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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Wilson disease: revision of diagnostic criteria in a clinical series with great genetic homogeneity

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Abstract

Background Wilson disease is an autosomal recessive disorder of copper metabolism caused by mutations in the *ATP7B* gene. An early diagnosis is crucial to prevent evolution of the disease, as implantation of early therapeutic measures fully prevents its symptoms. As population genetics data predict a higher than initially expected prevalence, it was important to define the basic diagnostic tools to approach population screening.

Methods A highly genetically homogeneous cohort of 70 patients, belonging to 50 unrelated families, has been selected as a framework to analyze all their clinical, biochemical and genetic characteristics, to define the disease in our population, with an estimated prevalence of 1 in 12,369, and determine the most useful features that reach diagnostic value.

Results Serum ceruloplasmin below 11.5 mg/dL and cupremia below 60 µg/mL, were the best analytical predictors of the disease in asymptomatic individuals, while cupruria or hepatic copper determination were less powerful. Genetic analysis reached a conclusive diagnosis in all 65 patients available for complete testing. Of them, 48 were carriers of at least one p.Leu708Pro mutant allele, with 24 homozygotes. Nine patients carried a promoter deletion mutation, revealing that extended sequencing beyond the *ATP7B* gene-coding region is essential. All mutations caused hepatic damage since early ages,

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increasing its severity as diagnosis was delayed, and neurological symptoms appear.
Conclusion Serum ceruloplasmin determination followed by genetic screening would reduce costs and favor the prioritization of non-invasive procedures to reach a definitive diagnosis, even for asymptomatic cases.

Keywords Wilson disease · Ceruloplasmin · Genetics

Introduction

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism caused by mutations in the *ATP7B* gene, encoding for a key regulator of copper homeostasis [1]. Toxic accumulation of free metal, initially in the liver followed by other organs, such as the brain, the kidney and the cornea, leads to liver disease, generally evolving to neurological and/or psychiatric manifestations [1–4]. Up to 995 different *ATP7B* gene mutations, including deletions, insertions, and substitutions, distributed along the entire coding region as well as in promoter and intronic regions, may affect the functionality and/or the expression of the *ATP7B* gene product [5–7].

An early diagnosis, difficult in the asymptomatic phase, is crucial to prevent evolution of the disease, as implantation of early therapeutic and dietetic measures fully prevents its symptoms. Typical analyses reveal low serum ceruloplasmin and copper levels accompanied by elevated urine copper excretion and hepatic copper content [1, 2]. Urinary copper excretion after a penicillamine challenge test (PCT) has been considered of great diagnostic value [8]. The presence of the Kayser–Fleischer (KF) ring around the cornea is a late event that often appears when the disease evolves as a neurological disorder [3]. The concurrence of all these symptoms is rare, and a definitive WD diagnosis is achieved using a balanced judgement among all tests, known as the Leipzig criteria [9], resulting in specific recommendations for the diagnosis and management of the disease [10].

WD is easily identified when patients present with typical symptoms, but may be missed in asymptomatic individuals, that may occur at a higher frequency than expected. Although it has been initially estimated that the incidence of WD is 1 every 30,000 births, meaning that 1 in every 90 are heterozygous carriers [1], recent studies suggest, based on population-wide genetic data, that this carrier frequency may be higher than expected [11–14]. Furthermore, the prevalence of the disease may be much higher in isolated populations as carrier frequency increases [15–18]. The possibility that many patients are missed due to the difficulty of an early diagnosis and the lack of awareness of the true extent of the disease by health care providers raises the importance of establishing reliable diagnosis protocols that are amenable to population screening.

One of the main difficulties in the evaluation of diagnostic features is the lack of genetically homogeneous populations to eliminate the issue of variable genetic penetrance. Therefore, we present a highly genetically homogeneous cohort as a framework to analyze all clinical, biochemical and genetic characteristics, to determine the most useful features that define the disease in our patient population and reach diagnostic value.

Methods

Patients

Seventy patients with a diagnosis of WD were identified through electronic and paper clinical records from both main hospitals in the island: Complejo Hospitalario Universitario Insular Materno Infantil and Hospital Universitario de Gran Canaria Dr Negrín ($n = 68$), and from a list of patients taking D-penicillamine, during years 2016 to 2018, provided by the Pharmacy department of primary care in Gran Canaria (*Atención Primaria*) ($n = 2$). Controls ($n = 42$) were represented by unaffected family members ($n = 35$) and patients with a suspected WD initial diagnosis ($n = 7$) but later discarded after detailed clinical, biochemical and genetic analyses. The study was approved by the ethical clinical research committee of our institution, and appropriate informed written consent was obtained from all participants.

Genetic analysis

Genomic DNA was extracted from white blood cells with the Puregene kit (QUIAGEN, Hilden, Germany), and initially screened for the p.Leu708Pro mutation (SNP Id: rs121908000) [15], using a Restriction-Fragment-Length-Polymorphism (RFLP) test [19]. Homozygotes were not further tested and, both heterozygotes and non-carriers of the p.Leu708Pro mutation, were subjected to extensive screening. Before year 2017, variants were found in the *ATP7B* gene-coding and flanking regions using PCR-aided heterozygote double-pass Sanger sequencing [15, 19] while, after that time, the 5′ end of the mRNA and the promoter region were also screened for variants [16], an approach extended, when possible, to patients that only had a mutant allele and were analyzed before 2017.

