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Focused review

Liver pathology in Wilson's disease: From copper overload to cirrhosis

C. Gerosa[a], D. Fanni[a], T. Congiu[a], M. Piras[a,*], F. Cau[b], M. Moi[a], G. Faa[a]

[a] Division of Pathological, University of Cagliari AOU Cagliari, Cagliari, Italy
[b] Division of Pathological, San Gavino Hospital, ATS, San Gavino, Italy

A B S T R A C T

Wilson's disease (WD) is a genetic metabolic disease strictly associated with liver cirrhosis. In this review, the genetic bases of the disease are discussed, with emphasis on the role of ATP7B (the Wilson disease protein) dysfunction as a determinant factor of systemic copper overload.

Regarding the different multiple mutations described in WD patients, the peculiarity of Sardinian population is highlighted, Sardinians carrying a rare deletion in the promoter (5′ UTR) of the WD gene.

The role of epigenetic changes in the clinical presentation and evolution of liver disease in WD patients is also discussed, nutrition probably representing a relevantly risk factor in WD patients. The role of transmission electron microscopy in the diagnosis of WD-related liver disease is underlined. Mitochondrial changes, increased peroxisomes fat droplets, lipolysosomes and intranuclear glycogen inclusions are reported as the most frequent ultrastructural changes in the liver of WD carriers.

The role of histochemical stains for copper is analyzed, and the Timm's method is suggested as the most sensitive one for revealing hepatic copper overload in all stage of WD.

The marked variability of the histological liver changes occurring in WD is underlined simple steatosis may represent the only pathological changes, frequently associated with glycogenated nuclei.

Mallory-Denk bodies lipogranulomas alcoholic and non-alcoholic fatty liver disease ending with bridging fibrosis and cirrhosis. Finally, the reversal of fibrosis as a possible therapeutic objective in WD is discussed.

1. Introduction

The disease nowadays generally known as Wilson's disease (WD), was first described in 1912 by the British neurologist Samuel Alexander Kinnier Wilson, who defined it as “progressive lenticular degeneration: a familiar nervous disease associated with liver cirrhosis” [1]. In his article, Wilson has not only delineated in detail the pathological profile of the disease, characterized by the association of damage in the lenticular nuclei of the brain with liver cirrhosis, but he also introduced for the first time the term extrapyramidal system, stressing the role of the basal ganglia in motility disorders [2]. Regarding the pathogenesis of brain and liver disease, Wilson formulated the hypothesis of an “unknown toxin”, that was a prophetic intuition. The role of excess copper, as the main pathogenetic mechanism responsible for cell pathology in WD, was later described in 1929. More than 100 years after its first description, WD is considered a multi-systemic disorder, in which hepatic, neurological and psychiatric symptoms are often associated with renal, endocrine, osteo-articular, corneal and myocardial disturbances, all related to abnormal copper metabolism ending with systemic accumulation of the metal [3]. Despite the significant progresses in the understanding of the mechanisms underlying WD, there are many open questions regarding the relationships between copper storage and liver disease. Firstly, copper overload should not be exclusively associated with liver cell damage, high levels of copper being physiologically present in the newborn liver, in the absence of any pathological change [4,5]. Moreover, the phenotypic expression of WD is highly variable, even among subjects with the same genetic changes and within the same family [6], with marked heterogeneity regarding age of onset and clinical presentation [7]. Among the several factors contributing to the phenotypic variability of WD, the multiple different WD-causing mutations produce a wide range of changes in stability, activity, intracellular localization, and trafficking of ATP7B (the Wilson disease protein) [8]. In any case, the marked variability in age of onset of WD may partly be explained by environmental factors as nutrition, copper-rich food representing an acquired potential risk factor for developing copper overload in ATP7B-deficient subjects [9]. On the other hand, some dental creams have been identified as an excess zinc source, and its frequent use could represent an involuntary therapeutic tool that might decrease copper absorption and delay clinical presentation in patients affected by WD [10]. Modulator genes are also probably involved in the phenotypic expression of the disease: inter-individual variability within genes encoding proteins involved in the antioxidant defense system has been hypothesized to modulate phenotypic expressions of WD [11].

* Corresponding author.
E-mail address: monipiras@hotmail.com (M. Piras).

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The aim of this article is to report the most important data regarding the linkage between copper storage in liver cells and the development of liver disease, focusing on the relationship between the type and site of copper overload and liver cell damage, insurgence of fibrosis and liver cirrhosis.

