Genetical and Clinical Studies in Wilson's Disease

ERIK WALDENSTRÖM
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**Abstract**


Wilson’s disease is a rare inborn error of metabolism caused by a defect in ATP7B, a protein necessary for proper copper excretion into bile. It is characterised by copper accumulation with hepatic and central nervous system dysfunction.

We investigated 24 Swedish families with Wilson’s disease by sequencing the entire coding sequence using a new technique called manifold sequencing. Disease causing mutations were found in 44 out of 48 alleles.

From data obtained in the first study, the two most common mutations (C3207A and C2930T) were sought in 2640 anonymous DNA samples from a Swedish population, using a pooling strategy and solid-phase minisequencing. Four C3207A and one C2930T were found. From the number of C3207A, a prevalence of Wilson’s disease in Sweden of about 1 in 110,000 could be estimated.

Four groups with three patients each had four different genotypes concerning mutations in ATP7B. The patients’ psychopathological symptoms were investigated, using the Karolinska Scales of Personality rating (KSP) and Comprehensive Psychopathological Rating Scale (CPRS). A trend towards lower CPRS scores was seen in the groups with mutations known to render ATP7B completely without activity.

Using $^{64}$Cu liver PET in patients homozygous for mutations in ATP7B, heterozygotes, normal individuals and two patients with alcoholic liver cirrhosis, significantly slower uptake was seen in the homozygotes as compared to the heterozygotes and normal individuals. The patients with cirrhosis had values in between. This implies that $^{64}$Cu liver PET might be used as an additional rapid and little invasive diagnostic tool in Wilson’s disease.

In a retrospectively studied cohort consisting of 363 patients followed in Sweden and the UK, nine cases of aggressive intra-abdominal malignancies were seen, which is more than expected. Caution should be taken in the follow-up of Wilson’s disease patients.

**Keywords:** copper radioisotopes, DNA sequence analyses, genotype, hepatolenticular degeneration, neoplasms, phenotype, positron-emission tomography, psychopathology

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urn:nbn:se:uu:diva-7779 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7779)
To my family

At vide,
hvad man ikke vêd,
er dog en slags
alvidenhed

Piet Hein
List of publications

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


III Kamilla Portala*, Erik Waldenström*, Lars von Knorring, Kerstin Westermark. Psychopathology and personality traits in patients with treated Wilson’s disease grouped according to gene mutations. Submitted

IV Joakim Tedroff*, Erik Waldenström*, Vladimir Tolmachev, Hans Lundqvist, Bengt Långström, Kerstin Westermark. 61Cu liver uptake in Wilson disease studied with PET. Manuscript


* The first two authors of papers III and IV respectively, contributed equally to the work.

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# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>9</td>
</tr>
<tr>
<td>Copper</td>
<td>12</td>
</tr>
<tr>
<td>Pathophysiology</td>
<td>15</td>
</tr>
<tr>
<td>Clinical presentation</td>
<td>15</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>17</td>
</tr>
<tr>
<td>Treatment</td>
<td>19</td>
</tr>
<tr>
<td>Aims of the investigation</td>
<td>20</td>
</tr>
<tr>
<td>Material and methods</td>
<td>21</td>
</tr>
<tr>
<td>Study I</td>
<td>21</td>
</tr>
<tr>
<td>Study II</td>
<td>22</td>
</tr>
<tr>
<td>Study III</td>
<td>24</td>
</tr>
<tr>
<td>Study IV</td>
<td>25</td>
</tr>
<tr>
<td>Study V</td>
<td>26</td>
</tr>
<tr>
<td>Results and discussion</td>
<td>27</td>
</tr>
<tr>
<td>Study I</td>
<td>27</td>
</tr>
<tr>
<td>Study II</td>
<td>28</td>
</tr>
<tr>
<td>Study III</td>
<td>30</td>
</tr>
<tr>
<td>Study IV</td>
<td>32</td>
</tr>
<tr>
<td>Study V</td>
<td>33</td>
</tr>
<tr>
<td>Conclusions</td>
<td>35</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>36</td>
</tr>
<tr>
<td>References</td>
<td>37</td>
</tr>
</tbody>
</table>
Abbreviations