Clinical and biochemical evaluation

Patients' data for evaluation included: history, date of birth, age at diagnosis, and findings from a full physical exam, ophthalmologic slit-lamp examination, and biochemical and liver function tests. Serum ceruloplasmin was measured by nephelometry. Total serum copper was measured by flame atomic absorption spectroscopy, while copper content in urine was determined using inductively coupled plasma mass spectrometry. Abdominal ultrasound imaging of the liver was performed on all patients, and percutaneous liver biopsy was performed in 35 patients after coagulation abnormalities had been corrected. Favorable response to a penicillamine challenge test (PCT) was detected by an increase of cupruria equal or superior to 5 times the levels detected before the administration. For the PCT, patients were given a single 250–500 mg dose of Penicillamine (depending on body weight) 12 to 24 h before the concentration of urine copper was measured, a dosing pattern sufficient to increase cupruria fivefold or higher in most of the patients tested.

Biopsy analysis

Hepatic copper content was measured using inductively coupled plasma mass spectrometry, and hepatic lesions were graded using a semi-quantitative evaluation protocol, based on a methodology previously developed for chronic hepatitis [20, 21], as: Grade 0, no fibrosis; grade 1, mild fibrosis in sinusoidal area (zone 3), subtle sinusoidal collagen deposits or delicate pericellular fibrosis; grade 2, incomplete septa, pericentral fibrosis (lobulillae) with incomplete septa toward pericellular areas or portal spaces; grade 3, complete septs (porto-portals or porto-central); grade 4, perinodular fibrosis or cirrhosis.

Statistical analysis

Categorical variables were expressed as frequencies and percentages and continuous as means and standard deviations (SD). The percentages were compared, as appropriate, using the Chi-square (χ^2) test or the exact Fisher test and the continuous variables by the *t*-test or a non-parametric test. The receiver operating characteristic curves (ROC curves) and their corresponding area under the curve (AUC) were used to obtain the optimal cut-off point between asymptomatic WD patients and no WD patients of the ceruloplasmin, serum copper and urine copper 24 h. Statistical significance was set at $p < 0.05$. Data were analyzed using the SPSS software v.19 (IBM Corporation, NY, USA) and R Core Team 2020, v. 4.0.2 [22].

Results

Wilson's disease in Gran Canaria

An extensive search revealed 70 patients (29 women and 41 men) who are, most likely, all known diagnosed WD patients in the island of Gran Canaria, belonging to 50 unrelated families. Therefore, according to these figures, the prevalence of WD in Gran Canaria, with 70 cases in a population of 865,864, was estimated as 1 in 12,369.

Thirteen asymptomatic patients were diagnosed after screening families with an index case. The youngest patient diagnosed, with hepatic symptoms (Supplementary Table 1), was 21 months old, while the oldest was 67 years old (Supplementary Table 4). Patients were classified into four groups (< 7, 8–14, 15–21, > 21 years old) to follow symptoms according to the age at diagnosis (Fig. 1). Most patients diagnosed at the earliest age group were asymptomatic (8/10), decreasing to 58.3% (14/24) in the 8–14, 27.8% (5/18) in the 15–21, and 44% (8/18) in the over 21-year-old group. Age at diagnosis was higher in patients with neurological symptoms compared with the ones with hepatic disease (18.9 ± 8.7 vs 15.7 ± 13.3 years, $p = 0.01$) and hepatic presentation was more frequent in males (25/35: 71.4%), while neurologic presentations appeared equally in both groups.

Digestive manifestations

Abdominal pain was the most common digestive symptom among our patients and, the most frequent finding, leading to further analysis, was the presence of hyper-transaminasemia (50 out of 69 at presentation, 72.8%), which helped to reach an earlier diagnosis (15.9 years old $\pm$ 11.6 vs 23.9 yo $\pm$ 14.5, $p = 0.020$). Among the rest, hepatomegaly was the most prevalent, especially among young patients belonging to the 8–14-year-old window (11/21; 52,4%) (Table 1).

Histological examination was performed on 57 patients, most of them (53/57) presenting with some degree of alterations in the hepatic biopsies at diagnosis, ranging from mild alterations with lobular inflammatory activity, micro- and macrovesicular steatosis to fibrosis (Table 1). In general, milder hepatic lesions (fibrosis grades 1 and 2) were more frequent in the 2 younger groups (74.2% vs 44%) and severe degrees (fibrosis grade 3 and cirrhosis) were more frequent in the eldest (56% vs 25.8%) (χ^2 test; $p = 0.021$) (Fig. 2; Supplementary Tables and Supplementary Fig. 1).

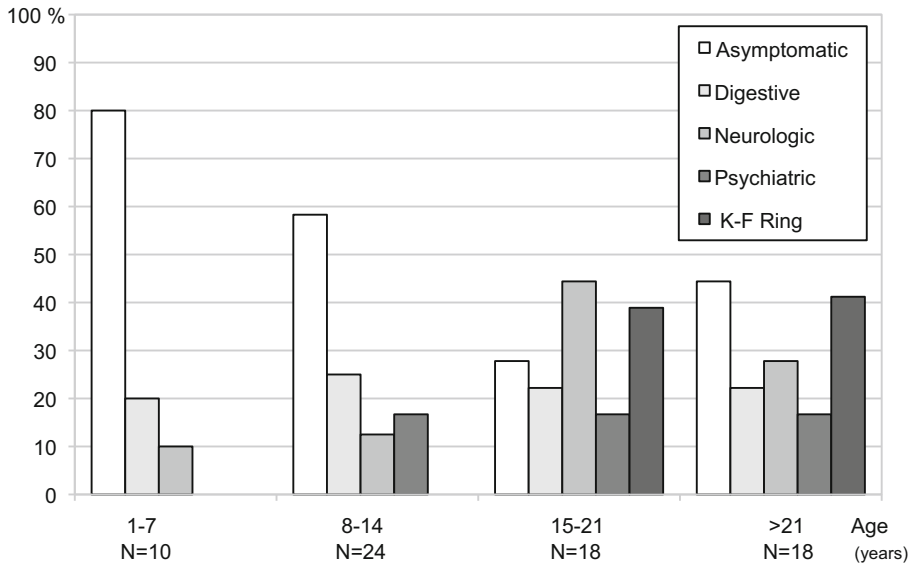


Fig. 1 Symptoms at diagnosis according to age. The number of patients per age group is indicated by N at the bottom

