EXTRAHEPATIC MANIFESTATIONS OF WILSON DISEASE

Dissertation for the Degree of Doctor of Philosophy (Faculty of Medicine)
in Internal Medicine

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2003
This work is dedicated to my brother, who helped me understand the minor details of his disease, and who inspired me to carry out this research to acquire the wisdom of Wilson disease on every aspect.
ABSTRACT

Wilson disease is a relatively rare inherited disorder of copper metabolism. Although it has been recognized and described for nearly a century, our knowledge and understanding of the disease is far from complete. Many patients remain undiagnosed, and diagnosed patients’ various symptoms and dysfunctions are often neglected.

The aim of this study was to investigate some extrahepatic manifestations, such as skeletal involvement and its mechanism, peripheral sensory and autonomic neuronal changes, and its impact on cardiovascular morbidity. We also determined the temperament and character profile of Wilson patients, and investigated the prevalence of various psychiatric disturbances, among them suicide. Furthermore, we measured the serum level of the newly discovered molecule called nociceptin, and investigated its clinical significance.

We found that osteoporosis is very common in young WD patients, most likely secondary to increased osteoclast activity. We also found early dysfunction of peripheral sensory neurons compared to the control group, and decreased heart rate variability only in patients with neurological symptoms, who therefore have increased risk for cardiovascular morbidity and mortality. The temperament and character profile deviated from healthy individuals on many scales, and psychiatric symptoms were very common in Wilson patients. We also found a 10% prevalence of attempted suicide among the patients. Nociceptin level was also significantly elevated.

In this study we pinpoint various unsolved questions about the disease, to which we attempted to provide some answers. However, the mysteries about this disease has not all been solved, rather, further questions are raised. I strongly believe, that there are many more interesting and exciting aspects and mechanisms related to the disease, waiting to be explored.
LIST OF PAPERS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals:


**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPM</td>
<td>24-hour ambulatory blood pressure monitoring</td>
</tr>
<tr>
<td>Anx</td>
<td>anxiety</td>
</tr>
<tr>
<td>APN</td>
<td>aminopeptidase N</td>
</tr>
<tr>
<td>β-CTx</td>
<td>β-crosslaps</td>
</tr>
<tr>
<td>BAL</td>
<td>British Antilewisite</td>
</tr>
<tr>
<td>BMC</td>
<td>bone mineral content</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BSEP</td>
<td>brain stem evoked potential</td>
</tr>
<tr>
<td>BUA</td>
<td>broadband ultrasound attenuation - heel</td>
</tr>
<tr>
<td>C</td>
<td>cooperativeness</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPT</td>
<td>current perception threshold</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>Dep</td>
<td>depression</td>
</tr>
<tr>
<td>DXA</td>
<td>dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>FMS</td>
<td>fibromyalgia syndrome</td>
</tr>
<tr>
<td>FN</td>
<td>femoral neck</td>
</tr>
<tr>
<td>GSI</td>
<td>global severity index</td>
</tr>
<tr>
<td>HA</td>
<td>harm avoidance</td>
</tr>
<tr>
<td>HBID</td>
<td>diastolic hyperbaric impact</td>
</tr>
<tr>
<td>HBIS</td>
<td>systolic hyperbaric impact</td>
</tr>
<tr>
<td>HF</td>
<td>high frequency</td>
</tr>
<tr>
<td>Host</td>
<td>hostility</td>
</tr>
<tr>
<td>HRV</td>
<td>heart rate variability</td>
</tr>
<tr>
<td>HTID</td>
<td>diastolic hypertensive time index</td>
</tr>
<tr>
<td>HTIS</td>
<td>systolic hypertensive time index</td>
</tr>
<tr>
<td>IS</td>
<td>interpersonal sensitivity</td>
</tr>
<tr>
<td>KF ring</td>
<td>Kayser-Fleischer ring</td>
</tr>
<tr>
<td>L2-4</td>
<td>lumbar 2-4 vertebrae</td>
</tr>
<tr>
<td>LF</td>
<td>low frequency</td>
</tr>
<tr>
<td>LFT</td>
<td>liver function test</td>
</tr>
<tr>
<td>MSA</td>
<td>multiple system atrophy</td>
</tr>
<tr>
<td>NC</td>
<td>nociceptin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NS</td>
<td>novelty seeking</td>
</tr>
<tr>
<td>OC</td>
<td>obsessive-compulsive</td>
</tr>
<tr>
<td>OCN</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>OP4</td>
<td>nociceptin</td>
</tr>
<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
</tr>
<tr>
<td>P</td>
<td>persistence</td>
</tr>
<tr>
<td>Para</td>
<td>paranoid ideation</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson disease</td>
</tr>
<tr>
<td>Phob</td>
<td>phobic anxiety</td>
</tr>
<tr>
<td>PINA</td>
<td>pineal night-specific ATPase</td>
</tr>
<tr>
<td>pNN50</td>
<td>proportion of adjacent RR intervals differentiated by more than 50 milliseconds</td>
</tr>
<tr>
<td>PSDI</td>
<td>positive symptom distress index</td>
</tr>
<tr>
<td>PST</td>
<td>total of positive symptoms</td>
</tr>
<tr>
<td>Psy</td>
<td>psychoticism</td>
</tr>
<tr>
<td>QUS</td>
<td>quantitative ultrasound</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of NF-kappaB ligand</td>
</tr>
<tr>
<td>RD</td>
<td>reward dependence</td>
</tr>
<tr>
<td>r-MSSD</td>
<td>root-mean square difference of successive RR intervals</td>
</tr>
<tr>
<td>RRD</td>
<td>diastolic blood pressure mean</td>
</tr>
<tr>
<td>RRS</td>
<td>systolic blood pressure mean</td>
</tr>
<tr>
<td>S</td>
<td>self-directedness</td>
</tr>
<tr>
<td>SCL-90-R</td>
<td>Symptom Check List</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDANN</td>
<td>standard deviation of the averages of RR intervals</td>
</tr>
<tr>
<td>SDNN</td>
<td>24-hour standard deviation of all RR intervals</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>Som</td>
<td>somatization</td>
</tr>
<tr>
<td>SOS</td>
<td>speed of sound</td>
</tr>
<tr>
<td>SPA</td>
<td>single photon absorptiometry</td>
</tr>
<tr>
<td>ST</td>
<td>self-transcendence</td>
</tr>
<tr>
<td>TCI</td>
<td>Temperament and Character Inventory</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TP</td>
<td>total power</td>
</tr>
<tr>
<td>WD</td>
<td>Wilson disease</td>
</tr>
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1 INTRODUCTION

1.1 Historical milestones

Wilson disease was first described by an American-born neurologist, Samuel Alexander Kinnier Wilson, who published the first paper on Wilson disease in the Brain in 1912 (1), and called it progressive lenticular degeneration. Many other physicians and investigators, both before and after Wilson’s paper, made contributions to various aspects of WD. The corneal copper deposition, seen in many of the patients, was observed by Kayser in 1902 (2), and Fleischer in 1903 (3), thus the Kayser-Fleischer ring bears the name of both physicians. However, there was no suggestion at this point that the disease was caused by toxic copper accumulation. The etiologic role of copper was first proposed by Rumpel in 1913, who reported excessive copper in the liver (4). In 1952 two groups of investigators independently reported low level of plasma cerulplasmin in Wilson patients (5, 6). The first significant but painful therapy was British Antilewisite (BAL) based upon the hypothesis that the disease was due to copper toxicity (7, 8). Shortly after, Walshe (9) reported significantly increased urinary copper excretion with d-penicillamine administration, which became lifesaving therapy for the patients. It was also Walshe (10) who published on an alternative treatment with trientine. Zinc was first used in 1961 by Schouwink as a therapeutic agent for WD (11). Over the next few decades investigations with zinc treatment became the center of attention; Brewer and his group (12) alone published 54 papers on zinc therapy. In 1993 the genetic cause of WD was discovered by three independent investigator groups (13, 14, 15). The gene is called ATP7B, which encodes a copper-binding, membrane-bound ATPase. Up to now over 200 mutations of this gene are described (16).

1.2 Epidemiology

WD is a relatively rare disease. Although reliable data on the prevalence of the disease is scarce, it is estimated to be 1/40 000 to 1/30 000. However, in most European countries this number is much lower – 12-18 per million – (17), whereas in countries where consanguineous marriages are common this number is relatively high. In Japan the prevalence is estimated to be 33/one million (18) and in Costa Rica this number exceeds 60/one million (19).
1.3 Etiology

1.3.1 Genetics

Wilson disease is a monogenic disorder. The mutation affecting the ATP7B gene is localized to chromosome 13q14.3-q21.1. The copper-binding ATPase protein, encoded by this gene, is a member of the CPx-ATPase family that is conserved from bacteria to humans (20). The ATP7B gene is expressed in the liver and brain, and the full-length mRNA transcript encodes the WD protein. However, alternative splicing of the same gene, particularly in the brain, have been reported (21). In 1999 Borjigin and his colleagues identified a pineal night-specific ATPase (PINA), a splice variant of the ATP7B gene in rats (22). The defect of the ATP7B gene in the liver results in reduced excretion of copper into bile, and consequently in the accumulation of copper and organ damage in the liver, brain and other organs (23). The copper transporting ATPase is needed for the biliary excretion of copper as well as the incorporation of copper into ceruloplasmin (24).

1.3.2 Inheritance

Wilson disease is a fully-penetrant, autosomal recessive, inherited disease, which means that the patient have to be homozygous or a compound heterozygote for harmful mutations in ATP7B gene, in order to develop the disease. However, not all mutations are complete gene knockouts, which accounts for the wide range of severity of the disease. Furthermore, it has recently been suggested that WD on occasion may become a dominant disease (25).

1.4 Pathophysiological mechanisms

1.4.1 Copper homeostasis versus toxicity

Copper is a trace element, which is essential for a number of biological reactions, including important metalloenzymes, such as tyrosinase and cytochrom oxidase; and copper also plays a role in many processes, including mitochondrial energy generation, melanin formation, scavenging of oxygen radicals and the cross-linkage of collagen and elastin (26). Copper is also a co-factor of Cu/Zn- superoxide-dismutase, which is essential for the cellular response to oxidative stress by scavenging reactive oxygen species. In addition, copper is a constituent of the dopamine-ß-hydroxylase, an important enzyme in the catecholamine biosynthetic pathway.

Normal copper homeostasis is maintained by the balance between intestinal absorption and biliary excretion. Metallothionein, which is a cystein-rich zinc- and copper-
binding protein, plays a crucial role in the regulation of copper absorption (27, 28). However, the regulation of copper excretion via bile is not completely understood.

Since copper is an essential trace element, a certain amount is required in the diet, which normally contains about 1.00 mg/day copper. However, the daily intake is a little more than it is required, and the excess copper must be eliminated. Under normal conditions, the majority of copper is tightly bound to the ceruloplasmin protein, whereas a small amount of copper is loosely bound to plasma albumin. Ceruloplasmin contains 0.3% copper. The concentration of plasma free copper is 0.1 mg/L (29).

In WD patients free copper concentration in plasma is approximately 0.30 mg/L (30). As a result of positive copper balance, copper accumulates in most organs, primarily in the liver and the CNS. The toxicity of copper is related to the induction of free radical formation, which causes cell injury, inflammation and finally cell death. Copper is also harmful to mitochondria and inhibits numerous enzyme activities, e.g. glutathione reductase (31).