5’ UTR    untranslated region upstream of a gene  
Atox1    antioxidant protein 1  
ATP7A    ATPase, Cu\textsuperscript{2+}-transporting, \( \alpha \) polypeptide – gene  
ATP7A    ATPase, Cu\textsuperscript{2+}-transporting, \( \alpha \) polypeptide – protein, syn. MNKP  
ATP7B    ATPase, Cu\textsuperscript{2+}-transporting, \( \beta \) polypeptide – gene  
ATP7B    ATPase, Cu\textsuperscript{2+}-transporting, \( \beta \) polypeptide – protein, syn. WNDP  
bp    base pair(s)  
CCS    copper chaperone for superoxide dismutase  
CMGP    cartilage matrix glycoprotein  
COMMD1    copper metabolism MURR1 domain-containing protein 1  
Cox    cytochrome c oxidase assembly protein  
Cu    copper  
Ctr1    copper transporter 1  
DMT1    divalent metal ion transporter 1  
H1069Q    histidine 1069 glutamine  
HCC    hepatocellular carcinoma  
kDa    kilodalton  
kbp    kilobases  
KF-rings    Kayser-Fleischer rings  
LEC rat    Long-Evans Cinnamon (coated) rat  
MNKP    Menkes’ disease protein  
MURR1    mouse U2af1-rs1 (human)  
nt    nucleotide  
PCR    polymerase chain reaction  
PET    positron emission tomography  
R1319X    arginine 1319 termination codon  
ROS    reactive oxygen species  
Sco    synthesis of cytochrome c oxidase  
SSCP    single-strand conformation polymorphism  
SNP    single nucleotide polymorphism  
T977M    threonine 977 methionine  
ULN    upper limit of normal  
W779M    tryptophane 779 methionine  
WD    Wilson’s disease  
WNDP    Wilson’s disease-associated protein
Introduction

Figure 1. SA Kinnier Wilson, (1978-1937)
(Courtesy of JVK Wilson, son of Dr. SAK Wilson, reprinted with permission from Läkartidningen)

S. A. Kinnear Wilson (fig. 1) published his seminal paper "Progressive lenticular degeneration: a familial nervous disease associated with cirrhosis of the liver" as his PhD thesis, for which he was awarded a gold medal at Edinburgh University in July 1911, and the following year in Brain (Wilson 1912, fig. 2). He presented six previously described cases (Gowers 1888; Homén 1890; Ormerod 1890; Homén 1892), one from hospital records, one from interviewing the deceased patient’s 70 year old mother and four cases of his own. Friedrich Theodor von Frerichs, while working in Breslau in 1854, saw a 10-year old boy with a movement disorder and cirrhosis of the liver, and presented it in his textbook “Treatise on Diseases of the Liver” (von Frerichs 1861). While Wilson’s article was in press, he noted von Frerich’s case report and added this as a note in his monograph. He also added a case presented by Völsch of a patient with “Pseudosclerosis” and liver cirrhosis (Völsch 1911). In 1883 Westphal and in 1898 Strümpell had presented cases with neurologic disease consistent with what should become part of WD (Westphal 1883; Strümpell 1898), but did not notice any pathology of the liver. Ernst Homén, professor of pathological anatomy in Helsinki, believed that his findings in three siblings (out of 11) with liver cirrhosis and lenticular degeneration, were due to congenital syphilis (Homén 1890; Homén 1892), which Wilson found highly unlikely. It thus became Kinnier Wilson who, by combining several cases of liver cirrhosis and lenticular degeneration, described it as a new disease entity.
Figure 2. Title page of the main article in Brain in March 1912, reproduced with permission from Oxford University Press.
The German ophthalmologists Bernhard Kayser and Bruno Fleischer described a pigmented ring in the periphery of the cornea in some patients with neurologic disease (Kayser 1902; Fleischer 1903). This ring was later shown to be a clinical hallmark in WD, and now bears their name. In the 1930s, Gerlach and Rohrschneider showed that the pigment of KF-rings is composed of copper, not silver, which was previously believed (Gerlach and Rohrschneider 1934). A rare ophthalmological finding in WD is the so called sunflower cataract, first described just 10 years after Wilson’s monograph (Siemerling and Olof 1922). These authors noticed the similarity between this cataract and that caused by a foreign body consisting of copper.

Already one year following the appearance of Wilson’s paper, Rumpel noted excess copper in the liver of a patient with WD (Rumpel 1913). Increased copper content in the brain was demonstrated as late as in 1948 by Cumings, who also proposed that dimercaprol, an agent that earlier the same year had been shown to increase copper excretion in urine in a patient with WD (Mandelbrote et al. 1948), might be used as a treatment for WD (Cumings 1948).

