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Genetic map of Wilson disease in Spain - A great tool to improve diagnosis and screening

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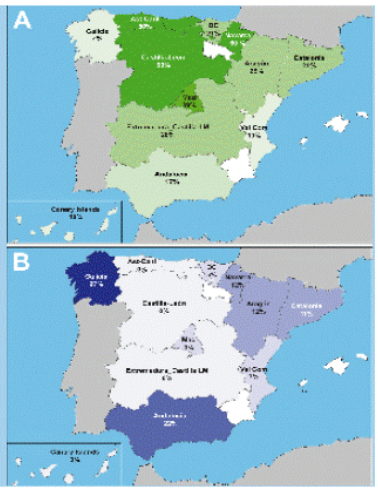
Genetic map of Wilson Disease in Spain: a great tool to improve diagnosis and screening

- Wilson disease is a genetic disease and more than 1000 different mutations have been found in patients around the world
- We had no national map of the mutations affecting our patients and the current study presents the first genetic map of mutations for Wilson disease
- This is a very useful tool to improve early diagnosis of patients, what is crucial to offer them the best outcomes
- The widespread use of national maps will also increase our knowledge of the disease and allow the best design of multicenter trials in the future

p.M645R distribution

p.H1069Q distribution



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Genetic map of Wilson disease in Spain - A great tool to improve diagnosis and screening

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** **P A-C and ZM** share first authorship

Authors' contributions

LGV and AT were responsible for the study design. ZM, MB and P A-C, revised the manuscript for important intellectual content. All the authors were responsible for acquisition and curation of data and for revisión of the manuscript. All the authors have read and agreed to the published version of the manuscript.

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List of abbreviations

WD: Wilson disease. AEEH: Spanish Association for Study of the Liver (Asociación Española para Estudio del Hígado. LOF: Loss of function. IQR: Interquartile range

Ethics approval

The protocol of the study was reviewed and approved by all the Ethical Committees of the participating centers. All included subjects provided their informed consent. The research was carried out pursuant to the ethical principles established in the Declaration of Helsinki

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ABSTRACT

Background: A National Registry for Wilson disease (WD) was recently started by the Spanish Association for Study of the Liver (AEEH). We evaluated the genetic data of the patients and the differences between regions and hospitals.

Method: Multicentric observational study from the WD Registry after the first year.

Results: Patients from 30 hospitals, in 13/17 Spanish regions (covering 80% of population) were included. Genetic data were available for 260/320 patients. More than 130 mutations in the *ATP7B* gene were registered, the majority in less than 4 alleles, being most prevalent p.Met645Arg (20% alleles, mainland and the Canary Islands), p.Leu708Pro (16.5%, Canary Islands) and p.His1069Gln (8.3%, mainland). Only 15 mutations occurred in homozygosis, 3 in more than 5 patients: p.Leu708Pro (24 patients), p.Met645Arg and p.His1069Gln (6 patients each). Genetic data availability ranged from 0-100% among regions; similarly, the difference among hospitals within a region was larger than 50%. Without a genetic test up to 45% of patients would not have reached Leipzig score >3. In screening cases, genetics was used in 45/58 (78%); without genetics, two thirds of them would not have reached a Leipzig score >3 (33/49).

Conclusion: Here we present the first genetic map of WD in Spain. The use of genetic test was highly heterogeneous, being higher in screening cases. Although a large variability of mutations was found, regional characteristics indicated that screening for a limited number of exons (6, 8, 10, 14 or 17) would detect more than 50% of alleles in a given region, thus enabling the design of better diagnosis/screening tailored strategies.

Keywords: Genetic screening. Rare disease. ATP7B gene. Misdiagnosis.

Lay Summary

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Wilson disease (WD) is a rare genetic disease secondary to mutations in the *ATP7B* gene, encoding a protein dealing with copper excretion. There are more than 1000 different mutations among patients and diagnosis is sometimes difficult. Without treatment WD leads to death but there are therapies that are so effective to offer a normal life to patients, if started early. Every population shows different mutations, so a genetic map of every country is very important for patients and physicians, to reach early diagnosis and therapy with the best prognosis for patients.

Conflict of interest

VV: Speaker fees, Alexion Pharma, Consultant (advisory board), Ipsen

AM: speaker fees from Orphalan

ZM: speaker fees from Orphalan and Gilead; advisory fees from Orphalan, Alexion and Deep Genomics; grants from Gilead.