1.4.2 Animal model of Wilson disease

Long-Evans-Cinnamon (LEC) rat, which is the animal model of Wilson disease, provides the possibility to study various aspects of WD. These rats are afflicted by copper toxicosis, which is inherited in an autosomal recessive fashion (32, 33). Kolberg (34) used this rat model to investigate therapeutic strategies, McQuaid and Mason (35) compared the effects of zinc, tetrathiomolybdate, penicillamine and trientine.

1.5 Clinical presentation

The three major types of clinical presentation for WD are hepatic, neurological and behavioral. However, the clinical presentation of one patient may completely differ from that of another patient. The relationship between genotype and phenotype is still under investigations.

1.5.1 Liver pathology

Since the copper accumulation occurs in the liver initially, the disease often starts with hepatic symptoms; therefore, during childhood and the teenage years it is the most common manifestation. The youngest case in the literature was a 3-year old girl with severe hepatic presentation (36). The liver involvement may range from mild hepatitis, fulminate hepatic failure to chronic hepatitis and cirrhosis.

One fourth of all Wilson patients experienced sometime in their lives an acute episode of hepatitis, presenting with non-specific symptoms (malaise, anorexia, epigastric pain, jaundice, elevated LFT) without viral markers or history of a toxic agent. It is
important to rule out Wilson disease in cases with non-viral acute hepatitis, especially if mild hemolysis and low uric acid level are also present. Fulminate hepatitis is a relatively rare, severe disease, which is often lethal. It usually presents during the teenage years or young adulthood with symptoms of rapidly progressing acute hepatitis (deep icterus, encephalopathy, bleeding disorders, terminal renal failure, hepatic coma). Chronic hepatitis is the most common liver pathology in WD. The liver biopsy specimen reveals non-specific changes of chronic inflammation, intranuclear glycogen and periportal steatosis. The staining of the copper associated protein is usually positive. Measurement of the hepatic copper content aids in establishing the definitive diagnosis. The hepatic disorder usually progresses to cirrhosis, which may be either silent or may show the signs of cirrhosis. All patients with cirrhosis under 50 should be screened for Wilson disease.

1.5.2 Neuropathology

The neurological symptoms of WD are numerous and diverse, which are present from childhood, but most commonly after the age 20. They may be present without hepatic involvement. The earliest presentation of neurological symptoms reported was in a 6 year-old patient (37).

In the CNS copper damages prominently the subcortical nuclei, resulting in dysarthria, movement disorders, choreoathetoid symptoms, tremor, etc. Patients with neurological symptoms are often misdiagnosed as juvenile Parkinson disease or multiple sclerosis. In addition, many patients present some of the following symptoms: rigidity, dysdiadochokinesia, posture abnormality, gait abnormality, micro- or macrographia, facial expression abnormality, drooling, dysphagia, bradykinesia, diplopia or other abnormalities of eye movements. Occasional signs, not useful diagnostically, may be headaches, seizures and unprovoked, exaggerated emotional responses (12). Patients under 50 with dysarthria, tremor, dystonia or incoordination should always be screened for Wilson disease.

1.5.3 Psychopathology

Most patients present with behavioural changes, which include loss of ability to focus mentally (difficulties in school and at work), loss of control of emotions (temper tantrums, anger, bouts of crying) depression, loss if inhibition (may lead to exhibitionism), insomnia, anxiety, psychotic manifestations (hallucinations, delusions, catatonia) (12). The frequency and the time point when patients exhibit psychiatric and psychological symptoms vary from study to study.

Moreover, a number of psychiatric disturbances have been reported, including anxiety, affective disorders and psychotic disorders. Personality changes, incongruous
behaviour, aggression, irritability, antisocial behavior and cognitive impairment have also been reported.

1.5.4 Pathology of the skeletal system

Skeletal changes often develop in Wilson patients. Mindelzung and his colleagues found demineralization, subarticular cysts, marginal fragmentation in 33 out of 38 patients (38). Aksoy reported that the bone changes ranged from mild to severe and appeared to be age related (39). The changes they observed included osteochondritis dissecans and subchondral changes, marginal bone fragmentation, cystic changes, renal rickets and Milkman pseudofractures. Other changes, such as osteomalacia, osteoporosis, osteochondritis of spine, osteoarthritis, spontaneous fractures and para-articular calcifications, angulation of carpal bones, squaring of metacarpal heads have also been described (38, 39, 40). Many patients develop a type of osteoarthritis, particularly of the knees, but also involving other joints (41, 42, 43, 44, 45). The pathologic changes are non-specific, but there are increased levels of copper present in the joint tissue (45).

1.6 Diagnosis

Although Wilson disease is present from the first day of life, the disorder usually remains unrecognized until the development of clinical symptoms. The symptoms develop due to the toxic effect of copper accumulating mainly in the liver and central nervous system. Physicians should have WD in the differential diagnosis when patients under 50 years of age present with elevated liver enzymes, and/or parkinsonian symptoms, and/or behavioral changes. Therefore, it is necessary to screen for WD every patient under 50 with neurological disorder (dysarthria, tremor and other involuntary movements, dystonia, incoordination), hepatic disorders (hepatitis: viral negative acute or chronic; cirrhosis: any patient under 50 even with history of alcoholism or hepatitis C infection; hepatic failure), and behavioral disturbances (loss of ability to focus mentally on tasks, loss of control of emotions, depression, loss of inhibitions, insomnia, anxiety and psychotic manifestations).

1.6.1 Screening methods

The 24-hour urine copper assay. This is a very useful screening tool, which is diagnostic approximately 75% of the time. Normal values range between 20 to 50 µg/24h. For symptomatic affected patients this value is usually above 100 µg/24hr, for presymptomatic affected patients > 65 µg/24h, and for carriers < 100 µg/24h (< 65 µg/24h in 90% of cases).
**Kayser-Fleischer ring exam.** This is also very useful; however the exam must be performed properly by an experienced ophthalmologist with slit-lamp examination. Kayser-Fleischer ring is present in 90% of patients with neurological symptoms, but only 30% of patients with hepatic symptoms. Therefore, the lack of the presence of this ring does not rule out WD. Moreover, KF ring may seldom be present with long standing cholestatic diseases, such as primary biliary cirrhosis.

**Serum ceruloplasmin.** This method is helpful if serum level is very low, and useful only to increase index of suspicion. Normal serum ceruloplasmin is 0.2-0.6 mg/L. For Wilson patients the level is often below 0.1 mg/L, however, in about 15% of patients the level is normal. Twenty percent of carriers have also low ceruloplasmin levels.

**Other tests.** Serum copper by itself is not very useful and sometimes confusing. The value can be normal, low or high. Most of our patients, however, had low levels of serum copper. Another test is the d-penicillamin provocative test. In this procedure 24-hour urinary copper is measured after a dose of d-penicillamine. Unfortunately, the test is not adequately standardized, and diagnostic values are not established.

1.6.2 **Definitive diagnosis**

**Haplotype analysis.** Since Wilson disease is inherited by an autosomal recessive pattern, showing the 2 mutations on each chromosome is adequate by itself in making the diagnosis. However, more than 200 genetic causing WD mutations have been identified (46); therefore without currently available DNA chip for WD it makes it rather difficult to identify all the possible mutations for each patient and each WD suspect. Nevertheless, the H1069Q mutation is the most common mutation of the region of Hungary. Thus, we determined the presence of this mutation for each patient. More than sixty percent of the patients were either homozygous or heterozygous for this mutation. Even if the patient was heterozygous, with the presence of typical clinical symptoms and signs, this test was extremely useful in establishing diagnosis.

**Liver biopsy.** This diagnostic tool with measurement of quantitative hepatic copper levels remains the gold standard for definitive diagnosis. Hepatic copper level of 200 µg/g dry weight of tissue or more is diagnostic of WD in the absence of long standing hepatic failure or obstruction (47). Normal levels are 20-50 µg/g, and carriers may have elevated levels, but never higher than 125 µg/g. However, this technique is not widely used, and is unavailable in Hungary. Another helpful method is a semi-quantitative rhodamine dye to determine the presence of copper in the biopsy specimen. This method should be used
cautiously, because it shows the copper only in the intracellular organelles and not in the cytoplasm, as it is in some patients. Therefore, negative finding does not rule out WD.

1.7 Treatment

Once the diagnosis is made, it is imperative that patients go on appropriate anticopper drug therapy and stay on such therapy for the rest of their lives. The available anticopper agents are summarized in Table 1.7.1.

In the literature, it is recommended to use a chelator agent as an initial therapy for 4-6 months, and continue with zinc for maintenance therapy (48). Although d-penicillamine was the gold standard of treatment for many years, it is not recommended any longer to use as a chelator agent due to its toxicity. Because of the difficulty of availability of other agents in Hungary we treated our patients with d-penicillamine and none of the patients developed serious drug toxicity. Elastosis serpiginosa cutis was observed in a patient, who was misdiagnosed and incorrectly treated with d-penicillamine for WD.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Adult dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc acetate</td>
<td>Intestinal metallothionein induction and prevention of copper absorption</td>
<td>Effective, non-toxic, easily monitored</td>
<td>Slow-acting, occasional gastric intolerance</td>
<td>3x50 mg, separate from food</td>
</tr>
<tr>
<td>(GALZIN)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trientine</td>
<td>Chelator, enhances urinary excretion of copper</td>
<td>Effective, moderately fast-acting</td>
<td>Moderately toxic, teratogenic in animals</td>
<td>1g/day in 2-4 doses, separate from food</td>
</tr>
<tr>
<td>(SYPRINE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetrathiomolybdate</td>
<td>Complexes copper with drug and protein, rendering it non-absorbable and non-toxic</td>
<td>Effective, very fast-acting, little toxicity</td>
<td>Not commercially available, no studies for maintenance use</td>
<td>3x20mg with meals, 3x20mg between meals</td>
</tr>
<tr>
<td>Penicillamine</td>
<td>Chelator, enhances urinary excretion of copper</td>
<td>Effective, fast-acting</td>
<td>Acute, subacute and chronic toxicities, neurologic symptoms may worsen on initial therapy</td>
<td>1g/day in 2-4 doses, separate from food</td>
</tr>
<tr>
<td>(BYANODINE, CUPRIMINE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.7.1 Anticopper drugs used in WD (12)
1.8 Prognosis

“It is unfortunate to have a genetic disease, but if you’re going to have one, Wilson’s is a good one to have, because it is so treatable”, according to Brewer (12). With the initiation of proper anti-copper agents, further toxicity of the copper is prevented; therefore, the disease will not progress. Patients with mild neurological symptoms often recover, and liver functions will also be normal. Patients even with cirrhosis can usually live an essentially normal life. However, patients with very severe neurological, psychiatric, or hepatic disability do not have good prognosis and has a greater risk of living less than the normal life span with poor quality of life. Thus, it cannot be emphasized enough how important it is to recognize the disease early.
2 AIMS

As I pointed out earlier the importance of early diagnosis of WD, we based our studies on the extrahepatic manifestation of the disease, contributing to make the correct diagnosis and aiding in screening and treating the already diagnosed patients as a whole. The first study involved skeletal changes and establishing possible mechanism of bone involvement; in the second study we investigated peripheral neuropathic changes in WD, which has just recently become the center of attention; in the third study with the aid of the Temperament and Character Inventory, and the Symptom Check List questionnaires we examined the temperament and character profile and psychiatric disturbances in WD; in the fourth study we measured the serum level of the newly discovered nociceptin and investigated its clinical significance.