In 1921, Hall stated that WD was an inherited disease and proposed that it was transmitted by two different genes (Hall 1921). His data were however consistent with a recessive heterozygous inheritance, a hypothesis that was finally sufficiently strengthened several decades later (André and van Bogaert 1950; Bearn 1960).

Caeruloplasmin was purified and characterised by Holmberg and Laurell in Lund, Sweden in 1948 (Holmberg and Laurell 1948). They believed that caeruloplasmin was involved in WD and tested their hypothesis in a patient with presumed WD, but found normal levels. This was alas due to the fact that the patient did not have WD (personal communication). Scheinberg and Gitlin found the true relationship in 1952, i.e. that low caeruloplasmin levels really characterised WD (Scheinberg and Gitlin 1952). When the WD gene was mapped to chromosome 13 in the mid 1980s (Frydman et al. 1985) and the caeruloplasmin gene the same year to chromosome 3 (Naylor et al. 1985), it was finally proven that a direct causal relationship did not exist. In 1993, three groups independently cloned a P-type ATPase (Bull et al. 1993; Tanzi et al. 1993; Yamaguchi et al. 1993) homologues with ATP7A, the Menkes’ disease gene, which when defect causes a recessive X-linked syndrome of copper deficiency (Menkes et al. 1962). The WD gene is called ATP7B, is 80 kilobases in length, consists of 21 exons and gives rise to a 165 kDa membrane bound protein with 1465 amino acids (Yang et al. 1997) showing 76% homology to ATP7A. Both proteins have eight hydrophobic transmembrane sequences and six copper binding domains, a transduction domain, a cation channel and phosphorylation domain, and a nucleotide-binding domain (fig. 3).
Copper

Copper is an essential trace element serving as an important catalytic cofactor in redox chemistry for a large number of proteins required for normal cell function (table 1). Copper deficiency gives rise to a multitude of symptoms, well illustrated by Menkes’ disease, an X-linked recessive disorder with a mutated ATP7A (Menkes et al. 1962). Dietary copper is primarily absorbed from the small intestine. The membrane protein copper transporter 1 (Ctr1) is essential in this process (Sharp 2003). The Fe²⁺ transporter DMT1 has also been implicated as a possible Cu¹⁺ transporter in the brush border (Arredondo and Núñez 2005). From the enterocyte, copper is transported into the portal circulation with the aid of the Menkes’ disease protein ATP7A. In the portal vein, Cu is bound with albumin, transcuprein and histidine. From portal vein plasma, copper is rapidly internalised in the hepatocytes (Peña et al. 1999). The exact process utilised is not known, but Ctr1 is also here believed to be the main transporter. Intracellularly, several copper chaperones mediate the transport of copper into different organelles, e.g. Atox1 which interacts with ATP7B, CCS for the delivery of copper to SOD (superoxide dismutase) and Cox17 which is required for cytochrome c oxi-
dase (CCO) functioning. Six other copper chaperones are involved in proper CCO performance: Sco1, Sco2, Cox11, Cox17, Cox19 and Cox23 (Prohaska and Gybina 2004; Banci et al. 2007). ATP7B has two known functions in the hepatocyte. Crucial for the pathogenesis of WD, it is necessary for proper excretion of copper surplus from the hepatocytes into bile. It is also required for incorporation of copper into apoceruloplasmin in the synthesis of holoceruloplasmin (Harris 2000). A recently discovered protein, MURR1 or COMMD1, which has a deletion of the second exon in copper toxicosis of Bedlington terriers (van de Sluis et al. 2002), directly interacts with ATP7B (Tao et al. 2003) and is believed to be of great importance in the final excretion of copper into bile. Interestingly, a very recent paper shows that COMMD1 binds copper in its cupric state (Narindrasorasak et al. 2007), whereas ATP7B binds it as Cu\(^{1+}\) (DiDonato et al. 1997). The mechanism of oxidation at this point is still poorly understood. For a schematic view of the copper metabolism of hepatocytes, see fig. 4.

