61 females) presented hepatic symptoms, 82 patients (36.3 %; 36 males and 46 females) had neurologic presentation and 30 (13.3 %; 22 males and 8 females) were diagnosed by family screening at a presymptomatic stage. The mean interval between first symptoms and diagnosis was earlier in patients with a hepatic presentation than in those with a neurologic presentation (1.7 ± 1.2 *vs* 4.2 ± 1.9 years; $p < 0.001$).

Among the 196 symptomatic patients, the most frequent hepatic manifestations were jaundice ($n = 86$) and abdominal distension ($n = 80$), followed by hepatomegaly ($n = 67$), and edema of the lower limbs ($n = 57$), ascites ($n = 90$), splenomegaly ($n = 73$) and esophageal varices ($n = 51$). The most frequent neurological symptoms were: tremor ($n = 55$) and gait disturbance ($n = 55$), followed by dystonia ($n = 50$), dysarthria ($n = 47$), psychiatric disorders ($n = 40$), and mental retardation ($n = 30$); no patient was diagnosed with psychiatric symptoms alone. Sixty-eight (34.7 %) patients had liver cirrhosis at diagnosis, and 24 (12.2 %) fulminant liver failure.

Table 1
Prevalence of Wilson's disease in Morocco.

Year	Total population			WD patients			Prevalence per 10^5 persons		
	F	M	T	F	M	T	F	M	T
2008	197 389	175 748	373 137	2	3	5	1.01	1.71	1.34
2009	202 611	189 669	392 280	3	4	7	1.48	2.11	1.78
2010	207 491	206 806	414 297	4	7	11	1.93	3.38	2.66
2011	212 452	226 024	438 476	6	5	11	2.82	2.21	2.51
2012	217 139	242 899	460 038	7	4	11	3.22	1.65	2.39
2013	220 657	253 150	473 807	11	4	15	4.99	1.58	3.17
2014	222 586	254 067	476 653	9	10	19	4.04	3.94	3.99
2015	223 170	248 080	471 250	12	11	23	5.38	4.43	4.88
2016	223 222	239 444	462 666	14	8	22	6.27	3.34	4.76
2017	222 873	232 110	454 983	9	14	23	4.04	6.03	5.06
2018	221 383	226 449	447 832	10	14	24	4.52	6.18	5.36
2019	218 656	224 021	442 677	10	16	26	4.57	7.14	5.87
2020	214 968	223 824	438 792	8	21	29	3.72	9.38	6.61

F: Female, M: Male, T: Total.

3.3. Diagnostic examination and function tests

Among the 226 patients, 193 were underwent slit-lamp examination by an experienced ophthalmologist and K-F rings were present in 131 (67.9 %) patients; these were found in 66/111 (59.5 %) patients who presented with the hepatic form and in 65/82 (79.3 %) with the neurologic form.

Serum ceruloplasmin determination was performed in 187 patients, and it was < 0.2 g/L in 167 (89.3 %) patients. Serum copper concentration was measured in 159 patients, and it was < 10 μmol/L in 126 (79.2 %) patients. The urinary copper level was measured in 209 patients, and it was > 1.6 μmol/24 h in 199 (95.2 %) patients.

Cerebral Magnetic Resonance Imaging (MRI) data were available for 85 patients, and it was abnormal in 53 patients (T2 and FLAIR hyperintensities involving bilateral thalami, midbrain, and upper pons); all had a neurological form.

All the clinical and biochemical features of these patients at the time of diagnosis are summarized in Table 2.

3.4. Treatment

Nine patients had a fulminant hepatic presentation and died shortly after admission without any treatment. For 207/217 (95.4 %) patients, the initial treatment was D-penicillamine, zinc acetate for five patients (2.3 %), and zinc sulfate for another five patients (2.3 %). Among those who started with D-penicillamine, 141/207 (68.1 %) patients continued treatment during the study period, while 42 (20.3 %) switched to zinc acetate, and 24 (11.6 %) switched to zinc sulfate. D-penicillamine was stopped because of adverse effects (thrombocytopenia in 32 patients, severe hypersensitivity with a skin rash in 13, neurological deterioration in 11, severe vomiting in 6 and disturbance of consciousness in 4 patients) that occurred within the first six months of treatment. No patient received a liver transplant.