2.1 Study I

As mentioned earlier, skeletal changes are common in WD. However, to describe the degree of the osteopathy, previous studies have not investigated the bone mineral density (BMD) and bone mineral content (BMC) in WD, which have recently become important parameters in the assessment of the bone quantity. Biochemical markers of bone turnover are also important in the assessment of osteoblastic and osteoclastic functions. Osteocalcin (OCN) is a non-collagenous protein secreted by osteoblasts and is widely accepted as a marker for osteoblastic activity (49) and hence, bone formation (50), whereas serum β-crosslaps (β-CTx), which is a collagen-degradation product is a marker of bone resorption (51).

The recent discovery of osteoprotegerin (OPG/OCIF/TNFR: osteoprotegerin, osteoclastogenesis inhibitory factor, tumor necrosis factor receptor) and its ligand (OPGL/ODF/RANKL: osteoprotegerin ligand, osteoclast differentiation factor, receptor activator of NF-kappaB ligand), which play a crucial role in the balance of bone remodeling, provides new insights into the regulation of osteoclastogenesis (52), bone formation and normal bone development. RANKL is an endogenous osteoclast-activating factor, secreted mostly by osteoblasts and T- and B-cells as well as monocytes. OPG acts as a decoy receptor for RANKL and prevents its function (53). Increased serum OPG levels are reported in renal osteodystrophy (54), rheumatoid arthritis (55), Cushing’s disease, HIV patients and common variable immunodeficiency (CVI) (56), in patients with advanced prostate cancer with bone metastasis (57) and in primary biliary cirrhosis (58). Reduced OPG levels were found in patients with multiple myeloma with lytic bone disease (59).
2.2 Study II

Wilson disease (WD) is characterized by toxic copper deposition in the liver and central nervous system (CNS). Although autonomic and peripheral neuropathy has been described earlier in chronic alcoholic (60, 61) and non-alcoholic hepatic disorders such as primary biliary cirrhosis (61, 62, 63), C viral hepatitis (61, 64) and extra-hepatic portal vein thrombosis (65), autonomic and peripheral nerve function in WD has just recently received attention (66, 67). The importance of impaired cardiovascular autonomic function lies in the fifth-fold increase in mortality (62). Cardiovascular autonomic neuropathy is associated with a high risk of unexpected death, possibly related to silent myocardial infarction, ischemia, cardiac arrhythmia and hypoxia (68). However, not only have the abovementioned, most severe complications attracted considerable attention, but also the less severe consequences of the impaired autonomic regulation, such as the attenuated circadian variation of blood pressure and heart rate due to sympathovagal imbalance (69, 70). The association between autonomic dysfunction, depressed heart rate variability (HRV) and cardiovascular diseases is obvious, and independent from other cardiovascular risk factors. Assessment of HRV is a sensitive method for early detection of autonomic neuropathy even if the standard cardiac tests are normal (71, 72).

Although copper toxicity in the CNS is well known and supported by many studies, the toxic effect of excessive copper in the peripheral neurons and the regulator centers of the autonomic nervous system have not yet been studied extensively. Our aim was to investigate whether Wilson disease is associated with peripheral and/or autonomic neuropathy and abnormal heart rate variability.

2.3 Study III

Patients usually present with hepatic or neuropsychiatric symptoms. The disease may manifest in any form of acute and chronic liver diseases. The neurological symptoms such as tremor, choreoathetoid movement disorders, dysarthria, and dysphagia are characteristic, but not disease specific. However, the initial presentation of WD may be limited to a wide range of psychiatric disorders such as anxiety (73), affective- (74, 75), psychotic-, and personality disorders (76, 77), hypersomnia (78), cognitive impairment (79, 80), abnormal behavior, such as aggression and hostility (81), without any other symptoms. These patients are often misdiagnosed and mistreated for years. On the other hand, correctly diagnosed Wilson patients’ emotional distress and other psychiatric conditions are often neglected.
In this study we investigated the temperament and character changes by Cloninger’s (82) Temperament and Character Inventory (TCI) and psychiatric symptoms by Derogatis’ (83) Symptom Check List (SCL-90-R) questionnaires. Furthermore, we studied the occurrence of manifested psychiatric diseases, such as anxiety, affective disorders and suicide. We also present 3 illustrative cases of Wilson disease with psychiatric disturbances, which were misdiagnosed at initial presentation.

2.4 Study IV

In animal studies localization of OP4 receptor in the central nervous system and the periphery (84) as well as pharmacological characterization of the orphanin FQ/nociceptin system in different animal tissues were extensively studied in the recent years (85). However, the role of nociceptin in human subjects and the clinical significance of the heptadecapeptide are still unclear. So far only four clinical studies on nociceptin were reported any of them dealing with chronic liver disorder patients. Although both the long and short splice variants of the OP4 receptor mRNA were demonstrated in rat liver by reverse transcriptase-polymerase chain reaction (86) there are no data about its physiological/pathophysiological role in the liver function. The aim of the present study was to examine whether the chronic liver damage in Wilson disease patients has any effect on plasma levels of nociceptin.
3 METHODOLOGY

3.1 Subjects

3.1.1 Patient population

Forty-two WD patients from the Hepatological Outpatient Unit of the Semmelweis University, Budapest were involved in the study. Abnormal liver tests and signs of liver disease without neurological disorder were present in 18; neurological symptoms, such as tremor, choreoathetoid movements, coordination disturbances, Parkinson like symptoms in 24, psychiatric and behavioral disturbances in 12 patients. In most patients characteristically more than one abnormalities were present. Kayser-Fleischer ring by slit-lamp examination, investigated by experienced ophthalmologist was present in 26 patients. The H1069Q gene mutation of ATP7B gene, which is the most common mutation in Hungary (87) among the more than 200 described mutations in Wilson disease (88) was assessed by a semi-nested polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay. 13 patients were homozygous, 21 patients were heterozygous and 8 were negative for H1069Q mutation.

All four study-protocols conform to the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the local Regional Committee of Science and Research Ethics.

3.1.2 The inclusion criteria

Patients with definitive diagnosis only were involved in the studies. The diagnosis of Wilson disease was based on characteristic clinical, laboratory findings, presence of Kayser-Fleischer ring and genetic testing. To confirm the diagnosis the presence of excessive copper accumulation in the liver biopsy specimen and increased 24-hour urinary copper excretion were required in some cases. Serum ceruloplasmin level was low in each patient.

3.1.3 Wilson patients in studies I-IV

I.

Twenty-one patients with Wilson disease (mean age: 30.8 yrs, range: 14-46) were involved in Study I. Abnormal liver tests and signs of liver disease without neurological disorder were present in 8; neurological symptoms, such as tremor, choreoathetoid movements, coordination disturbances, Parkinson like symptoms in 13, psychiatric and behavioral disturbances in 5 patients. 8 patients were homozygous, 7 patients were heterozygous and 6 were negative for H1069Q mutation.
II.

Twenty-two patients with Wilson disease (mean age: 32.4±11.8, range: 15-52) were involved in the study. Abnormal liver tests and signs of liver disease without neurological disorder were present in 8; neurological symptoms, such as tremor, choreoathetoid movements, coordination disturbances, Parkinsonian symptoms in 14; psychiatric and behavioral disturbances in 6 patients. All but 1 patient demonstrated hepatic damage with laboratory testing, abdominal ultrasound or liver biopsy. Six patients were homozygous, 11 patients were heterozygous and 5 were negative for H1069Q mutation.

III.

Thirty patients with Wilson disease (mean age: 33±12 years, range: 14-55; 13 females and 17 males) were involved in the study. Five patients were homozygous, 14 patients were heterozygous and 11 were negative for H1069Q mutation. In 11 patients abnormal liver tests and/or signs of liver disease were present without neurological disorder. In 19 patients predominantly neurological symptoms, such as tremor, choreoathetoid movements, coordination disturbances, Parkinson like symptoms were present with or without signs of liver disease. Psychiatric and behavioral disturbances with social malfunctioning were observed in 7 patients.

IV.

Twenty patients with Wilson disease (7 female and 13 male, age 32±13 years, mean duration of d-penicillamine treatment 110.7±163.6 months) were involved in this study. Abnormal liver tests and signs of liver disease were present in 12, neurological symptoms in 6, psychiatric and behavioral disturbances in 3 patients.

3.1.4 Comparison groups in studies I-IV

I.

The control group was recruited from 20 age and gender matched (10 males and 10 females) individuals with mean age of 28.3±8 years (range: 19-51). We also involved a second control group, which comprised 74 post-menopausal osteopenic, otherwise healthy women (mean age: 55.7, range: 47-71). The reason for the latter control group was to assess whether elevated osteoprotegerin level is associated merely with osteopenia, or it is a consequence of WD.

II.

The control group was recruited from 15 age and gender matched individuals with mean age of 28.3±8 years (range: 19-51). The controls included in this study did not suffer
from cardiac disease, hypertension or diabetes mellitus, nor did they take beta-blockers, anti-arrhythmics or ACE-inhibitors.

III.

Thirty healthy age and gender-matched volunteers with different social economic status and educational background, without any history of psychiatric disorders from the medical staff and from healthy volunteers, who appeared for a physical check-up, were recruited (15 female and 15 male, mean age 36.6±11.2 years).

IV.

The group of twenty-five healthy subjects (11 female and 14 male, age 36?14 years) were recruited from blood donors and members of the staff served as control.

3.2 Methods

3.2.1 Bone densitometry and quantitative ultrasound

Bone mineral density (g/cm2), assessed by Norland XR26 (USA) dual energy x-ray absorptiometry (DXA), was performed at the lumbar 2-4 vertebrae (L2-4) and the left femoral neck (FN); BMD, Z-score, T-score were determined for each patient and control. The distal radius was investigated by measurements of bone mineral content (g/cm) by single photon absorptiometry (SPA) method (Gamma NK-364, Hungary). Heel broadband ultrasound attenuation (BUA) and speed of sound (SOS) was assessed by DTU-1 (Osteometer MediTech, Hawthorne, CA, USA) device.

According to the WHO guidelines (89), osteoporosis is defined as the BMD or BMC values being 2 standard deviation or more below the average values of the age matched healthy population (Z-score=-2) (90). The same criterion (Z-score=-2) was used for the QUS parameters. However, there are no QUS guidelines for diagnosing secondary osteoporosis in young patients, although there are several studies involving postmenopausal osteoporosis (91, 92, 93).

3.2.2 Laboratory testing of liver enzymes, osteocalcin β-crosslaps

Laboratory testing was performed by Olympus AU600 autoanalyser (Olympus Ltd, Japan). Serum bilirubin and the liver function tests (AST, ALT, GGT and alkaline phosphatase) as well as the serum calcium and phosphate levels were investigated in each patient. We also measured the osteocalcin level for each patient and control with a commercially available N-MID Osteocalcin Electrochemiluminescence Immunoassay (ECLIA) kit (Roche Diagnostics GmbH, Mannheim, Germany), as well as determined the serum levels of type 1 collagen cross-linked C-telopeptide (β-CTx) by the Elecsys β-
Crosslaps CalSet commercially available immunoassay kit (Roche Diagnostics GmbH, Mannheim, Germany). Both measurements were performed for patients and controls by Elecsys 2010 immunoassay system (Hitachi, Japan).

3.2.3 OPG level measurement

For the measurement of serum OPG blood was drawn from fasting subjects into 5ml vacuum containers with added potassium-EDTA as anticoagulant. Blood was centrifuged at 3000 rpm for 10 minutes at 4°C (Rotina 35 R, Germany) and the plasma was collected in 2 mL plastic tubes. After the separation of the plasma, we stored the samples at –20 °C until the measurement. Serum OPG level was measured with a commercially available ELISA kit according to the protocol of the manufacturer. (Osteoprotegerin Kit; Biomedica GmbH, Germany). The assay was performed blind to the subject group. The absorption was determined with an ELISA reader (Labsystems Multiscan MS, Finland) at 450 nm against 690/620 nm.