\[\text{Figure 4. Schematic presentation of the pathways of copper in the hepatocyte.}\]
\[\text{aCp denotes apocaeruloplasmin, CCO cytochrome c oxidase, hCp holocaeruloplasmin, MT metallothionein, SOD superoxide dismutase and TGN trans-Golgi network.}\]
Table 1. Copper-dependent proteins in mammals. Modified from (Tapiero et al. 2003)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Amidating enzyme</td>
<td>Modifies C-terminal ends of hypothalamic peptide hormones ending in glycine, (hormone maturation)</td>
</tr>
<tr>
<td>Amine oxidase (extracellular)</td>
<td>Inactivation of histamine, tyramine, dopamine, serotonin?</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>Induction of blood vessel formation</td>
</tr>
<tr>
<td>β-Amyloid precursor protein</td>
<td>Normal function currently unknown</td>
</tr>
<tr>
<td>Blood clotting factors V and VIII</td>
<td>Blood clotting</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Ferroxidase, promotes flow of Fe from liver to blood. Scavenger of ROS. Cu transport</td>
</tr>
<tr>
<td>CMGP</td>
<td>Ferroxidase/amine oxidase, homologous to ceruloplasmin (chondrocytes and eye ciliary epithelia)</td>
</tr>
<tr>
<td>Cu/Zn-SOD</td>
<td>Free radical detoxification</td>
</tr>
<tr>
<td>Cytochrome-c oxidase</td>
<td>Electron transport in mitochondria</td>
</tr>
<tr>
<td>Diamine oxidase</td>
<td>Inactivation of histamine and polyamines? (cellular and extracellular)</td>
</tr>
<tr>
<td>Dopamine-β-monooxygenase</td>
<td>Catecholamines production</td>
</tr>
<tr>
<td>Hephaestin</td>
<td>Ferroxidase, in trans-golgi of enterocytes; aids iron absorption</td>
</tr>
<tr>
<td>Metallothionein</td>
<td>Storage of excess Cu and other divalent metal ions. Possible donor of Cu to certain apoproteins</td>
</tr>
<tr>
<td>Peptidylglycine monoxygenase</td>
<td>Bioactivation of peptide hormones</td>
</tr>
<tr>
<td>Prion protein (PrPC)</td>
<td>Cu binding properties suggests that it may protect against ROS; may return copper to neurons at synapses</td>
</tr>
<tr>
<td>Protein-lysine-6-oxidase</td>
<td>Cross-linking of collagen and elastin</td>
</tr>
<tr>
<td>S-Adenosylhomocysteine</td>
<td>Sulfur amino acid metabolism hydrolase</td>
</tr>
<tr>
<td>Tyrosinase (catechol oxidase)</td>
<td>Formation of melanin</td>
</tr>
</tbody>
</table>
Pathophysiology

Already in the mid 1950s several studies showed a decreased recovery of i.v. injected $^{64}$Cu in the stool of patients with WD (Bearn and Kunkel 1955; Bush et al. 1955). One and a half decade went by until it was unambiguously shown that this was due to decreased excretion of copper into bile (O'Reilly et al. 1971). As the typical daily intake of copper, normally believed to amount to about 0.6–1.6 mg a day (Linder and Hazegh-Azam 1996), is more than the human body needs, a well functioning excretion process must exist, in order to keep the organism from copper overindulgence leading to toxic effects. This is solved by the liver protein ATP7B, that is defect in WD. Not only is ATP7B required for proper incorporation of copper into caeruloplasmin, for release to the circulation, but also necessary for excreting copper into bile. The exact mechanism of this process is not known, but ATP7B has in many studies been shown to move to the canalicular membrane (Bartee and Lutsenko 2007). It is believed that COMMD1 (previously called MURR1) probably in some way is involved in that process. COMMD1 is mutated in copper toxicosis of Bedlington terriers, a canine model of WD (van de Sluis et al. 2002).

Copper toxicosis is not always caused by mutations of $ATP7B$. Some additional diseases characterised by copper accumulation with early hepatic cirrhosis are Indian childhood cirrhosis, the analogous endemic Tyrolean infantile cirrhosis, and idiopathic copper toxicosis (Müller et al. 2003).

