Università degli Studi di Cagliari

PhD Programme

MOLECULAR MEDICINE (Cycle XXIX)
Medical Sciences and Public Health
Unit of 2° Pediatric Clinic

Wilson disease: comparison among regional, national and international molecular pathologies defined at a Sardinian reference center

Scientific Disciplinary Code
MED/03

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Final exam 2015 – 2016
Thesis discussed on Aprile 2017

Index:

pag.

1. Introduction 3

1. 1 Wilson's Disease 3

1.2 Epidemiology 4

1.3 Pathogenesis 4

1.4 ATP7B protein 7

1.5 Genotype-phenotype correlation 8

1.6 Clinical aspects of Wilson disease 9

1.7 Diagnostic aspects of Wilson Disease 10

1.8 Treatment 12

1.9 Geographical distribution of ATP7B mutations 13

2. Materials and methods 16

2.1 Mutational and sequencing analysis 16

2.2 Multiple Ligation-dependent Probe Amplification (MLPA) 17

2.3 Single Strand Conformation Polymorphism (SSCP) 17

2.4 Functional prediction and mutations pathogenetic meaning: use of Polyphen and SIFT softwares 18

2.5 Method for the identification and definition of extended deletions/duplications: array-CGH 19

3. Results 20

3.1 Wilson disease patients analyzed 20

3.2 Geographical distribution of ATP7B gene mutations in Italy: our experience 20

3.3 Worldwide geographical distribution of ATP7B gene mutations: our experience 24

3.4 Using MLPA technique on negative ATP7B patients 30

4. Discussion 35

5. Bibliografy 40

Introduction

1.1 Wilson's desease

Wilson disease is an inherited autosomal recessive disorder of copper metabolism resulting in copper toxicity. The disease was first described in 1912 by Kinnier Wilson as ‘progressive lenticular degeneration (Wilson S.A.K, 1912).

Subsequently, in the early 1990's the role of copper in the pathogenesis of Wilson disease was established. The causative gene, ATP7B responsible for Wilson disease, was identified in 1993 and encodes a copper-transporting P-type ATPase that functions in the transmembrane transport of copper within hepatocytes.

The worldwide prevalence of WD is 1 in 30,000-100,000, with a carrier frequency of 1 in 90. It is higher in populations isolated and/or with high frequency of consanguinity.

In a healthy organism, ATP7B is expressed in various tissues, liver, central nervous system, kidney, mammary gland, and others (Lutsenko et al. 2007) but the major physiologic role of ATP7B is in the liver, a key homeostatic organ for copper metabolism. In hepatocytes, ATP7B transports copper from the cytosol into the lumen of the *trans*-Golgi network (TGN), where copper is incorporated into secreted copper-dependent enzymes, such as ceruloplasmin. In addition, ATP7B maintains copper levels in the cytosol where, when cytosolic copper exceeds a certain threshold, ATP7B traffics towards the canalicular membrane and sequesters excess copper into vesicles, which subsequently fuse with the canalicular membrane excreting excess copper into the bile (Bartee and Lutsenko 2007).

In WD, ATP7B expression, function, and/or intracellular trafficking are disrupted by mutations. As a result, copper delivery to the TGN and copper excretion are both impaired, and copper accumulates to very high levels. It is thought that a tissue’s inability to adequately protect itself from excess of redox active copper causes the development of WD pathology (Ferenci et al. 2005).

Homozygous, or, more commonly, compound heterozygous mutations in ATP7B lead to defective incorporation of copper into apo-ceruloplasmin, the subsequent formation of holoceruloplasmin and hamper the normal excretion of copper into bile. Consequences of this defect are the impaired copper metabolism and consequent copper intoxication. Copper overload, and actually free copper as the main acting element, exerts its toxicity through two main mechanisms: direct oxidative stress, with lipid peroxidation of membranes, damage of DNA, and mitochondria, as well as unregulated apoptosis leading to cell death because of the loss of inhibitory control of caspase-3.

ATP7B belongs to the P-type ATPase family of genes and is located on the chromosome 13q.14.3. The WD gene was identified almost simultaneously by three separate laboratories (Bull et al., 1993; Tanzi et al., 1993; Yamaguchi et al., 1993). The ATP7B gene consists of 21 exons spanning 100 kb with a 4.3 kb open reading frame encoding a 165-kDa, 1465-amino acid protein designated ATP7B (Petrukhin et al., 1994).

ATP7B mutations are located along the whole gene consisting of 21 exons and 20 introns and most of them are extremely rare. Worldwide, nearly 650 mutations in ATP7B have been identified an deposited in the “ Wilson disease mutation Database-University of Alberta” (http://www.wilsondisease.med.ualberta.ca).

Mutations may occur at any position of the gene, including exons, splicing sites, introns and promoter regions. The most common forms of ATP7B mutation are missense and nonsense mutation (60%), followed by insertions/deletions (26%) and splicesite mutations (9%). Generally, frameshift and missense mutation are associated with a more severe phenotype of WD but as we shall discuss later the phenotype-genotype correlation is still not clear. Compound heterozygous are much more common than homozygous states. The mutations differ greatly in different geographic regions and show significant allelic heterogeneity except for some isolated populations.

The clinical phenotype is highly variable and includes myriad presentations varying from asymptomatic states to chronic liver disease, neuropsychiatric manifestations or acute liver failure, hence a low threshold for suspecting the disease is important. Individuals usually becomes symptomatic between the ages of 5 and 35 years, which reflects the different potential of the liver to store excess copper, resulting in symptoms at both younge and older ages .

Early diagnosis and treatment with copper chelators (D-penicillamine, trientine and tetrathiomolybdate) and/or zinc salts are associated with good prognosis; long-term follow-up shows a satisfactory response in the great majority of adequately treated patients with WD and survival coincides with that of the general population (Bruha et al., 2011), whereas delay in therapy can result in incomplete recovery and even mortality. Hence, the treatment of WD must,, be continued lifelong, as, the prolonged theraoy withdrawal exposes to the risk of death from acute liver failure.

1.2 Epidemiology

The estimated prevalence of WD worldwide is between 1/30,000 and 1/100,000 individuals, and the carrier rate is about 1 in 90 people (Roberts, E.A. 2011). The prevalence of WD can be considerably higher in isolated populations such as Sardinia (Zappu A. et al. 2008, Gialluisi et al. 2013) where the carrier rate is approximately, 1/26 and the disease 1/3000 live births, one of the highest worldwide. The marked discrepancy between our calculated genetic prevalence data for WD in Sardinia and the considerably lower number of clinically diagnosed WD patients is likely to be at least partially due to a reduced penetrance of ATP7B mutations. However, our study also raises concerns that WD may still be unrecognized in a substantial proportion of affected individuals.

1.3 Pathogenesis

Copper, an important microelement of the human body, participates in numerous physiological processes including the mitochondrial respiratory chain, neurotransmitter synthesis, and iron metabolism. When the level of copper in the hepatocytes is normal or slightly low, Cu is transported from the cytoplasm into the TGN and binds to ceruloplasmin, which is secreted into plasma where it accounts for over 95% of the

circulating copper.
However, when the binding capacity of the proteins is exceeded, the free copper accelerates the formation of reactive oxygen species, inducing oxidative DNA damage and accelerated apoptotic cell death by the inhibition of IAPs (Inhibitor of Apoptosis Proteins)(Mufti A.R. et al. 2006). To avoid overloaded copper environment and toxic accumulation of Cu, Cu ions are secreted by hepatocytes into the bile capillary and then discharged through the bile out of the human body, maintaining intracellular copper homeostasis. This mechanism is regulated by a complex network that operate both to satisfy the metabolic demand for Cu and to control Cu levels at the cellular and systemic levels. Infact, high affinity transporter CTR1 imports Cu from extracellular space into the cytosol, where the metal is captured by cytosolic copper chaperones and shuttled towards different intracellular destinations. The copper chaperone ATOX1 carries copper to the trans-Golgi network (TGN), where it is transferred to ATP7B, which loades Cu into newly synthesized ceruloplasmin, activating the secretory pathway.
Once the function and structure of ATP7B are impaired by mutations, the level of ceruloplasmin is decreased and copper overload occurs in the liver, as result of the failure of ATP7B protein to traffic to the sites of Cu excretion, resulting in various clinical presentations of WD.
Despite the fundamental role of ATP7B trafficking in Cu homeostasis, the intracellular itinerary of ATP7B transport remains poorly understood and controversial. Recently, it has been shown that an increase in Cu concentration induces direct ATP7B trafficking from the TGN to a subset of lysosomes, where ATP7B imports Cu for storage in the lysosome lumen and through the interaction with p62 subunit of dynactin complex enables lysosomes for polarized exocytosis at the canalicular surface of hepatocytes. Activation of lysosomal exocytosis stimulates the delivery of ATP7B to the canalicular membrane domains of hepatocytes and the release of excess Cu into the bile (Polishchuk E.V et al. 2014).
The ATP7B-mediated transport of copper involves several steps. First, ATP7B becomes transiently phosphorylated at the residue D1027 located in the P-domain (catalytic phosphorylation). Subsequent dephosphorylation releases the energy necessary to transfer copper across membrane (transport step). Each of these steps can be affected by WD-causing mutations.The effect could be severe, resulting in complete loss of ATP7B function, if mutated residues are critical for binding of ATP or copper and/or conformational transitions during catalysis. The inactivation of ATP7B could also be partial if mutations diminish the affinity for substrates, slow down conformational transitions or interfere with precise protein targeting to TGN or vesicles. Understanding the phenotypic diversity in WD requires knowledge of how causative mutations alter protein stability, activity, and localization in the cell (Huster et al. 2012).

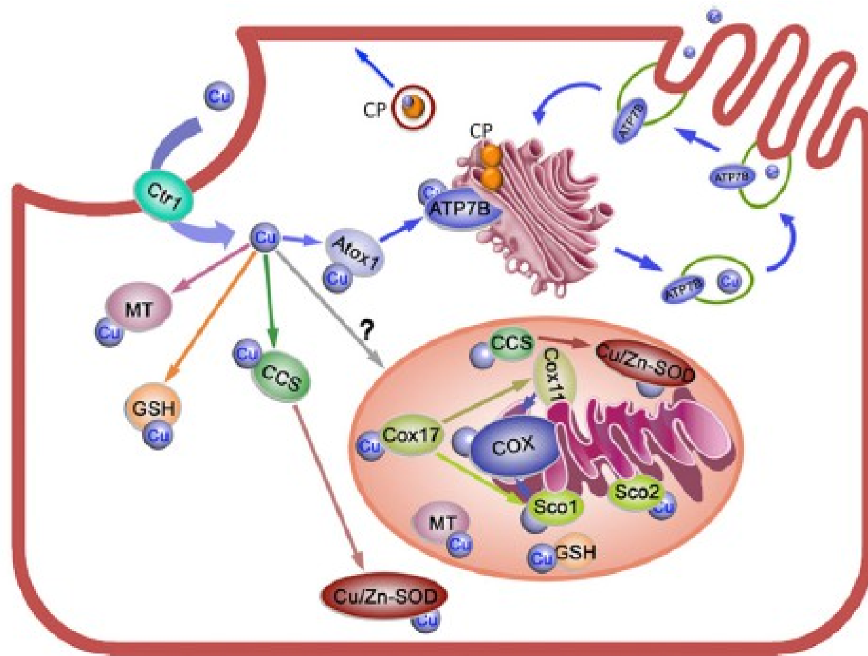

Figure 1. Copper distribution pathways in the hepatocyties. Copper enters a hepatocyte through the high affinity copper transporter Ctr1 located at the basolateral membrane. In the cytosol, copper binds to copper chaperones Atox1 which delivers it to ATP7B protein, located in the trans-Golgi network, to transfer copper into the TGN lumen. Copper is then incorporated into ceruloplasmin and then released into bloodstream. When cellular copper is elevated, ATP7B also transports it from the TGN to vesicles whose fusion with apical membrane results in the release of copper into the bile. Cu: Copper; Ctr: copper transporter; GSH: glutathione; Atox1 and CCS: copper chaperones; Cos11, Cox17, Sco1 and Sco2: a set of chaperones in the mitochondria; COX: cytochrome C oxidase; TGN: the *trans*-Golgi network; CP: ceruloplasmin protein; MT: metallothionein protein. (Lutsenko 2014, modified).

1.4 ATP7B protein

ATP7B is a large multidomain protein with eight transmembrane helices, which form a channel that pumps Cu from the cytosol at the expense of ATP hydrolysis. ATP7B is highly expressed in liver, where it normally resides in the trans-Golgi network (TGN) of hepatocytes.

ATP7B protein belongs to P-type ATPase superfamily. The characteristic domains of P-type ATPase of class IB (PIB) include: Phosphatase-domain (A-domain), the place where the acyl-phosphate gets dephosphorylated. Phosphorylation domain (P-domain), the room for phosphorylation of Asp residue from the sequence DKTGT. ATP-binding domain (N-domain), where ATP binds to. The transmembrane channel (M-domain) which is composed of eight transmembranespanning helices and contributes to copper transport. The metal-binding domain (MBD domain) contains six copper-binding motifs (MBD1-6), that can bind to Cu at the N-terminus in citosol.

The core structures of ATP7B protein are highly conserved: A-domain contains a Thr–Gly–Glu sequence motif (TGE) in which the Glu residue is required for the phosphatase to function. The P-domain contains a highly conserved sequence motif DKTGT which is critical for enzyme phosphorylation infact as a P-type ATPases, ATP7B undergoes auto-phosphorylation of a key conserved aspartic acid (D) residue in the DKTGT motif. The ATP binding to the protein initiates the reaction and copper binds to the transmembrane region. Then phosphorylation occurs at the aspartic acid residue in the DKTGT motif with Cu release. Later dephosphorylation of the aspartic acid residue regenerates a protein ready for the next transport (Banci et al. 2010).

There is a unique amino acid motif that is highly conserved in the N-domain, the histidine 1069 in the SEHPL motif. The mutation of this motif (His1069Q) represents the most common mutation of WD in northern European populations. The transmembrane domain contains the Cys–Pro–Cys (CPC) sequence motif , the key residue that confers metal ion selectivety. The *N*-terminal metal-binding domains (MBDs) comprises six heavy metal-associated sites, each of which contains the repetitive sequence motif GMXCXXC. The MBDs have a high affinity for copper and play a major role in the acceptance of copper from ATOX1 by special protein–protein interactions. Mutations may occur at any position of the gene, causing accumulation of copper.

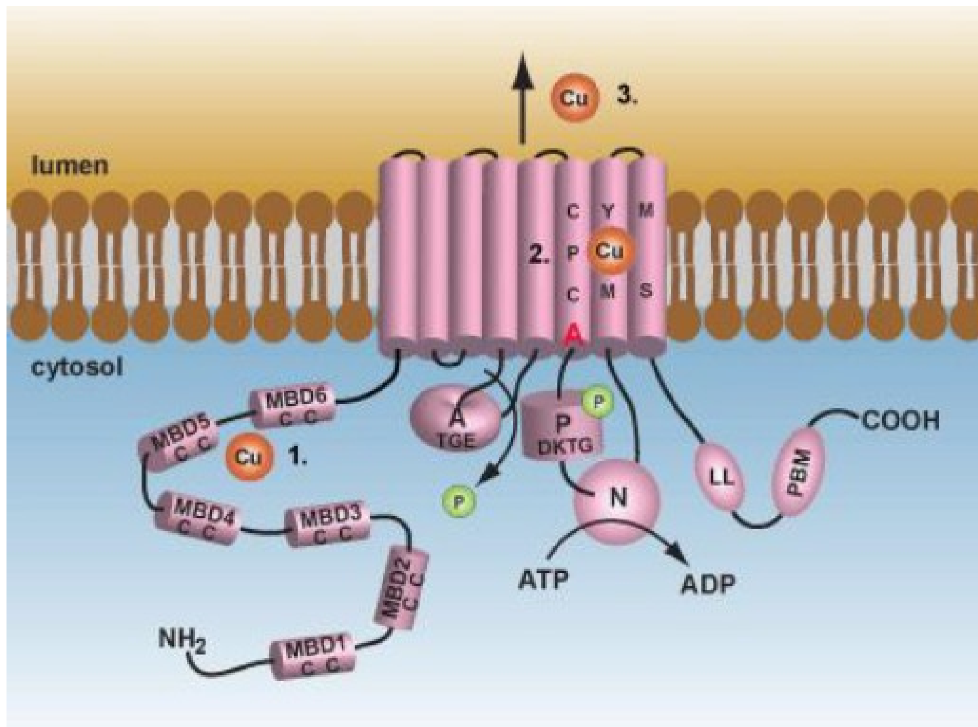

Figure 2. ATP7B protein: topological model proposed for the localization of the ATP7B protein in the region trans-Golgi. Cu: copper; MBD1-6: six Cu-binding domain; A-TGE: Phosphatase-domain; P-DKTG: phosphorylation domain; N: ATP-binding domain; LL-PBM: DiLeucine and PDZ-Binding Motifs for subcellular localization; CPC: critical residues binding for copper of the transmembrana domain.