Table 1 Digestive manifestations and liver pathology by age group at diagnosis

Age group (years)	Digestive manifestations (%)	Abdominal pain (%)	Ascites (%)	Jaundice (%)	Hepatomegaly (%)	Choluria (%)	Lobular activity	Steatosis	Fibrosis
1–7	20 (2/10)	20 (2/10)	0 (0/10)	0 (0/10)	10 (1/10)	0 (0/10)	8/10 (80)	9/10 (90)	7/10 (70)
8–14	25 (6/24)	16.7 (4/24)	4.2 (1/24)	12.5 (3/24)	52.4 (11/21)	12.5 (3/24)	16/22 (72.7)	13/22 (59)	15/22 (68.1)
15–21	22.2 (4/18)	22.2 (4/18)	11.1 (2/18)	11.1 (2/18)	17.6 (3/17)	16.7 (3/18)	6/12 (50)	6/12 (50)	12/13 (92.3)
> 21	22.2 (4/18)	22.2 (4/18)	11.1 (2/18)	5.6 (1/18)	16.7 (3/18)	5.6 (1/18)	8/12 (66.6)	2/12 (16.7)	8/12 (66.6)
Total	22.9 (16/70)	20 (14/70)	7.1 (5/70)	8.6 (6/70)	27.3 (18/66)	10 (7/70)	38/56 (67.9)	30/56 (53.6)	42/57[a] (73.6)

Not all patients were subject to biopsy analysis, and the numbers are indicated

[a]In one patient, only fibrosis was scored and other pathological features were not evaluated

Central nervous system involvement

Neurological symptoms were present in 17 patients (24.3%), being extrapyramidal syndrome the most frequent (11/17, 64.7%), followed by dysarthria (9/17, 52.9%) (Table 2). Seven patients presented with both symptoms at once. Language delay and dyslalia were only evident in a single child below 8 years of age, and only one of the patients from the 8–14, and one in the 15-to-21 year-old group presented with dysphagia. In the elder group dysarthria, found in 2 patients, was always associated with extrapyramidal syndrome. Neurological presentations were more prevalent in patients diagnosed after their 15th birthday (81.3% vs 42.6%, $p = 0.01$), and was associated with a higher incidence of cirrhosis (88.9% vs 15.6%, $p < 0.001$).

Ten patients, none of them younger than 8 years old, presented with psychiatric symptoms. Conduct disorders were most frequent: four affected in the 8–14 years old group, accompanied by educational delay, and three in the 15–21 years old group. Depression was suffered by one and two patients in the 8–14 and the over 21-year-old groups, respectively. One patient over 21 years of age was diagnosed with schizophrenia.

Although not a neurological manifestation per se, there was a significant correlation between the presence of neurological symptoms with the detection of the KF ring: 80% (12/15) of patients with neurological symptoms at diagnosis versus only 9.6% (5/52) of those without them presented with the KF ring (χ^2 test; $p < 0.001$), and detectable, mostly, when a diagnosis was reached after 14 years of age (40% vs 9.1%, $p = 0.003$). The KF ring was also observed in half (4/8) of the patients with psychiatric symptoms that were evaluated (8/10). Given that 4 of them also have neurological symptoms associated with

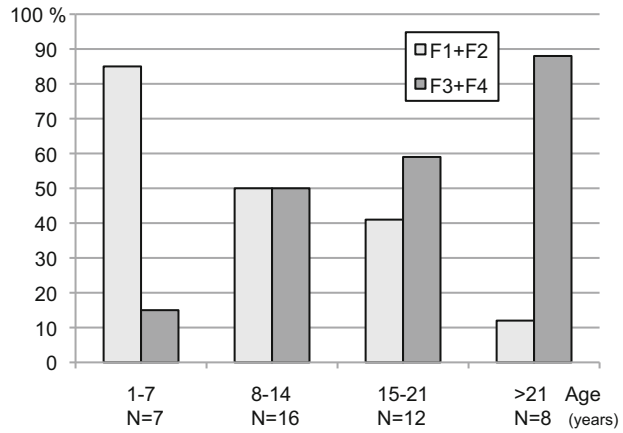


Fig. 2 Fibrosis grades according to age at diagnosis after pathological examination of liver biopsies. Fibrosis grades are described in the materials and methods section, under biopsy analysis, and have been grouped according to similar severity. Mild grades of fibrosis (F1 + F2) versus severe liver disease (F3 + F4) are represented. Not all patients had a biopsy performed, and the number per group is indicated by N at the bottom

them, there was only one patient (1/4) with exclusive psychiatric symptoms presenting with the KF ring.

ATP7B gene mutations in Gran Canaria

Patients (67 from 50 families) were screened for *ATP7B* gene mutations; three other patients were no longer available for genetic screening. Sequencing of the 5′ UTR and the promoter was not available for five of them, which are listed as heterozygous carriers of a single mutation, even though they fulfill diagnostic criteria. Therefore, out of 140 possible alleles (70 patients), 11 remained to be identified.