Clinical presentation

WD, in its classical form, consists of hepatopathy of different severity in combination with neurologic deficits of extrapyramidal character in conjunction with psychopathology ranging from personality changes to overt psychosis (Roberts and Schilsky 2003). Kidney involvement is sometimes found, i.e. tubular defects causing aminoaciduria and renal glucosuria and even renal calcification has been described (Litin et al. 1959). Coombs negative haemolytic anemia, often intermittent, in combination with raised liver transaminases is highly suggestive of WD (Dacie 1995). Cardiac involvement is mild, with increased prevalence of left ventricular hypertrophy with concentric remodelling and a relatively high frequency of benign supraventricular tachycardias and extrasystolic beats (Kuan 1987). Skeletal changes have been reported in WD, among others a high prevalence of osteoporosis resulting from increased bone resorption of unknown causes (Golding and Walshe 1977).
The liver disease in WD may be subclinical, not showing any aberrations in liver blood tests, but a histological picture of slight steatosis is often observed. Marked steatosis can however also be found. Most variants of liver abnormalities can be seen; from asymptomatic elevations of liver transaminases to acute or chronic hepatitis, fulminant hepatic failure or end-stage cirrhosis (Ala et al. 2007). A cholestatic presentation is suggestive of a non-Wilsonian disease. In cases of fulminant hepatic failure, a low ratio of serum alkaline phosphatase concentration to serum bilirubin concentration is believed to strengthen a possible diagnosis of WD (Sallie et al. 1992).

Neurologic symptoms are entirely coupled to the motor system and could be divided in four main subgroups: parkinsonian (akinesia and rigidity), pseudosclerotic (tremor and dysarthria), dystonic and choreic (Walshe and Yealland 1995). The neurologic symptoms can be illustrated by Wilson’s first case (fig.5), which was first described by Gowers (1888).

*Figure 5.* 10-year old boy with tremor, spasticity, and contractures characteristic of WD. From a silver print dated 1886. Patient one in SAK Wilson (1912), published with permission from Oxford University Press.
Diagnosis

The diagnosis of WD is based on a combination of findings. If KF-rings are present in a patient with hepatopathy (of non-cholestatic origin) and/or neurologic motor deficits and high urinary copper with low plasma caeruloplasmin are found, the diagnosis of WD is easily established. This situation is however not always the case. Ferenci et al. have proposed a scoring system for the diagnosis of WD (table 2). KF-rings are almost always present in patients with neurologic symptoms. The same is true for MRI of the brain where pathological changes are seen in the basal ganglia, but also in other parts of the brain, e.g. pontine lesions (Thuomas et al. 1993). In hepatic WD though, KF-rings may be absent in up to 50% of cases (Roberts and Schilsky 2003). Caeruloplasmin is an acute-phase reactant and plasma caeruloplasmin could be in the normal, albeit lower range, in up to 40% of patients with liver disease (Steindl et al. 1997). Urinary copper measurements are not uncomplicated, due to the risk of copper contamination from vessels used for collection, resulting in false positively elevated levels. The golden standard for diagnosis has so far been the performance of quantitative copper analysis in a liver biopsy. A value higher than 250 µg copper per gram dry liver (normal value less than 55) is considered supportive of WD. However, this method is invasive and copper could be unevenly distributed in the liver parenchyma. In addition, cholestatic liver disease is also known to result in increased liver copper levels. Radio-copper uptake has been used to distinguish between patients with WD, heterozygotes and healthy individuals (Sternlieb and Scheinberg 1979), but the method is not completely accurate in distinguishing between diseased and carriers, is labour intensive and the patient needs to be referred to a centre were the investigation can be done. As the gene is known and many mutations have been found – around 300 mutations had been described in January 2006 in the Wilson disease mutation database (http://www.medicalgenetics.med.ualberta.ca/wilson/index.php), genetic evaluation is a possible way of confirming the diagnosis of WD. In particular, this is an applicable method for the performance of family screening once the mutations of the index patient are known (Waldenström et al. 1996).
Table 2. A scoring system for the diagnosis of Wilson’s disease.
Modified from (Ferenci et al. 2003)

<table>
<thead>
<tr>
<th>KF-rings – present</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>KF-rings – absent</td>
<td>0</td>
</tr>
<tr>
<td>Neuropsychiatric symptoms suggestive of WD (or typical brain MRI)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>2</td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Coombs negative haemolytic anaemia (+ high serum copper)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>1</td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Urinary copper (in the absence of acute hepatitis)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>1-2 x upper limit of normal (ULN)</td>
<td>1</td>
</tr>
<tr>
<td>&gt;2 x ULN</td>
<td>2</td>
</tr>
<tr>
<td>Normal, but &gt;5 x ULN one day after challenge with 2 x 0.5 g D-penicillamine</td>
<td>2</td>
</tr>
<tr>
<td>Liver copper quantitative</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>-1</td>
</tr>
<tr>
<td>Up to 5 x ULN</td>
<td>1</td>
</tr>
<tr>
<td>&gt;5 x ULN</td>
<td>2</td>
</tr>
<tr>
<td>Rhodanine pos. hepatocytes (only if quantitative Cu measurement is not available)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Present</td>
<td>1</td>
</tr>
<tr>
<td>Serum caeruloplasmin (nephelometric assay, normal: &gt;20 mg/dL)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>10-20</td>
<td>1</td>
</tr>
<tr>
<td>&lt;10</td>
<td>2</td>
</tr>
<tr>
<td>Disease causing mutations on both chromosomes</td>
<td>4</td>
</tr>
<tr>
<td>Disease causing mutation on one chromosome</td>
<td>1</td>
</tr>
<tr>
<td>No disease causing mutation detected</td>
<td>0</td>
</tr>
</tbody>
</table>