1.5 Genotype-phenotype correlation

Wilson's disease has complex clinical phenotypes, including a wide range in age of onset, diverse clinical presentations, and greatly different metabolic disorders. The over 650 mutations identified in ATP7B gene exert widely varying effects on the ATP7B protein structure (loss of ATP7B integrity, misfolding, impaired interaction between proteins) and function (phosphorylation, abnormal copper transportation, decreased ATP binding affinity, and abnormal intracellular transport).The majority of studies on genotype-phenotype correlations in WD have been inconclusive. Recently, in a study involving two pairs of monozygotic twins with WD who were ATP7B H1069Q homozygous, it was shown that their clinical manifestations were different (Czlonkowska A. et al. 2009), suggesting that in addition to the allelic heterogeneity, there must be other factors contributing to the phenotypic diversity in patients with WD. In a recent study conducted in a mountainous population with a high prevalence of WD, it was also shown that the onset of disease, predominant manifestations and time of diagnosis were similar among the individuals tested, indicating there might be an environmental factor involved in the disease phenotypes (Cocoş R, et al. 2014). Evidently, the genotype–phenotype relationship in the patients with WD is multifactorial, and it is difficult to identify a direct genotype–phenotype correlation in all patients with WD.

Although some studies made significant effort in examining genotype-phenotype correlations and revealed some trends (Merle et al. 2010, Wu et al. 2001, Stapelbroek et al. 2004), other studies comparing various mutations showed no correlations with a phenotype (Nicastro et al. 2009; Hermann et al. 2002). In fact, the clinical presentations of WD cannot be simply attributed to specific genotypes as they are a manifestation of dynamic complex factors at the subcellular level. Furthermore, there has been a growing appreciation that epigenetic, environmental and or dietary factors as well as modifiers genes (Chen Chen et al. 2015), may play a very important role in regulating the WD phenotype even though we still know little about such factors.

1.6 Clinical aspects of Wilson disease

Clinical manifestations of WD may be of several kinds, but usually the symptoms of presentation are hepatic or neuropsychiatric, with a vast range of heterogeneity for both groups of symptoms. Although copper is always elevated in the WD liver, major disease manifestations vary, and either neurologic or hepatic phenotype may dominate (Table I). Wilson's disease can manifest with an impressive spectrum of neurological, behaviour or psychiatric disorders, which may be its first clinical manifestation, appearing simultaneously with hepatic signs, or years later.

In the liver, copper overload triggers development of hepatitis, which may progress to cirrhosis and liver failure (Huster et al. 2010) but there are WD patients who show only mild hepatic manifestations such as asymptomatic minor biochemical disturbances. In the brain, globus pallidus, putamen, thalamus, mesencephalon, pons, and corpus collosum are mainly affected (Trocello et al. 2010). The development of neuropsychiatric symptoms (involuntary movements, ataxia, depression) usually occurs in older patients and is thought to be secondary to liver malfunction.

The age of WD onset varies significantly. However, the disease usually presents in children or young adults. Neurological symptoms in WD typically begin in the second or third decade, but late onset with WD manifesting over 70 years of age is also well documented. Thus, the diagnosis of WD should never be excluded because a patient is "too old". Conversely, WD with onset in early infancy has also been reported, the youngest age of onset being 9 months (Kim J. et al.2013). In children clinical symptoms may be absent, and typical features, such as the Kayser-Fleischer rings, are rarely seen before the age of 7 years, making the diagnosis of the disease more difficult than in adults. However, all children diagnosed in early infancy with genetically confirmed WD, presented with hepatic symptoms. The clinical hallmark of Wilson's disease is the Kayser-Fleischer ring, which is present in 95% of patients with neurological symptoms and somewhat over half of those without neurological symptoms (Roberts EA. et al., 2008). They are not entirely specific for Wilson's disease, since they may be also found in patients with chronic cholestatic diseases including children with neonatal cholestasis.

Hepatic	Subclinical	Abnormal liver function test
	Acute	Mild, self-limiting hepatitis
		Acute/fulminant liver failure with/without haemolysis
	Chronic	Chronic active hepatitis with progressive fibrosis
		Liver cirrhosis
Neurologic	Movement and coordination	Tremor
		Dysarthria,dysphagia
		Ataxia, dystonia, choreathetosis
		Rigidity, bradykinesia, hypomimia, parkinsonism
		Micro-/macrographia
	Other	Concentration and cognitive impairment
		Hypersalivation, drooling
		Epilepsy
Psychiatric	Generally	Personality change (aggressive behavior, impairment of emotional control etc.)
		Disorders of mood
		Sleeplessness, anxiety etc.
	Psychotic	Hallucinations,
		Catatonia
		Delusions
Other organs	Eyes	Sunflower cataract; Kayser-fleischer ring
	Fertility	Amenorrhoea, ovarian dysfunction, infertility, abortion
	Musculoskeletal	Stiffness, back pain, osteoarthritis, osteoporosis
	Kidneys	Tubular dysfuncion, aminoaciduria
	Heart	Cardiomyopathy, arrhythmias
	Haematologic	Anaemia, thrombocytopaenia
	Other	Gall stones, endocrine disturbances

Table I. Signs and symptoms of Wilson's disease (Loudianos et al. 2014, modified).

1.7 Diagnostic aspects of Wilson disease

Early diagnosis and prompt treatment of Wilson disease is critical for complete recovery and to avoid morbidity and mortality. The diagnosis of WD may be made readily in presence of the classic signs such as liver disease, neurological signs, Kayser-Fleischer rings and laboratory data of low serum ceruloplasmin levels and elevated urinary copper excretion (Table II). However, since not all the symptoms are always present, it is difficult to establish the diagnosis, expecially in children. No single test can confirm the diagnosis with 100 percent accuracy. In fact, is the combination of clinical and family history, physical examination, and certain key laboratory tests that collectively establish the diagnosis. A scoring system for WD diagnosis was developed by an international consensus of experts, the Leipzig scoring system (Ferenci et al. 2003). This approach collates biochemical, clinical and molecular genetic data from individual patients to provide a quantitative score (Table III).

Genetic tests are useful to ascertain the diagnosis in suspected patients and to screen asymptomatic siblings. In fact, in the Leipzig scoring system, the highest weight is assigned to the detection of ATP7B mutations. Molecular testing for ATP7B mutations has therefore become an important advance in the diagnostic tools of clinicians especially when routine testing is equivocal for WD. However, with over 600 ATP7B mutations reported and with no common set of mutation across regions, its role in the diagnosis can be considered limited in some populations.

Typically, the presence of Kayser-Fleischer rings (KFR) and serum ceruloplasmin (CP) < 10 mg/dl are sufficient to establish the diagnosis. CP levels can also be low in other conditions such as hepatic

insufficiency due to advanced liver disease. Laboratory findings that further support the diagnosis of WD include low serum copper levels, elevated hepatic transaminase levels, aminoaciduria, and hemolytic anemia. However transaminase levels may be normal in WD patients who present with neurological symptoms. Analysis of 24 hr urine copper excretion in urine is an easily performed and important diagnostic test for WD. Urinary copper excretion greater than 100 µg per 24 hr in the absence of cholestatic liver disease is typical for WD. A liver biopsy for measurement of hepatic copper may rarely be indicated in patients with the neurological presentation of WD in whom other investigations are ambiguous. Hepatic copper values greater than 250 micrograms per gram of dry weight (normal 20–50) are characteristic of WD. Intermediate values (50–200 microgram per gram of dry weight liver tissue) suggest heterozygote ATP7B mutation carrier status. (Bandmann O. et al. 2015).

Test	Typical finding
Serum eruloplasmine	Decreased by 50% of lower normal value
24-hour urinary copper	> 1.6 µmol/24 h > 4 µmol/24 h in children
Serum "free" copper	> 1.6 µmol/L
Hepatic copper	> 4 µmol/g dry weight
Kayser-Fleischer rings By slit lamp examination	Present

Table II. Routine tests for diagnosis of Wilson's Disease (Loudianos et al. 2014, modified).

Typical clinical symptoms and signs		Other tests	
KF rings		Liver copper (in the absence of cholestasis)	
Present	2	> 5 × ULN (> 4 µmol/g)	2
Absent	0	0.8–4 µmol/g	1
Neurologic symptoms**		Normal (< 0.8 µmol/g)	-1
Severe	2	Rhodanine-positive granules*	
Mild	1	Urinary copper (in the absence of acute hepatitis)	
Absent	0	Normal	0
Serum ceruloplasmin		1–2 × ULN	1
Normal (> 0.2 g/L)	0	> 2 × ULN	2
0.1–0.2 g/L	1	Normal, but > 5 × ULN after D-penicillamine	2
< 0.1 g/L	2	Mutation analysis	
Coombs-negative hemolytic anemia		On both chromosomes detected	4
Present	1	On 1 chromosomes detected	1
Absent	0	No mutations detected	0

TOTAL SCORE	Evaluation:
4 or more	Diagnosis established
3	Diagnosis possible, more tests needed
2 or less	Diagnosis very unlikely

Table III. Scoring System developed at the 8 International Meeting on Wilson's Disease, Leipzig 2001 (Loudianos et al.2014, modified).

1.8 Treatment

All Wilson's disease patients, even in the presymptomatic stage, need lifelong drug therapy. If treatment is initiated opportunely, deterioration can be prevented and life expectancy can be comparable to subjects without the disease (Skowronska M. et al. 2013). Prognosis for WD patients is excellent as long as compliance to therapy is adequate. On the contrary, the natural course of the disease is characterized by progressive deterioration leading to death due to liver or neurological disease.

The objectives of treatment therefore are to prevent appearance of symptoms in asymptomatic subjects, prevent clinical deterioration in affected subjects, and can also be life-saving in cases of acute-on-chronic hepatitis. Currently only liver transplantation is able to cure the disease in the hepatic form, but in general treatment options include the copper chelators (D-penicillamine, trientine and tetrathiomolybdate) and/or zinc salts. While chelating agents bind copper directly in blood and tissues and facilitate its excretion, zinc interferes with the intestinal uptake of copper. So Zinc is used mostly for maintenance therapy or the treatment of asymptomatic WD patients. A different chelating agent, tetrathiomolybdate (TTM) may be a promising alternative as it appears to be superior in reducing the circulating free copper pool. However, clinical experience with this drug remains limited. Elevated amounts of copper are naturally found in numerous food products and, although dietary restriction of copper-rich food is no sufficient therapy for WD, its is nevertheless an important part of WD management.

1.9 Geographical distribution of ATP7B mutations

More than 600 worldwide mutations have been identified to date in the ATP7B gene. We used the following data sources to obtain information on known genetic mutations: 1)The Wilson Disease Mutation Database of the University of Alberta unfortunately updated until 2009, 2) Literature searches using PubMed. An understanding of the regional distribution of ATP7B gene mutations can help to design shortcuts for genetic diagnosis of WD (Ferenci et al. 2006). Figure 3 graphically illustrates the worldwide distribution of the most common mutations seen in different countries.

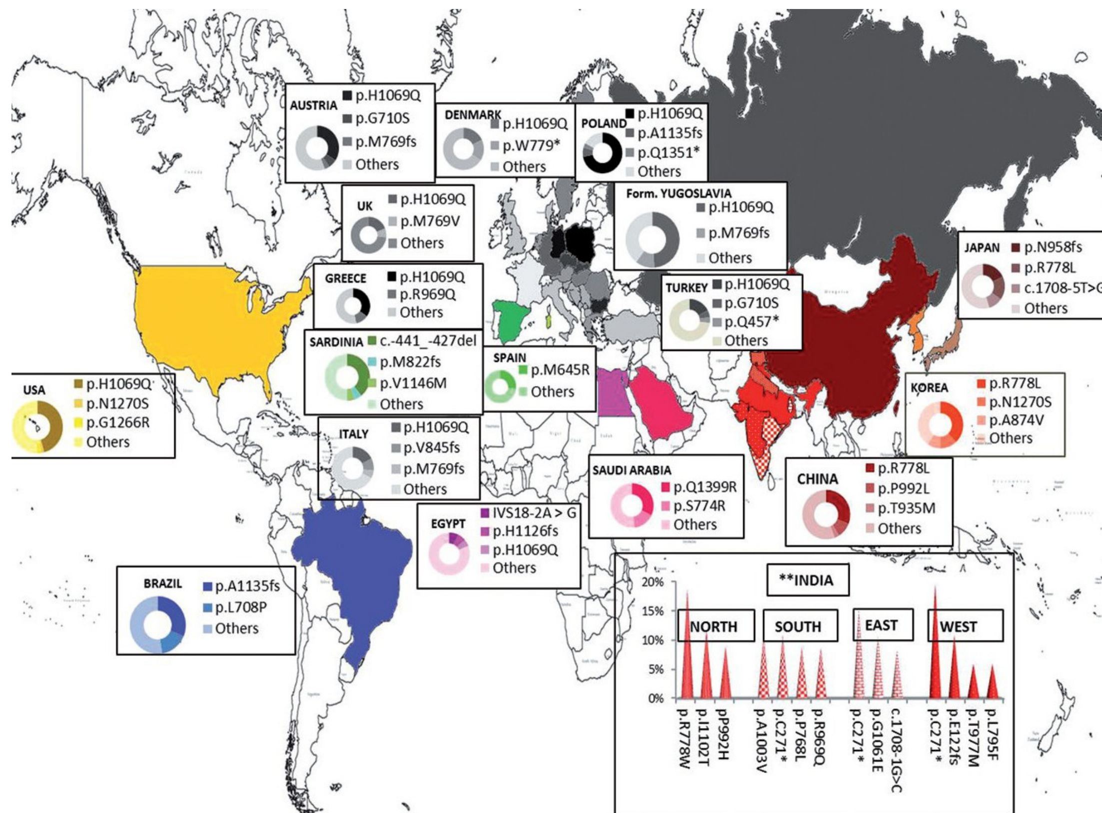

Figure 3. Worldwide geographical distribution of the most common ATP7B mutations (Gomes et al. 2016, modified).

Europe

Mutation p.His1069Gln is considered to be the principal mutation in Central and Eastern Europe and a number of groups have suggested that this mutation could have arisen originally from Eastern Europe (Firneisz et al., 2002). There is a large difference in the prevalence of this mutation among WD patients depending on their geographic area and ethnic background (Ferenci, 2006). From the European countries, its highest allele frequency is seen in Poland (72%), Former East Germany (63%) and Bulgaria (55.8%).