Forty-eight patients were carriers of at least one p.Leu708Pro mutant allele, while 24 of them, representing 20 families, were homozygotes (Table 3), in agreement with its founder effect [15]. The second most prevalent mutation, found in 11 compound heterozygotes from 7 families was the p.Met645Arg variant, most common in mainland Spain [16, 23, 24]. The well-extended p.Ala1135GlnfsX13 frameshift [6] was found in seven compound heterozygotes belonging to six different families. The extension of genetic screening to the promoter region identified nine patients, belonging to 3 different families, carrying the Sardinian 15 base pair deletion within the promoter region of the gene (c.-436_-422del15) [25]. One family, with six affected members, carried the Sardinian allele in combination with a mutation first described as I13_T-12A [15], later defined as c.3061−12T>A, and shown to affect *ATP7B* transcript splicing [26].

Other mutations found were: p.Asn1270Ser, p.Gly1099Ser, likely pathogenic [6, 23, 27]; p.Met769Val mutation with conflicting interpretations of pathogenicity [6]; p.Thr1232Pro [23, 28] found in two patients originating from Western Sahara, one of them also carrying the c.51+4A>T substitution at the 5′ UTR, affecting a splicing site [23]; two different missense substitutions affecting arginine at position 778: p.Arg778Trp and p.Arg778Gly; the frameshift mutation p.Met769HisfsX26 and, finally, p.Gly869Arg, with conflicting interpretations of pathogenicity [6] Table 3.

Analytical findings

Serum ceruloplasmin was below 11.5 mg/dL in almost all patients analyzed (98.6%; 69/70), being above this value in a single asymptomatic patient with the p.Leu708Pro/p.Gly869Arg genotype.

Total serum copper was below 60 μg/mL in all patients diagnosed before 15 and after 21 years of age, while only 83.3% (15/18) had pathological levels in the 15-to-21 age group. In younger patients (< 18 yo), there was a correlation between cupremia and age ($r = 0.44$, $p < 0.01$), being lower in patients under 10 compared with patients between 10 and 18 yo (20.3 + 8.8 vs 33.6 + 23.3, $p < 0.01$).

Urine copper excretion was above 100 μg/24 h in 41 out 67 patients tested, distributed as follows: 3/10 in the younger group 1, 13/2 (59.1%) in the second group (8–14 years), 13/17 (76.5%) in the 15–21 years old group and 12/18 (66.7%) in the patients diagnosed at ages 21 or over, with a linear correlation between cupruria and the age

Table 2 Neurological symptoms by age group at diagnosis

Age group (years)	Neurological symptoms (%)	Extrapyramidal symptoms (%)	Dysarthria (%)	Extrapyramidal + dysarthria (%)	Others[a] (%)
1–7	10 (1/10)	0 (0/10)	0 (0/10)	0 (0/10)	10 (1/10)
8–14	12.5 (3/24)	0 (0/24)	4.2 (1/24)	0 (0/24)	8.3 (2/24)
15–21	44.4 (8/18)	33.3 (6/18)	33.3 (6/18)	27.8 (5/18)	5.6 (1/18)
> 21	27.8 (5/18)	27.8 (5/18)	11.1 (2/18)	11.1 (2/18)	0 (0/18)
Total	24.3 (17/70)	15.7 (11/70)	12.9 (9/70)	10 (7/70)	5.7 (4/70)

[a]Any additional symptom, such as language delay, dislalia and dysphagia, either alone or in combination with others

Table 3 ATP7B mutations in patients from Gran Canaria

Genotype	SNP ID	Families	Patients
p.Leu708Pro/p.Leu708Pro	rs121908000/rs121908000	20	24
p.Leu708Pro/p.Met645Arg	rs121908000/rs121907998	6	9
p.Leu708Pro/wt[a]	rs121908000/–	3	3
p.Leu708Pro/p.Met769Val	rs121908000/rs193922103	2	2
p.Leu708Pro/p.Gly1099Ser	rs121908000/rs761632029	2	3
p.Leu708Pro/Ala1135GlnfsX13	rs121908000/rs137853281	2	2
p.Leu708Pro/p.Asn1270Ser	rs121908000/rs121907990	2	2
p.Leu708Pro/p.Arg778Gly	rs121908000/rs137853284	1	1
p.Leu708Pro/p.Gly869Arg[b]	rs121908000/rs191312027	1	1
p.Leu708Pro/p.Gly1266Trp	rs121908000/rs121907992[c]	1	1
Subtotal		39[a]	48
c.-436-422del15/c.3061-12T>A	rs1484840087[d]/rs1045194246	1	6
c.-436-422del15/c.-436-422del15	rs1484840087/rs1484840087	1	2
p.Ala1135GlnfsX13/c.-436-422del15	rs137853281/rs1484840087	1	1
p.Ala1135GlnfsX13/p.Met645Arg	rs137853281/rs121907998	1	2
p.Ala1135GlnfsX13/p.Met769HisfsX26	rs137853281/rs137853287	1	1
p.Ala1135GlnfsX13/wt[a]	rs137853281/–	1	1
p.Arg778Trp[e]/p.Asn1270Ser	rs137853284/rs121907990	1	3
p.Thr1031Ile[f]/p.Gly1099Ser	novel/rs761632029[c]	1	1
p.Thr1232Pro/wt[a]	rs568009639/–	1	1
p.Thr1232Pro/c.51+4A>T	rs568009639/rs369488210	1	1
Total		50[a]	67

Genotypes found are shown along with the number of families and patients carrying each. All aminoacid substitutions are referred to peptide NP_000044.2, while nucleotide substitutions are according to reference transcript NM_000053.3