3.5. Outcome

The course of WD was marked under treatment by clinical improvement in 77/114 hepatic forms and 47/82 neurologic ones (a total of 124 (54.9 %) patients). The thirty asymptomatic patients (13.3 %) evolved favorably and followed their treatment without any complications.

Seventy two patients died during the course of the disease (31.8 %). Early death due to fulminant hepatic disease occurred in 20 patients and late death in 52 patients (decompensated cirrhosis in 41 and neurological deterioration in 11 patients). The mean age at death was 15.7 ± 4.5 years (range 7–28 years).

3.6. Mutational analysis

Targeted sequencing of the *ATP7B* gene by NGS was performed in the 18 WD patients identified in the Marrakesh university hospital (from 17 unrelated families), their parents and siblings; a total of 56 individuals were investigated. The parents of 11 WD patients were first degree relatives Table 3.

Pathogenic or likely pathogenic variants within the *ATP7B* gene were identified in 14 of them, at homozygous state in all patients; 8 different variants (4 nonsense, 3 missense and 1 splice-site variants; Table 4).

Of these eight variants, two had not been reported previously. The first was a transversion, c.3310T > C (exon 15), which led to an amino acid change from cysteine to arginine (p.Cys1104Arg) in the ATP loop domain of the ATP7B protein. This variant is not reported in the GnomAD population database. It is predicted as probably damaged by the