The others authors reported no competing interests

INTRODUCTION

The Wilson disease (WD) is an autosomal recessive disorder of copper metabolism, caused by mutations in the *ATP7B* gene (1). Over 650 mutations are known to be pathogenic (2), according to the standards established by the American College of Medical Genetics and Genomics (ACMG) (3). While most reported mutations occur in single families, a few ones are typically frequent in certain populations.

The consequences of copper accumulation include the development of hepatic and/or neurological conditions of variable severity. However, consequences can be prevented by starting therapy at an early stage, even before the onset of symptoms. Therefore, knowing the geographical distribution of WD mutations is paramount to design appropriate screening strategies and to improve proper diagnosis in symptomatic cases. (4)

The prevalence of the disease is variable among regions. It has been estimated to be 1:29,000 to 1:40,000, although can be much higher in specific populations (5). Prevalence estimates based on the number of mutant allele carriers in the general

population predict much higher prevalence values, indicating that the disease might be underdiagnosed (6-12). Thus, genetic screening appears as a powerful tool when clinical hallmarks are not clearly defined for a given patient.

Genetic studies performed with central European patients have reported p.His1069Gln as the most prevalent mutation in WD patients, defining a general phenotype mostly based on this prevalent mutation. However, such studies did not include data from Spain (4), and preliminary data from earlier reports suggested that the prevalence in Spain could be different, since p.Met645Arg was found to be the most prevalent in WD patients in our country (13,14). The island of Gran Canaria is considered a WD hot spot with a strikingly high prevalence of the disease due to a specific mutation, p.Leu708Pro, associated with a founder effect (15).

The Spanish Association for the Study of the Liver (AEEH) recently established a registry of WD patients in our country, the AEEH WD Registry. This tool now offers the possibility to verify the true prevalences and establish a real map of genetic mutations in Spanish patients. On this background, the objectives of this study were:

- To describe the genetic characteristics of WD patients in Spain, showing most frequent mutations and their homozygote/heterozygote frequencies
- To evaluate the proportion and distribution of main mutation groups: Missense and Loss of Function.
- To evaluate regional differences in the occurrence of most prevalent mutations and in the use of genetic diagnosis.
- To identify exons carrying the highest proportion of mutations in patients from each region.
- To verify the feasibility and usefulness of genetic testing for diagnosis and screening of patients in our registry.

To the best of our knowledge, this is the first genetic analysis of ATP7B mutations covering almost all the regions of Spain. We postulate that our results could contribute to design the most suitable WD screening strategies for Spain, thus enhancing early diagnosis, which is critical in WD patients.

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PATIENTS AND METHODS

Patients

This multicenter retrospective study was based on data of WD patients included in the AEEH-WD Registry between December 2021 (date of creation of the registry) and November 2022, attended at one of 28 public health care centers in Spain (covering approximately 80 % of the population). The protocol of the study was reviewed and approved by all the Ethical Committees of the participating centers. All patients included in the Registry had provided written informed consent to the use of their clinical data for research purposes.

Clinical and biochemical parameters

Patient data were collected from Hospitals' registries on a retrospective basis. Patients were scored following the Leipzig criteria (16). We collected their clinical, demographic, analytical and genetic information.

Genetic analysis

Genetic analysis to search for *ATP7B* gene variants included double pass heterozygous Sanger sequencing of *ATP7B* coding and flanking sequences, and MLPA analyses when appropriate and available. Next-generation-sequencing was not used. The Ensembl database was used for identification of already known mutations. Missense variants were defined as a substitution in a coding base pair predicted to result in an amino acid change. Loss-of-function (LOF) variants were defined as nonsense (substitution in a coding base pair predicted to result in a premature stop codon), frameshift (insertion or deletion predicted to result in a shift in the reading frame) and splice site (substitutions predicted to involve a splice site resulting in splicing abnormalities).

RESULTS

Baseline patients' characteristics

One year after its creation, the registry included a total of 320 WD patients (with Leipzig score >3), from 14 different Spanish regions, whose health services covered approximately 40 million people; 167 were men (52.2%) and the median age at diagnosis was 16 years (IQR_{25-75} 10-27.8). One third of patients (32.2%) reported a positive family history. The predominant phenotype at diagnosis was chronic liver disease (n=158, 49%), followed by pre-symptomatic cases (n=66, 20.6%), neurological presentations (n=32, 10.1%), mixed phenotypes (n=29, 9.1%) and acute liver disease (n=29, 9.1%).

Genetic data were available for 260 out of 320 patients (81%), including 66 homozygotes, 154 compound heterozygotes and 40 patients with only one mutation.

The number of patients from different regions was quite heterogeneous, being higher in Catalonia (80 patients) and the Canary Islands (79 patients), and very low in Extremadura and Cantabria (1 patient each). Regions with more than one participating hospital also showed high internal variability: Catalonia ranged 4-63 patients per center and the Canary Islands, 1-71 per center (Table 1).