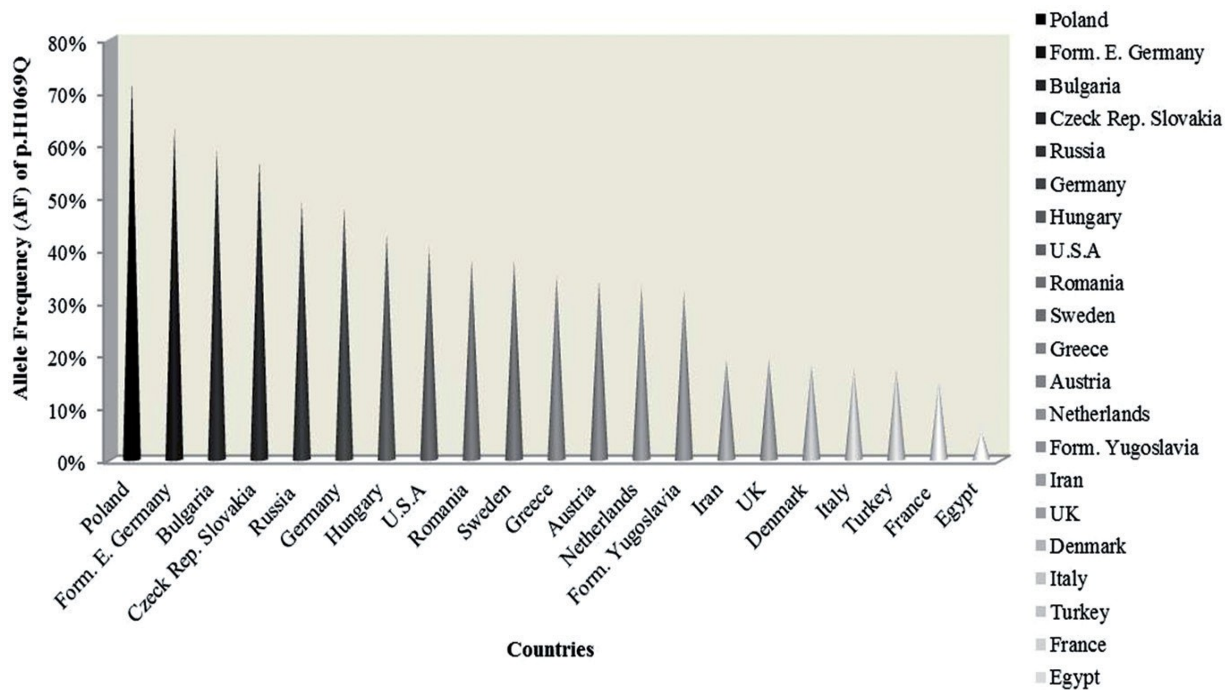

Figure 4. Gradient distribution of H1069Q mutation in several countries (Gomes et al. 2016, modified).

In other European countries, WD mutation data have become available only recently and show the following allele frequencies of p.His1069Gln: Czech Republic Slovakia (57%), Russia (49%), Former Yugoslavia (48.9%), Germany (47.9%), Hungary (42.9%), Romania (38.1%), Sweden (38%), Greece (35%), Austria (34.1%), The Netherlands (33%), UK (19%), Denmark (18%), continental Italy (17.5%), Turkey (17.4%) and France (15%) (Gomes et al. 2015). In Mediterranean countries there is a wide range of mutations, the frequency of each of them varies considerably from country to country: in Spain, the missense mutation p.Met645Arg (27%) is the most prevalent, whereas in the Canary Islands, the mutation p.Leu708Pro is the predominant mutation (64%). Another exception is Sardinia, which is reported to have a high WD prevalence. In this region a unique deletion mutation in the 5' UTR (c.-441_427del) is seen at a frequency of 65.5% followed by p.Met822fs (8.5%) and p.Val1146Met (7.9%). The sardinian deletion c.-441_427del was not reported from anywhere else. Interestingly, this mutation was observed also in an isolated valley in Costa Rica reflecting the genetic sardinian origin of the spanish soldier (Ferenci et al. 2006). In Turkey, there is no very common mutation and are frequently located in exons 8 and 13.

Asia

India

The distribution of WD mutations in the different regions of India suggests high genetic heterogeneity and the absence of a single or a limited number of common founder mutations. This reflects the ethnic diversity of the country. In the north of India, the most prevalent mutations are p.Arg778Trp (19%), p.Ile1102Thr

(12%) and p.Pro992His (9%), while in the south, p.Ala1003Val (11%), p.Cys271* (11%), p.Pro768Leu (9%) and p.Arg969Gln (9%) have high prevalence. In the east, p.Cys271* (16%), p.Gly1061Glu (11%) and c.1708- 1G>C (8.5%) are the most commonly observed mutations, whereas in the west, p.Cys271* (20%), p.Glu122fs (11%), p.Thr977Met and p.Leu795Phe (6%) predominate. Although its presence has not been reported in the north of India, the p.Cys271* mutation is the most frequent mutation observed in the Indian population (Aggarwal et al., 2013; Gupta et al., 2005; Kumar et al., 2005; Santhosh et al., 2006).

Far East Asia

Data from the East Asian region is mainly from China, Japan and Korea. In China, 67% of the WD mutations lie in exons 8, 12 and 13 (Li et al., 2011), with p.Arg778Leu resulting the most prevalent (28.4–33.8%). The next two are p.Pro992Leu (8.8 – 11.2%) and p.Thr935Met (0.86 – 6.8%). In Japan the mutations are concentrated in exons 8, 13 and intron 4, accounting for 49% of them and the mutations p.Asn958fs (15.9–20%) followed by p.Arg778Leu (13.4–20%) and c.1708-5T4G (10–11%) are the most frequent (Okada et al., 2000). In Korea, the predominant mutations are p.Arg778Leu (37.9%), p.Asn1270Ser (12.1%) and p.Ala874Val (9.4%). Thus, the p.Arg778Leu mutation is widespread in the whole of this region.

Middle East

In Saudi Arabia, genetic analysis showed disease causing mutations in three exons (exons 8, 19 and 21) of the ATP7B gene in 50% of the patients (Al Jumah et al. 2004). The two most common mutations are p.Gln1399Arg (32%) followed by p.Ser774Arg (16%). In an Iranian population was reported by a 2001 study that p.His1069Gln mutation is the most prevalent mutation with an allele frequency of 19%. However, it is important to note that all the homozygous patients were found to have hepatic symptoms and were between 5–40 years of age. This result disagrees with what is reported in relation to the p.His1069Gln mutation being associated with central nervous system diseases and a later onset of disease (Gomes et al. 2015). Furthermore, a recent study in the southern Iranian population showed 20% WD patients had mutations within exons 8 and 14 and only a lower frequency of the most common mutations of exons 8 and 14 was present (Dastsooz et al. 2013).

Africa

In the African continent, Egypt is the only country for which WD mutation data are available. The most common mutation found was IVS18-2A>G (7%), followed by p.His1126fs (6.25%) and p.His1069Gln (5.46%). The mutational spectrum identified differs from that observed in other countries. The high rate of homozygous mutations reflects the high rate of consanguinity (Ghaffar et al.2011).

America

Studies in Brazilian population found 62.5% of the mutations in exons 8 and 15 and showed that the most predominant mutations were p.Ala1135fs (30.8-31.7%) and p.Leu708Pro (15.8-16.7%). The mutational spectrum seen in Brazil is compatible with the Brazilian history of Mediterranean immigration (Deguti et al. 2004; Machado et al. 2008). The p.His1069Gln was seen in three patients in the Brazilian study, but was not a prevalent mutation, as we'll see in North America. In the US the study was carried out on Wilson disease patients predominantly with European–American origin. Infact, the p.His1069Gln mutation had the highest prevalence (40.3%) followed by p.Asn1270Ser (1.9%) and Gly1266Arg (1.9%) revealing that 84% of the mutations lay in exons 14 and 18. This shows that the spectrum of WD mutations in North America and South America are vastly different (Kuppala et al. 2009; Machado et al. 2008).
The country with the highest incidence in the world, however, is Costa Rica (4.9/100.000 inhabitants), possibly due to elevated degree of consanguinity and a possible founder effect, the most frequent mutant being p.Asn1270Ser, previously described only in Sicilian, Lebanese and Turkish populations (Shah AB et al. 1997). Unfortunately recent data about mutation prevalence are lacking.

2. Materials and methods

2.1 Mutational and sequencing analysis

DNA was extracted from peripheral blood using both salt and automatic extraction (NorDiag Arrow) technologies. Mutation detection was performed by DNA amplification of the 21 exons and of the known 5' UTR region of the ATP7B gene by several PCR reactions (Polymerase Chain Reaction), using oligonucleotide probe pairs localized about 25 nucleotides from both exon/intron bounderies of each exon.
The amplification product was subsequently subjected to purification treatment by the use of ExoSAP-IT reagent (USB Corporation, Cleveland, Ohio, USA) or by the automatic Biomek NXP Workstation. The sequence of the purified amplification product was obtained with the use of BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, California, USA), subsequently purified through the use of the automatic Biomek NXP Workstation again, and availing of 3130 DNA Analyser (Applied Biosystems) for elettrophoretic runnning step. All mutations were confirmed by an independent second test and, when samples were at our disposal, by evaluating family segregation. The description of the mutations and the corresponding nomenclature has been assigned following the recommendations of the Human Genomic Variation Society (HGVS May 2016), while the search of mutations described by other authors was obtained thanks to the Wilson Disease Mutation Database of University of Alberta and by a bibliographic search on PubMed.

2.2 Multiple Ligation-dependent Probe Amplification (MLPA)

The MLPA method was used to identify the possible presence of deletions/duplications in WD patients negative to mutation analysis of ATP7B gene. The MLPA provides four phases of DNA genomic analysis: denaturation and hybridization of the two synthesized oligonucleotide probes specific for each region to be analyzed, ligation of the same probes located in a position adjacent to the complementary target sequences, amplification of fragments from 130 to 427 nucleotides, electrophoretic separation through the 3130 DNA Analyzer, and finally by displaying the peaks in the form of histograms, whose height will be directly proportional to the number of copies of each fragment analyzed. The main advantages of this method are the possibity to analyze very small amounts of DNA (20ng) and the high efficiency of the technique ensured by a double control intelligence system represented by the hybridization and ligation steps of the probes; in fact only the probes perfectly paired to the complementary sequence will give a signal amplification. Even a mismatch of a single nucleotide prevents the amplification reaction. In particular we used the commercial available kit (P098, MRC Holland, Amsterdam, the Netherlands) made up of 37 probes, four of which are specific for the wild-type sequence of four mutational sites located in the ATP7B gene (R778L, A874V, H1069Q and N1270S); there are also nine other probes that work as control for experimental efficiency (amount of DNA denaturation, chromosome X and Y).

2.3 Single Strand Conformation Polymorphism (SSCP)

Previously the method of choice for mutation analysis of the ATP7B gene was the SSCP. Indeed, this method applies well in the presence of a high and contemporary number of samples to be analyzed, as in our case.

It also allows to limit the use of the DNA sequence step only for samples which show an altered electrophoretic pattern, discovered by this method. In fact the SSCP provides a DNA fragments electrophoretic run, previously amplified and denatured, containing in our case the ATP7B gene portions. The next step, a staining with a silver nitrate solution which selectively binds to the DNA molecule, highlights the electrophoretic migration changes in the single DNA chain, resulting in a conformational change due to the presence of a sequence variant. Thereafter, the samples were submitted to sequencing to define the altered sequence. Subsequently, with the advent of new sequencing methods always more efficient and rapid, and the possibility of use sequencer 3130 DNA Analyser, mutational analysis has been carried out directly through the sequencing of exon regions of the ATP7B gene, except for the promoter region which is still analyzed in Sardinian patients through electrophoretic run of a PCR fragment containing the Sardinian deletion (- 441_427del).

2.4 Functional prediction and mutations pathogenetic meaning: use of Polyphen and SIFT softwares

In the last decade several specific functional prediction software have been created for amino acid substitutions of the coding portions of the genome. In fact, with the evolution of the whole-genome sequencing technology (next generation sequence), it's possible to analyze a large number of sequence variants from each sample in a relatively quick way, compared to previous sequencing methods; however, identification of a sequence variation in cases of missense mutations does not allow by itself to draw conclusion about the functional significance and pathogenesis of most of the mutations. The challenge is to be able to discriminate between common/rare polymorphic variants, which don't cause phenotypic consequence in individuals, from rare pathogenic variations, whose impact on protein results in pathological phenotypic alteration, without necessarily relying on expensive and laborious in vitro functional experiments. For this purpose worldwide bioinformatics teams have developed specific algorithms for the analysis of altered protein function.

Polyphen (Polymorphism Phenotyping): PolyPhen is an automatic tool for prediction of possible impact of an amino acid substitution on the structure and function of a human protein. This prediction is based on a number of features comprising the sequence and phylogenetic and structural information characterizing the substitution:

1. Sequence annotation/prediction: the tested sequence is analyzed if already described or predicted as component of a protein functional domain (trans-membrane, signal-peptide, binding etc ..).
2. Multiple alignment: the protein under examination is compared with the other proteins of the same family to calculate the percentage of amino acid "identity" investigated. It is then calculated the Position Specific Independent Counts (PSIC) to assess the likelihood of a given amino acid in a given position. The reliability of the data depends on the number of aligned sequences and the length of the aligned fragment (Not less than 50 amino acids).
3. Structure and interactions parameters: this tool investigates if the amino acid substitution eliminates the hydrophobic center of the protein and the presence and possible alteration of electrostatic interactions with the protein ligands.

A final score classifies the amino acid substitution studied in "probably damaging" (high probability of being pathogenic), "possibly damaging" (average probability of being pathogenic), "benign" (without pathogenic effects),

"Unknown" (insufficient data to perform the prediction).

SIFT (Sorting Intolerant From Tolerant): Using the information obtained by multiple alignment of more sequences, SIFT software can predict whether an amino acid substitution is tolerated or deleterious, through the following steps:

1. Search for similar sequences
2. The sequences closely related to that investigated are selected for possible sharing of similar functions
3. choices sequence alignment

4. Calculation of the probability of all possible amino acid substitutions for the investigated position, and transformation into a final score.
A score below 0.005 identifies a "deleterious" prediction, but if this is over 0.005 the prediction is "Tolerated". In addition, the SIFT also provides the Median info, which measures the rate of diversity of sequences used for the prediction. Its ideal value is between 2.75 and 3.5, so higher values indicate that the sequences used are too closely correlated with the sequence in question and "deleterious" prediction is no longer trusted because does not discuss a possible replacement evolutionarily tolerated.
The number of sequences used for each position (coverage) also provides us the reliability degree of obtained score.
Despite these prediction tool, obtained thanks to years of study and testing, it is however necessary to remind that these are not completely reliable predictions and always imply the presence of a certain percentage of false positives and negatives.

2.5 Method for the identification and definition of extended deletions/duplications: array-CGH

Comparative Genomic Hybridization is a technique developed to identify numerical chromosomal abnormalities (aneuploidy) in charge of 22 autosomes and the sex chromosomes (X and Y), or even variations (changes in the number of copies “CNV”) content of small chromosomal portions, as duplications, amplifications, or deletions.
The principle on which is based the technique, the array CGH, is the quantitative comparison of the DNA under examination or test DNA, and genomic DNA reference coming from a healthy subject. During the analytical process these DNA are differentially labeled with fluorescent molecules and, subsequently, are hybridized on a microarray, composed by a glass holder, whose surface is covered with DNA probes. The more the number of probes the greater the effectiveness of the array in the identification of number of copies changes. The resolution power of the platform used can vary in function of the density and type of probes used; currently the array used for diagnostic purposes have a resolution power between 1 Mb and 100 kb. At the end of the above incubation, the DNA tested will bind to the control clones on the array. The result will be the emission of two distinct fluorescent signals, the intensity of which will be measured by a specific reading tool. A comparative analysis on the image obtained is then performed between the intensity of fluorescence emitted by the two DNAs, in order to highlight any changes in the number of copies of the tested DNA. In case of normal chromosomal structure, the relationship between the two emissions is balanced (1: 1). If the DNA examinated shows any deletions, the relationship between the tested DNA and the control DNA will be 1: 2. In case of duplication the relationship between the examined DNA and that control will be 2: 1. The great advantage of this method is that it allows to assess the presence of chromosomal abnormalities at the level of the entire genome in a single experiment, without knowing at the biginning what to look for. In addition, compared to other methods of investigation, such as the traditional karyotyping, the analysis of array-based genome has a much higher resolution (100 times and more).