3.2.4 RANKL level measurements

The same serum samples were used for RANKL measurements as for OPG measurements. Serum RANKL level was also measured with a commercially available ELISA kit according to the protocol of the manufacturer. (sRANK Kit; Biomedica GmbH, Germany). The assay was performed blind to the subject group. The absorption was determined with an ELISA reader (Labsystems Multiscan MS, Finland) at 450 nm against 690/620 nm.

3.2.5 Autonomic function assessment

The autonomic nervous system was assessed by the five standard cardiovascular reflex tests. The parasympathetic function was investigated by heart rate variations during deep breathing, Valsalva-maneuver and after standing up (30/15 ratio = the ratio of 30th and 15th RR intervals after standing up from supine position). The sympathetic function was measured by the blood pressure changes during sustained handgrip test and after standing up (94).

3.2.6 Peripheral sensory nerve function assessment

The sensory function was studied by the Neurometer® device (Neuroton Incorporated, Baltimore, USA). This method provides a simple, non-invasive and quantitative measurement of peripheral sensory nerve function. Detection thresholds (Current Perception Threshold, CPT) for constant current electric sine wave stimulation were measured at three different frequencies (2000, 250, 5 Hz) on index finger (median nerve) and on great toe (peroneal nerve) on both left and right sides. According to previous studies high
frequency CPT correlates best with tests of large fiber function, while low frequency CPT correlates with tests of small fiber function: 250 Hz with small myelinated, 5 Hz with small unmyelinated fibers (95, 96). We examined the peroneal nerve and the median nerve by putting a pair of (1 cm in diameter) electrodes symmetrically on the distal phalanx of the hallux and the index finger. The current being used was between 0.01 and 9.99 mA. During the test the current was slowly and gradually increased until the patient indicated perception. The measurements were repeated several times to assess the CPT. Incorrectness resulting from the subjectivity was significantly decreased by a built in, double blind evaluation (“forced choice method”).

3.2.7  **Heart Rate Variability (HRV)**

24-hour ambulatory blood pressure monitoring (ABPM) and HRV analysis were performed by Cardiotens 1.34 (Meditech, Hungary) device. HRV was characterized by time domain and frequency domain methods. In the time domain we calculated 24-hour standard deviation of all RR intervals (SDNN), the standard deviation of the averages of RR intervals in all 5-minute segments of the entire recording (SDANN), the proportion of adjacent RR intervals differentiated by more than 50 milliseconds (pNN50), and the root-mean square difference (r-MSSD) of successive RR intervals. The latter two parameters are considered to reflect the vagal tone of the heart (97).

In the frequency domain we evaluated the total power (TP), the low frequency (LF) and the high frequency (HF) components, as well as the LF/HF ratio. The efferent vagal activity is a major contributor to the HF component, while LF component is influenced by both sympathetic and parasympathetic stimuli. The LF/HF ratio is considered to be a marker for sympathovagal balance.

The following ABPM parameters were evaluated: the systolic and diastolic blood pressure means (RRS and RRD), the systolic and diastolic hypertensive time indices (HTIS and HTID), and the systolic and diastolic hyperbaric impact (HBIS and HBID) values.

3.2.8  **Temperament and Character Inventory (TCI)**

We determined the temperament and character features according to the Hungarian version of the TCI questionnaire (82, 98), which consists of 240 statements. The patients had to give “Yes” or “No” answers to each, according to which we analyzed 4 main temperament (novelty seeking=NS, harm avoidance=HA, reward dependence=RD, persistence=P), and 3 main character (self-directedness=S, cooperativeness=C, self-transcendence=ST) profiles. The temperament and character profiles are summarized in Table 3.2.8.
<table>
<thead>
<tr>
<th>Temperament and character profile</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NS</strong>  ?</td>
<td>difficult to inspire, stoical, considerate, systematic, prefers monotony</td>
</tr>
<tr>
<td>NS1  ?</td>
<td>prefers well known places, cases and people; avoids uncertainty; resistant for new ideas; has difficulty in changing the routine; seldom gets bored</td>
</tr>
<tr>
<td>NS2  ?</td>
<td>considerate, rarely acts based on emotions or insights; carefully examines and analyses the information before making a decision or opinion</td>
</tr>
<tr>
<td>NS3  ?</td>
<td>reserved, formal, does not waste money, energy or time</td>
</tr>
<tr>
<td>NS4  ?</td>
<td>orderly, punctual, systematic; likes to act according to rules</td>
</tr>
<tr>
<td><strong>HA</strong>  ?</td>
<td>timid, worrisome, over-anxious, indecisive, shy, rejecting, passive, negativistic, pessimistic, has low energy level, easily gets exhausted</td>
</tr>
<tr>
<td>HA1  ?</td>
<td>pessimistic, worries about the future, feel uncertain when getting in a strange situation; has difficulty getting over unpleasant experiences</td>
</tr>
<tr>
<td>HA2  ?</td>
<td>does not tolerate doubtfulness; does not take risks</td>
</tr>
<tr>
<td>HA3  ?</td>
<td>timid in most social events; often avoids meeting strangers; has difficulty in establishing relationships</td>
</tr>
<tr>
<td>HA4  ?</td>
<td>weak, has less energy than others; needs extra rest, gets tired easily; needs more time to recover from illnesses and stress</td>
</tr>
<tr>
<td><strong>RD</strong>  ?</td>
<td>not flexible, cool, socially not sensitive, independent, not willing to make contacts</td>
</tr>
<tr>
<td>RD1  ?</td>
<td>practical, undistractable, sometimes cynical, seldom sentimental</td>
</tr>
<tr>
<td>RD2  ?</td>
<td>unsociable, indifferent; prefers not to share his feelings and experiences with others</td>
</tr>
<tr>
<td>RD3  ?</td>
<td>does not consider other people’s opinion; does not care what others do</td>
</tr>
<tr>
<td><strong>P</strong>  ?</td>
<td>does not strain himself even if there is a reward; does only what is absolutely necessary, has difficulty in starting something new; easily gives it up; usually satisfied with his situation; often willing to compromise</td>
</tr>
<tr>
<td><strong>S</strong>  ?</td>
<td>has low self-esteem; does not care about other people’s problem; has no goals, has no new ideas; often depends on others; sometimes feels inferior</td>
</tr>
<tr>
<td>S1  ?</td>
<td>tries to escape responsibility; often blames the environment</td>
</tr>
<tr>
<td>S2  ?</td>
<td>less capable of giving a reason, goal and direction to his life; lives for the moment</td>
</tr>
<tr>
<td>S3  ?</td>
<td>lacks inventiveness; shows helplessness; relies on others</td>
</tr>
<tr>
<td>S4  ?</td>
<td>not satisfied with his own physical and intellectual state</td>
</tr>
<tr>
<td>S5  ?</td>
<td>undetermined, even though he knows what to do</td>
</tr>
<tr>
<td><strong>C</strong>  ?</td>
<td>less cooperative, intolerant</td>
</tr>
<tr>
<td>C1  ?</td>
<td>intolerant, unfriendly, critical toward others</td>
</tr>
<tr>
<td>C2  ?</td>
<td>not interested in other people’s feelings; incapable of sharing happiness or suffering</td>
</tr>
<tr>
<td>C3  ?</td>
<td>self-centered, egoistic, only cares about himself in a group setting</td>
</tr>
<tr>
<td>C4  ?</td>
<td>enjoys avenge; can be actively or passively aggressive</td>
</tr>
<tr>
<td>C5  ?</td>
<td>opportunistic; acts in accordance with his own interests</td>
</tr>
<tr>
<td><strong>ST</strong>  ?</td>
<td>proud, does not rely on imagination, impatient, cannot tolerate natural things, surprises</td>
</tr>
<tr>
<td>ST1  ?</td>
<td>incapable of abstraction, does not notice the natural beauty of thing, does not appreciate art, his imagination is limited</td>
</tr>
<tr>
<td>ST2  ?</td>
<td>does not like nature; does not feel that he can change the world</td>
</tr>
<tr>
<td>ST3  ?</td>
<td>materialistic, thinks empirically, does not accept things that cannot be explained scientifically</td>
</tr>
</tbody>
</table>

**Table 3.2.8. Temperament and character profiles according to the TCI (NS=novelty seeking, HA=harm avoidance, RD=reward dependence, P=persistence, S=self-directedness, C=cooperativeness, ST=self-transcendence)**
3.2.9 Symptom Check List (SCL-90-R)

The clinical symptom profile was assessed by the Hungarian version of the 90-item containing SCL-90-R questionnaire to screen for 9 primary scales: somatization (Som), obsessive-compulsive (OC), interpersonal sensitivity (IS), depression (Dep), anxiety (Anx), hostility (Host), phobic anxiety (Phob), paranoid ideation (Para) and psychoticism (Psy) (83). It is a widely used questionnaire for self-report of psychological distress and multiple aspects of psychopathology. The patients had to give 0-4 numbers to each question and statement (0:”not at all”, 4:”extremely”). By combining the 9 scales we assessed the Global Severity Index (GSI), which is a global index of distress and psychological dysfunction. We also report the Total of Positive Symptoms (PST) and the Positive Symptom Distress Index (PSDI). In every scale a score higher than 1 indicates psychopathological distress in that area, which is severe if the score exceeds 2.

3.2.10 Extraction and radioimmunoassay of nociceptin

Blood drawn from fasting subjects between 8.00-10.00 am was collected in 6 ml vacutainers containing K-EDTA as anticoagulant. Aprotinin (0.6 TIU/ml of blood, Calbiochem) was added immediately as a protease inhibitor. Blood was centrifuged at 1600 g for 15 minutes at ?4°C (Janetzky K70, Germany) and the plasma was collected in mini-sorb tubes (OMKER, Hungary). Samples were kept frozen at ?80°C until direct analysis by radioimmunoassay. 1.0 ml aliquots of plasma samples were mixed with equal volume of trifluoroacetic acid (TFA; 1? v/v) and centrifuged at 1600g for 20 minutes at ?4°C. Acidified plasma samples were loaded onto C18 Sep-Pack cartridges (ABL? E JASCO Magyarország Kft) and washed twice with 1.0% TFA, eluted with 60% acetonitrile in 0.1? TFA, then freeze dried (SAVANT, USA). The reconstituted elute was subjected to radioimmunoassay using a commercially available 125I-Nociceptin kit (DRG, Germany) with minimum sensitivity of 1pg/ml. The assay was performed blind to the subject group.

3.2.11 Statistics

OPG levels were compared with Kruskal-Wallis one way ANOVA on ranks and Dunn’s method was used for the pair-wise multiple comparison procedures. OCN, β-CTx and RANKL levels in Wilson patients were compared to the healthy controls with Mann Whitney Rank Sum Test. Associations between age, bilirubin, liver function tests, osteocalcin, β-crosslaps, osteoprotegerin, RANKL level, and bone mineral density were tested with Spearman Rank Order correlation. Data are presented as mean ± standard deviation if otherwise not stated.
The significance of the difference of the CPT, the autonomic reflex tests and heart rate variability between Wilson patients and the control group was assessed by the t-test and the Mann-Whitney Rank Sum Test. The comparison of the patients with neurological, the patients without neurological symptoms and the controls was done by one way ANOVA and Kruskal-Wallis one way ANOVA on ranks. In the first case the Bonferroni t-test, in the second case the Dunn’s method was used for the pairwise multiple comparison procedures. Data are presented as mean ± standard deviation if otherwise not stated. Correlations were determined by Kendall Tau Correlations. Tau and p is indicated.