2. Genetic bases of WD

WD is an inherited, autosomal recessive disorder of copper metabolism, originating from a genetic defect in the copper-transporting ATPase ATP7B, that is required for biliary copper excretion and loading of ceruloplasmin and other cupro-enzymes with copper [12]. The implicated gene at 13q14.3 encodes for a transmembrane protein, ATP7B, which is located in the Golgi apparatus and in the endosomal vesicles, depending on different intracellular copper concentrations [13]. A large number of mutations have been detected in WD patients, scattered over the 17 exons of the ATP7B gene. As we already know, more than 600 pathogenic variants in ATP7B have been identified. Among them, single-nucleotide missense and nonsense mutations are the most common, followed by insertions/deletions, and, rarely, splice site mutations [7]. The worldwide most common mutation, found in the majority of WD carriers in Europe and USA, is H1069Q, but this mutation is not present in several countries, including patients from Lebanon [14]. In the population from Sardinia, an island of the Mediterranean Sea in which WD shows a very high incidence (about 1:7000 live births), molecular analysis of the WD chromosomes often fails to detect any disease-causing mutation. In Sardinians, the most frequent change is represented by a 15 nt deletion in the promoter and the 5′ UTR of the WD gene [15]. Further studies have demonstrated that mutations in regulatory elements of ATP7B may be detected even in WD carriers from other geographical areas, but variations in the promoter and 5′ UTR are uncommon, except in Sardinians [16]. Some mutations have been correlated with a particular clinical presentation in WD carriers. For example, Arg778Leu has been related to younger age onset and lower serum levels of ceruloplasmin (Cp) and copper [17]. ATP7B dysfunction leads to copper storage inside the hepatocytes, a consequence of the inability of ATP7B to transport copper ions into biliary canaliculi [18]. In studies aimed at performing screening for WD in a large series of subjects, denaturing high-performance liquid chromatography (DHPLC) has been proposed as a fast and useful tool, superior to traditional haplotyping [19].

3. ATP7B dysfunction in WD

Mutations in the ATP7B gene result in changes in the expression or in the function of the enzyme. The main molecular players operating in the quality control pathways, leading to copper overload, remain to be discovered yet. For example, the most frequent ATP7B mutant, H1069Q, still shows a significant copper-transporting activity, but the protein is rapidly degraded. Expression of the H1069Q ATP7B mutant has been reported to activate the p38 and JNK stress kinase pathways, which, in turn, facilitate the sequestration of ATP7B-H1069Q in the Endoplasmic Reticulum, ending with the acceleration of its degradation [20]. Regarding the 5′ UTR mutation, the most frequent mutation in Sardinians, since this mutation is located in the regulatory elements of the ATP7B gene, the sequence of the encoded protein is normal, but the altered regulation of its secretion is at the basis of its dysfunction [15].

The N-terminal part of ATP7B constitutes six metal binding domains (MBDs), each of them can bind a copper ion, interact with other ATP7B domains as with several other proteins. Many WD causing missense mutations are found in these domains, but it remains unclear how these domains modulate the overall structure and function of ATP7B [21]. In WD patients carrying the G85 V or G591D mutation, a reduced stability and enhanced dynamics of Metal-Binding Domains (MBD1 and MBD6) has been identified at the origin of ATP7B dysfunction [22].

A better knowledge of the molecular mechanisms regulating ATP7B expression might be important for the development of new therapeutic approaches, in WD patients. Recently a major role inside the hepatocytes has been assigned to Atox1 (the copper metallochaperone), which shuttles copper ions from the main copper transporter, hCtr1 (copper transporter 1), to ATP7B in the Golgi apparatus [23]. Atox1 delivers copper to the first three metal-binding domains (MBD1-3), and it is central to regulation of the activity of ATP7B. Atox1-mediated copper binding to ATP7B activates ATP hydrolysis, and regulates its activity (2n) [24].

ATP7B expression is not restricted to liver cells. Recently, it has been reported that ATP7B is localized to intestinal cells, where it regulates vesicular storage of copper ions, buffering copper levels in enterocytes participating in the formation of chylomicrons [25]. According with these findings, changes in ATP7B expression might be responsible for misbalance of copper and lipid in the intestine and could account for gastrointestinal disease occasionally reported in WD patients. ATP7B is also expressed in the resting and lactating human breast [26]. In the lactating toxic milk mouse, a murine model of WD, ATP7B has been found to be dislocated, being mostly documented in perinuclear areas, in comparison with the diffuse cytoplasmic localization typical of the lactating normal mammary gland, ending with impaired copper transport from the mammary gland into milk [27]. These data suggest that ATP7B dysfunction might be responsible for the systemic disturbances of copper trafficking in the whole human body, with bad consequences in all organs [28].