[a]Wt alleles in the context of an incomplete genetic screening, as the 5′ UTR end of the mRNA and the promoter region were not tested in these patients

[b]One family had three disease alleles, resulting in the presence of siblings with two possible genotypes: p.Leu708Pro/p.Leu708Pro y p.Leu708Pro/p.Gly869Arg

[c]The p.Gly1266Trp (c.3796G>T) is not annotated as such in dbSNP but shares location with SNP Id: rs121907992

[d]There is an annotation uncertainty with the deletion encompassing 15 base pairs in the promoter region of the gene. Although in the initial report this mutation was referred as c.-441_-427del15 [25], the nucleotides missing, numbered according to the current annotation of the + 1 site for the NM_000053.3 transcript turn to c.-436_-422del15 and, therefore, the deletion corresponds to rs1484840087

[e]Two different missense substitutions affecting arginine at position 778: p.Arg778Trp and p.Arg778Gly, are both identified by the same SNP Id: rs137853284

[f]The variant p.Thr1031Ile (c.3092C>T; GRCh38_13:51944260C>T), has not been reported previously but has known mutations at this position: p.Thr1031Ala [29, 30] and p.Thr1031Ser [31]

at diagnosis ($r = 0.4$; $p < 0.05$). Meanwhile, out of 33 patients tested, 25 showed a positive response to a PCT, and were distributed similarly in all age groups: 1/2 in the youngest, 10/15 (66.7%) in group 2, 7/8 (87.5%) in group 3 and 7/8 (87.5%) among the eldest.

Hepatic copper was above 250 μg of copper per gram of dry tissue in 25 out of 35 patients tested, evenly distributed in all age groups: 7/9 below 7 years old (77.8%), 8/13 in the group from 8 to 14 years old (61.5%), 6/8 (75%) in the 15-to-21 age window and 4/6 (66.7%) in those diagnosed over 21 years of age. Among the patients presenting with hepatic copper below diagnostic levels, half of them were below 50 μg/g (Supplementary Table 6).

A comparison of these parameters by age group is shown on Fig. 3.

Genotype–phenotype correlations

Five groups were selected for comparison: p.Leu708Pro homozygotes ($n = 24$), p.Leu708Pro heterozygotes ($n = 24$), p.Met645Arg heterozygotes ($n = 11$), c.-436_-422del15 promoter deletion carriers ($n = 8$) and, lastly,

frameshift carriers ($n = 7$). Data collected for each of these patients are all shown in Supplementary Tables 1 to 5, and summarized in Supplementary Fig. 1.

All mutations caused hepatic damage since early ages. However, severe phenotypes, like cirrhosis, were more restricted to patients diagnosed at later ages, as well as those homozygous for the p.Leu708Pro mutation (37.5% vs 14.3% heterozygotes or 33.3% with other mutations). Neurological symptoms were also significantly more frequent in the group of p.Leu708Pro homozygotes (50% vs 17% in p.Leu708Pro heterozygotes and close to 10% in M645R heterozygotes and promoter mutation carriers; $p < 0.05$), while the fraction of patients presenting with digestive symptoms was smaller than in other groups, being significant when compared with those without any p.Leu708Pro mutant allele (8.7% vs 42.1%; $p < 0.05$). Additionally, diagnosis was made at later ages in the p.Leu708Pro homozygote group with neurological presentation (18 + 3.9 vs 11.4 + 4.9 years old, $p = 0.003$). All patients in this group presented with ceruloplasmin levels well below the cut-off value, cupremia was low in all but two young patients (15–21 age group) and the fraction of patients with elevated cupruria was steadily increasing with age from 50 up to 100%. Transaminases were elevated in 60% of cases, being this fraction younger than those presenting with normal values (11.3 + 5.1 vs 18.0 + 3.9 years; $p = 0.004$). Liver disease was also more severe in the L708P homozygote group. Biopsy analysis revealed advanced liver disease (F3 + F4) in almost half of the patients, finding cirrhosis in 6 out of 16 tested, being more frequent in those patients with neurological symptoms (80.0% vs 18.2%, $p = 0.036$).

The group of patients with the p.Met645Arg mutation was analyzed due to its high frequency in both mainland Spain and Gran Canaria. These patients were diagnosed older (18.5 + 11.6 vs 14 + 5.5 years). There was only one case with neurological presentation with cirrhosis, while the rest had hepatic presentations or were screened because they were siblings of the index case. Hepatic copper was lower in this group of patients versus the rest, with 5 out of 9 cases below the classic diagnostic value of 250 mg/g and normal levels, without any diagnostic value, in three of them.

Patients bearing the c.-436_-422del15 promoter deletion, with an estimated 25% wild type ATP7B activity left [25], had all presentations and no distinctive features when compared to the previous heterozygous groups.

Finally, all patients with mutant alleles leading to frameshifts were diagnosed relatively early, all with moderate hepatic presentations and no neurological manifestations (Supplementary Fig. 1).

Evaluation of diagnostic criteria

To optimize health care strategies, we wished to determine, in this cohort, the value of diagnostic criteria as a whole, as determined by the Leipzig criteria [9] or individually: All patients fulfilled Leipzig diagnostic scores when all diagnostic tests were considered.