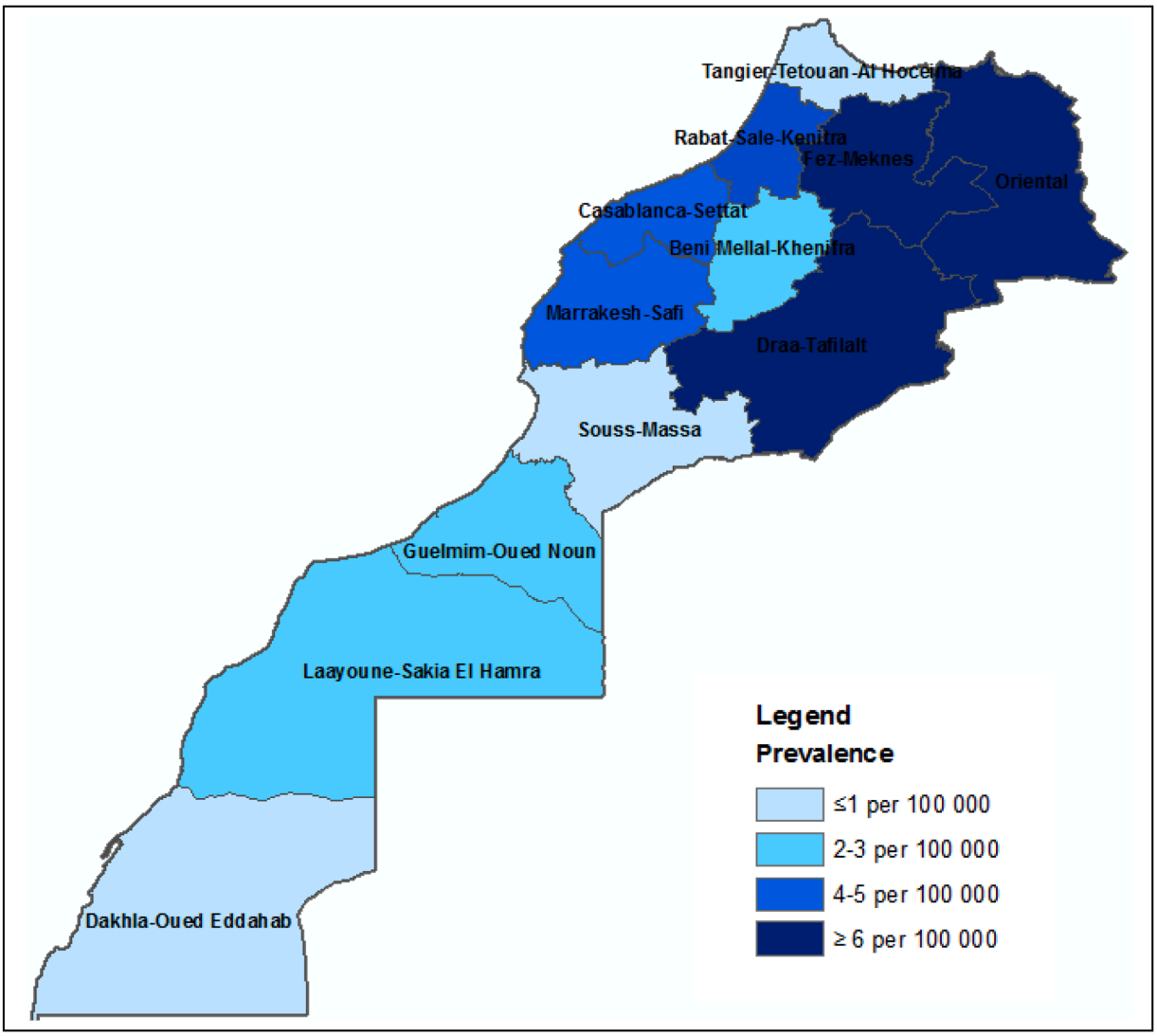

Fig. 1. Standardized Moroccan prevalence of WD patients by region.

Table 2
Clinical characteristic and laboratory parameters in 226 patients with Wilson's disease in Morocco.

	Neurological presentation	Hepatic presentation	Asymptomatic patients	p-value neurological *vs* hepatic presentation
Patients n	82	114	30	
Males/females n	36/46	53/61	22/8	0.027
Age at testing years, mean ± SD	16.1 ± 6.1	11.6 ± 3.5	10.1 ± 3.3	< 0.001
Age of manifestations onset years, mean ± SD	13.5 ± 6.1	10.5 ± 3.6	-	< 0.001
Time to diagnosis years, mean ± SD	4.2 ± 1.9	1.7 ± 1.2	-	< 0.001
K-F ring present n (%) of patients	65 (79.3)	66 (59.5)	-	< 0.001
Ceruloplasmin g/L, mean ± SD	0.09 ± 0.2	0.12 ± 0.13	0.1 ± 0.05	0.775
Serum copper µmol/L, mean ± SD	7.1 ± 4.3	10.7 ± 10.6	7.1 ± 3.6	0.001
Cu in urine µmol/24 h, mean ± SD	11.5 ± 9.3	10.6 ± 9.9	5.6 ± 3.8	0.926
Cirrhosis n (%) of patients	25 (30.5)	43 (37.7)	-	< 0.001
Fulminant hepatic failure n (%) of patients	18 (21.9)	6 (5.3)	-	< 0.001
AST IU/L, mean ± SD	305.3 ± 368.2	342.1 ± 375.2	95 ± 94.3	0.509
ALT IU/L, mean ± SD	220.8 ± 345.6	239.1 ± 339.1	101.5 ± 92.8	0.521
ALP IU/L, mean ± SD	316.2 ± 218.3	451.6 ± 344.7	216.7 ± 75.2	0.003
GGT IU/L, mean ± SD	103.1 ± 72.2	140.1 ± 88.5	50.6 ± 25.2	0.451

m/f: male/female, K-F ring: Kayser-Fleischer ring, SD: standard deviation, Cu: copper, AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: gamma-glutamyltransferase, ALP: alkaline phosphatase, ALB: albumin, TB: total bilirubin, PR: prothrombin rate, INR: international normalized ratio, Hb: hemoglobin, n: total.

Polphen2 algorithm. It was identified as homozygous in a 20-year-old woman who had hepatic and neurological manifestations with a K-F ring. The second was a frameshift variant, c.3831_3835delinsC (p. Gln1277Hisfs*52), which occurred in exon 18. It was also not reported in the gnomAD population database. This variant was identified as homozygous in two patients who were 10 and 13 years of age. They were from two unrelated families and both were presented with a chronic hepatitis without neurological manifestations.

Table 3
Phenotype and genotype of 18 WD patients from Marrakesh University Hospital.