In the CGH-array experiment we performed, we used “SurePrint G3 Human CGH Microarray kit” (4x180K Agilent Tecnologies) that is characterized by a 13 kb overall median probe spacing (11 KB in Refseq genes).

3. Results

3.1 Wilson disease patients analyzed:

Since nearly twenty years the Liver Genetic Diseases Laboratory is responsible for the molecular diagnosis of WD patients coming from all Italian regions and mainly from Sardinia. Moreover, thanks to many International cooperations, it has been possible to carry out ATP7B gene molecular diagnosis on large series of WD patients coming from Greece, Turkey, Serbia, Croatia and Romania. Also, many sporadic WD patients from all over the world came to the attention of the mentioned center. Over the years, it were collected and analyzed 806 families each containing one or more affected, and identified 217 of the 650 total mutations described by the official database of Wilson disease (http://www.wilsondisease.med.ualberta.ca), thus offering an extraordinary contribution to the molecular characterization of ATP7B gene. Therefore, with a such large patients group it was possible to observe and describe epidemiological distribution and allele frequency data of the different mutations and then program the strategies of targeted diagnosis for several populations.

3.2 Geographical distribution of ATP7B gene mutations in Italy: our experience

Italy:

As previously mentioned the Genetic Liver Diseases Laboratory of Microcitemico Hospital in Cagliari is carrying out molecular diagnosis of Wilson's disease on ATP7B gene since 1995. In more than twenty years the Molecular Diagnostics Centre, being one of the few national centers recognized and certified, has collected a very large series of WD patients mostly from Italy. To date, 316 WD families were analyzed, each one with one or more affected individuals, coming from all Italian regions except from Sardinia, which as we'll discuss afterwards, represents a special case. A number of 617/632 alleles were characterized, therefore reaching a "mutational detection rate" of 97%.
This broad experience has made possible to process the data in our possession and to propose an overview of the frequency, distribution and type of mutations identified to date in the Italian population. The molecular study has allowed the identification, in the only Italian population to the exclusion of that of Sardinia, of 139 different mutations which belong to all categories, missense, nonsense, deletions, insertions and splice site; all data combined suggest the presence of high allelic heterogeneity (Table IV).

N.	MUTATION		EXON	DOMAIN	CHR	%
1	c.3207C>A	p.H1069Q	14	SEHPL	95	15
2	c.1772G>A	p.G591D	5	Cu5	30	4,7
3	c.2906G>A	p.R969Q	13	Tm6	30	4,7
4	c.3955C>T	p.R1319X	19	Tm8	29	4,6
5	c.2532delA	p.Val845SerfsX28	10	Tm4	29	4,6
6	c.2304dupC	p.Met769HisfsX26	8	Tm4	26	4,1
7	c.1877G>C	p.G626A	6	Cu6	13	2
8	c.-441_427del		5' UTR	PROMOTER	13	2
9	c.3904-2A>G		19	Tm8	12	1,9
10	c.2930C>T	p.T977M	13	Tm5	11	1,7
11	c.3649_3654del	p.Val1217_Leu1218del	17	Tm6	11	1,7

Table IV. Most common ATP7B mutations in Italian Population.

The only mutation found at high frequency is the p.H1069Q (14.8%), which as we have already observed, it is the most prevalent mutation in the European population; therefore in the Italian population is not possible to identify others frequent mutations, in fact the sum of the ten most frequent mutations constitutes just 43% of the total. Furthemore in recent years the Genetic Liver Diseases Laboratory identified in WD Italian patients, 10 mutations already described, but only in WD patients coming from other populations (Table V); this data confirm the great allelic heterogeneity present in the Italian population, with few common mutations and a majority of rare and very rare mutations. Therefore, the different mutations more likely arose by the presence of hotspot regions in the ATP7B gene than in relationship to the ethnic or geographical origin.

N.	MUTATION		EXON	MUTATION-POSITIVE POPULATION
1	c.1846C>T	p.R616W	5	German
2	c.2121+3A>G		7	Polish
3	c.2222A>G	p.Y741C	8	German
4	c.2605G>T	p.G869X	11	Chinese
5	c.2817G>C	p.W939C	12	Hungarian
6	c.3182G>A	p.G1061E	14	Pakistan,Turkish,Greek,Spanish,Indian
7	c.3646G>A	p.V1216M	17	Turkish,German,Spanish,Chinese,Taiwanese
8	c.3694A>C	p.T1232P	17	Brazilian,Spanish
9	c.3859G>A	p.G1287S	18	British
10	c.3818C>T	p.P1273L	18	Turkish,Polish,Hungarian,Korean,Egyptian,Chinese

Table V. ATP7B mutations identified in Italian WD patients but already described in other populations.

The Genetic Liver Diseases Laboratory has also recently identified in the Italian population 16 different novel mutations not yet described in any other population, on which we proceeded with an in silico functional analysis method to predict the potential pathogenic level of mutations (Table VI).

N.	NOVEL MUTATION		EXON	CHR	STATE	GEOGRAPHIC ORIGIN	SIFT PREDICTION	POLYPHEN PREDICTION	MUTATION TASTER PREDICTION
1	c.522_523insT	p.K175X	2	2	COMPOUND	PUGLIA			
2	c.1546G>T	p.V516F	4	1	COMPOUND	LAZIO	NOT TOLERATED	POSSIBLY DAMAGING	POLYMORPHISM
3	c.1708-6T>G		5	1	COMPOUND	EMILIA ROMAGNA			POLYMORPHISM
4	c.1947-31A>T		7	1	COMPOUND	LAZIO			POLYMORPHISM
5	c.2078C>T	p.S693F	7	2	COMPOUND	TURKEY/SICILY	DAMAGING	PROBABLY DAMAGING	DISEASE CAUSING
6	c.2186T>A	p.M729K	8	1	COMPOUND	PIEMONTE/VENETO	NOT TOLERATED	PROBABLY DAMAGING	DISEASE CAUSING
7	c.2195T>G	p.L732R	8	2	OMOZIGOTE	PUGLIA	NOT TOLERATED	PROBABLY DAMAGING	DISEASE CAUSING
8	c.2512A>G	p.K838E	10	1	COMPOUND	CALABRIA	NOT TOLERATED	PROBABLY DAMAGING	DISEASE CAUSING
9	c.3023T>C	p.L1008P	13	1	COMPOUND	SICILY	NOT TOLERATED	PROBABLY DAMAGING	DISEASE CAUSING
10	c.2953delT	p.C985AfsX37	13	1	COMPOUND	LIGURIA			
11	c.2963G>A	p.G988E	13	2	OMOZIGOTE	CAMPANIA	NOT TOLERATED	PROBABLY DAMAGING	DISEASE CAUSING
12	c.3244G>T	p.E1082X	15	1	COMPOUND	TUSCANY			
13	c.3671G>T	p.R1224L	17	1	COMPOUND	SARDINIA/VENETO	TOLERATED	POSSIBLY DAMAGING	DISEASE CAUSING
14	c.3895C>A	p.L1299I	18	1	COMPOUND	CAMPANIA	NOT TOLERATED	PROBABLY DAMAGING	DISEASE CAUSING
15	c.3970A>G	p.N1324D	19	1	COMPOUND	SICILY	NOT TOLERATED	PROBABLY DAMAGING	DISEASE CAUSING
16	c.4124+6T>C		20	2	COMPOUND	SICILY			DISEASE CAUSING

Table VI. Novel ATP7B mutations recently identified in Italian population.

However, we also found a prevalence of regional distribution for some mutations that prevailed in geographical areas inhabited by so called "isolated" populations. The most typical examples are the Apulian and Sardinian populations.

Apulian population

A study in 27 Apulaian families showed that 5 mutations are more frequently present and represent 80% of the alleles. Of these, the most frequent is p.G591D, being in 38% of the alleles, followed by p.H1069Q that with a frequency of 20% is approaching to the European average (Table VII).

N.	MUTATION		EXON	DOMAIN	CHR	%
1	c.1772G>A	p.G591D	5	Cu5	23	42,5
2	c.3207C>A	p.H1069Q	14	SEHPL	10	18,5
3	c.3928A>C	p.S1310R	19	ATPloop	4	7,4
4	c.2304dupC	p.Met769HisfsX26	8	Tm4	3	5,5
5	c.2930C>T	p.T977M	13	Tm6	3	5,5
6	c.917T>C	p.I306T	2	Cu3	2	3,7
7	c.1934T>G	p.M645R	6	Tm1	2	3,7
8	c.2121+3A>G		7	Tm2	2	3,7
9	c.2128G>A	p.G710S	8	Tm2	1	1,8
10	c.2906G>A	p.R969Q	13	Ch/Tm6	1	1,8
11	c.3007G>A	p.A1003T	13	Ch/Tm6	1	1,8
12	c.3284A>C	p.Q1095P	15	ATPloop	1	1,8
13	c.3863C>T	p.T1288M	18	ATPloop	1	1,8

Table VII. ATP7B Mutations identified in Apulaian population.

Sardinian Population

The Sardinian population establishes a unique case in all the world. In fact to date the Genetic Liver Diseases Laboratory of Microcitemico Hospital analyzed 171 families with a total of 215 WD patients. Following molecular diagnosis, 339/344 alleles were characterized reaching a "mutational detection rate" of 98.5%. The study of the molecular basis of the WD showed the presence of 29 different mutations of which the most common, the deletion -441_427del localized in the promoter of the gene ATP7B, constitutes about 64% of the alleles. This high allele frequency suggests the presence of a founder effect due to the isolation and inbreeding of the Sardinian population that favored the spread of several other genetic disorders including thalassemia, cystic fibrosis, APECED and other mendelian diseases as well as multifactorial diseases with a strong genetic component, such as Type I Diabetes, Celiac Disease and Multiple Sclerosis.

The 6 most common mutations identified in the ATP7B gene in Sardinian population represent about 85% of the total (Table VIII). These data suggest a high allelic homogeneity and allow an effective strategy for the genetic study. The Sardinian population exibits a very high WD incidence, which until a few years ago it was thought to be 1:7000 individuals. However, these data are likely underestimated. For this reason, in our laboratory we calculated the incidence of WD in Sardinian by two different methods: 1) a pilot screening for the -441_427del mutation in 5290 newborns revealed the presence of 122 heterozygotes with an allelic frequency of 1.15%. Assuming the same distribution of WD mutations in the general Sardinian population, it was also possible to infer an allelic frequency of 0.77% for the other mutations, which accounts for a total frequency of any WD mutation of 1.92%. These data, assuming Hardy-Weinberg equilibrium, could be translated into a WD incidence of 1 in 2707 live births (Zappu et al. 2008). In a second study recently published, we used a new approach that makes it possible to estimate the allelic frequency of an autosomal recessive disorder if one knows both the proportion between homozygous and compound heterozygous patients (the homozygosity index) and the inbreeding coefficient , in a sample of affected individuals. It was applied this method to a set of 178 Sardinian individuals each with a clinical and molecular diagnosis of WD and was obtained a incidence data of 1:2732 (Gialluisi et al. 2013), perfectly matching with the result obtained by the other method previously tested. Both results confirm that the prevalence of WD was largely underestimated in Sardinia and set the basis for a genetic mass screening aimed at diagnosis and early treatment of the disease (Zappu et al., 2008).

The use of the MLPA technique in the few Sardinian WD patients not yet characterized, allowed us to discover three, heterozygotes for the -441_427del mutation, that were also carriers of the c.52-2671_368del previously found in one Italian patient.

N.	MUTATION		EXON	DOMAIN	CHR	%
1	c.-441_427del		5'UTR	PROMOTER	219	63.7%
2	c.3436G>A	p.V1146M	16	ATPloop	27	7.8%
3	c.2463delC	p.Met822TrpfsX51	10	Td	26	7.5%
4	c.213_214delAT	p.Val73GlufsX4	2	Cu1	8	2.3%
5	c.2332C>T	p.R778W	8	Tm4	6	1.7%
6	c.2998G>A	p.G1000R	13	Ch/Tm6	6	1.7%
7	c.3053C>T	p.A1018V	13	ATPloop	6	1.7%
8	c.3207C>A	p.H1069Q	14	SEHPL	6	1.7%
9	c.2605G>A	p.G869R	11	Td	5	1.4%
10	c.1512dupT	p.Asn505Stop	3	Cu5	4	1.2%
11	c.52-2671_368del		IVS1_EX2	Cu1_Cu4	3	0.9%
12	c.2304dupC	p.Met769HisfsX26	8	Tm4	3	0.9%
13	c.2035delC	p.His679ThrfsX17	7	Tm1-Tm2	2	0.6%
14	c.2762G>A	p.S921N	12	Tm5	2	0.6%
15	c.2978C>T	p.T993M	13	Ch/Tm6	2	0.6%
16	c.3809A>G	p.N1270S	18	ATPhinge	2	0.6%
17	c.19_20del	p.Gln7AspfsX15	1	N-Tail	1	0.3%
18	c.1285+5G>T		2	Cu4	1	0.3%
19	c.2122-8T>G		8	Tm3	1	0.3%
20	c.2239A>T	p.I747F	8	Tm3	1	0.3%
21	c.2591T>G	p.V864G	11	Td	1	0.3%
22	c.2668G>A	p.V890M	11	A-domain	1	0.3%
23	c.2755C>T	p.R919W	12	Tm5	1	0.3%
24	c.2827G>A	p.G943S	12	Tm5	1	0.3%
25	c.2906G>A	p.R969Q	13	Ch/Tm6	1	0.3%
26	c.3128T>C	p.L1043P	14	ATPloop	1	0.3%
27	c.3266G>T	p.G1089V	15	ATPloop	1	0.3%
28	c.3451C>T	p.R1151C	16	ATPloop	1	0.3%
29	c.3852_75del	p.Gly1285_Ile1292del	18	ATPhinge	1	0.3%
	Unknown				5	1.4%

Table VIII. ATP7B mutations and related allelic frequencies identified in Sardinian population

3.2 Worldwide geographical distribution of ATP7B gene mutations: our experience

Thanks to the numerous international collaborations established by our Laboratory at the Microcitemico Hospital in Cagliari e were able to expand our series of WD patients from non Italian origin. Among those, the population most studied through molecular diagnosis of ATP7B gene is the Turkish one, represented by 96 families. Croatia has contributed to expand the WD patient's series with 77 families, Greece with 53 families, Yugoslavia (Serbia) with 48 families, Romania with 16 families an Albania con 10 families. Several sporadic patients coming from other populations were also subjected to molecular diagnosis but they were not numerically significant to show a particular mutational distribution (Table IX).

NATIONALITY	WD FAMILIES
TURKEY	96
CROAZIA	77
GREECE	53
SERBIA	48
ROMANIA	16
ALBANIA	10
SPAIN	5
SWITZERLAND	2
PHILIPPINES	2
MACEDONIA	2
SLOVENIA	1
ERITREA	1
COLOMBIA	1
MAROCCO	1
EQUADOR	1
INDIA	1
CHINA	1
CRIMEA	1
RUSSIA/ITALIA	1
GERMANY	1
TOT.	321

Table IX. Worldwide WD families analyzed by Genetic Liver Diseases Laboratory

Turkish population

The Turkish population comprises a special situation due to number and distribution of the mutations. In fact, among the 96 families characterized in the ATP7B gene, we identified 63 different mutations (Table X); this shows a peculiar allelic heterogeneity that makes rare mutations be defined "familiar", namely present within a single family. Another feature emerged from the molecular study of the ATP7B gene in Turkish population is that the vast majority of patients are homozygous for a single mutation; this result is justified by the widespread cultural tradition of consanguineous marriage that increases the likelihood that both partners are carriers of the same mutation which is transmitted in an homozygous form to the progeny.
Moreover, given the high representation of the Turkish origin, it was possible to observe the distribution of the mutations along the ATP7B gene and the highest occurrence in some exons; in particular exons 7, 8, 13, 14, 17 and 19 show both a greater number of different mutations and a greater number of alleles affected by the same mutations. These regions likely represent "mutational hotspots" that facilitate the molecular diagnosis allowing a priority screening of the most frequent mutations.