T-test and Mann-Whitney Rank Sum Test were performed for statistical analysis of the temperament and character parameters and of the symptom scales between the patients and the controls, and in the male and the female groups. The comparison of the predominantly neurological, predominantly hepatic symptoms presenting patients and the controls was done by one way ANOVA and Kruskal-Wallis one way ANOVA on ranks. In the first case the Bonferroni t-test, in the second case the Dunn’s method was used for the pair-wise multiple comparison procedures. Data are presented as mean ± standard error of mean (SEM) if otherwise not stated.

Comparison of the plasma nociceptin concentration between patients and controls was made using Mann-Whitney Wilcoxon Rank Sum Test. The level of significance was set at p<0.05. Correlation between NC level and liver function test results was evaluated by Spearman Rank Order correlation.
4 RESULTS

4.1 Skeletal disorders

4.1.1 Densitometry and quantitative ultrasound

According to the densitometric measurements, osteoporosis was found in 9 (43%) out of 21 Wilson patients. In patients <40 years 4/14 (29%), in patients =40 years 4/7 (71%) had Z-score=-2. According to the QUS values 7/21 (33%) patients had BUA Z-score=-2. Heel SOS Z-scores were in the normal range for each patient. The mean ± standard deviation for BMD and BMC score is summarized in Table 4.1.1a, and the QUS parameters are summarized in Table 4.1.1b. Bone density parameters did not correlate with age or liver function tests.

4.1.2 Laboratory liver tests

Most patients had normal or mildly elevated serum liver enzyme level, the serum bilirubin was in the normal range. The calcium and phosphate levels were in the reference range for each patient.

<table>
<thead>
<tr>
<th>Densitometric parameters</th>
<th>mean±SD</th>
<th>No. of patients with Z-score=-2 (age&gt;40) (n=7)</th>
<th>No. of patients with Z-score=-2 (age&lt;40) (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumbal 2-4 BMD (g/cm²)</td>
<td>1.08±0.22</td>
<td>5/7 (71%)</td>
<td>4/14 (29%)</td>
</tr>
<tr>
<td>T-score</td>
<td>-0.59±1.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-score</td>
<td>-0.51±1.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur BMD (g/cm²)</td>
<td>0.99±0.10</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>T-score</td>
<td>-0.53±1.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-score</td>
<td>-0.31±1.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radius BMC (g/cm)</td>
<td>0.96±0.29</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>T-score</td>
<td>-0.92±1.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-score</td>
<td>-0.87±1.37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1.1a Bone mineral density (g/cm²) and bone mineral content (g/cm) parameters in Wilson disease patients (n=21) sorted according to age
<table>
<thead>
<tr>
<th>QUS parameters (measured in 21 Wilson patients)</th>
<th>mean±SD</th>
<th>No. of patients with Z-score=-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUA (dB/MHz)</td>
<td>44.1±7.1</td>
<td></td>
</tr>
<tr>
<td>T-score</td>
<td>-1.84±1.07</td>
<td></td>
</tr>
<tr>
<td>Z-score</td>
<td>-0.92±1.72</td>
<td>7/21 (33%)</td>
</tr>
<tr>
<td>SOS (m/s)</td>
<td>1562±9</td>
<td></td>
</tr>
<tr>
<td>T-score</td>
<td>-0.10±0.69</td>
<td></td>
</tr>
<tr>
<td>Z-score</td>
<td>0.29±0.76</td>
<td>0/21</td>
</tr>
</tbody>
</table>

**Table 4.1.1b** Quantitative ultrasound parameters in patients with Wilson disease (n=21).

### 4.1.3 Osteocalcin and β-crosslaps

Serum levels of osteocalcin did not differ statistically in patients and controls (29.93 ± 24.65 ng/mL, 29.84 ± 6.89 ng/mL, respectively). However, β-CTx levels were significantly higher in Wilson patients (625.4 ± 312.3 pg/mL, p=0.022) compared to the controls (423.6 ± 144.3 pg/mL). Moreover, OCN levels correlated with β-CTx levels in Wilson patients (r=0.55, p=0.01). The correlation is illustrated in Figure 4.1.3.

![Figure 4.1.3](image)

**Figure 4.1.3** Correlation between serum osteocalcin (OCN) and serum β-crosslaps (β-CTx) in Wilson patients (Spearman correlation, r=0.55, p=0.01)

### 4.1.4 Osteoprotegerin

We found that serum osteoprotegerin level was significantly elevated in Wilson patients (7.2 ± 3.4 pmol/L, p<0.001) compared to both healthy controls (3.5 ± 1.0 pmol/L)
and osteopenic, otherwise healthy controls (4.0 ± 1.0 pmol/L p<0.001) Results are illustrated in Figure 4.1.4. OPG did not show correlation with neither bone density scores, nor OCN and ß-CTx levels.

4.1.5 RANKL

On the other hand, there was no statistical difference in serum RANKL level in Wilson patients (0.6 ± 1.2 pmol/L) compared to healthy subjects (0.2 ± 0.4 pmol/L). In 15 individuals from the 21 Wilson patients and in 14 controls RANKL level was undetectable, what we considered 0 pmol/L RANKL concentration. There was no correlation between RANKL levels and OPG levels, bone density parameters, OCN and ß-CTx levels. No RANKL measurements were performed in the osteopenic, otherwise healthy control group. Osteocalcin, ß-crosslaps, osteoprotegerin and RANKL values are summarized in Table 4.1.5.

![Figure 4.1.4](image-url)

**Figure 4.1.4** Serum OPG in the healthy controls (3.5±1.0 pmol/L), osteopenic otherwise healthy controls (4.0±1.0 pmol/L) and Wilson patients (7.2±3.4 pmol/L)

<table>
<thead>
<tr>
<th></th>
<th><strong>WD patients</strong> (n=21)</th>
<th><strong>Controls</strong> (n=20)</th>
<th><strong>p</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>OCN (NG/ML)</td>
<td>29.93 ± 24.65</td>
<td>29.84 ± 6.89</td>
<td>ns</td>
</tr>
<tr>
<td>ß-CTx (pg/mL)</td>
<td>625.4 ± 312.3</td>
<td>423.6 ± 144.3</td>
<td>0.022</td>
</tr>
<tr>
<td>OPG (pmol/L)</td>
<td>7.2 ± 3.4</td>
<td>3.5 ± 1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RANKL (pmol/L)</td>
<td>0.6 ± 1.2</td>
<td>0.2 ± 0.4</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Table 4.1.5** Osteocalcin (OCN), ß-crosslaps (β-CTx), osteoprotegerin (OPG) and RANKL serum concentrations in Wilson patients and healthy controls (ns=not significant)
4.2 Peripheral and autonomic neuropathy

Two patients had abnormal cardiovascular reflexes in the autonomic function assessment: one of them showed parasympathetic, the other showed both, sympathetic and parasympathetic damage. One patient presented with damage of the large myelinated fibers on both upper extremities without autonomic function impairment.

When we compared all patients with WD to the healthy subjects, we found that, although the results were in the normal range in most cases, the sensory function of the lower extremities were reduced in WD compared to the healthy controls. The patients demonstrated an increased CPT, indicating hypoesthesia on 2000, 250 and 5 Hz (p=0.002) at the peroneal nerve testing. Results are shown in figure 4.2a. We found no differences in the cardiovascular reflex tests, the blood pressure parameters, or the HRV measurements between Wilson patients and the controls.

* p=0.002

**Figure 4.2.a** Peripheral sensory nerve function studies in WD and controls (CPT=current perception threshold) at different frequencies
To evaluate the possible relationship between the neurological symptoms and the autonomic dysfunction we divided the patients into 2 groups (patients with neurological symptoms n=14, and patients without neurological symptoms n=8). Both time domain and frequency domain parameters of HRV were significantly reduced in patients with neurological symptoms compared to those without these symptoms (p<0.05). The 30/15 ratio and the blood pressure response to sustained handgrip were also significantly lower in patients with neurological symptoms (p<0.05). Results are summarized in table 4.2b. There was no difference noted in the peripheral nerve conduction studies between the 2 groups with WD.

There was no difference in the blood pressure parameters between Wilson patients and the controls. However, in WD the LF/HF ratio positively correlated with the diastolic ABPM parameters (RRD and HTID: p=0.01; HBID: p=0.005) (table 4.2c).

<table>
<thead>
<tr>
<th></th>
<th>Patients with neurological symptoms (n=14)</th>
<th>Patients without neurological symptoms (n=8)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Handgrip (Hgmm)</td>
<td>21.5±5.5</td>
<td>28.7±3.5</td>
<td>0.022</td>
</tr>
<tr>
<td>30/15 (ratio)</td>
<td>1.2±0.2</td>
<td>1.4±0.2</td>
<td>0.034</td>
</tr>
<tr>
<td>LF (ms²)</td>
<td>924±670</td>
<td>1729±751</td>
<td>0.049</td>
</tr>
<tr>
<td>HF (ms²)</td>
<td>557±995</td>
<td>987±382</td>
<td>0.006</td>
</tr>
<tr>
<td>TP (ms²)</td>
<td>3413±2590</td>
<td>6489±2701</td>
<td>0.019</td>
</tr>
<tr>
<td>pNN50 (%)</td>
<td>9.9±11.3</td>
<td>19.6±9.4</td>
<td>0.041</td>
</tr>
</tbody>
</table>

**Table 4.2b** Comparison of HRV parameters between Wilson patients with neurological symptoms and patients without neurological symptoms (data±SD)

<table>
<thead>
<tr>
<th>ABPM parameters</th>
<th>HRV parameters</th>
<th>Kendall Tau</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRD</td>
<td>LF/HF</td>
<td>0.39</td>
<td>0.01</td>
</tr>
<tr>
<td>HTID</td>
<td>LF/HF</td>
<td>0.38</td>
<td>0.01</td>
</tr>
<tr>
<td>HBID</td>
<td>LF/HF</td>
<td>0.43</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Table 4.2c** Kendall Tau Correlations between heart rate variability (LF/HF ratio) and the diastolic ABPM parameters (RRD, HTID, HBID) in Wilson patients (n=22)
4.3 TCI and SCL-90

4.3.1 TCI

From the TCI questionnaire 2 main temperament (harm avoidance, persistence) and all 3 main character (self-directedness, cooperativeness, self-transcendence) parameters differed in Wilson patients from that of the healthy controls. Furthermore, we have found significant differences in 10 sub-parameters. There were no significant differences in 2 main temperament scales (novelty seeking, reward dependence) compared to the control group. Results are summarized in Figure 4.3.1a.

![Figure 4.3.1a](image)

**Figure 4.3.1a** TCI scores in Wilson patients and the control group (NS=novelty seeking, HA=harm avoidance, RD=reward dependence, P=persistence, S=self-directedness, C=cooperativeness, ST=self-transcendence)

We also compared the patients with predominantly neurological (n=19) symptoms to the patients with predominantly hepatic symptoms (n=11) and also to the control group. In this comparison we found significant differences in the harm avoidance, persistence, self-directedness and self-transcendence main parameters, and the HA4, RD1, S2, S3, C1, C3, C5 sub-parameters. Interestingly, in harm avoidance and HA4 scales the difference was significant between the predominantly neurological presentation of Wilson patients and the control group. However, in the self-directedness, cooperativeness and self-transcendence the differences were significant in the predominantly hepatic presentation of the patients compared to the control group. In persistence the difference was significant between the
neurological symptoms presenting patients and the hepatic symptoms presenting patients. The results are summarized in Table 4.3.1b.