The rate of utilization of genetic testing was very variable among the different regions of Spain; it was more used in the Valencia Community and Navarra (100% both) and poorly used in Cantabria and Extremadura. Within regions with more than one participating hospital the use was also highly variable, with differences among centers in more than 80% of cases in Madrid or Canary Islands, for instance (Table 1).

Observed mutations, frequencies and homozygosity

After examining 448 alleles, 130 different mutations were found, most of them (91%) affecting less than 4 alleles. The most frequent mutations were: p.Met645Arg (92 alleles i.e. 20% alleles, found both in mainland Spain and the Canary Islands), p.Leu708Pro (75 alleles, 16.5%, only in the Canary Islands), p.His1069Gln (38 alleles, 8.3%,only in mainland Spain), promoter region (14 alleles, 3.3%, both in mainland Spain and the Canary Islands) and c.1708-1G>A (12 alleles, 2.6%, only in mainland Spain). Fifteen mutations appeared in homozygosis, but only four of them occurred in more than 3 patients: p.Leu708Pro (n=24), p.Met645Arg (n=6), p.His1069Gln (n=6) and c.1708-1G>A (n=5), the latter one restricted to patients belonging to the gipsy ethnicity

from mainland Spain.

Type of mutations

In terms of the type and localization of genetic alterations, most of them (74%) corresponded to missense mutations (332/448 alleles), while nonsense, deletion, insertion, splice sites and frameshift mutations affected 14% of alleles (61/448). Outside the coding regions 14 mutated alleles were found at the promoter region and 41 at introns: intron 1: n=8; intron 3: n=3; intron 4: n=15; intron 9: n=1; intron 13: n=11; intron 14: n=1; intron 17: n=2).

Localization along exons

The distribution of mutations along the DNA sequence was also very heterogeneous, with only 6 out of the 21 exons presenting more than 10 different mutations: exon 8 (109/448: 24%, mainly p.Leu708Pro occurring in 80 alleles), exon 6 (93/448: 20% with 92 alleles presenting p.Met645Arg), exon 14 (54/448: 12%, 38 alleles with p.His1069Gln), exon 15 (31/448: 7%), exon 2 (24/448:5%) and exon 19 (12/448: 3%). No mutations were found at exons 3 and 21.

Geographical distribution of the most prevalent mutations and affected exons

The distribution of the most frequent mutations was not homogeneous throughout the country, as shown along with the number of cases detected (Table 2). The most prevalent mutation in Spain, p.Met645Arg, was found in all regions, though with different frequencies, more abundant in regions away from the seashore (Figure 1A) , while p.Leu708Pro was exclusively found in the Canary Islands, mainly in Gran Canaria, and p.His1069Gln was mainly found in northern regions (31/38 alleles, 82%) (Figure 1B). The change affecting the promoter region (c.-436-422del15) was restricted to the regions along Mediterranean coast and the Canary Islands. Mutation c.1708-1G>A was only found in the regions along Mediterranean coast and was restricted to people of gypsy ethnicity. Also shown is the geographic distribution of most affected exons. As it can be observed, in many regions, 2 or 3 exons include more than 50% of mutations in WD patients (Table 2).

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Value of genetic data for diagnosis

A review of data from registered patients with available genetic information (260 of 320 patients) showed that, if the score points obtained from genetic information were eliminated from the diagnostic procedure, almost half of them (126/260) would not have reached a Leipzig score higher than 3.

In the sub-group of patients who had been diagnosed after family screening, 78% of them had undergone a genetic test. If such information were removed, 60% would have showed a Leipzig score lower than 4. Conversely, only 130 (50%) out of the 260 patients with genetic information available, had undergone histologic evaluation. If biopsy information was removed and genetic testing kept, most of them (118/130: 90%) still had a Leipzig score higher than 3.

DISCUSSION

Several studies on WD in Spain including genetic data been published (13-15, 17) but information covering almost the entire country was not available until now, and allowed us to produce the first WD genetic map of Spain.