Family			Index case phenotype											*ATP7B* genotype		
Id	Con (Dg)	Origin	Clinical form	Age at onset years	Sex	AST UI/L	ALT UI/L	PR %	Cp g/L	SCu µmol/L	UCu µmol/24h	K-F ring	MRI	Under treatment (D-P)	Variant	Patient genotype	Family genotype
WDM 1	Yes (1)	M-S	H (jaundice, asthenia, hepatomegaly, cirrhosis, portal hypertension)	10	M	144	278	16	0.04	5.9	22.7	Yes	_	Moderate regression of clinical signs	p.Lys1020Arg	+/+	Father: +/-Mother: +/-
WDM 2	Yes (1)	D-T	N (jaundice, asthenia, hepatomegaly, dysarthria)	14	F	68	49	24	0.05	3.1	9.1	No	Anormal	Low regression of clinical signs	p.Gln289Ter	+/+	Father: ND Mother: ND,Brother: +/+
WDM 3	Yes (1)	M-S	N (dysarthria, dystonia, tremor, hepatomegaly)	13	F	42	32	38	_	_	33.8	No	Anormal	High regression of clinical signs	c.51+4A>T	+/+	Father: +/-, Mother: +/-, Sister: +/+
WDM 4	Yes (3)	G-O.N	N (jaundice, hepato-splenomegaly, cirrhosis, portal hypertension)	8	M	74	44	28	_	_	7.9	Yes	Anormal	Moderate regression of clinical signs	No pathogenic variants		
WDM 5	Yes (2)	M-S	N (dysarthria, dystonia, tremor, psychiatric signs, Parkinsonism, hepatomegaly)	12	M	51	47	50	0.05	3.7	5	Yes	Anormal	High regression of clinical signs	p. Glu583ArgfsTer25	+/+	Father: +/-, Mother: +/-, Brother: +/+
WDM 6	Yes (1)	M-S	N (asthenia, dysarthria)	11	M	1413	1489	50	0.03	_	9.2	Yes	Anormal	High regression of clinical signs	No pathogenic variants		
WDM 7	Yes (1)	M-S	H (jaundice, chronic hepatitis, portal hypertension, splenomegaly)	13	M	237	121	40	0.2	12.2	2.7	Yes	_	High regression of clinical signs	No pathogenic variants		Brother: +/-, Brother: +/-
WDM 8	Yes (1)	D-T	H (jaundice, hepato-splenomegaly)	6	F	272	128	13	_	_	26.6	No	_	High regression of clinical signs	p.Gln289Ter	+/+	Father: ND, Mother: +/-
WDM 9	Yes (1)	M-S	H (jaundice, hepatomegaly	9	F	181	61	42	0.03	8.9	7.9	No	_	High regression of clinical signs	p.Thr1232Pro	+/+	Father: ND, Mother: +/-
WDM 10	Yes (1)	S-M	H (jaundice, chronic hepatitis)	8	M	171	376	_	0.03	1.6	9.1	No	_	High regression of clinical signs	p.Lys1020Arg	+/+	Father: +/-, Mother: +/-
WDM 11	Yes (1)	D-T	H (jaundice, asthenia, chronic hepatitis splenomegaly)	10	F	72	52	19	_	_	2	Yes	_	High regression of clinical signs	p.Gln1277Hisfs*52	+/+	Father: +/-, Mother: +/-
WDM 12	No	M-S	H (cirrhosis)	15	F	161	74	44	_	_	7.9	Yes	_	High regression of clinical signs	No pathogenic variants		
WDM 13	Yes (1)	M-S	H (jaundice, asthenia, cirrhosis, portal hypertension)	16	M	171	76	_	0.02	1.4	359	Yes	_	Moderate regression of clinical signs	p. Glu583ArgfsTer25	+/+	Father: ND, Mother: +/-
WDM 14	No	M-S	N (dysarthria, dystonia, tremor, chronic hepatitis)	20	F	49	28	_	0.05	4	5.6	Yes	Anormal	Moderate regression of clinical signs	p.Cys1104Arg	+/+	Father: +/-, Mother: +/-
WDM 15	Yes (2)	M-S	N (dysarthria, tremor, psychiatric signs, chronic hepatitis)	24	F	56	16	_	0.05	7.5	2.4	No	Anormal	High regression of clinical signs	p.Cys305Ter	+/+	Father: +/-, Mother: ND
WDM 16	Yes (2)	D-T	H (hepatomegaly)	9	F	81	57	34	0.03	3.8	4.9	No	_	High regression of clinical signs	p.Thr1232Pro	+/+	Father: +/-, Mother: +/-