<table>
<thead>
<tr>
<th></th>
<th>Controls mean±SEM</th>
<th>Predominantly neurological WD mean±SEM</th>
<th>Predominantly hepatic WD mean±SEM</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=30</td>
<td>n=19</td>
<td>n=11</td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>0.52±0.03</td>
<td>0.51±0.03</td>
<td>0.5±0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>HA</td>
<td>0.34±0.05</td>
<td>0.51±0.04</td>
<td>0.48±0.04</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>RD</td>
<td>0.65±0.03</td>
<td>0.61±0.03</td>
<td>0.59±0.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>P</td>
<td>0.51±0.04</td>
<td>0.45±0.06</td>
<td>0.21±0.06</td>
<td>p&lt;0.005⁺, c</td>
</tr>
<tr>
<td>S</td>
<td>0.75±0.03</td>
<td>0.64±0.04</td>
<td>0.60±0.04</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>C</td>
<td>0.82±0.01</td>
<td>0.71±0.03</td>
<td>0.72±0.03</td>
<td>p&lt;0.001⁺, c</td>
</tr>
<tr>
<td>ST</td>
<td>0.45±0.03</td>
<td>0.40±0.03</td>
<td>0.29±0.05</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

a: neurological vs hepatic, b: neurological vs controls, c: hepatic vs controls, n.s.: not significant

Table 4.3.1b. TCI scores in controls and subgroups of patients with Wilson disease. For abbreviations see Table 3.2.8.

We have also divided the patients according to their gender, and compared the male Wilson patients (n=17) to the male healthy volunteers, and the female patients (n=13) to the female controls. In the female group the difference was significant only in persistence, cooperativeness and C1 subscale, whereas in the male group the significant differences between the healthy individuals and the patients were numerous in various parameters (harm avoidance, HA4, reward dependence, RD3, self-directedness, S3, S4, cooperativeness, C1, C3, self-transcendence, ST2, ST3). The results are also summarized in Table 4.3.1c.

<table>
<thead>
<tr>
<th></th>
<th>Male WD patients mean±SEM</th>
<th>p-values (male WD patients vs male controls)</th>
<th>Female WD patients mean±SEM</th>
<th>p-values (female WD patients vs female controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=17</td>
<td></td>
<td>n=13</td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>0.51±0.04</td>
<td>n.s.</td>
<td>0.51±0.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>HA</td>
<td>0.46±0.04</td>
<td>p&lt;0.05</td>
<td>0.55±0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>RD</td>
<td>0.56±0.03</td>
<td>p&lt;0.05</td>
<td>0.65±0.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>P</td>
<td>0.38±0.07</td>
<td>n.s.</td>
<td>0.33±0.07</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>S</td>
<td>0.63±0.04</td>
<td>p&lt;0.005</td>
<td>0.61±0.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>C</td>
<td>0.70±0.03</td>
<td>p&lt;0.05</td>
<td>0.72±0.02</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>ST</td>
<td>0.35±0.04</td>
<td>p&lt;0.05</td>
<td>0.38±0.04</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

⁺ All male WD patients were compared to all male healthy controls (n=15)  
⁺⁺ All female WD patients were compared to all female healthy controls (n=15) (data of male and female controls are not shown).  
n.s.: not significant

Table 4.3.1c TCI scores in male and female WD patients. For abbreviations see Table 3.2.8.
4.3.2 SCL-90-R

In the SCL-90-R questionnaire we found significantly increased scores in Wilson patients in all but one symptom (Table 4.3.2b): somatization, obsessive-compulsive, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety and paranoid ideation (Figure 4.3.2a). Among the 30 patients 16 (53%) presented with somatization symptoms; 16 (53%) with obsessive-compulsive signs; 12 (40%) showed increased interpersonal sensitivity, 17 (57%) patients were found to have depression according to the SCL-90-R, 7 of whom had clinically manifest, previously diagnosed affective disorder, such as bipolar affective disorder or major depression, and 3 attempted suicide (10%); 17 (57%) patients were found to have anxiety, only one of whom was treated for it; 10 showed hostility (33%), 8 (27%) patient presented with phobic anxiety; 15 (50%) patients presented with paranoid ideation and 9 (30%) with psychoticism. Moreover, from the 30 patients 16 had elevated GSI scores, 4 of whom had scores higher than 2. When we divided the patients into groups with predominantly hepatic and predominantly neurological symptoms, the difference between the groups were not significant neither in the different symptoms nor in the GSI and PST scores. Results are shown in Table 4.3.2c.

Figure 4.3.2a SCL dimensions in Wilson patients and healthy controls (Som=somatization, OC=obsessive-compulsive, IS=interpersonal sensitivity, Dep=depression, Anx=anxiety, Host=hostility, Phob=phobic anxiety, Para=paranoid ideations, Psy=psychoticism, GSI=Global Severity Index)
Table 4.3.2b. Number of WD patients (n=30) with $1<\text{Score}<2$, and score=2 of the SCL-90-R (GSI=Global Severity Index).

<table>
<thead>
<tr>
<th></th>
<th>All Wilson mean±SEM $n=30$</th>
<th>Hepatic mean±SEM $n=11$</th>
<th>Neurological mean±SEM $n=19$</th>
<th>p-values (hepatic vs neurological)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSI</td>
<td>1.19±0.13</td>
<td>0.89±0.17</td>
<td>1.41±0.16</td>
<td>n.s.</td>
</tr>
<tr>
<td>PST</td>
<td>45.93±3.06</td>
<td>39±4.67</td>
<td>50±3.79</td>
<td>n.s.</td>
</tr>
<tr>
<td>PSDI</td>
<td>2.16±0.12</td>
<td>1.79±0.18</td>
<td>2.38±0.14</td>
<td>p=0.006</td>
</tr>
</tbody>
</table>

n.s.: not significant

Table 4.3.2c  Global Severity Index (GSI), Total of Positive Symptoms (PST) and Positive Symptom Distress Index (PSDI) scores in all Wilson patients and the subgroups.

Three out of 30 Wilson patients attempted suicide, which is a relatively large number (10%) even though there is a high rate of suicide in the Hungarian population. The reason for all 3 attempts was depression due to worsening of the clinical presentation. One of the cases is presented below, the other two involved 2 young women with debilitating neurological symptoms. One of the women and the man had high scores (=2) in depression, obsessive-compulsive and anxiety; the other woman however, presented with high (=2) hostility and obsessive-compulsive scores. All 3 patients had GSI scores higher than 1, and one of them had higher than 2.

4.3.3 Illustrative cases with psychiatric symptoms misdiagnosed at initial presentation

Case 1

A 21-year old man was diagnosed with major depression, and was treated with imipramine, without any success. The following year cryptogenic liver cirrhosis and major depression was diagnosed. No etiological diagnosis was established at that time. Kayser-Fleischer ring was undetectable. His depression
worsened and he was given 20 mg sertaline a day. A year later his affective disorder deteriorated (apathy, devoid of interest, restrained activity, and lack of initiation) and was admitted to the psychiatry unit. The MRI of the brain revealed pontin and extrapontin myelinosis, and basal ganglia sclerosis bilaterally; and the SPECT showed cortical atrophy. The laboratory findings were consistent with the Wilson diagnosis. A second liver biopsy was performed, which showed excessive copper accumulation. The PCR revealed that the patient is homozygous for the H1069Q gene mutation. D-penicillamine treatment was introduced. Three years have passed and most of the neurological symptoms disappeared. Although the patient is properly treated for Wilson disease, he still shows some aggressive behavior, hostility, antisocial behavior and difficulty in finding his place in the society, but in less extent than prior to proper treatment.

Case 2

A 26 year-old man was diagnosed with bipolar affective disorder with maniac phase (history of depressive episodes for 2 years, increased activity, irritability, insomnia, uncritical behavior, which was alien to his normal personality), and was started on anxiolytic and antidepressant therapy, after which his depressive symptoms seized. He had worked as a firefighter until his symptoms started. Two years later, on examination he was found to have tremor, dysarthria and mild ataxia. The abdominal ultrasound showed minimal hepatomegaly and hepatic inhomogeneity. The CT scan of the head was unremarkable and the brain stem evoked potential (BSEP) suggested abnormality in the ponto-mesencephalic function bilaterally. The MRI of the brain showed sclerotic changes in the basal ganglia. Liver function test was normal; serum ceruloplasmin and copper levels were low. The patient was found to have Kayser-Fleischer ring, and the PCR revealed, that he was homozygous for the H1069Q mutation. After the initiation of d-penicillamine treatment his neurological symptoms improved, and the maniac outbreaks were milder and less frequent.

Case 3

A 33-year-old white man, a former sportsman was admitted to the hospital because of a suicide attempt, which was carried out by drinking hypochloric acid. His life has been saved by complex intervention at the intensive care unit.
Careful history taking revealed an accidental finding of liver enzyme elevation at the age of 13. Liver enzyme elevation was also detected in the past year, but thought to be a consequence of his accidental alcohol consumption. Emotional lability and Parkinson-like symptoms had started about a year before with mild tremor of his hands progressing to the stage of whole body stiffness treated with anti-Parkinson drugs. He revealed to have coordination problems even before he was 13. That time he was competing in the Hungarian national youth team of canoeing, and since his performance deteriorated it made him to leave the team. During the years his coordination problems and gait disturbances increased. The abovementioned deterioration led him to attempt suicide.

The diagnosis of Wilson disease has been established based on the typical clinical picture, the low ceruloplasmin level, the detection of Kayser-Fleischer ring, and the homozygous mutation of H1069Q gene. After the initiation of d-penicillamine therapy, the patient symptoms improved rapidly. Mild tremor and mild dysphasia remained, but his affect is stable; and after one year he is able to live a complete life working as a sport manager.

4.4 Nociceptin

In healthy controls the mean SD plasma NC level amounted to 9.18 ± 1.63 pg/ml, whereas in the group of age-matched patients with Wilson disease significantly higher level was measured (13.98 ± 2.44 pg/ml, p < 0.001). Data summarized in figure 4.4. No correlation was found between NC level and any liver function test, serum copper level, presence and severity of neurological symptoms, duration of the disease or d-penicillamine treatment (data not shown).

![Figure 4.4](image-url) *Figure 4.4 Plasma nociceptin concentrations (pg/ml ±SD) in healthy subjects and in patients with Wilson disease. * indicates significance (p < 0.001)
5 DISCUSSION

5.1 Study I.

Major findings of the present study are the high serum OPG and β-CTx levels without change in RANKL and osteocalcin levels, the significantly decreased bone mineral density by osteodensitometric measurements, and reduced quality of bone by quantitative bone ultrasound. This is the first report on bone mineral density and bone ultrasound in Wilson disease. Furthermore, there have been no OPG, RANKL, osteocalcin and β-CTx measurements reported in WD.