Firstly, the study shows that the use of genetic testing is not homogeneous among the regions that participated in the study. The reasons behind such variability are not clear. The tradition of the management of WD may be an important one. The size of the city may be a further factor influencing such heterogeneity, since hospitals in smaller cities often need to send their samples to other hospitals in larger cities. Many cases were diagnosed by clinical criteria only, so genetic data were not necessary. However, there was also a great variability between centers within the same region, suggesting that genetic testing accessibility did not seem to depend on autonomic region´s diagnostic strategies, but rather linked to specific procedures adopted at each center, probably based on local expertise stressing the value of genetic testing. As it becomes cheaper, it seems that test price might not be as important as stressing its value. Indeed, we show that genetic testing was of great value during diagnosis, even more than intensively invasive procedures. We consider that the work presented will induce the

universal use of genetic testing for WD patients as a powerful tool to increase our knowledge and contribute to improve treatment and prognosis of patients.

The increasing use of total genome studies might reveal individuals with mutations in both alleles, who however remain asymptomatic or show atypical manifestations (21-23). The sooner we gain information on all possible mutations, the sooner we will be able to provide all patients with suitable diagnosis and treatment.

A strategy that may even make genetic testing more accessible is the customization to the screening process according to the territory being under scrutiny. Certain genetic variants are linked to specific populations, and that is also the case for disease causing mutations, sometimes simplifying genetic screening (4, 24). This first Spanish country-wide WD genetic evaluation revealed strikingly a high variability of disease causing mutations: Out of 448 alleles tested, up to 130 different mutations were found. On the other hand, we found remarkable homogeneity in certain subpopulations, which may evidence a founder effect. This is the case of Gran Canaria, where the mutation p.Leu708Pro is highly prevalent with a well-known founder effect dating several centuries back (15), or the intron 4 mutation (c.1708-1G>A), restricted to gypsy families along the Mediterranean coastline. Finally, mutation c.-436-422del15 affecting the promoter sequence, originally from Sardinia (24), was only found in the regions along the Mediterranean coast and in Gran Canaria, especially in families with ancestry on the most eastern islands Fuerteventura and Lanzarote (12) revealing specific population movements, since Sardinia was a part of the Spanish Kingdom for several decades and the commercial maritime exchange with the Spanish Mediterranean coast was intense. It should be noticed that this mutation has not been included in routine genetic testing for some years, so this might explain cases with only one or no mutant alleles when mutations were only looked at within exons and immediate flanking regions. Therefore, the extent of the Sardinian promoter mutation might may be redefined in the future as more data are gathered.

Our results confirmed that mutation p.Met645Arg, found both in mainland Spain and the Canary Islands, is the most prevalent one in our country (13, 18), with a great number of carriers in the population (12). According to global databases, this variant is poorly represented in other European populations but its frequency is remarkably

higher in Ashkenazis (20). The later finding might be related to the long-lasting presence several centuries ago of an important Jewish population in Spain, mainly Sephardic, but probably sharing common ancestors with Ashkenazi.

The pathogenicity of the p.Met645Arg mutation has been evaluated in several articles. In the first one (13), the authors suggested low pathogenicity, since they could not find homozygous patients. However, the registry included 6 homozygotes, with clinical and analytical confirmation. In a recent study, a second pathogenic mechanism, involving the splicing of the *ATP7B* transcript, has been proposed for this mutation (19). Its high allele frequency, both in the general Spanish population (12) and in the WD patient registry, should lead clinicians to consider it in WD diagnosis or in any patient presenting with liver disease of unknown etiology.

Our data confirmed the occurrence in our country of the most prevalent mutation in Eastern Europe, p.His1069Gln (4), which might be due to immigration from those countries, something reinforced by the fact that it was mainly concentrated in Northern regions and was restricted to Spain mainland territory.

In summary, genetic testing in Spain might be efficiently reduced to a few most prevalent variants, detecting most of the cases. These variants are found in a limited number of exons, making a first pass screening approach sensibly cheaper, considering that the most accepted screening technique is Sanger sequencing of individual exons. This is especially important as access to genetic testing, with results reaching almost the 100% of alleles in some centers, may change the WD diagnosis process. Performing a liver biopsy to measure hepatic copper content is an invasive and expensive procedure, often with inconclusive results due to the heterogeneous copper tissue distribution (25). Evaluating fibrosis with not invasive tools like elastography (26), could be a better follow-up method when properly evaluated. As expected, the most widespread use of genetic testing concerns family screenings where only the mutations of the index case are looked for. However, this can only be done if genetic tests have been previously carried out inpatients, which is a further reason to recommend a wider adoption of genetic studies.

The main limitations of the present work were, on the one hand, reduced recruitment of both patients and hospitals and, on the other hand, the lack of genetic information

from approximately 25% of the patients included. However, it should be noticed that the largest earlier WD national study (27) included less patients and did not address genetic information. Another issue to consider when evaluating the number of cases diagnosed per region is the fact that communities such as Catalonia, Galicia or the Valencia Community receive samples to genotype from other regions lacking resources, a fact that is known but not finally registered in the database.