Hepatic copper content was determined in 35 patients (50%). From these, 25 had hepatic copper values above 250 μg/g. If the biopsy information had been absent in this group, 11 patients would have not reached the Leipzig threshold without genetic testing. Among the patients tested, 10 cases were below 250 μg/g (Supplementary Table 6). If biopsy had not been performed in 4 cases with hepatic copper levels below 50 μg/g, three of them would have reached 4 diagnostic points even in the absence of a genetic test. In patients with levels between 50 and 250 μg/g (6 cases), if biopsy had not been performed in any of them,

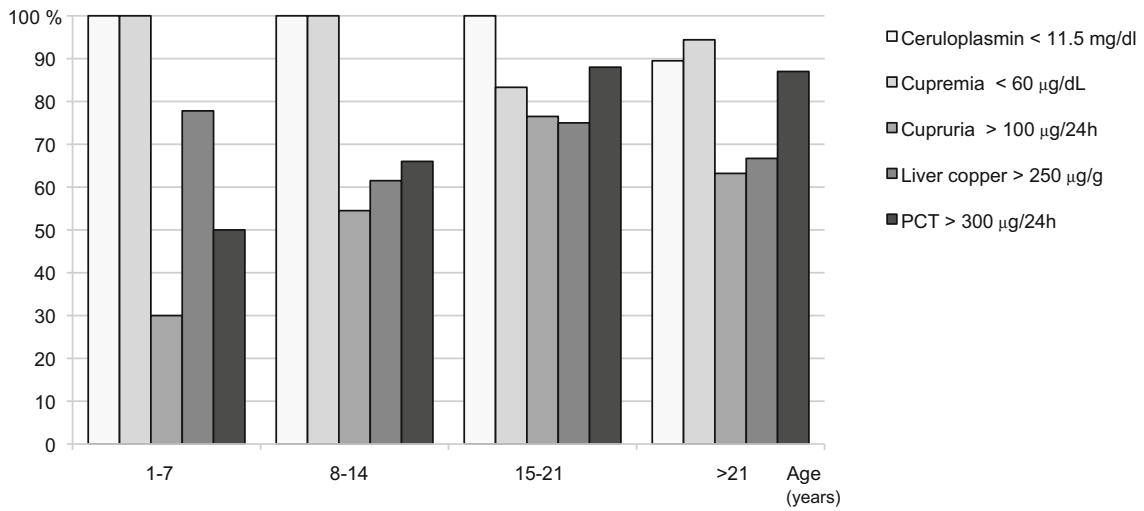


Fig. 3 Analytical parameters according to the age at diagnosis

three of them already reached 4 points, and the other three would have done so after a positive PCT, if performed.

Serum ceruloplasmin was tested in all patients, and was between 10 and 19 mg/dL (only one Leipzig point) in only five of them. Being the most consistent of all tests, the sensitivity and specificity of ceruloplasmin values were tested and compared to serum copper (total) and 24 h urinary copper in the asymptomatic cases, to define which tests were most suitable to detect disease in the asymptomatic stage. To perform this analysis, analytical data from 35 asymptomatic cases, as defined by those presenting at first visit without disease-specific symptoms besides the elevation of serum trans-aminases or identified through family screening, 35 relatives from affected families, and 7 WD suspect patients, all fully genotyped for *ATP7B*, were included as controls. The suitability of this heterogeneous group of heterozygotes and wild type *ATP7B* genotypes to serve as controls was evaluated by comparing the values obtained for both serum ceruloplasmin (Supplementary Fig. 2a) and serum copper content (Supplementary Fig. 2b), showing that both control groups, wild type and heterozygous carriers, were equivalent.

Receiver operating characteristic (ROC) analyses revealed first that serum ceruloplasmin, at a cut-off value of 11,5 mg/dL, showed an area under the curve (AUC) equal to 0.99 (95% CI 0.97–1), with a specificity of 1 and a sensibility of 0.97. At this cut-off value, the positive predictive value (PPV) is 1, and the negative predictive value (NPV) is 0.977, versus PPV = 0.92 and NPV = 0.975 for the 15 mg/dL threshold (Fig. 4a).

Second, serum copper, at values above 62 µg/dL, showed the highest specificity of 1 along with a sensibility of 0.971, also displaying an AUC of 0.99 (95% CI 0.97–1). In this case, PPV = 1 while NPV = 0.96 (Fig. 4c).

Finally, copper content in urine collected during a 24 h period was also included in the analyses, revealing that a cut-off value of 21.5 µg/24 h was necessary to discriminate between cases and asymptomatic individuals, with a specificity of 1 and a sensibility of 0.909, being the AUC equal to 0.993 (95% CI 0.981 -1). Sensitivity of the test drops to 0.455 when the cut-off value is set at 100 µg/24 h, being PPV = 1 in both cases, and NPV = 0.88 at the 21.5 µg/24 h and 0.55 at the 100 µg cut-off values (Fig. 4e).

A PCT was performed in 33 patients, 2 of them without basal urine copper excretion data, and 17 with basal levels below twice the normal values. The rest (14 cases) should not have been evaluated as basal cupruria was above the informative values. The PCT was diagnostic in 25 patients and, from these, a hepatic biopsy could have been spared in 10 of them, as they reached 4 points, even without genetic testing.