4. Epigenetics of WD

The marked variability in clinical presentation frequently observed even among subjects carrying the same mutation, suggests the existence of epigenetic factors able to influence the degree of copper overload. The report of WD patients with a late clinical onset, initial symptoms occurring in late adulthood, suggests the presence of other factors influencing the progression of liver changes in this disease [29]. Epigenetic changes are alterations that take place at the transcriptional level without altering the DNA sequence. DNA methylation, histone modifications and microRNA are generally considered the most common epigenetic modifications occurring in human cells [30]. Epigenetic alterations are involved in the regulation of lipid metabolism in liver cells, oxidative stress response, mitochondrial damage, and the release of inflammatory cytokines, all of them being implicated in the development and progression of liver disease in WD patients [31].

The epigenetic factors acting in liver pathology due to WD, include nutrition, representing one of the most relevant epigenetic elements to be analyzed. Nutrients rich in copper represent a risk factor in WD patients, excess copper absorbed by the intestine inducing mitochondrial oxidative stress and liver cell damage. A copper-rich diet might lead to the progression from steatosis to steatohepatitis, a more severe liver disease possibly ending with cirrhosis. The negative role of copper overload in nutrition, has been reported in patients with non-alcoholic fatty liver disease (NAFLD), a liver disease very similar to Wilson's hepatitis, both being characterized by steatosis, inflammation and cell death [32]. In the last years, the evolution of NAFLD into non-alcoholic steatohepatitis (NASH) has been correlated to unbalanced dietary copper levels and, actually, some clinical trials are examining the use of copper chelating agents to stop this progression [33]. Thus, the strict control of alimentary copper could be considered a target point for counteracting the progression of liver disease in WD carriers.

Another factor that should be taken into consideration regarding nutrition and WD, is represented by methionine. In experimental models of liver steatosis, methionine administration has been shown to reverse liver steatosis, by promoting fatty acid oxidation, increasing the export of lipids and reducing obesity-related inflammatory responses [34].

Dietary polyphenols have been recently introduced in prevention of obesity and obesity-related chronic diseases. Polyphenols reduce

viability of adipocytes and proliferation of preadipocytes, suppress adipocyte differentiation and triglyceride accumulation, stimulate lipolysis and fatty acid β-oxidation, reducing inflammation [35]. Even though no data is available to the best of our knowledge, on the effects of a polyphenol-rich diet in the progression of Wilsonian liver disease, all these data suggest that the amount of dietary polyphenols, might influence the evolution of WD-related liver disease, by protecting liver cells against copper-induced oxidative stress [36].

Among the anti-oxidant agents present in our diet, zinc represents one of the most important ones, due to its antioxidant activity associated with a competition for intestinal absorption of copper ions [37]. Zinc ions act by directly competing with copper ions for binding with histidine, suggesting that metal binding competition can be important in the more general context of trace metal homeostasis [38]. Thanks to the ability of the ion to reduce copper absorption, and lower systemic copper overload, zinc has been introduced in clinical practice, for controlling WD-related liver disease [39]. In other mild studies, zinc as first-line therapy, demonstrated poor efficacy to control liver disease in a large part of children with WD, associated with a high incidence of gastrointestinal adverse effects [40].

5. Main targets of copper overload in liver cells

Ultrastructural changes in liver biopsies from WD patients are characterized by severe mitochondrial changes, associated with an increased number of peroxisomes and with the presence of lipolysosomes, characteristic cytoplasmic bodies formed by lipid vacuoles surrounded by electron-dense lysosomes [41]. Moreover, hepatocytic nuclei are frequently involved in WD, with disorganization of the nucleoplasm and glycogen inclusions, giving rise to the typical “glycogen nuclei”. In the cytoplasm of hepatocytes, the most frequent pathological finding is represented by fat droplets, clearly indicating a disarrangement in lipid metabolism. The major effect of copper overload on lipid metabolism suggests the role of fat and cholesterol metabolism as modifying factors in WD [8]. Microanalysis may be more sensitive than histology and histochemistry, detecting copper and iron accumulation in the hepatocellular lipofuscin particles, whose presence is considered a typical marker of WD, particularly in young subjects [42].