In conclusion, this study offers the first nationwide genetic map of Wilson disease in Spain, which may contribute to improve the diagnosis of future cases. The different mutations are depicted with their particularities throughout regions, which could help reduce the costs of genetic testing; namely, although *ATP7B* is a large gene with 21 exons, we showed that over 50% of mutations can be detected by analyzing 2 to 3 exons, which reduces costs and increases feasibility (6). Our results showed that genetic diagnosis can be better tailored to particular target populations, something considered of paramount importance in genetic screening, especially in cases like WD, involving almost 1000 possible mutations.

WD is a well known severe disease, for which an effective therapy is available, that has serious consequences, leading to irreversible damage, preventable through early diagnosis. The two main factors influencing the cost-effectiveness of a screening protocol are the cost of the diagnostic test and the prevalence of the disease: first, genetic test procedures have progressed remarkably, with the consequent reduction in prices, which could be even lower in the future; second, WD prevalence has been largely debated in recent years, with conflicting results from clinical versus genetic studies, the former yielding lower prevalences than the latter (6-12). One reason might be the low penetrance of certain frequent mutations, like p.Met645Arg in Spain (28), a finding that emphasizes the importance of large registries, like the AEEH WD Registry, for more accurate prevalence estimations, associated to the publication of the corresponding genetic map in every country to facilitate genetic testing in every suspected case.

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Figure legend

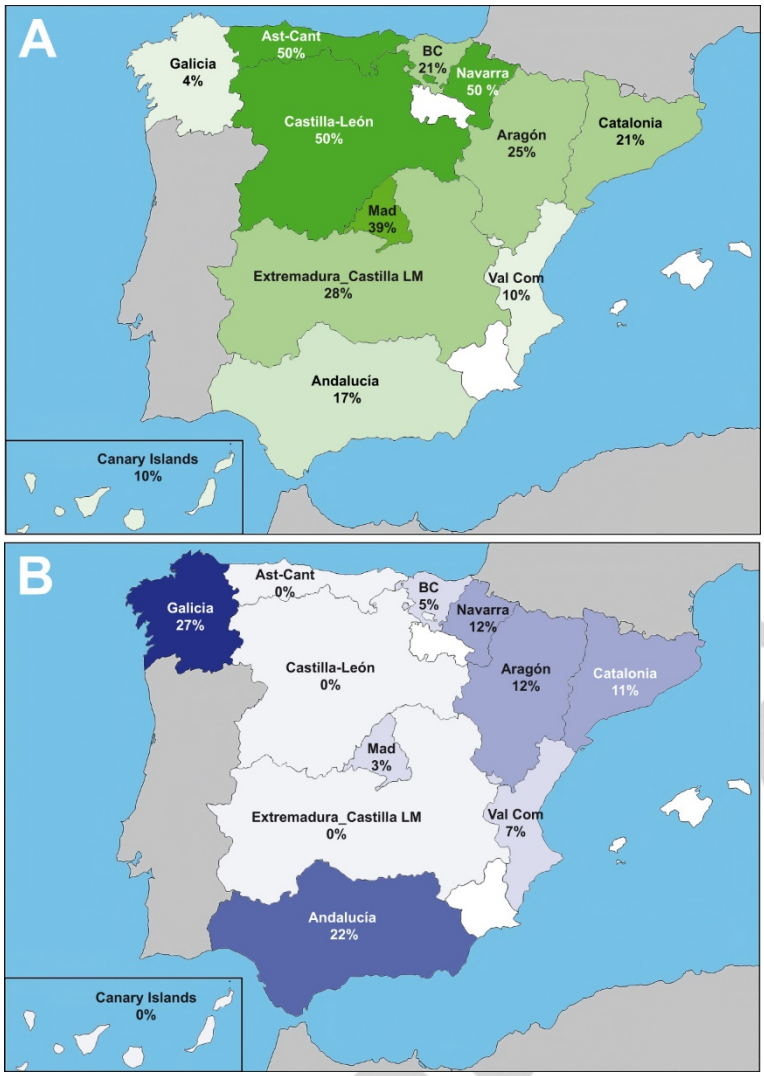

Figure 1. Geographical distribution of the most prevalent ATP7B mutations in Spanish patients. Shown are the percentages of alleles with the p. Met645Arg (A) and p.His1069Gln (B) mutations in the Spanish regions. Some have been united due to the low number of patients. Ast-Cant: Asturias-Cantabria; BC: Basque Country; Val Com: Valencian Community. Basic map by Daniel Dalet provided by https://d-maps.com/.