As in previous reports, our study confirms the high rate of skeletal abnormalities in Wilson disease. In 48% of patients with WD (10/21) had decreased bone mineral density, 8 of whom (38%) had osteoporosis according to the densitometric parameters. The ultrasound BUA values showed abnormalities in 14 patients (67%). All 10 patients with decreased densitometric results also had abnormal quantitative bone ultrasound values. The large number of abnormal BUA findings may support that Wilson patients have damaged bone microarchitecture apart from decreased bone density, therefore the bone quality in WD is also impaired. However, the damage in the bone elasticity is not prominent, which is reflected in the normal SOS T-scores. As a result of abnormal densitometric and ultrasound values there is an increased fracture risk in Wilson disease. Although Aksoy suggested, that the duration of the disease may be an important factor in the occurrence of bone lesions with the symptoms being milder in younger patients (39), we did not find correlation between the bone mineral densities of the patients and their ages, which finding is in concordance with that of other studies (38, 99).

The reason for the decreased bone mineral density may be the toxic effect of copper damaging the tissues. Skeletal copper content is reported to be increased about four times (40). Menerey described that the excessive toxic copper in the skeletal system may mediate oxygen free radical release thus tissue damage (99). Some studies propose, changes in the calcium and phosphate plasma levels may contribute to high incidence of osteoporosis (40, 100, 101). However, all of our patients had serum calcium and phosphate levels in the normal range.

To investigate the underlying mechanism of decreased bone mineral density and bone mineral content in Wilson disease, we measured the serum levels of the newly discovered osteoprotegerin and RANKL, as well as the serum level of osteocalcin and β-crosslaps, which are markers of bone turnover. The mature OPG is a hydrophobic polypeptide consisting of 380 amino acids (102). Although OPG is secreted mainly by osteoblasts and
immune-competent cells (T- and B-cells, monocyctic cells), high mRNA levels were detected in fibroblasts, endothelial cells and smooth muscle cells as well (103). The biological effect of OPG is inhibition of both: the terminal stages of osteoclastogenesis from osteoclast precursors and activity of mature osteoclasts (104, 105, 106, 107). High levels of OPG mRNA have been detected in lung, heart, kidney, liver, stomach, intestine, skin, brain and spinal cord, thyroid gland, and bone (102, 104). RANKL, which was discovered later (105, 108), is a 317-aminoacid containing ligand in the form of a type II transmembrane protein, as well as a soluble molecule derived form the cell-associated form by post-translational processing. RANKL gene expression by marrow stromal cells and osteoblasts is most abundant in the skeleton and lymphoid tissues (105, 108, 109, 110) and plays a role in the differentiation and activation of osteoclasts by binding to its high affinity receptor (ODAR/RANK: osteoclast differentiation and activation receptor/receptor activator of NF-kappaB) located on the surface of osteoclasts (111, 112). The effect is counterbalanced by OPG, which acts as a decoy receptor competing with RANK for RANKL.

The insignificant statistical difference in the OCN levels between the patients and the controls reflects, that there is normal osteoblastic function in Wilson disease. However, β-crosslaps level was significantly increased in WD, which reflects increased bone resorption in the patients. The strong positive correlation between osteocalcin and β-CTx may suggest that by increasing their activity, osteoblasts attempt to compensate for decreased bone mass.

The high OPG level in WD found in our study may implicate that other tissues, such as immune competent cells or fibroblasts in the cirrhotic liver, may contribute to the production of the molecule as a consequence of the inflammatory process (53). Although most patients had normal liver function tests and other laboratory parameters as a result of proper treatment and good control over the disease, we observed, that patients with more severe liver damage – based on initial presentation and/or liver biopsy findings – had higher serum OPG concentrations. The correlation between OPG and liver function tests was not significant, but as mentioned before, all patients were treated and the disease was well controlled in each patient, therefore liver function tests were normal.

Another explanation for high OPG level in WD is, that osteoblasts alone produce increased amount of OPG as a compensatory response to bone loss, which is neither effective nor adequate, as it is reflected in the decreased bone densitometric values. Ueland (56) also hypothesized that increased serum OPG levels found in Cushing’s syndrome may reflect a compensatory response to enhanced osteoclastic activity or negative bone remodeling balance, or even an enhanced activity in the OPG system possibly correlated to increased
activity of other members of the TNF family. We propose that the increased osteoprotegerin level with decreased bone mass in WD might have a similar pathomechanism.

To further assess the underlying mechanism, we examined whether OPG is produced as a result of a compensatory reaction to high RANKL level and activity in Wilson disease by measuring serum RANKL. However, the insignificant difference of RANKL level between WD patients and controls contradicts the hypothesis, that osteoporosis results from increased activation of RANKL, which may be insufficiently counterbalanced by OPG. Therefore, it seems, that RANKL production in WD is not affected, whereas OPG serum levels are elevated. Based on the normal serum RANKL level, we may conclude, that increased bone resorption is not a consequence of increased RANKL production by osteoblasts. However, it is interesting to note, that both WD gene and RANKL gene are localized to chromosome 13q14. Although RANKL level was not elevated, disturbances in this area of the 13th chromosome may cause altered RANKL transcription or regulation as well. Another interesting observation is that the cleavage of the membrane bound RANKL and the release in the soluble form is carried out by a specific metalloprotease. Altered function of metalloproteases as a result of copper-zinc imbalance may also play an important role in Wilson disease. (113).

In summary, we found a high rate of osseous disorders in Wilson disease. Based on the high serum ß-CTx and OPG level with normal osteocalcin and RANKL levels found in our study, we conclude that decreased bone density in WD is the consequence of increased bone resorption unrelated to RANKL production; and the underlying mechanism is still unknown. We also hypothesize that either normally functioning osteoblasts and/or immune competent cells produce increased amount of OPG as a compensatory reaction to increased bone loss in Wilson disease. Further studies are warranted to disclose the underlying mechanism of bone disorders in Wilson disease.

5.2 Study II.

Polyneuropathy as a consequence of chronic alcohol consumption has been observed for a long time. In the last decade, both sensory and autonomic damage have often been reported in non-alcoholic chronic liver diseases as well. In case of liver disease coexisting with other risk factors for neuropathy, the damage is more severe and accentuated. The reason why we find the investigation of peripheral neuropathy interesting in Wilson disease, is the fact that (1) one of the consequences of WD is chronic liver damage; (2) all patients are treated with d-penicillamine, which may cause peripheral neuropathy itself, or may worsen the possibly existing damage; (3) copper is shown to directly damage neurons in the central
nervous system, but the toxic effect of copper in the peripheral nervous system is not clear. In the literature of WD 2 cases with optic neuropathy (114, 115) and 2 cases with peripheral neuropathy has been reported. However, the peripheral neuropathy in both cases was a consequence of another underlying disorder: in one of the cases sensorimotor neuropathy due to vitamin B deficiency was observed in a 13-year-old girl, who underwent living related liver transplantation (116), and in the other case peripheral neuropathy was present together with hepatocellular carcinoma (117).

Recently, 2 studies (66, 67) have been published regarding autonomic changes in WD, however no systematic study of peripheral sensory neuropathy has been performed. In our study higher CPT values, indicating hypesthesia of large and small sensory nerve fibres were detected at all three frequencies at the peroneal nerve in patients with WD compared to the healthy volunteers, while there was no difference in the CPT on the upper extremity between the two groups. This observation is in concordance with previous studies, which describe, that longer fibres are damaged earlier (118). Therefore, in the lower extremities the damage precedes the upper peripheral neuropathy. In WD all 3 sensory nerve fibre-types were impaired only on the lower extremity, which may be due to a relatively young patient population. When we compared the patients with neurological symptoms to the rest of the patients, we found no difference in the function of the peripheral sensory neurons, which supports the hypothesis that the peripheral sensory neuropathy may be the result of the chronic hepatic disorder, the effect of continuous d-penicillamine treatment or direct toxic effect of copper on the peripheral neurons, and is independent from the presence of CNS damage.

D-penicillamine is a chelator agent, used in the treatment of Wilson disease. It has a vitamin B6 depletion effect, as a result of which, peripheral sensory and motor neuropathy due to vitamin B6 deficiency may occur. In the literature there are studies describing d-penicillamine induced neuropathy in rheumatoid arthritis (119, 120); as well as neuropathy, as a complication of other conditions, such as Sjogren’s syndrome (121), rheumatoid vasculitis (122), and arsenic poisoning (123), which is successfully treated with d-penicillamine. D-penicillamine induced optic neuropathy has also been described in rheumatoid arthritis (124), and also in Wilson disease (114). Since our patients were supplemented with vitamin B6, the d-penicillamine induced vitamin deficiency mechanism of sensory dysfunction is unlikely.

Unlike in previous studies (66, 67) there was no difference in the autonomic function between WD patients and the control group; however, patients with signs of CNS
impairment significantly differed from patients without neurological symptoms. This comparison helps to elucidate the subtle changes of autonomic functions in patients with predominantly neurological dysfunctions. Even the less sensitive cardiovascular reflex tests revealed simultaneous damage of both parasympathetic and sympathetic systems, which was supported by the very sensitive HRV analysis. These results confirm the findings of that of the 2 previous studies (66, 67). According to the frequency domain analysis both LF and HF were lower in the neurological symptoms presenting patients. The changes in the two parameters indicate simultaneous damage of the sympathetic and parasympathetic fibers.

The characteristics of the autonomic nervous system impairment are similar to that in Parkinson syndrome. Impaired HRV and severe hypotensive responses in Parkinson disease (PD), and in Multiple System Atrophy (MSA) have been reported by many authors (125, 126, 127). This similarity may suggest that the basis of the dysfunction is located in the autonomic centers of the CNS. In PD, post-mortem studies revealed Lewy bodies and neuronal degeneration in the brainstem and in the autonomic nuclei of the central nervous system, such as hypothalamus, dorsal motor nucleus of the vagus, and the sympathetic ganglia (128, 129, 130), as well as in the peripheral autonomic cardiac plexus (131). Some of these areas may be directly damaged by toxic copper accumulation in Wilson patients as well. Furthermore, in animal studies a potential role of rhythmic copper metabolism in pineal circadian function has been suggested (132), which might be compromised in WD. Although these data were not confirmed in humans yet, and Wilson disease usually affects the basal ganglia, on the analogy it is possible that the altered copper transport may also alter the circadian rhythm (133) and the autonomic centers.

It has been reported that the poor prognosis of the autonomic neuropathy may be the result of parasympathetic dysfunction. In WD patients with neurological symptoms the 30/15, pNN50 and the HF parameters were significantly lower than in the patients lacking these symptoms. The changes in these parameters prove the impairment of the parasympathetic system. Nowadays, there is increasing evidence that sympathetic hyperactivity plays an important role in the pathogenesis of hypertension (134). The positive correlation between LF/HF ratio and diastolic parameters suggest, that the parasympathetic impairment and the imbalance of parasympathetic and sympathetic integrity may lead to increased diastolic blood pressure values even in WD. Further studies and follow-ups in the peripheral neuropathy and HRV are required to assess the prognostic importance of these factors in Wilson disease.
In summary, this is the first systemic study of peripheral sensory neuropathy involving an age and gender matched control group. Furthermore, this is the first study for autonomic dysfunction involving a sensitive 24-hour heart rate variability analysis comparing WD patients with predominantly hepatic and predominantly neurological symptoms, which aids in the understanding of the underlying mechanism. Our novel finding is the increased current perception threshold of peripheral sensory nerves in Wilson disease. We also found decreased heart rate variability indicating impairment of autonomic regulation in Wilson patients with characteristic neurological symptoms. Since decreased HRV is a risk factor for increased morbidity and mortality due to cardiovascular disease, we strongly believe that patients should be screened and monitored. Prospective studies and follow-ups are required to clarify the pathomechanism and to further assess the clinical significance of these alterations.