Fig. 4 Receiver operating characteristic (ROC) curves for serum ceruloplasmin, serum copper and 24 h urinary copper values. On the left column, ROC curves for serum ceruloplasmin (**a**), serum copper (**c**) and 24 h urine copper (**e**) are shown. Their corresponding AUC and IC values are indicated, and their cut-offs, specificity and sensitivity are shown at the top of the plots. On the right, violin and point plots show the distribution of values for serum ceruloplasmin (**b**), serum copper (**d**) and 24 h urine copper (**f**) used in the ROC analysis. In these, the long dash line represents the cut-off obtained in the ROC curves and the dot dash line indicates consensus cut-off values for each analyte: 15 mg/dL for serum cerulolasmin, 60 µg/dL for serum copper, and 100 µg copper in a 24 h urine collection

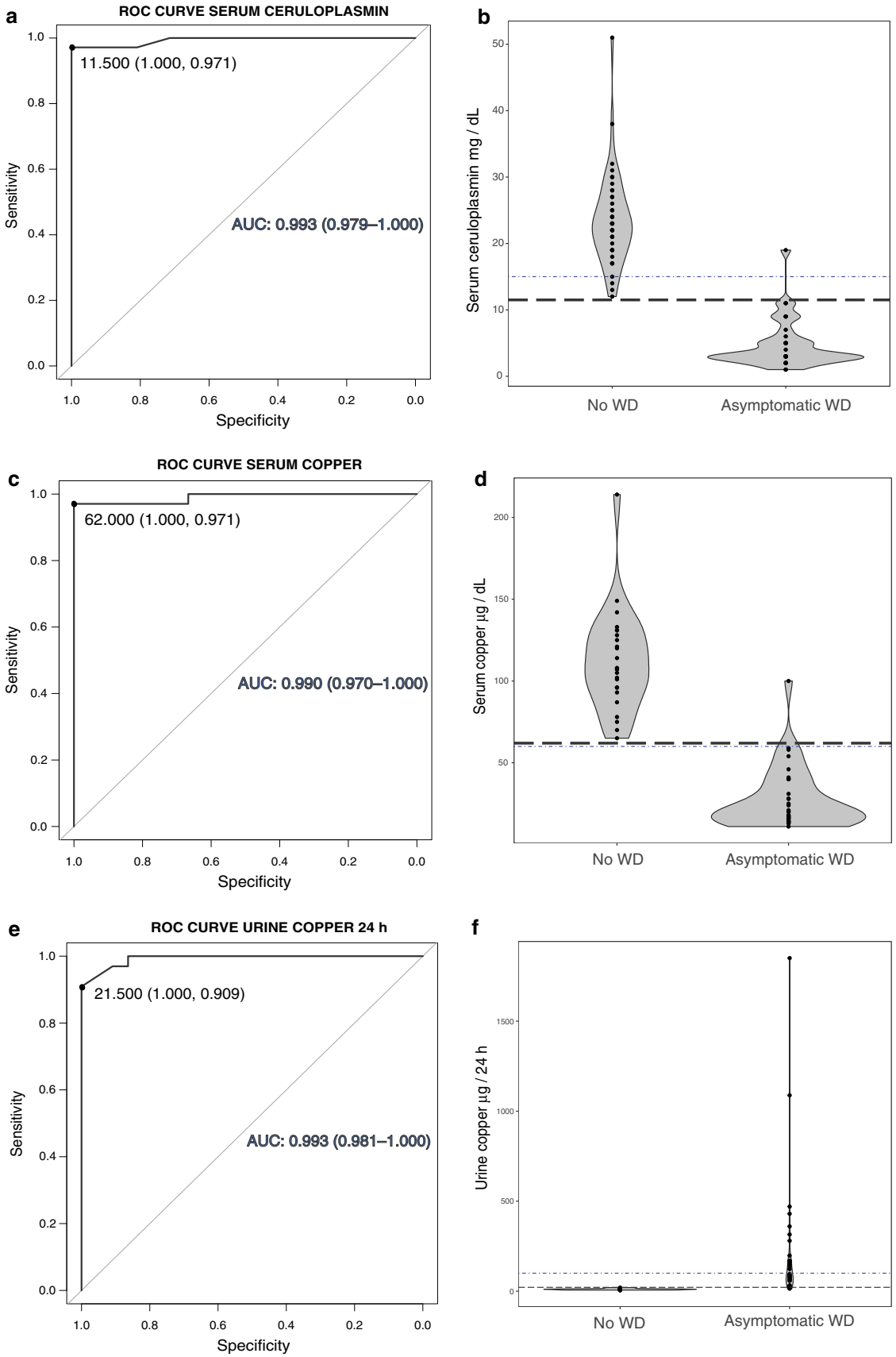
Finally, if genetic tests were excluded, we observed that 60/70 (85.7%) patients reached 4 or more points. From the 10 cases that did not reach the diagnostic threshold without a genetic test, two cases presented with hepatic copper content below 50 µg/g, and would have reached 4 points with a genetic test, even if a biopsy had not been performed.

Discussion

We present a thorough clinical and genetic analysis and evaluated diagnostic parameters for WD in a highly genetically homogeneous cohort where over 70% of the patients carry a common founder p.Leu708Pro mutation. This is, to the best of our knowledge, the largest fully defined clinical series of a homozygous group after the one carrying the most common p.His1069Gln mutation [32].

When available, genetic testing is a powerful diagnostic tool [9], although it might not be complete [32], may detect variants of unknown significance that may require further validation [33]. In addition, weak hypomorphic alleles may lead to mild forms of the disease that remain undetected, so the true prevalence could be underestimated [11–14]. This effect might be inferred by the increase of the asymptomatic group among patients diagnosed later than 21 years of age, a "rebound effect" shown in Fig. 1, revealing the asymptomatic course of the disease in this group of patients. In addition, a recent study shows three cases, with a Leipzig score > 4, where mutation detection in the *ATP7B* gene has been inconclusive. One of these patients, with hepatic copper levels of 2,057 µg/g and a Leipzig score of 5, is really a congenital metabolic disease due to mutations in the *CCDC115* gene [16]. These observations emphasize the need of powerful diagnostic tools, especially in populations with a high carrier frequency.