(*continued on next page*)

Table 3 (*continued*)

Family			Index case phenotype										*ATP7B* genotype		
Id	Con (Dg)	Origin	Clinical form	Age at onset years	Sex	AST UI/L	ALT UI/L	PR %	Cp g/L	SCu µmol/L	UCu µmol/24h	K-F ring	MRI	Under treatment (D-P)	Variant	Patient genotype	Family genotype
WDM 17	Yes (1)	D-T	N (dysarthria, psychiatric signs, chronic hepatitis)	15	M	47	55	-	0.03	-	2.1	Yes	Anormal	High regression of clinical signs	p.Thr1232Pro	+/+	Father: +/-, Mother: +/-, Sister: +/+, Brother: +/+, Sister: +/-, Brother: -/-
WDM 18	Yes (2)	D-T	H (asthenia, chronic hepatitis, splenomegaly)	13	M	140	70	42	0.04	-	8.3	No	Anormal	High regression of clinical signs	p.Gln1277Hisfs*52	+/+	Father: ND, Mother: +/-

F: female, M: male, Cong (dg): consanguinity (degree), H: hepatic, N: neurologic, AS: asymptomatic, M-S: Marrakesh-Safi, D-T: Draa-Tafilalet, S-M: Souss-Massa, G-O.N: Guelmim-Oued Noun, Cp: ceruloplasmin, SCu: serum copper, UCu: urinary copper, K-F ring: Kayser-Fleischer ring, AST: aspartate aminotransferase, ALT: alanine aminotransferase, PR: prothrombin rate, MRI: magnetic resonance imaging, D-P: D-penicillamine, ND: Not determined.

Three patients were homozygous for a previously published missense mutation c.3694A>C (p.Thr1232Pro) in exon 17. Two patients presented with a hepatic disease at the age of 9, and one patient with a neurological manifestation at the age of 15. These patients came from three unrelated families (WDM 9, 16, and 17). WDM 17 proband had two affected siblings (15 and 8 years of age), who were asymptomatic but screened by sequençing of the *ATP7B* gene, and two unaffected siblings; the parents were heterozygous. Another previously published missense mutation c.3059A>G (p.Lys1020Arg) was detected in exon 13 in homozygous form in two patients (WDM 1; 10 years of age and WDM 10; 8 years of age); both presented with a hepatic failure.

Five of the 17 unrelated families had nonsense mutation homozygous; these were p.Gln289Ter in two patients (WDM 2 and WDM 8), p.Glu583ArgfsTer25 in two patients (WDM 5 and WDM 13), and p.Cys305Ter in one patient (WDM 15). The WDM 2 family had two affected siblings (14 and 18 years of age) presenting with neurological manifestations. WDM 5 family had two affected siblings (10 and 12 years of age), one had a hepatic disease and the other neurological manifestations, both had a K-F ring; the parents were heterozygous.

One splice site mutation (c.51+4A>T) was detected in two patients' siblings (WDM 3). The age at onset was 8 and 13 years. One had a hepatic disease and the other neurological manifestations; the parents were heterozygous.

In the present study, the variants were localised on exon 2 ($n = 3$), 5 ($n = 2$), 13 ($n = 2$), 15 ($n = 1$), 17 ($n = 3$), 18 ($n = 2$), and exon 1 - intron 1 junction ($n = 1$).

Genetic investigation remained inconclusive in 4 patients (who had been diagnosed based on K-F rings, clinical, and biochemical exams), even after CNV analysis, an extensive evaluation of intronic variants in *ATP7B*.