5.3 Study III.

One of the major findings in our study is, that treated Wilson patients have changes in the scores of the temperament and character scales. The other is, that numerous Wilson patients deviated from the healthy controls on almost all scales of the SCL-90-R. Moreover, according to the psychometric tests and the clinical presentations more than half of the patients had psychological or psychiatric disturbances, which should be explored and treated. We can conclude that the occurrence of personality disorders with emotional distress is high; affective and anxiety disorders is the most common psychopathology in the Hungarian WD patients.

Although personality disorders and trait changes have been described earlier (73, 77, 81, 135, 136), detailed personality profile was not reported. To the best of our knowledge this is the first report on detailed temperament and character investigations for personality traits of patients with Wilson disease.

In the development of personality both genes and the environment are important factors. Personality is determined by temperament, character and intelligence. The temperament is biological; the character is social-cultural aspect of the personality. According to Cloninger the personality traits are genetically homogenous and independent of each other (137). Goldstein (73) stated that the personality disturbances antedated the overt manifestation of the disease, and the slowly accumulating copper in the brain alters the development of the Wilson patients’ personality reactions to the psychological stresses and strains. In the pathomechanism of the psychiatric disturbances the abnormal metabolism of neurotransmitters, particularly dopamine, noradrenalin and serotonin (138, 139, 140, 141) in
the basal ganglia (putamen, globus pallidus), caudate, internal capsule, substantia nigra, claustrum, thalamus subcortical white matter, subthalamic nucleus, cerebellum, medulla oblongata and pons (142) has been suggested.

The scores in the TCI for WD significantly differed from that of the healthy controls. Higher scores in the harm avoidance dimension show that the WD population are a more timid, worried, undetermined, nervous, rejecting, passive, negativistic or pessimistic population than the controls. Changes in the HA2 scale indicate that the patients worry more about uncertainty and challenge. Higher scores in the HA4 dimension shows that these patients have less energy, and are more tired than the healthy controls. Decreased scores in persistence, which is a main temperament parameter, reveals that the patients are less persistent, they rarely overload themselves with work even if there is a reward for it. They do not like to do more than is absolutely necessary; they easily give things up. Most of the time they are satisfied with their situation; they are often willing to compromise. Changes in self-directedness indicate that the patients have less willpower, and often change their decisions. Lower S1 scale reveals that the patients try to avoid responsibilities more than the control group; lower S2 shows that patients are less goal-orientated; decreased S3 scores suggest increased helplessness; and low S4 scores show less flexibility and less acceptance toward new ideas. Cooperativeness scores in patients with WD are also decreased. The low C1 score shows that the patients are more intolerant and critical toward other people; lower C3 scores suggests that the Wilson patients are more self-centered, and are more involved in themselves than in others; changes in C5 scores show a decreased tendency toward self-sacrifice. The lower self-transcendence main character dimension indicates that patients less tolerate and accept transcendence and spiritual life. Changes in ST3 suggest that the patients are more materialistic than the volunteers, and are less likely to accept things that cannot be explained scientifically.

Differences in the clinical presentation and differences in gender exhibit differences in the temperament and character scales. It was interesting to see that male patients have deviated from the male controls more than the female patients. This finding is similar to Portala’s (77), who found that female patients had results more similar to those of female healthy volunteers than the male WD patients.

In the study of Svrakic and his colleagues (143) it was found that patients with personality disorders had higher harm avoidance and lower self-directedness scores than the general community, suggesting the possibility that these are the risk factors or indicators of personality disorders. They also suggest that high scores in cooperativeness and reward
dependence may be protective factors against personality disorders. Since the self-transcendence scores were low in psychiatric disorders regardless of personality disorders, they hypothesize a possible relationship between low self-transcendence scores and psychiatric disturbance in general. In our study the TCI scores were very similar in the WD population as in Svrakic’s patients suggesting a higher frequency of personality disorders in Wilson disease than in the general population. However, this difference is more prominent in the male Wilson population.

We have found significant differences in the SCL-90-R inventory in 8 parameters (somatization, obsessive-compulsive, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety and paranoid ideations). Fontana (144) described global emotional stress and increased frequency of somatization, obsessive-compulsive, depression, anxiety and psychoticism in patients with chronic hepatitis C infection not receiving antiviral therapy. The elevated somatization, anxiety and depression are consistent with other studies involving patients with chronic illnesses (145, 146). Fontana and Slaughter (144, 147) suggested that the elevated obsessive-compulsive scores reflect subtle changes in cognitive function, as it is often the case in Wilson disease. However, the higher scores in obsessive-compulsive symptoms can also be explained by anatomical background. In the pathomechanism of OC symptoms the disorders in the circle of the orbitofrontal cortex – thalamus – pallidum – caudate nucleus play a crucial role, which was studied by Baxter (148), who used PET examinations. In the area of the caudate nucleus and the orbital surface of the frontal lobe, he found changes in glucose metabolism. This is in accordance with excessive copper accumulation in the caudate nucleus, pallidum and thalamus and frontal lobe resulting in hypometabolism shown on SPECT investigations (142), which may play a crucial role in the OC symptoms in WD. In the pathomechanism of depression and affective disorders the limbic system, the basal ganglia and the hypothalamus play a role, where excessive copper accumulate in WD. This might be a possible explanation for affective symptoms in WD, because PET and SPECT examinations showed hypometabolism in these areas. Fontana did not find increased scores in the paranoid ideations in untreated patients with hepatitis C infection. The paranoid symptoms in Wilson disease can be explained by the impairment of the basal ganglia and the limbic system (149), therefore, this may also be the result of the toxic effect of the copper in the in these nuclei.

Some studies (75, 76, 77) found that the evaluation of Wilson patients by experts and family members differ from that in self-report inventories. While the self-rating showed low scores on aggression and hostility, family members and caretakers often reported aggression
and irritability. However, in the SCL-90-R self-inventory the hostility levels were significantly elevated in WD patients. It has also been reported that psychiatric symptomatology is related to neurological symptoms (74). However, our study seems to confirm Portala’s findings (75): we did not find any difference between the group with predominantly hepatic manifestation and the other group with predominantly neurological presentation on the SCL-90-R.

Reports on suicide in WD are scarce. Kassam (150) described a patient who committed suicide after successful liver transplantation. In a prospective study of 45 Wilson patients Oder reported a history of an attempted suicide in 7 patients (151). Therefore, the high rate of suicide is not a feature of the Hungarian Wilson population only. The increased rate of suicide is probably due to anxiety and feeling of helplessness as a result of the deteriorating symptoms and signs. None of the 3 attempts were well planned, and all patients described this action impulsive, which they are ashamed of. Thus, it is very important to pay attention to the warning signs and support the patients psychologically or even involve an expert during the critical times.

In conclusion, the psychopathology of WD is important for gastroenterologists, neuropsychiatrists as well as general practitioners from two aspects. The initial presentation of the disease may be limited to a wide range of psychiatric disorders without any other symptoms. These patients are often misdiagnosed and mistreated for years. On the other hand, correctly diagnosed Wilson patients’ psychological distress and other psychiatric conditions are often neglected. Therefore, we strongly believe that careful psychiatric evaluation is indicated for each patient. In this study we emphasize the importance of treating the accompanied psychiatric disorders and paying attention to latent symptoms, in order to avoid the serious consequences, such as personality disorders with impaired social functioning, life debilitating affective disorders or suicide.

5.4 Study IV.

This is the first report on plasma nociceptin level in patients with Wilson disease characterized by toxic copper accumulation in the liver and brain. The nociceptin/OP4 receptor was demonstrated in rat liver (86) but the role of nociceptin in liver physiology/pathophysiology is still unknown.

So far only four clinical studies on nociceptin has been reported, none of them dealt with chronic liver disease patients. Examining the plasma nociceptin level in patients with fibromyalgia syndrome (FMS) Anderberg et al. (152) found that NC level of female cyclic FMS patients in luteal phase was significantly lower than in corresponding controls. They
concluded that perturbed plasma NC levels may be linked to both the sex hormones and to the stress system. Brooks et al. (153) was the first identifying nociceptin in human cerebrospinal fluid (CSF) and he observed significantly higher CSF NC concentrations than in plasma. They also reported that acute pain of labor had no association with the nociceptin levels. Kumar et al. (154) found that neither NC nor OP4 receptors could be detected in human synovial fluid and tissue. Intravesical instillation of nociceptin to neurogenic incontinence patients produced a significant increase in mean bladder capacity and volume threshold (155).

The novel finding of the study is that plasma NC level of patients with Wilson disease is significantly higher (p < 0.001) than in age matched healthy controls and NC plasma levels have no correlation with liver function test results. The plasma NC level measured in healthy controls (9.18 ± 1.63 pg/ml) was in concordance with previous studies (153, 154) and shows no sex-related difference. Patients with Wilson disease are characterized by impaired hepatic extraction of copper and toxic accumulation of the metal principally in the liver and brain because of the autosomal recessive abnormality in gene ATP7B located in chromosome 13q14-q21 and also by deficiency of the plasma copper protein ceruloplasmin.

It has been shown that nociceptin is processed from prepronociceptin. The human NC precursor mRNA is expressed in all parts of the brain at different levels (156) and the expression of NC mRNA in spleen and in peripheral blood leucocytes is comparable with the amount in adult brain (156) Human fetal kidney and fetal brain show similar levels of the NC mRNA expression, however no signal could be found in adult kidney and liver by Northern analysis.

In vitro studies on rat brain slices (157) showed that inactivation of the NC heptadecapeptide is going on by hydrolysis essentially at the Phe1-Gly3 and Ala7-Arg8 peptide bonds by the membrane-bound aminopeptidase N (APN) and the cytosolic endopeptidase 24.15. The biological relevance of these metabolic pathways was confirmed in mice treated by specific inhibitors of the enzymes. It is known, that both APN and endopeptidase 24.15 belong to the group of Zn-metallopeptidases. Replacement studies of metal ions in metallopeptidases (158) showed, that replacement of Zn by Cu in porcine aminopeptidase results in very strong, approximately 50% inhibition in the enzyme activity.

D-penicillamine, generally used the life-long therapy of Wilson disease, is reported to be an inhibitor of the collagenase that also belongs to the group of Zn-metallopeptidases (159, 160). However no data are available about its inhibitory effect for aminopeptidase-N or endopeptidase 24.15.
We suggest that significantly elevated plasma nociceptin level in patients with Wilson disease may be due to inhibition of nociceptin metabolism caused by the toxic copper deposits in liver and/or brain. It is known that the membrane-bound aminopeptidase N is present in liver and removes the N-terminal amino acid of peptide substrates, however, endopeptidase 24.15 is a cytosolic enzyme with a minor (10-20%) membrane-associated component which accounts for the involvement of this peptidase in the in vivo metabolism of several neuropeptides (157). Due to the complementary role of APN and endopeptidase 24.15 in the inactivation of nociceptin found in animal tissues, further studies are needed to clarify their role in human metabolism of the heptadecapeptide and also in chronic liver damage such as Wilson disease.
6 MAIN CONCLUSIONS

Although WD was described nearly a century ago, and has been studied extensively ever since, the findings of our study has pointed out interesting results, which raise many other questions. Wilson disease is one of the most complex diseases, where symptoms and signs of this disorder are very colorful and not fully understood. Even though we know much about WD, our knowledge is far from being complete. I strongly believe, that further understanding of the mechanism and symptoms are necessary, in order to recognize the disease early, which is a crucial point in the prognosis and quality of life of the patients. Moreover, the patients should not be treated for only one symptom. As we see in this study, primary caretakers should pay attention to musculoskeletal problems, neuropathic changes and psychiatric disorders as well.
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