N.	MUTATION		EXON	CHR	N.	MUTATION		EXON	CHR
1	c.254G>T	p.G85V	2	2	33	c.2977A>C	p.T993P	13	2
2	c.267dupG	p.Lys90GlufsX73	2	1	34	c.3008C>T	p.A1003V	13	4
3	c.328C>T	p.Q110X	2	4	35	c.3061-1G>A		14	4
4	c.1543+1 G>T		3	2	36	c.3106G>A	p.V1036I	14	2
5	c.1512dupT	p.Asn505Stop	3	2	37	c.3182G>A	p.G1061E	14	3
6	c.1625T>C, p.I542T	p.I542T	4	1	38	c.3190G>A	p.E1064K	14	4
7	c.1707+3 ins T		4	1	39	c.3207C>A	p.H1069Q	14	12
8	c.1707+2dupT		4	2	40	c.3266G>A	p.G1089E	15	3
9	c.1709T>C	p.I570N	5	2	41	c.3305T>C	p.I1102T	15	2
10	c.1846C>T	p.R616W	5	2	42	c.3311G>T	p.C1104F	15	3
11	c.1883_1884delAT	p.His628ArgfsX126	6	2	43	c.3336_3337delCCinsA	p.Leu1113TrpfsX8	15	1
12	c.1924G>C	p.D642H	6	5	44	c.3402delC	p.Ala1135GlnfsX13	15	3
13	c.2071G>A	p.G691R	7	7	45	c.3403G>A	p.A1135T	15	1
14	c.2078C>T	p.S693F	7	1	46	c.3452G>A	p.R1151H	16	1
15	c.2128G>A	p.G710S	8	6	47	c.3506T>C	p.M1169T	16	1
16	c.2138A>G	p.Y713C	8	1	48	c.3547G>A	p.A1183T	16	1
17	c.2293G>A	p.D765N	8	4	49	c.3620A>G	p.H1207R	17	2
18	c.2304dupC	p.Met769HisfsX26	8	5	50	c.3645C>A	p.D1215E	17	1
19	c.2333G>A	p.R778G	8	18	51	c.3646G>A	p.V1216M	17	2
20	c.2332C>T	p.R778W	8	4	52	c.3659C>T	p.T1220M	17	8
21	c.2480G>A	p.R827Q	10	1	53	c.3665A>T	p.D1222V	17	1
22	c.2519C>T	p.P840L	10	1	54	c.3704G>A	p.G1235D	18	2
23	c.2532delA	p.Val845SerfsX28	10	3	55	c.3763C>T	p.L1255F	18	1
24	c.2570T>C	p.I857T	10	5	56	c.3809A>G	p.N1270S	18	2
25	c.2621C>T	p.A874V	11	3	57	c.3818C>T	p.P1273L	18	2
26	c.2668G>A	p.V890M	11	2	58	c.3904-2A>G		19	1
27	c.2752G>A	p.D918N	12	2	59	c.3955C>T	p.R1319X	19	2
28	c.2755C>T	p.R919W	12	3	60	c.3979C>G	p.L1327V	19	11
29	c.2807T>A	p.L936X	12	1	61	c.4051C>T	p.Q1351X	20	1
30	c.2827G>A	p.G943S	12	5	62	c.4088C>T	p.S1363F	20	2
31	c.2906G>A	p.R969Q	13	7	63	c.4021+87_4125-2del		21	2
32	c.2975C>T	p.P992L	13	1					

Table X. ATP7B mutations identified in Turkish WD patients**.**

Croatian and Serbian populations

WD patients from 77 families coming from Croatia were characterized identifying 20 different mutations uniformly distributed along the ATP7B gene, except for a higher density in exon 14 in which the p.H1069Q showed a frequency of 55%, similar to the frequency observed in the rest of Europe (Table XI).

Furthermore through a partnership with a Croatian researchers group, we subjected to the MLPA method (Multiple Ligation-dependent Probe Amplification) 37 WD patients coming from Croatia, previously completely sequenced and found ATP7B negative, heterozygous or homozygous and suspected hemizygous due to the presence of extended deletion in ATP7B gene. By MLPA we identified a novel deletion in one patient that extends from exon 9 to exon 21 and over, as shown by the data obtained from CGH-array. In fact this technique confirmes the presence of the deletion from exon 9 of ATP7B gene, already resulted from the MLPA method, but as we will discuss later, defines the 3' deletion's boundary beyond the ATP7B gene.

In 48 WD Yugoslavian families, coming mainly from Serbia, we identified 15 different mutations (Table XII) that show an allele frequency similar to that of WD patients coming from Croatia. In fact, in both populations, the three most common mutations are the p.H1069Q, the p.A1003T and the c.2304dupC with a allele frequency of 55% -34%, 11% and 7.5% -5% -8% respectively.

N.	MUTATION		EXON	CHR	%
1	c.3207C>A	p.His1069Gln	14	67	43,5
2	c.3007G>A	p.Ala1003Thr	13	13	8,4
3	c.2304dupC	p.Met769HisfsX26	8	9	5,8
4	c.1847G>A	p.Arg616Gln	5	7	4,5
5	c.3402delC	p.Ala1135GlnGlnfsX13	15	7	4,5
6	c.4374_4375delCA	p.Arg1459GlyfsX2	21	4	2,6
7	c.1772G>A	p.Gly591Asp	5	3	1,9
8	c.2906G>A	p.Arg969Gln	13	3	1,9
9	c.2532delA	p.Val845SerfsX28	10	2	1,3
10	c.2648_2649delTG	p.Val883AlafsX3	11	2	1,3
11	c.3088G>A	p.Gly1030Ser	14	2	1,3
13	c.1877G>C	p.Gly626Ala	6	1	0,6
14	c.2304delC	p.Met769CysfsX38	8	1	0,6
12	c.3079G>A	p.Asp1027Asn	14	1	0,6
15	c.3556+1G>A		IVS16	1	0,6
16	c.3809A>G	p.Asn1270Ser	18	1	0,6
17	c.3809A>C	p.Asn1270Thr	18	1	0,6
18	c.4051C>T	p.Gln1351X	20	1	0,6
19	c.4295C>T	p.Ser1432Phe	21	1	0,6
20	exon9_exon21del		9_21	1	0,6

Table XI. ATP7B mutations identified in Croatian WD patients.

N.	MUTATION		EXON	CHR	%
1	c.3207C>A	p.H1069Q	14	33	34,4
2	c.2304dupC	p.M769HfsX26	8	8	8,3
3	c.3007G>A	p.A1003T	13	4	4,2
4	c.1847G>A	p.R616Q	5	4	4,2
5	c.343C>T	p.Q115X	2	1	1
6	c.562C>T	p.Q188X	2	1	1
7	c.2447+1G >T		9	1	1
8	c.2463delC	p.Met822TrpfsX51	10	1	1
9	c.2648_2649delTG	p.Val883AlafsX3	11	1	1
10	c.2906G>A	p.R969Q	13	1	1
11	c.2930C>T	p.T977M	13	1	1
12	c.3402delC	p.Ala1135GlnfsX13	15	1	1
13	c.3305T>C	p.I1102T	15	1	1
14	c.4022G>A	p.G1341D	20	1	1
15	c.4374_4375delCA	p.Arg1459GlyfsX2	21	1	1

Table XII. ATP7B mutations identified in Serbian WD patients

Greek population

WD patients from 53 families coming from Greece were characterized and 22 different mutations were identified (Table XIII). As elsewhere in Europe, the predominant mutation is p.H1069Q with a frequency of 38%, followed by p.R969Q with a frequency of 22%. Even in Greece, exists a population that represents a genetic isolate similar to Sardinia in the island of Kalymnos. In fact, it was recently shown through a screening of 397 newborns carried out in our Laboratory, that the allele frequency of the most frequent

mutations present in the population (p.H1069Q and p.R969Q) was relatively underestimated and was recalculated to be 3.7%, which means an estimated carrier frequency of 7% and one of the highest in the world (Zappu et al. 2008). This estimate of frequency candidates the population of the Kalymnos island's to a mass screening for the diagnosis and early treatment of Wilson disease .

N.	MUTATION		EXON	CHR	%
1	c.845delT	p.Leu282ProfsX2	2	2	1,9
2	c.865C>T	p.Gln289ter	2	9	8,5
3	c.779_780insC	p.Gln260HisfsX10	2	1	0,9
4	c.1456G>T	p.Ala486Ser	3	1	0,9
5	c.1707+3 insT		4	1	0,9
6	c.1782delT	p.Tyr594Stop	5	1	0,9
7	c.1708-1G>A		5	3	2,8
8	c.2101_2102delAT	p.Ile701LeufsX53	7	1	0,9
9	c.2332C>G	p.Arg778Gly	8	1	0,9
10	c.2299insG	p.Pro767ArgfsX28	8	4	3,8
11	c.2532delA	p.Val845SerfsX28	10	4	3,8
12	c.2668G>A	p.Val890Met	11	1	0,9
13	c.2807T>A	p.Leu936ter	12	8	7,5
14	c.2906G>A	p.Arg969Gln	13	15	14,1
15	c.3034G>C	p.Gly1012Arg	13	1	0,9
16	c.3182G>A	p.Gly1061Glu	14	1	0,9
17	c.3207C>A	p.His1069Gln	14	28	26,4
18	c.3295G>A	p.Gly1099Ser	15	1	0,9
19	c.3443T>C	p.Ile1148Thr	16	3	2,8
20	c.3904-2A>G		19	1	0,9
21	c.4125-1G>A		20	1	0,9
22	c.4396T>A	p.Ter1466Arg	21	2	1,9

Table XIII. ATP7B mutations identified in Greek WD patients.

Romanian and Albanian population

16 WD patients coming from Romania were characterized through mutational analysis of ATP7B gene and ten different mutations have been identified (Table XIV). The allele frequency shows, despite the small sample size, the prevalence of p.H1069Q mutation with a frequency of 60% that places it just below the 70% frequency found for the same mutation in Poland, where it reaches the maximum allelic frequency in Europe.

N.	MUTATION		EXON	CHR	%
1	c.1995G>A	p.M665I	7	1	3,1
2	c.2304dupC	p.M769HfsX26	8	1	3,1
3	c.2532delA	p.V845SfsX28	10	2	6,2
4	c.2621C>T	p.A874V	11	1	3,1
5	c.2817G>T	p.W939C	12	2	6,2
6	c.3008C>T	p.A1003V	13	2	6,2
7	c.3190G>A	p.E1064K	14	1	3,1
8	c.3207C>A	p.H1069Q	14	19	59,4
9	c.4022G>A	p.G1341D	20	1	3,1
10	c.4301C>T	p.T1434M	21	1	3,1

Table XIV. ATP7B mutations identified in Romanian WD patients.

N.	MUTATION		EXON
1	c.1995G>A	p.Met665Ile	7
2	c.2304dupC	p.Met769HisfsX26	8
3	c.2532delA	p.Val845SerfsX28	10
4	c.3207C>A	p.His1069Gln	14
5	c.4022G>A	p.Gly1341Asp	20

Table XV. ATP7B mutations identified in Albanian WD patients.

Other Nationalities

As mentioned above, the contribution of samples to our laboratory from many other world countries although sample was not sufficient to draw conclusion about the frequency of WD mutations in these countries (Table XV). However, these scattered data can be useful for enriching the mutational framework of each country of patient's origin, in the event that further mutations not yet described in the original population will be later identified.

MUTATION		EXON	CHR	NATIONALITY
c.1934T>G	p.M645R	6	2	Colombia
c.1934T>G	p.M645R	6	1	Spain
c.1934T>G	p.M645R	6	1	Marocco
c.1934T>G	p.M645R	6	1	Equador
c.1946+6 T>C		6	2	Switzerland
c.2337G>A	p.W779X	8	1	Eritrea
c.2304dupC	p.M769HfsX26	8	1	Crimea
c.2507G>A	p.G836E	10	3	Marocco
c.2533delA	p.V845SfsX28	10	1	Albania
c.2816G>A	p.W939X	12	1	Switzerland
c.2975C>T	p.P992L	13	1	China
c.3207C>A	p.H1069Q	14	3	Macedonia
c.3207C>A	p.H1069Q	14	1	Spain
c.3207C>A	p.H1069Q	14	1	Russia
c.3207C>A	p.H1069Q	14	2	Germany
c.3446G>C	p.G1149A	16	4	Philippines
c.3877G>A	p.E1293K	18	2	China
c.3833C>T	p.A1278V	18	1	Equador
c.3842G>A	p.G1281D	18	1	Switzerland
c.3889G>A	p.V1297I	18	1	China
c.3809A>G	p.N1270S	18	1	Macedonia
c.4022G>T	p.G1341V	20	1	Crimea
c.4022G>A	p.G1341D	20	1	Albania

Table XV. ATP7B mutations identified in sporadic WD patients coming from all over the world.

3.3 Using MLPA technique on negative ATP7B patients

The direct sequencing is the method that is routinely used to confirm the diagnosis of WD; neverthless, it's possible that partial or even entire deletions of the ATP7B gene escape identification by sequencing analysis of the normal allele gene. that "mask" the presence of the possible deletion. Thus the molecular diagnosis could fail in many cases and justify the high number of WD patients without or with only a single mutation identified.

Taking into account these considerations, the WD patients who by sequencing were negative or heterozygous through mutational analysis of the whole ATP7B gene, underwent a subsequent deletion/duplication investigation by the previously described MLPA technique (Multiple Ligation-dependent Probe Amplification), method of choice for the identification of deletions/duplications. In particular 34 Italian patients were analyzed of which 11 with no mutation and the remaining 23 with a single mutation identified; 15 Sardinian patients, of which two patients without mutations and 13 with a single mutation; 37 Croatian patients, of which 13 without any mutation and the remaining 24 apparently homozygous for the p.H1069Q mutation.

In the Italian patients we did not detect any deletion or duplication. In three unrelated Sardinian patients, heterozygous for -441_427del we identified the deletion c.52-2671_368del (Figure 5), previously found in only one Italian patient in homozygous state (Figure 6). In the Croatian patients, we identified in a patient heterozygous for the mutation p.R969Q, a deletion that spans from exon 9 to exon 21 of the ATP7B gene (Figure 7); successively we have observed, through the array-CGH method, that the deletion extends beyond the 3'UTR region of the ATP7B gene and involves several adjacent genes configuring a contiguous gene syndrome.

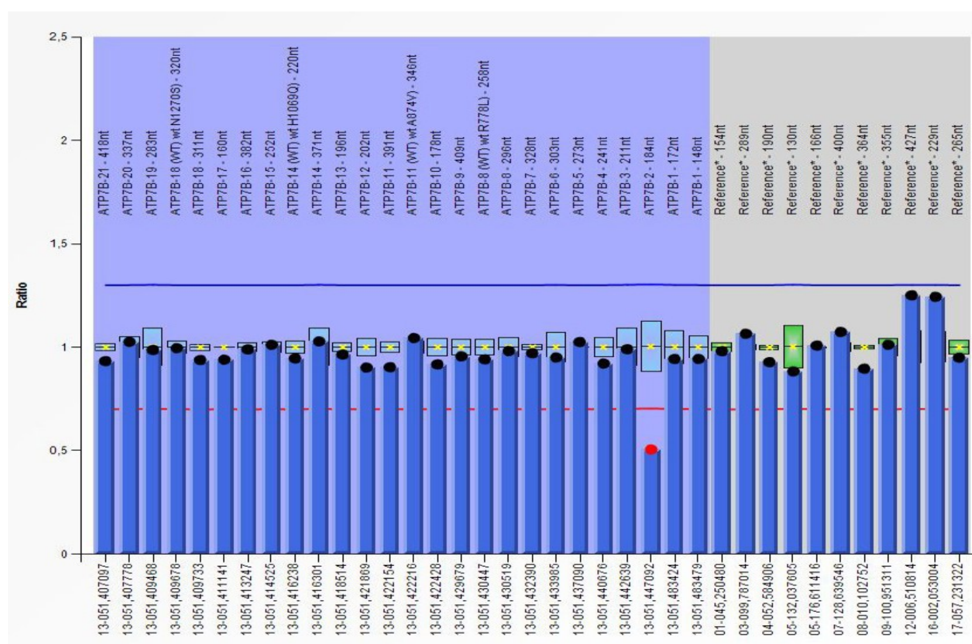

Figure 5. Graphical representation of "c.52-2671_368del" deletion found by MLPA technique (heterozigous state).