4. Discussion

The present study is the first population-based epidemiological study on WD in the Maghreb, estimating the prevalence of WD and addressing all aspects of WD: epidemiology, clinical forms, diagnosis, genetic analysis, treatment and mortality rate. We detected a high prevalence of WD in Morocco, and this was greater than that reported by Cheung *et a1.* [24] in China (1.8 per 100,000), and by Poujois *et a1.* [25] in France (1.5 per 100,000), which is probably due to the high frequency of consanguinity in Morroco (22,79 %) [11] compared to these countries (1.8 % [26] and 0.8 % [27], respectively). This hypothesis was reinforced by the fact that consanguineous marriages were more frequent (29.58 % in the oriental, 25 % in Draa-Tafilalet and 25 % in Fez-Meknes) compared to other Moroccan regions [11]. The prevalence increased during the study period, which is probably correlated with an improvement in diagnostic tools and more efficient health services in university hospitals in Morocco [28]. However, Machado et al. [29] reported that the mean interval between the initial symptom and the definitive diagnosis of the neurological form was 1.1 ± 1.2 years. Herein, the mean gap in the present study was nearly 4-fold longer, which demonstrates a significantly long period for the correct diagnosis of WD to be established. In Morroco, the diagnostic testing for WD includes liver tests (serum transaminases, total bilirubin, alkaline phosphatase and prothrombin time/INR), serum ceruloplasmin, and 24-hour urinary copper, and the medication usually prescribed for WD is D-penicillamine, in compliance with ESPGHAN guidelines on the management of these patients [30]. We detected a high mortality rate in Moroccan patients, and this was 2-fold higher than that reported in Egypt (15 %) [31] and 15-fold higher than in France (2 %) [5]. This high mortality is in a context of late and difficulty for the diagnosis of WD in Morroco (for example, genetic anlaysis is not widely available), the absence of liver transplantation, and the unavailability of WD treatment. Genetic analysis is one of the most important diagnostics for WD. Variants are distributed throughout the *ATP7B* gene, and more than 900 are reported [5–8]. However, the frequency of variants varies greatly among different ethnic groups. For

Table 4
ATP7B variants detected in patients with WD identified in the Marrakesh university hospital.

Protein change	cDNA change	ClinVar accession	Variant classification (source)	Exon or Intron	Variant type	Protein domain	GnomAD Variant allele frequency (%)			Age of onset -mean - (range in years)	Clinical form - H, N, AS (n)
							Africa	Europe	All		
p.Gln289Ter	c.865C>T	VCV000003864	5 (CV, HGMD) [31,44]	Ex 2	NS	Cu 3	0	0.0054	0.0028	10 (6–14)	$H = 1$ $N = 1$
-	c.51+4A>T	VCV000312401	5 (CV, HGMD) [5,36]	I 1	Spl	before Cu 1	0	0.003	0.002	13	$N = 1$
p.Cys305Ter	c.915T>A	VCV000092391	5 (CV, HGMD) [35]	Ex 2	NS	Cu 3	0	0.0009	0.0004	24	$N = 1$
p.Thr1232Pro	c.3694A>C	VCV000555144	5 (CV, HGMD) [34]	Ex 17	MS	ATP bind	0	0	0.0004	11 (9–15)	$H = 2$ $N = 1$
p.Lys1020Arg	c.3059A>G	VCV001162209	5 (CV, HGMD) [5,31]	Ex 13	MS	Ph / ATP loop	0	0	0	9 (8–10)	$H = 2$
p.Glu583ArgfsTer25	c.1746dupA	pending	5 (HGMD) [37]	Ex 5	NS	Cu 6	0	0	0	14 (12–16)	$H = 1$ $N = 1$
p.Cys1104Arg	c.3310T>C	pending	4 (this study)	Ex 15	MS	ATP loop	0	0	0	20	$N = 1$
p.Gln1277Hisfs*52	c.3831_3835delinsC	pending	4 (this study)	Ex 18	NS	ATP hinge	0	0	0	11.5 (10–13)	$H = 2$

Ex: exon, I: intron, MS: missense, NS: nonsense, Spl: splice region variant, Cu: copper-binding domain, ATP bind: ATP binding domain, Ph: Phosphorylation domain, H: hepatic, N: neurologic, AS: asymptomatic, CV: ClinVar database, HGMD: Human Gene Mutation Database.