Mitochondria represent the principal target for copper toxicity in WD patients, being the first responders to imbalanced copper homeostasis. The typical mitochondrial changes are the earliest observable pathological features in the liver cells of WD patients [43]. In the advanced phase of WD, when copper is massively deposited in mitochondria, over-production of reactive oxygen species causes the mitochondrial membranes to disintegrate, triggering hepatocytic cell death. Reactive oxygen species (ROS) play a relevant role in the maintenance of physiological functions, a process termed redox biology. On the other hand, excessive intracellular levels of ROS produce oxidative stress, that causes damages to lipids, proteins and DNA, ending with cell death [44]. Many biochemical reactions may generate ROS. Reactions mediated by transition metals, including iron and copper, are particularly effective in catalyzing the formation of hydroxyl radicals and other ROS [45]. The sensitivity of mitochondria to copper overload is likely due to a copper-dependent attack on mitochondrial protein thiols, which probably represent the major target of copper toxicity [46]. In contrast, an increased production of ROS might represent a late-stage event in Wilsonian liver disease, only occurring in destroyed mitochondria. Cu-induced liver toxicity and Cu-induced ROS formation, since as with iron, the toxic species is the cuprous (Cu^{+}) form rather than the cupric (Cu^{++}) form. [47].

6. Electron microscopy: mitochondrial changes in WD

Wilson's Disease is characterized by the uneven copper accumulation in liver cells [48] that results in different degrees of mitochondrial damage, so the typical pattern of mitochondria may be observed in defined part of the whole liver. Copper-stressed mitochondria show changes in dimension, shape and arrangement of matrix. The mitochondrial matrix is highly electron-dense, and it pushes the cristae against the inner membrane. The cristae acquire a leaf-like shape and they are short and apparently deformed by the matrix (Fig. 3). These damaged mitochondria are in close relationship with the enlargement of smooth endoplasmic reticulum, well visible in transmission Electron Microscopy as translucent vesicles.

7. Histochemistry for copper in WD

Excess copper storage in the hepatocytes is a relevant sign of Wilson's disease, and determination of hepatic copper content in the liver biopsy, is important in the diagnosis of WD [48]. Hepatic copper overload should not be considered specific of WD, high concentrations of liver copper being detectable in cholestatic liver diseases [49], primary biliary cholangitis [50], alcoholic liver disease [51] and idiopathic copper toxicosis [52]. Multiple histochemical methods have been proposed, during the years, for the detection of copper stored in the cytoplasm of hepatocytes [53]. A number of histochemical methods have been developed to reveal copper overload: I) orcein, that reveals the accumulation of metallothioneins, the proteins involved in excess copper sequestration, appearing as large irregular granules dark-brown in colour [54]; II) Timm silver stain, that shows free copper deposits in the cytoplasm of hepatocytes, appearing as small black granules [55]; III) rubeanic acid, that reveals copper deposits as cytoplasmic green granules [56]; IV) rhodanine, that shows copper overload inside lysosomes as red granules scattered in the cytoplasm of hepatocytes [57]. A quick histochemical stain is able to reveal copper overload in liver biopsies in a very short time (10 min), thanks to the use of microwave treatment [58]. A study from our group on a large series of liver biopsies from WD patients, showed that the use of three histochemical stains for copper, allows the diagnosis of more cases than using only one method. From a practical point of view, our findings suggest the use, in clinical practice, of multiple histochemical stains in order to increase the diagnostic utility of histochemistry for copper. In the same study, the Timm's method appeared to be the most effective method for the demonstration of copper overload in all stages of WD [59].

Fig. 1. Schematic representation of steatosis. Macrovesicular (a) steatosis is characterized by a unique unstained large vacuole, occupying all cytoplasm of the hepatocyte, dislocating and deforming the nucleus; thus that the nucleus is pushed at the periphery of the cell. In microvesicular (b) steatosis vacuoles have a diameter not exceeding the nucleus and usually more than one, as many as less is the diameter.

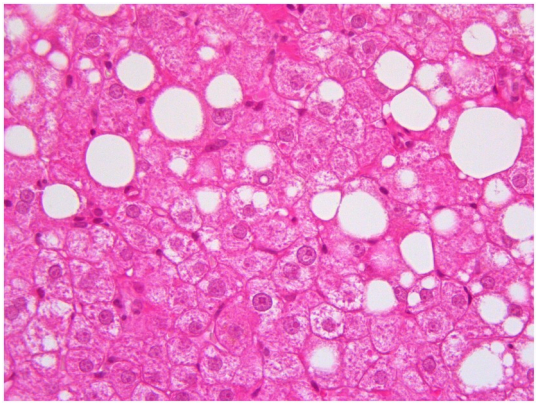

Fig. 2. Histological hematoxylin-eosin stained section of a liver with Wilson disease. In the picture, the microvascular (a) and microvascular (b) steatosis are easily recognised and apparently randomly distributed. Middle size vacuoles, with a diameter between those of macrovesicular and microvascular steatosis, may be observed.