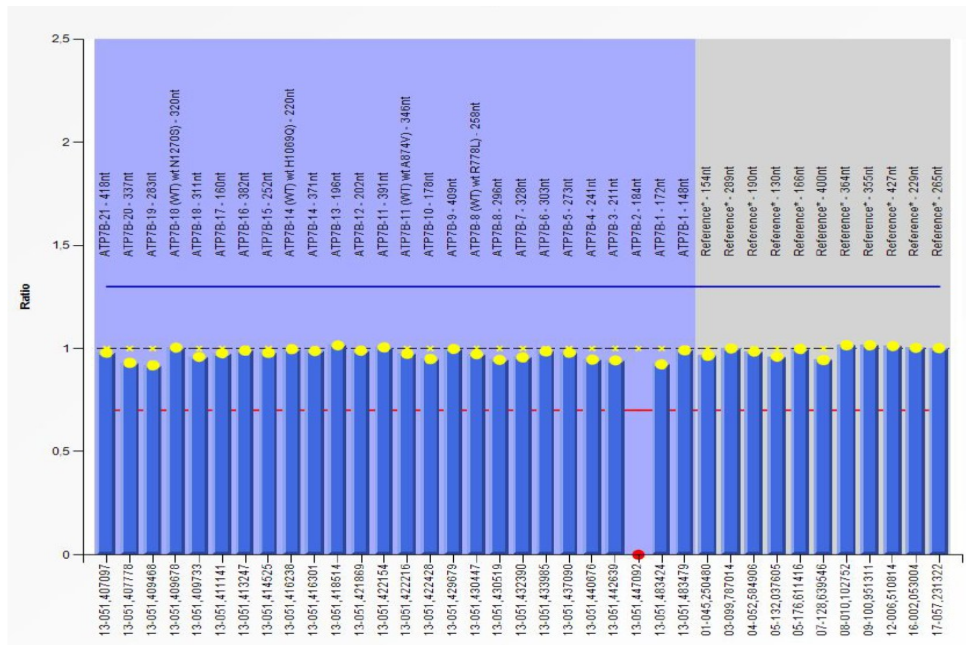

Figure 6. Graphical representation of "c.52-2671_368del" deletion found by MLPA technique (homozygous state).

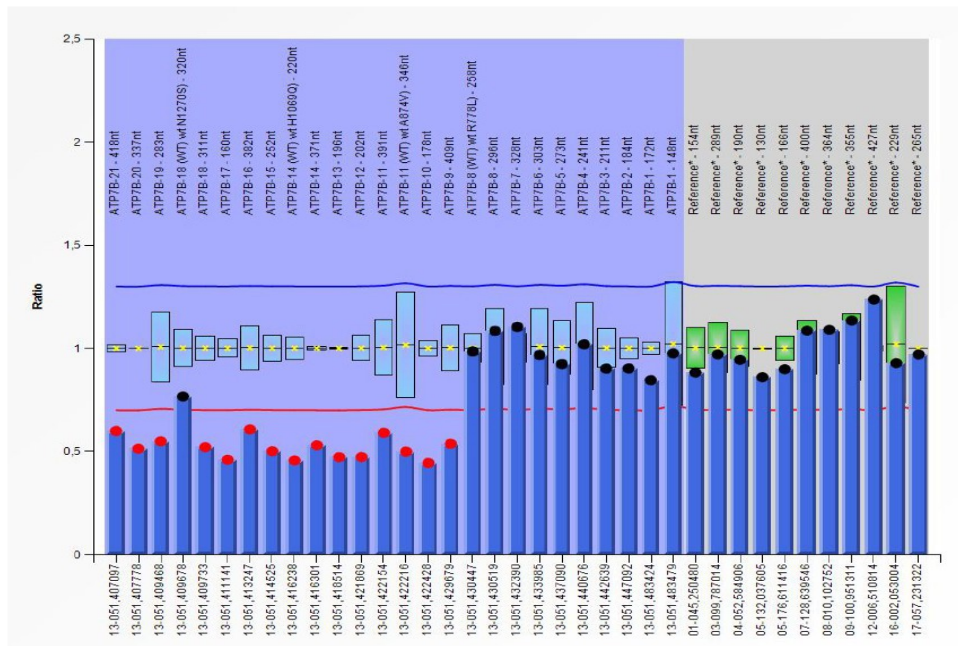

Figure 7. Graphical representation of "exon9?_exon21?del" mutation found by MLPA technique

In particular, the experiment of array-CGH (Figure 8) showed that the deletion extends up to the gene NCRNA00282 (LINC00282) to about 80kb away from ATP7B gene, including the CCDC70 gene.

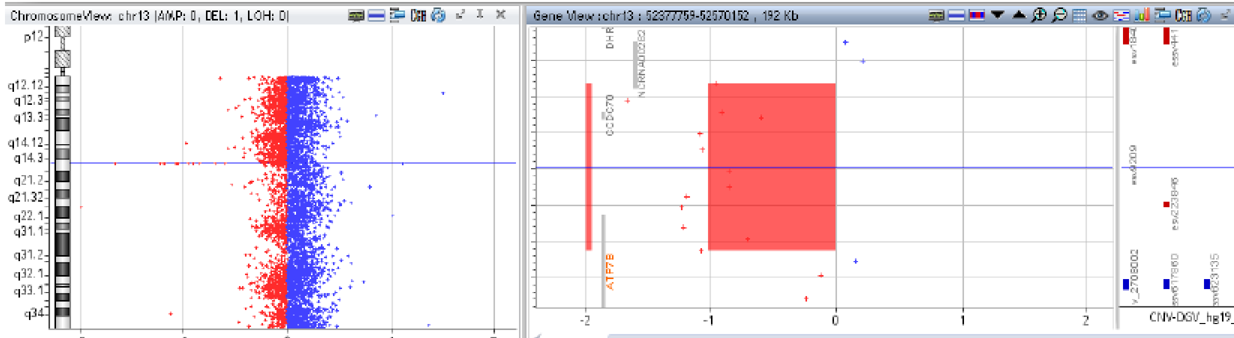

Figure 8. Graphical representation of CGH-array result for the "exon9?_exon21?del".

We used MLPA to confirm some deletions previously suspected through other methods: the c.334_1708-953del (Figure 9) and the c.52-2671_368del (Figure 6) in one Italian patient, and the c.4021 + 87_4125-2del described by other authors for the first time in a homozygous Turkish patient (Figure 11) and also confirmed by us in other family's heterozygous members (Figure 12).

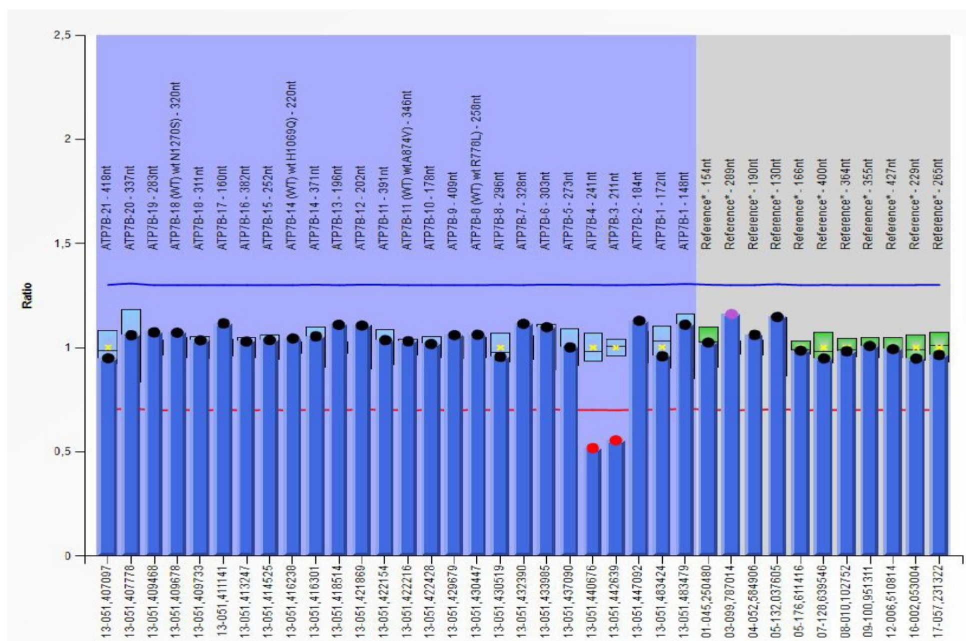

Figure 9. Graphical representation of c.334_1708-953del deletion found by MLPA technique (heterozygous state).

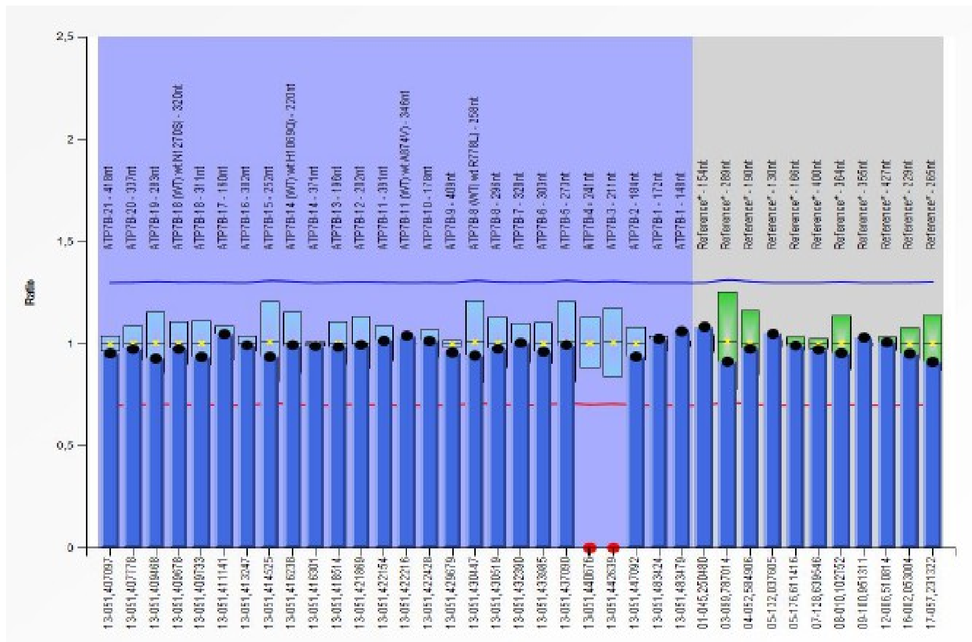

Figure 10. Graphical representation of c.334_1708-953del deletion found by MLPA technique (homozygous state).

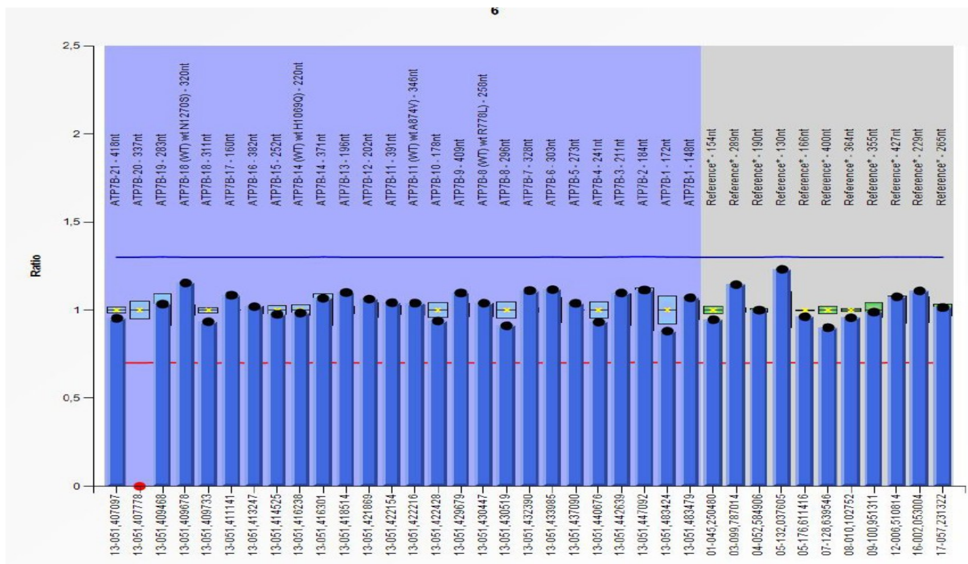

Figure 11. Graphical representation of c.4021 + 87_4125-2del deletion found by MLPA technique (homozygote state).

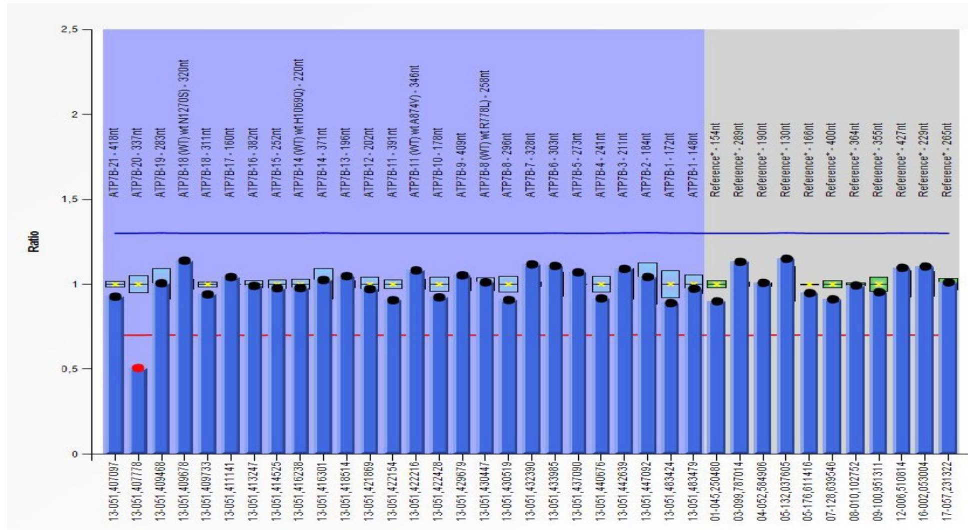

Figure 12. Graphical representation of c.4021 + 87_4125-2del deletion found by MLPA technique (heterozygote state).

Discussion

Wilson's disease is an autosomal recessive genetic disorder caused by the presence of pathogenic mutations in both alleles of the gene ATP7B. The Genetic Liver Diseases Laboratory since many years is a reference center for the molecular diagnosis of WD patients coming from Sardinia, Italy and the rest of the world.

One of the first goals of a genetic study within a population is the detection of any correlation between the molecular alteration localized in the gene responsible and the clinical features presented by the patients examined. In Wilson disease to date it has not been possible to associate a defined clinical phenotype with mutations present in the ATP7B gene. In fact, although in some regions of the world is a fairly common disease, the great allelic heterogeneity encountered does not allow a clear and reliable genotype-phenotype correlation.