example, p.His1069Gln is the most frequent variant in Caucasian populations, and which occurs in 15 % to 63 % of patients with WD [6]. p. Arg778Leu and p.Pro992Leu are the most frequent variants in the Asian population, and they occur in 50 % of patients with WD [7]. In Morocco, the spectrum of *ATP7B* gene has, before the present study, been studied in only 8 patients [32–34]. The variants detected in the present study were shared with other countries, such as p.Thr1232Pro in Brazil [35]; p.Lys1020Arg in France [5]; p.Cys305Ter in Italy [36]; c.51+4A>*T* in both France and Italy [5,37]; and p.Glu583ArgfsTer25 in UK [38]. p. Lys1020Arg and p.Gln289Ter have been described in two Moroccan families [32]. None of the identified mutations were shared with those of the Arab countries (Algeria [33], Tunisia [39], Saudi Arabia [40], Egypt [41], Iran [42], Oman [43], and Lebanon [44]). Herein, the variants were mainly detected in exons 2, 5, 13, 15, 17, 18, and intron 1. This has to be confirmed by a wider investigation of WD patients in Morocco in order to help with genetic diagnosis. In the present study, remarkable differences in phenotypes were noted among patients and even among family members carrying the same genotype. For example, p. Glu583ArgfsTer25 was detected in three patients from two unrelated families (WDM 5; $n = 2$ and WDM 13; $n = 1$). For the WDM 5 family, the index patient presented with neurological manifestations at 12 years of age and his brother presented with hepatic manifestations at 10 years of age; WDM 13 presented with cirrhosis without neurological manifestations at 16 years of age. However, there is a need to increase the number of studied WD patients to define a phenotype-genotype correlation in Moroccan patients. Four of the WD patients who were included in the present study had no identifiable pathogenic or likely pathogenic variant (or even a compelling variant of unknown significance) in the entire sequence of the *ATP7B* gene, including deep intronic and proximal promoter (up to c.−1200) regions. Causative variants may be present in the distal promoter region or a non-covered, deep-intronic region (approximately 20 % in panel design). Alternatively, a differential diagnosis may explain the manifestations. In line with this hypothesis, four genes were reported to be associated with impaired copper balance (*CP, SLC33A1, NPC1*, and *NPC2*), although with distinct clinical phenotypes, were also observed. In such patients, detailed clinical and biochemical examination and family history may be of help for the diagnosis; for example, the diagnosis of these patients was based on clinical and biochemical assessment and we found a high urinary copper and a presence of K-F rings in the context of hepatic or neurological manifestation. All of the patients showed a regression of clinical signs under D-penicillamine. However, the complete cupric balance (exchangeable copper, relative exchangeable copper [REC in % = exchangeable copper / serum copper], hepatic copper …) is still necessary for these patients.

The present study has some limitations, such as under-diagnosis and non-exhaustive copper assessment that did not allow us to study all the diagnostic tests for the total population. In addition, the genetic study was only done on a small number of patients, which precluded investigation of a potential phenotype-genotype correlation.

In conclusion, a high prevalence due to consanguinity and a high mortality rate due to the difficulty of diagnosis and lack of treatment were observed in WD patients from Morroco. NGS sequencing identified *ATP7B* variants in WD patients from Morocco. Therefore, it is necessary to consider this disease for any child who is presented with liver disease or non-specific neurological disorders and to do a family screening.

CRediT authorship contribution statement

Nadia Abbassi: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Aicha Bourrahouat:** Resources, Supervision, Validation, Writing – review & editing. **Eduardo Couchonnal Bedoya:** Formal analysis, Methodology, Supervision, Validation, Writing – review & editing. **Cécile Pagan:** Methodology, Supervision, Validation, Writing – review & editing.

Meriem El Qabli: Resources. **Sana Maidoumi:** Conceptualization, Validation. **Abdelouahed Belmalih:** Supervision. **Olivier Guillaud:** Supervision. **Najib Kissani:** Resources. **Abdelhak Abkari:** Resources. **Imane Chahid:** Resources. **Mohammed Abdoh Rafai:** Resources. **Nezha Mouane:** Resources. **Yamna Kriouile:** Resources. **Saadia Aidi:** Resources. **Moustpha Hida:** Resources. **Mounia Lakhdar Idrissi:** Resources. **Mohammed Faouzi Belahsen:** Resources. **Mohammed El Abkari:** Resources. **Maria Rkain:** Resources. **Zahi Ismaili:** Resources. **Azeddine Sedki:** Project administration, Supervision, Writing – review & editing. **Muriel Bost:** Project administration, Supervision, Writing – review & editing, Methodology. **Nisrine Aboussair:** Methodology, Resources, Validation. **Alain Lachaux:** Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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