8. Histology: variability of liver changes in WD

Liver disease occurring in WD patients is characterized by a striking variability in pathological changes. Historically, the elementary liver lesions generally considered typical of WD were: cytoplasmic lipofuscin granules hepatocytic nuclear ballooning due to glycogen intranuclear inclusions; fine fat droplets (Fig. 1), patchy cytoplasmic glycogen deposition [60]. The spectrum of hepatic pathological changes occurring in WD is very broad, ranging from elementary changes typical of a toxic pathology, to inflammatory changes typical of viral or autoimmune etiology [61]. In the first steps, hepatocyte dysfunction may initially manifest as simple steatosis (Fig. 2), frequently associated with glycogenated nuclei. Steatosis, Mallory-Denk bodies, lipogranulomas and glycogenated nuclei have been reported as characteristic findings in Wilsonian liver biopsies, giving rise to a picture that often mimics alcoholic and non-alcoholic fatty liver disease [62]. In other patients, the histological picture may overlap that typical of chronic active viral hepatitis or that of autoimmune liver disease [63,64]. Liver disease may also progress to other hepatic phenotypes like acute liver failure, chronic steatohepatitis with bridging fibrosis, ending with cirrhosis [12].

9. Is regression of fibrosis a possible therapeutic objective in WD?

The relevance of an early diagnosis of WD is mainly related to the fact that, when early diagnosed and properly treated, WD may have a favorable prognosis [65]. In a recent study from our group, 33/40 (83%) patients affected by WD, after years of treatment, showed stable hepatic histology or improvement. Only 7 of 40 (17%) showed worsening of fibrosis in spite of treatment with zinc or penicillamine [66]. These data contrast with previous clinical studies, in which the number of WD patients undergoing significant improvement following therapy was much lower than cases where patients showed worsening of liver fibrosis [67]. Previous studies evidenced that D-penicillamine may reduce the rate of liver fibrogenesis in patients with Wilson disease [68]. In a study carried out on a large cohort (380 subjects) of WD patients, treated with penicillamine or with trientine, hepatic improvement was observed in more than 90% of patients [69]. Unfortunately, whereas the efficacy of the commonly used drugs is satisfactory for hepatic disease, on the contrary, it appears disappointing in neurologic patients, where the risk of neurologic deterioration persists even after the initiation of chelation therapy [70].

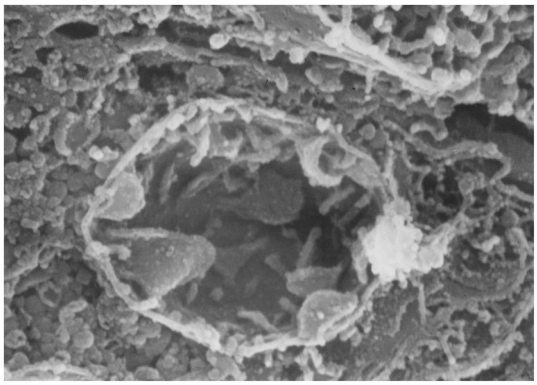

Fig. 3. (30.000 x) The mitochondria show different shape and volume due mainly to enlargement of the matrix. This feeling is confirmed by the cristae pressed against the inner membrane. Note the typical leaf-like pattern of the cristae.

10. Conclusions

During the last century, Wilson's disease has evolved from a disease presenting insurmountable challenges for the clinicians, to a disorder of copper metabolism which is diagnosable, treatable successfully, and even preventable [71]. Unfortunately, in spite of all progresses, the diagnosis of WD appears often challenging, due to the variability of its clinical presentation and to the complexity of the histological lesions found in the liver biopsy. Liver histology, in fact, does not show a specific pattern, but it may show multiple patterns, with overlapping features between WD and alcoholic hepatitis, non-alcoholic liver disease (NAFLD) and viral hepatitis. This clinical and pathological complexity is associated with a very high number of mutations in the ATP7B gene [72], and as well as with the multiple epigenetic factors that may interfere, on the clinical presentation and progression of the disease. The postponed diagnosis of many patients affected by WD represents bad news, given that Wilsonian liver disease, when diagnosed in its initial pre-fibrotic stages, is easily curable, and patients may have a “normal” live, taking one or few pills per day. It may be long before the genetic defect becomes amenable to correction and a cure of the disease becomes possible.

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