Several studies in the past have attempted to establish this correlation, but the results have been contradictory without agreement regarding the age of onset of the disease and the prevailing clinical manifestations of either hepatic or neurological origin. Accordingly, it is thought that other factors are likely to account for the clinical heterogeneity. In particular, environmental factors such as, the kind of prevailing food habits or yet unknown genetic modifiers that could determine differential expression of the ATP7 gene even in the presence of the same genetic mutations like it has been observed in twins.

Our patient collection of WD, which is probably the largest in the world, reflects the high allelic heterogeneity of the ATP7B gene, characterized by the presence of 650 mutations described up to 2009 throughout the world. To update the mutational archive (Wilson Disease Mutation Database, University of Alberta) until 2016 it was necessary to examine all the international publications via PubMed. This search increased the number of mutations described by another 270, reaching a total of 920 mutations.

The distribution of the mutations within the gene is widespread, but with mutational hotspots regions in exons 8, 13, 14, 15,16, 18 and 19, in which reside the most important functional protein domains such as the transmembrane, phosphorylation and ATP-binding domain. This aspect is independent from the geographic and ethnic origin of the WD patients examined and confirms that there is a clustering of mutations in the ATP7B gene regardless of the ethno-geographic origin of the patients. The knowledge of the prevailing mutations guides the genetic testing indicating the study of hotspot regions as the first target analysis. This strategy is confirmed, as we'll discuss later, by other observations set forth below.

Another important implication concerns the allelic frequency of mutations in the different populations. From the data we have about the mutational distribution in the Italian population emerges as this is characterized by an high allelic heterogeneity, having to date identified 139 different mutations in 316 families analyzed. Furthermore, most of the mutations occur at very low allelic frequency, and therefore belong to the category of rare and very rare mutations, with the exception of the p.H1069Q (14.8%). Combining the data of the hotspot distribution with those of the prevailing geographical frequency is the basis for an effective and efficient strategy for the molecular diagnosis of WD patients. This strategy allowed us to develop a diagnostic flowchart that provides a first step of analysis by sequencing of exons 5, 6, 8, 10, 12, 13, 14, 15, 16, 17, 18, 19 with the achievement of a detection rate of 85% for the Italian mutations. In the event of

failure to identify one or two mutations, it is necessary to proceed with the second step of mutational analysis and the sequencing of the remaining exons of the ATP7B gene to achieve a 97% of mutation detection rate.

The third step relies on the MLPA technique to identify possible deletions and duplications. By this means, we could in fact find two different large deletions in Italian WD patients previously suspected by traditional methods, that identified a region of extended homozygosity.

The technology evolution utilized for the molecular analysis of ATP7B gene has evolved in the past years through a succession of techniques now in disuse such as SSCP, HPLC, Reverse Dot Blot, with the final resolution by the direct sequencing analysis, at first limited only to the regions found to be "altered" on SSCP, subsequently with the advent of the automatic ABI 3130 sequencers, applied to all the coding portions of the ATP7B gene. Thus, up to now it has been fundamental for the rapid and efficient molecular diagnosis, the knowledge of the regions most suitable for analysis by sequencing. With the advent of the new platforms of Next Generation Sequencing this algorithm is likely to be substituted by a targeted gene sequence analysis of the full gene.

Nevertheless, in some populations exemplified by Sardinian, the use of these new platforms may not be necessary as it will be too expensive and counterproductive compared to the traditional approach.. In fact, we know that, in certain cases, as in Sardinian, is sufficient the electrophoretic analysis of the promoter region to identify the most prevalent Sardinian mutation in 64% of the analysis. It would therefore be much more complex and costly to process all Sardinian samples by directly sequencing all the ATP7B gene.

It's also important to note that in recent years the detection rate of the methods used has greatly increased, becoming more efficient and effective. This is confirmed by the fact that in only seven years, from 2009 to 2016, about 270 worldwide ATP7B new mutations have been described.

In our case, the advent of direct sequencing, has effectively allowed the identification of a greater number of mutations that probably escaped to the analysis with methods previously in use (SSCP, DHPLC). In fact, in the last few years, we have identified 16 novel mutations in the Italian population, including 10 missense, 3 splicing site, 2 nonsense and one point deletion. Of course the nonsense and frameshift mutations do not need to be functionally validated as pathogenetic. However in order to better evaluate the pathogenetic potential of the missense and non invariant splicing site mutations not yet described in other populations, it has been necessary to perform a functional analysis in silico, using the SIFT, Poliphen and Mutation Taster softwares. This predictive analysis has given in some cases unclear outcomes, in particular: the p.V516F mutation identified in exon 4 in one chromosome, gave contradictory results (not tolerated, possibly damaging, and polymorphism) with the three different software used, as well as the missense p.R1224L identified in exon 17 in a single chromosome. For these two mutations it will be necessary in the future to do more in-depth functional assessments. All remaining missense mutations show a concordant result with all software used, with a prediction of high pathogenicity, in good agreement with the clinical phenotype of severe Wilson disease

We have also identified in the last years, in the Italian population, 10 more mutations described in other populations, but not yet in Italians. This assumed a particularly important significance both from the genetic

and practical point of view.
In fact, despite extensive analysis new mutations continue to be described in the well studied Italian population that were not considered to be country specific, probably reflecting new population migrations or novel technology developments. This new mutations should be included in the Italian mutations panel to be used for future mutational screening.
In recent years, we are witnessing new waves of migration, , coming mainly from the regions of the Middle East, Africa and the Far East that for different reasons, migrate to Central and Western Europe. In addition, the age distribution of migrants shows a high prevalence of young people in reproductive age, and this consideration is important from the genetic point of view, because of the heterogeneous composition of the future new populations. It is therefore necessary the knowledge of the molecular pathology data that characterize the different populations, to integrate the traditional mutation panels.
The knowledge of the spectre of mutations is particularly useful in the isolated populations with prevailing founder mutations such as the Sardinian and Apulian Italian populations, the Kalymnos island's population in Greece and the Romanian population in Europe. All these will benefit of a first pass analysis with the single prevailing mutations that will discover between 60 and 70% of the mutations.
As an example in Sardinian patients, we use a diagnostic flowchart that provides a first step with the electrophoretic analysis of the promoter region carrying the Sardinian mutation -441_427del followed by a second step with the sequencing analysis of exons 2, 3, 8, 10, 13 and 16. Using this approach it is possible to reach a 90% mutation detection rate.
At the end of this second phase, if the two mutations object of the molecular study are not yet identified, we proceed with a third step that provides for the sequencing analysis of exons 12, 14, 15 and 18 with attainment of 97% of mutational identification. The fourth step is represented by the use of the MLPA technique which by identifying the large ATP7B deletion (c.52-2671_368del) will further extend the detection rate in Sardinian up to 98.5%.
In the Apulian population the two most frequent mutations, the p.G591D and p.H1069Q, account for almost 60% of total alleles; hence, even in this population it is preferable to perform a first step analysis for the two most frequent mutations and in the negative cases proceed with the analysis of the remaining part of the ATP7B gene. The same is true for the Greek population of the Kalymnos Island, where through a newborn screening only two mutations have been described (p.H1069Q, p.R969Q) at very high allele frequencies with a estimated frequency of carriers of 7%. Although the sample size analyzed is small, we have observed in the Romanian population a very high allelic frequency for p.H1069Q mutation (60%), data that justify an initial step of analysis for the detection of this mutation, reserving the subsequent sequencing of all the ATP7B gene only to the patients negative for the prevailing mutation.
The Turkish population has a rather complex situation that can not be afforded in simple way. Indeed, the presence of such a high allelic heterogeneity, never found in other populations, does not allow a focused approach on a small portion of coding regions. In fact, as we previously saw, only a few exons show an higher mutational allelic frequency (exons 8, 14, and 19) than the others. It will therefore be necessary, in the event of a negative outcome in the mutational analysis of the mentioned exons, to extend to every exon of

the ATP7B gene the sequencing analysis. Such a procedure, however, is expensive and time-consuming. In this case, as in other similar ones, the next generation sequencing platform may be a more convenient choice.
The Croatian and Serbian populations show a mutational distribution rather similar, in fact, despite of the low number of families analyzed, the data obtained show a sharing of the three most frequent mutations in the two populations (p.H1069Q, p.A1003T and c.2304dupC). The two populations are from a genetic point of view more similar to each other, than what is commonly thought.
Most of the mutations identified in the ATP7B gene in the world are represented by point mutations, therefore they are easily identified by the direct sequencing technique. However, this method is not indicated for the identification of rearrangements such as extended deletions and duplications. Many studies that rely on the sequencing technique in fact describe a rate of mutational identification that oscillates between 80% and 95%.
In our laboratory using our approach, we offer a mutational detection rate for the Italian and Sardinan population of approximately 97-98,5% using three combined methods of PCR amplification, direct sequencing and MLPA
Differently from what has been described for Menkes disease, in which there is a deletion rate up to 17% of the mutations (Moller LB et al. 2009), we found only few intragenic deletions in the ATP7B gene by MLPA analysis: a deletion in both alleles of 8798bp (c.334_1708-953del) extending from exon 2 to intron 4 (Incollu et al.2011), a homozygous deletion of 3039 bp (c.52-2671_368del) which extends from intron 1 to exon 2 (Mameli et al. 2015). Both these deletions have been identified by us in Italian patients. The deletions were not identified during the mutational analysis because the PCR reaction performed on the involved exons failed to amplify the deleted regions. The deletion was further defined by amplifying the truncated cDNA.
Furthermore other deletions have been described by several authors: the c.4021 + 87_4125-2del (ex20del) (Moller MB et al. 2005) in a Turkish patient and the c.1708? _1946 +? of (ex4_5del) (Moller MB et al. 2011) in a Danish patient where, however, the deletion's boundaries have not been characterized at the genomic level. More recently it has been identified the deletion c.1544? _ 1708? (ex4del) (Bost M et al. 2012) in a French patient but also in this case the boundaries of the deletion were not characterized at the genomic level.
In a very recent work two novel ATP7B gene deletions have been described: the c.3134_3556 + 689del which extends from exon 14 to intron 16, identified in a Bulgarian patient and the c.3556 + 281_4001del which involves the ATP7B gene from intron 16 to exon 19, described in a patient Polish (Todorov T et al. 2016). It is quite unexpected the low frequency of partial or complete deletions found in ATP7B gene until now as the homologous Menkes gene, ATP7A, is often site of intragenic deletions in up to 17% of the mutations.
Following these findings we used the MLPA technique (Multiple Ligation-dependent Probe Amplification) to analyze the WD patients that escaped definition in the first 2 steps of the analysis of the ATP7B gene. Despite our initial expectations, the results were not encouraging; in fact, out of 49 Italian and Sardinian patients ATP7B-negatives, we found deletions only in 3 Sardinian patients, who were all compound

heterozygous for the common -441_427del and carriers of the same deletion c.52-2671_368del already identified previously in one Italian patient.

This result led us to draw the following conclusions: the first is that the intragenic deletions of ATP7B gene are uncommon in the Italian and Sardinian population; the second is that despite the Sardinian population is known to be isolated with a high allelic homogeneity, it also shows some very rare mutations that are shared with the Italian population, especially from the southern regions. In fact the Italian patient who presented the same deletion found in the three Sardinian patients (c.52-2671_368del), came from Campania. However, even though the MLPA results appear unexpected, they are quite comparable with the data coming from a large sample of WD Europeans and Indians patients ATP7B negatives, in which MLPA analysis identified a deletions in only 0.49% of the patient sample (T. Todorov et al. 2016). Our experience indicates a similar percentage of 0.6%. Thus, the mechanisms that are active in the ATP7A gene, responsible for the high rate of deletions in Menkes disease, should not be present in the homologous ATP7B gene. Despite the low mutation yield, MLPA analysis should be performed to allow an increase of the detection rate up to 98.5% The residual 1.5% of undetected mutations should be completely defined by applying a next generation approach targeted to the sequence of the whole ATP7B gene.

Despite obvious advances in the understanding of the function of the ATP7B protein domains furthered by the clustering of point mutations along the gene, a question still unresolved and controversial is the identification of a reliable correlation between genotype and phenotype that would give support to the clinical and molecular diagnosis providing an indication of the diverse clinical evolution of Wilson's disease.

In conclusion, we have analysed the molecular genetics of WD patients referred to our laboratory from worldwide countries for the genetic analysis of the ATP7B gene.

The gathered data suggest three important considerations. The first is the importance of extending the analysis of the ATP7B gene in the different countries to permit the development of country specific mutation panels with higher detection rates. The second is that the discovery of new mutations should improve our knowledge of novel protein domains as well as potentially increasing the possibility of a resolutive cure for the WD . The third conclusion is that the work conducted in our laboratory in the last decades has allowed an increase in the detection rate of WD and discovered the high prevalence of the disease in Sardinia that can now be exploited to set up a genetic screening for WD in the neonatal age and to allow the easy discovery of patients in the asymptomatic phase, preventing the development of the severe complications which will invariably affect the patients with undetected Wilson disease. For this reason we can now recommend, more than suggest, the need to perform not only the molecular diagnosis of individual cases but also a mass screening for the diagnosis and early treatment of the disease.

At last but not least, we've highlighted the fundamental role of the MLPA technique as the final step in the diagnostic flowchart to complete the molecular definition of the yet undefined WD patients. This new approach has not yet been described neither in the Italian nor in the Sardinian population.

"Francesca Chiappe gratefully acknowledges Sardinia Regional Government for the financial support of her PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007-2013 - Axis IV Human Resources, Objective l.3, Line of Activity l.3.1.)".

Bibliography

Aggarwal A, Chandhok G et al. *Wilson disease mutation pattern with genotype-phenotype correlations from Western India: confirmation of p.C271* as a common Indian mutation and identification of 14 novel mutations.* Ann Hum Genet. 2013 Jul;77(4):299-307.

Ala A, Walker AP, Ashkan K, et al. *Wilson's disease*. Lancet 2007;369:397-408.

Al Jumah M1, Majumdar R, Al Rajeh S et al. *A clinical and genetic study of 56 Saudi Wilson disease patients: identification of Saudi-specific mutations.* Eur J Neurol. 2004 Feb;11(2):121-4.

Banci L, Bertini I, Cantini F, Ciofi-Baffoni S (Aug 2010). "*Cellular copper distribution: a mechanistic systems biology approach". Cellular and Molecular Life Sciences*. 67(15): 2563–89.

O Bandmann, Karl Heinz Weiss,Stephen G. Kaler. *Wilson's disease and other neurological copper disorders.Lancet Neurol*. 2015 January ; 14(1): 103–113.

Bartee MY, Lutsenko S. *Hepatic copper-transporting ATPase ATP7B: function and inactivation at the molecular and cellular level.* Biometals. 2007 Jun;20(3-4):627-37.

Bost M, PiguetLacroix G, Parant F, Wilson CM. *Molecular analysis of Wilson patients: direct sequencing and MLPA analysis in the ATP7B gene and Atox1 and COMMD1 gene analysis*. J Trace Elem Med Biol. 2012; 26(2–3):97–101.

Brewer GJ, Yuzbasiyan-Gurkan V. *Wilson disease*. Medicine 1992;71:139-64.

Bruha R, Marecek L, Pospisilova L, et al. *Long-term follow-up of Wilson Disease: natural history, treatment, mutation analysis and phenotypic correlation*. Liver Int 2011;31:83-91.

Bull PC, Thomas GR, Rommens JM, et al. *The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene*. Nat Genet 1993;5:327-37.

Caca K, Ferenci P, Khun HJ, et al. *High prevalence of the His1069Gln mutation in East German patients with Wilson disease. Rapid detection of mutations by limited sequencing and phenotype- genotype correlation analysis.* J Hepatol 2001; 35:575-81.

Calvo PL, Pagliardini S, Baldi M, et al. *Long-standing mild hypertransaminasemia caused by congenital disorder of glycosylation (CDG) type IIx*. J Inherit Metab Dis 2008: doi:10.1007/s10545-008-1004-9.