All mutations caused hepatic damage since early ages in our patients, increasing its severity, in most cases, as diagnosis was delayed, and neurological symptoms appear.


a
ROC CURVE SERUM CERULOPLASMIN
11.500 (1.000, 0.971)
AUC: 0.993 (0.979–1.000)
Sensitivity
Specificity
b
Serum ceruloplasmin mg / dL
No WD
Asymptomatic WD
c
ROC CURVE SERUM COPPER
62.000 (1.000, 0.971)
AUC: 0.990 (0.970–1.000)
Sensitivity
Specificity
d
Serum copper µg / dL
No WD
Asymptomatic WD
e
ROC CURVE URINE COPPER 24 h
21.500 (1.000, 0.909)
AUC: 0.993 (0.981–1.000)
Sensitivity
Specificity
f
Urine copper µg / 24 h
No WD
Asymptomatic WD

Among them, p.Leu708Pro homozygotes had the most severe phenotype, while other patients, including p.Leu708Pro heterozygotes, appeared asymptomatic even at later ages, and were detected as part of a family screening approach. A singular case was patient 5005, a 27 years old female with the genotype p.Leu708Pro/p.Gly869Arg, the second allele with conflicting interpretations about its pathogenicity [6]. The patient appeared asymptomatic, and was screened because her siblings were affected, but careful examination resulted in 4 points in the Leipzig score, confirming the difficulty of the diagnosis in the least penetrant cases or with milder mutations. While p.Leu708Pro homozygotes showed the most severe neurological and hepatic symptoms, patients with the p.Met645Arg mutation had milder presentations: less hepatic copper content, one patient with neurological presentation diagnosed at an advanced age, another asymptomatic screened because of family history, and seven with mild hepatic symptoms, two of them heterozygotes with frameshift mutations. In fact, a previous study of the Spanish population [23] suggests that this must be a mild mutation given that, even though it is rather prevalent in their patient sample, not a single homozygote was found. Furthermore, all patients bearing this mutation presented with mild hepatic symptoms, in agreement with our findings. Thus, they assume that p.Met645Arg homozygotes escape diagnosis because their symptoms would be mild. Indeed, in silico analysis of this variant predicts that it is not pathogenic [12] or with reduced pathogenicity [13], although segregation with the disease [15, 16, 19, 23, 24], and recent functional evidence [34], further support this variant as a disease-causing mutation, evidencing the limitations of computational prediction methods.

The c.-436_-422del15 promoter mutation also appeared milder, leading to delayed diagnosis and less hepatic and neurological symptoms than the previous groups. Most surprisingly, patients bearing frameshift mutations, leading to truncated polypeptides, did not show a severe phenotype, in disagreement with what should be expected based on their effect on protein structure and functional effect [35, 36]. In these cases, the second mutant allele was quite heterogeneous and appeared as irrelevant: from the severe p.Leu708Pro, and even other frameshifts (p.Met769HisfsX26), to milder mutations (p.Met645Arg or the promoter deletion), strong phenotypic differences were not observed, with milder hepatic presentations and no neurological manifestations, although with earlier diagnostic ages, as previously shown [35, 36]. Maybe frameshifts occurring at the carboxyl end of the ATP7B polypeptide could be less severe, but in both Ala1135GlnfsX13 and p.Met769HisfsX26, the ATP-binding domain is lost, along with the rest of the carboxyl end. Therefore, we cannot explain the rather benign phenotype observed in these patients.

Among all diagnostic tests used, besides genetics, we found serum ceruloplasmin and total serum copper determination to be the best predictors of the disease in asymptomatic individuals, above other primary parameters, such as 24 h urinary copper excretion, liver copper content or the presence of KF rings. We show that, at a cut-off value of 11.5 mg/dL, serum ceruloplasmin determination was a highly sensitive and specific diagnostic test in our population, in agreement with recent reports [37–39] but in disagreement with others, that state a superior value for urinary copper excretion [38] or hepatic copper content [39–41], or none, giving a much better value to the combination of the previous [42]. Our cut-off value of 11.5 mg/dL was below previous recommendations but was obtained exclusively through the observations of asymptomatic individuals, as defined by those without evident symptoms at first visit beyond the elevation of transaminases or siblings identified through family screening.

Among quantitative copper analyses, serum total copper levels below 62 μg/mL were also found to have, after ceruloplasmin determination, the greatest diagnostic value in the asymptomatic population. In this case, the cut-off value is similar to what has been established previously (60 μg/mL) [42, 43], but may not be compared to the Leipzig criteria as this parameter is neither included in the list [9], nor is considered by current clinical practice guidelines [10].

Meanwhile, although cupruria has been previously proven a very useful diagnostic parameter, either alone or in combination with a PCT [8], we found that this was not the case with our patients, in agreement with others [40]. We believe that this type of test relies on the capacity of patients to follow specific instructions for the collection of the sample and, therefore, are subject to sampling errors. Such errors may also independently apply to the determination of liver copper content [44, 45]. Finally, the presence of KF rings, segregated mostly with neurological phenotypes [41, 43], therefore being of limited use for the initial diagnostic stage in most cases.

In summary, being aware of the variability observed in WD patients, our study gives great value to ceruloplasmin testing in our patient population, followed by a genetic analysis consisting of screening for the most common mutation, that could need, if negative, a complete *ATP7B* gene sequencing approach, including the promoter region. A broad sequencing approach, combined with specific analyses to detect micro-deletions at the locus, could substantially reduce the observed rate for patients with only one or no ATP7B mutant alleles [32, 33], as shown recently [16]. These observations strengthen the view that ceruloplasmin determination at 3 years of age could be a useful approach for population screening [46] which, combined with ATP7B gene sequencing in suspicious

cases, would reduce substantially laboratory costs and favors the prioritization of non-invasive procedures to approach a definitive diagnosis.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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