Chen Chen et al.*Currently Clinical Views on Genetics of Wilson's Disease,* Chin Med J 2015 Jul 5; 128(13): 1826–1830.

Cocoş R, Şendroiu A, Schipor S, Bohîlţea LC, Şendroiu I, Raicu F *Genotype-phenotype correlations in a mountain population community with high prevalence of Wilson's disease: genetic and clinical homogeneity.*. PLoS One. 2014 Jun 4;9(6).

Czlonkowska A, Gromadzka G, Chabik G. *Monozygotic female twins discordant for phenotype of Wilson's disease*. Mov Disord. 2009;24:1066–9.

Dastsooz H ,Imanieh MH et al. *Multiplex ARMS PCR to Detect 8 Common Mutations of ATP7B Gene in Patients With Wilson Disease.* Hepat Mon. 2013 May 16;13(5).

Dastsooz H1, Dehghani SM et al. *A new ATP7B gene mutation with severe condition in two unrelated Iranian families with Wilson disease.*Gene. 2013 Feb 1;514(1):48-53.

D.M Danks: "Disorders of copper transport" In: A.L. Beaudet, W.S Sly, D. Valle, editors. Metabolioc basis of inherited disease, New York: McGraw-Hill (1989) 1416-1422.

De Bie P, van de Sluis B, Klomp L, et al. *The many faces of the copper metabolism protein MURR1/COMMD1*. J Hered 2005;96:803-11.

Deguti MM, Genschel J et al. *Wilson disease: novel mutations in the ATP7B gene and clinical correlation in Brazilian patients.*Hum Mutat. 2004 Apr;23(4):398.

Dhawan A, Taylor RM, Cheeseman P, et al. *Wilson's disease in children; 37-year experience and revised King's score for liver transplantation*. Liver Transpl 2005;11:441-8.

Ferenci P, Caca K, Loudianos G, et al. *Diagnosis and phenotypic classification of WD*. Liver Int 2003;23:139-42.

Ferenci P, Steindl-Munda P, Vogel W, et al. *Diagnostic value of quantitative hepatic copper determination in patients with Wilson's disease*. Clin Gastroenterol Hepatol 2005;3:811-8.

Ferenci P. *Regional distribution of mutations of the ATP7B gene in patients with Wilson disease: impact on genetictesting*. Hum Genet 2006;120:151-9.

Ferenci P, Członkowska A, Merle U, et al. *Late-onset Wilson's disease*. Gastroenterology 2007;1294-8.

Field LS, Luk E, Culotta VC. *Copper chaperones: personal escorts for metal ions.* J Bioenerg Biomembr 2002;34:373-9.

Firneisz G, Lakatos PL et al. *Common mutations of ATP7B in Wilson disease patients from Hungary.*Am J Med Genet. 2002 Feb 15;108(1):23-8.

Abdel Ghaffar TY, Elsayed SM et al. *Phenotypic and genetic characterization of a cohort of pediatric Wilson disease patients.*BMC Pediatr. 2011 Jun 17;11:56.

J.D. Gitlin: *"Wilson Disease"* Gastroenterology 125 (2003) 1868-1877.

Gomes A, V.G. Dedoussis. *Geographic distribution of ATP7B mutations in Wilson disease.* Ann Hum Biol. 2016;43(1):1-8.

Gromadzka G, Schmidt HH, Genschel J, et al. *Frameshift and nonsense mutations in the gene for ATPase7B are associated with severe impairment of copper metabolism and with an early clinical manifestation of Wilson's disease*. Clin Genet 2005;68:524-32.

Gupta A, Aikath D et al. *Molecular pathogenesis of Wilson disease: haplotype analysis, detection of prevalent mutations and genotype-phenotype correlation in Indian patients.* Hum Genet. 2005 Oct;118(1):49-57.

Gupta A, S. Lutsenko: *"Human copper transporters: mechanism,role in human diseases and therapeutic potential"* Future Med Chem. 6 (2009) 1125-1142.

Hellman NE, Gitlin JD. *Ceruloplasmin metabolism and function.* Annu Rev Nutr 2002;22:439-58.

Hermann W, Caca K, Eggers B et al. *Genotype correlation with fine motor symptoms in patients with Wilson's disease*. Eur Neurol. 2002;48(2):97-101.

Hamza, A. Faisst, J.Prohaska, J. Chen, P. Gruss, J. D. Gitlin: *"The metallochaperone Atox1 plays a critical role in perinatal copper homeostasis"* Proc. Natl. Acad. Sci. U S A. 98 (2001) 6848-6852.

I.H. Hung, M. Suzuki, Y. Yamaguchi, D.S Yuan, R.D. Klausner, J.D. Gitlin: *"Biochemical characterization of the Wilson disease protein and functional expression in the yeast Saccharomyces cerevisiae"* J Biol Chem 272 (1997) 21461-21466.

Huster D. *Wilson disease.* Best Pract Res Clin Gastroenterol. 2010 Oct;24(5):531-9.

Huster D, Kühne A et al. *Diverse functional properties of Wilson disease ATP7B variants.* Gastroenterology. 2012 Apr;142(4):947-956.

Incollu S, Lepori MB, Zappu A, Dessì V, Noli MC, Mameli E, Iorio R, Ranucci G, Cao A, Loudianos G. *DNA and RNA studies for molecular characterization of a gross deletion detected in homozygosity in the NH2-terminal region of the ATP7B gene in a Wilson disease patient. M*oll Cell Probes 2011 Oct-Dec;25(5-6):195-8.

Iorio R, Porzio S, Mazzarella G, et al. *Wilson disease:diagnostic dilemma?* J Pediatr Gastroenterol Nutr 2000;31:93.

Iorio R, D'Ambrosi M, Marcellini M, et al. *Serum transaminases in children with Wilson's disease*. J Pediatr Gastroenterol Nutr 2004;39:331-6.

Kim JW, Kim JH, Seo JK, Ko JS, Chang JY, Yang HR, et al. *Genetically confirmed Wilson disease in a 9-month old boy with elevations of aminotransferases*. World J Hepatol. 2013; 5(3):156–9).

Kumar S, Thapa BR et al. *Identification and molecular characterization of 18novel mutations in the ATP7B gene from Indian Wilson disease patients: genotype.* Clin Genet. 2005 May;67(5):443-5.

Kuppala D, Deng J, Brewer GJ, Wang MM, Borjigin J. 2009. *Wilsondisease mutations in the American population?: identification of five novel mutations in ATP7B.* Open Hepatol J 18:1–4.

Lepori MB, Lovicu M, Dessi V, et al. *Twenty-Four Novel mutations in Wilson disease patients of predominantly Italian origin.* Genet testing 2007;11:328-32.

Li XH , Lu Y, Ling Y et al. *Clinical and molecular characterization of Wilson's disease in China: identification of 14 novel mutations.* BMC Med Genet. 2011 Jan 11;12:6.

Loudianos G, Dessi V, Lovicu M, et al. *Further delineation of the molecular pathology of Wilson disease in the mediterranean population*. Hum Mut 1998; 12:89-94.

Loudianos G, Dessi V, Lovicu M, et al. *Mutation analysis in patients of Mediterranean descent with wilson disease. Identification of nineteen novel mutations.* J Med Genet 1999;36:833-6.

Loudianos G, Lepori MB, et al. Wilson's Disease. Prilozi XXXV 1, 2014.

Lutsenko S, Efremov RG, Tsivkovskii R, et al. *Human copper-transporting ATPase ATP7B (the Wilson's disease protein): biochemical properties and regulation*. J Bioenerg Biomembr 2002;34:351-62.

S. Lutsenko, M.J. Petris: *"Function and regulation of the mammalian copper-transporting ATPase: insights from biochemical and cell biological approaches"* J. Membr. Biol. 191 (2003) 1-12.

Lutsenko S., Barnes N.L., Bartee M.Y., et al. *Function and Regulation of Human Copper-Transporting ATPases* Physiol Rev. 2007 Jul;87(3):1011-46.

Lutsenko S. *Modifying factors and phenotypic diversity in Wilson's disease*. Ann N Y Acad Sci. 2014 May;1315:56-63.

Machado AA, Deguti MM et al. *Neurological manifestations and ATP7B mutations in Wilson's disease.* Parkinsonism Relat Disord. 2008;14(3):246-9.

Mak CM, Lam CW, Tam S. *Diagnostic accuracy of serum ceruloplasmin in Wilson disease: determination of sensitivity and specificity by ROC curve analysis among ATP7B-genotyped subjects*. Clin Chem 2008;54:1356-62.

Mameli E, Lepori MB, Chiappe F, Ranucci G, Di Dato F, Iorio R, Loudianos G. *Wilson's disease caused by alternative splicing and Alu exonization due to a homozygous 3039-bp deletion spanning from intron 1 to exon 2 of the ATP7B gene*. Gene 2015 Sep 15;569(2):276-9.

Mandato C, Brive L, Miura Y, et al. *Cryptogenic liver disease in four children: a novel congenital disorder of glycosylation*. Pediatr Res 2006;59:293-8.

Merle U, Schaefer M, Ferenci P, et al. *Clinical presentation, diagnosis and longterm outcome of Wilson's disease: a cohort study*. Gut 2007;56:115-20.

Merle U, Weiss KH, Eisenbach C, et al. *Truncating mutations in the Wilson disease gene ATP7B are associated with very low serum ceruloplasmin oxidase activity and an early onset of Wilson disease*. BMC Gastroenterol 2010;18:10-18.

Møller LB, Ott P, Lund C, Horn N. *Homozygosity for a gross partial gene deletion of the Cterminal end of ATP7B in a Wilson patient with hepatic and no neurological manifestations*. Am J Med Genet A. 2005; 138(4):340–343.

Møller LB, Mongensen M,Horn N. *Molecular diagnosis of Menkes disease: genotype-phenotype correlation.*Biochimie. 2009 Oct;91(10):1273-7.

Møller LB, Horn N, Jeppesen TD, Vissing J, Wibrand F, Jennum P, et al. *Clinical presentation and mutations in Danish patients with Wilson disease*. Eur J Hum Genet. 2011; 19(9):935–94.

A.R. Mufti, E. Burstein, R.A. Csomos, P.C. Graf, J.C. Wilkinson, R.D. Dick, *et al.* XIAP *Is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders* Mol Cell, 21 (6) (2006), pp. 775–785.

Muller T, Koppikar S, Taylor RM, et al. *Re-evaluation of the penicillamine challenge test in the diagnosis of Wilson's disease in children*. J Hepatol 2007;47:270-76.

Nicastro E, Loudianos G, Zancan L, et al. *Genotype-phenotype correlation in Italian children with Wilson's disease*. J Hepatol 2009;50:555-61.

Nicastro E, Ranucci G, Vajro P, et al. *Re-evaluation of the diagnostic criteria for Wilson disease in children with mild liver disease.* Hepatology 2010;6:1948-56.

O'Connor JA, Sokol RJ. *Copper metabolism and copper storage disorders*. In: Suchy FJ, Sokol RJ, Balistreri WF eds. Liver disease in children. New York, NY USA:Cambridge University Press, 2007;626-59.

Okada T, Shiono Y, Hayashi H. et al. *Mutational analysis of ATP7B and genotype-phenotype correlation in Japanese with Wilson's disease.*Hum Mutat. 2000;15(5):454-62.

Pena MM, Lee J, Yhiele DJ: *"A delicate balance: Homeostatic control of copper uptake and distribution"* J Nutr 129 (1999) 1251-1260.

Petrukhin K, Lutsenko S, Chernov, I, et al. *Characterization of the Wilson disease gene encoding a P-Type copper transporting ATPase: genomic organization, alternative splicing, and structure/function predictions.* Hum. Mol Gene 1994;3:1647-56.

Polishchuk EV, Concilli M, Iacobacci S et al. *Wilson disease protein ATP7B utilizes lysosomal exocytosis to maintain copper homeostasis.* Dev Cell. 2014 Jun 23;29(6):686-700.

Roberts E, Schilsky ML. *Diagnosis and treatment of Wilson disease: an update*. Hepatology 2008;47:2089-111.

Roberts, E.A. Wilson's disease. *Medicine* 2011, *39*, 602–604.

Santhosh S1, Eapen CE et al. *Gene symbol: ATP7B. Disease: Wilson disease.* Hum Genet. 2006 Apr;119(3):361.

Scheinberg I.H, I. Sternlieb: Wilson's Disease. In: H. Lloyd, J. Smith editors. *"Major problems in internal medicine"* vol.23. Philadelphia: W.B. Saunders (1984) 10-12.

M. Schaefer, J.D. Gitlin: *"Genetic disorder of membrane transport. Wilson's disease and Menkes disease"* Am J Physiol 276 (1999) G311-G314.

Shah AB1, Chernov I, Zhang HT et al. *Identification and analysis of mutations in the Wilson disease gene (ATP7B): population frequencies, genotype-phenotype correlation and functional analyses*. Am J Hum Genet.1997 Aug;61(2):317-28.

Skowronska M, Litwin T, Dziezyc K, Wierzchowska A, Czlonkowska A. *Does Brain degeneration in Wilson disease involve not only copper but also iron accumulation?* Neurol Neurochir Pol. 2013; 47(6):542–6

R.J. Sokol: "*Antioxidant defenses in metal-induced liver damage"* Semin Liver Dis 16 (1996) 39-46.

Stapelbroek JM, Bollen CW, van Amstel JK, et al. *The H1069Q mutation in ATP7B is associated with late and neurologic presentation in Wilson disease: results of a meta-analysis*. J Hepatol 2004;41:758-63.

S. Tanner: *"Disorders of copper metabolism in disease of the liver and biliary system in children"* Kelly, Blackwell Science, editors (1999) 167-185.

Tanzi RE, Petrukhin K, Chernov I, et al. *The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene*. Nat Genet 1993;5:344-50.

Tapiero H, Townsend DM, Tew KD. *Trace elements in human physiology and pathology*. Copper Biomed Pharmacother 2003;57:386-98.

Todorov T, Balakrishnan P, Savov A, Socha P, Schmidt HH. *Intragenic Deletions in ATP7B as an Unusual Molecular Genetics Mechanism of Wilson's DiseasePathogenesis.PLoS One . 2016 Dec 19;11(12).*

Trocello JM, Guichard JP et al. *Corpus callosum abnormalities in Wilson's disease.*, J Neurol Neurosurg Psychiatry. 2011 Oct;82(10):1119-21.

Wilson SAK. *Progressive lenticular de generation: a familial nervous disease associated with cirrhosis of the liver*. Brain 1912;34:295-7.

Wu ZY , Wang N, et al. Arch Neurol. *Mutation analysis and the correlation between genotype and phenotype of Arg778Leu mutation in chinese patients with Wilson disease.* 2001 Jun;58(6):971-6.

Yamaguchi Y, Heiny ME, Gitlin JD. *Isolation and characterization of a human liver cDNA as a candidate gene for Wilson disease.* Biochem Biophys Res Commun. 1993 Nov 30;197(1):271-7.

Zappu A, Magli O, Dessi V, et al. *High incidence and allelic homogeneity of Wilson disease in two isolated populations. A prerequisite for efficient disease prevention programs*. JPGN 2008;47:334-8.

Ringraziamenti:

Un primo ringraziamento alla mia famiglia che mi ha sempre sostenuto in questi anni di studio e ricerca. Inoltre non posso che essere riconoscente a tutte le colleghe e colleghi che negli anni hanno svolto un importante e prezioso lavoro sulla malattia di Wilson: Mario, Antonella, Simona, Valentina, Cristina, Valeria ed Eva. Un sentito ringraziamento al Dott. Jorgos Loudianos, responsabile del laboratorio di Epatopatie Genetiche e mio referente e al Prof. Moi, mio tutor di Dottorato, che in questi anni mi hanno guidato e sostenuto. Ultimo ma non meno importante, tutta la mia gratitudine va alla collega e amica Barbara con la quale ho condiviso momenti bui e di gioia, in questi ultimi tre anni di Dottorato.