Assessment of the WD-diagnosis score:
4 or more: diagnosis of WD highly likely
2-3: diagnosis of WD probable, do more investigations
0-1: diagnosis of WD unlikely
Treatment

The treatment is aimed at reducing the copper load in the body and thereafter to keep copper at a normal level, without inducing copper deficiency. For the last 50 years, penicillamine (Walshe 1956) has been the prime drug used world-wide. Another copper chelator, trientine (\(N,N'\)-bis(2-aminoethyl)-1,2-ethanediamine dihydrochloride), primarily developed for use in patients with penicillamine intolerance (Walshe 1969), has become an alternative for use also as initial therapy because of fewer severe side-effects and similar therapeutic efficacy (Dahlman et al. 1995; Merle et al. 2007). For maintenance therapy, the patient could be transferred to zinc, an excellent inducer of metallothionein, which binds copper at high affinity, both in the intestinal cells, that are subsequently sloughed off and removed in the faeces, and in the liver were it can bind copper, making it harmless for the tissue (Schouwink 1961; Hoogenraad et al. 1978; Hoogenraad et al. 1979). Tetrathiomolybdate seems to be a good, albeit still not commercially available, option in treating patients with neurological presenting symptoms, as chelating treatment may worsen the patient’s deficits. It acts by complexing copper in the intestinal lumen together with ingested food and by making copper unaccesible in the circulation by complexing it together with albumin. Tetrathiomolybdate was the third therapeutic regimen for WD proposed by John Walshe (Walshe 1984). A recent trial showed promising results concerning the risk of neurologic deterioration which were less frequent in patients treated with tetrathiomolybdate as compared with those treated with trientine (Brewer et al. 2006).

In severe liver decompensation, due to subacute fulminant hepatitis – often with severe haemolysis – or in end-stage liver cirrhosis, liver transplantation has become a curative option for the last 25 years (Starzl et al. 1982).
Aims of the investigation

The aims of the present investigation were

- To study the mutational spectrum in Swedish WD patients using a newly developed solid phase sequencing method for sample handling (Manifold sequencing) and further, to investigate its usefulness as a diagnostic tool.

- To investigate the prevalence of WD in Sweden by analyzing the occurrence of the two most common WD mutations in an anonymous Swedish blood sample material and further, to explore the usefulness of a mini-sequencing method to study single nucleotide polymorphisms (SNPs) in pooled DNA samples.

- To study the possibility of a genotype-phenotype correlation in WD, in particular regarding psychopathological symptoms but also concerning age of onset, clinical presentation and laboratory parameters such as ceruloplasmin levels.

- To study liver copper metabolism, in particular copper uptake, in vivo in humans by using $^{64}$Cu and positron emission tomography (PET), comparing patients with WD, heterozygotes for WD, patients with liver cirrhosis of non-Wilsonian origin and healthy volunteers.

- To investigate the occurrence of abdominal malignancies, above all hepatocellular carcinomas, in patients with treated WD with regard to the type and duration of treatment.
Material and methods

The Department of Internal Medicine at Uppsala University Hospital has since the 1980s been a referral centre for patients with WD. The estimated number of patients known in Sweden amounts to around 50 and a little more than half of them are currently cared for in Uppsala.

Study I

In the first study, 26 patients were analysed concerning sequence variations in \textit{ATP7B}. The study was performed with a new method of processing large sets of sequencing reactions, so called manifold sequencing (Lagerkvist et al. 1994), now commercially available from GE Healthcare. When performing PCR reactions, one primer is 5’-biotinylated and the other has the sequence of the M13 universal sequencing primer as a 5’ addition. The biotinylated PCR products are then bound to a set of solid supports consisting of plastic combs with streptavidin-coated teeth, for preparing sets of sequencing templates, performing sequencing reactions and direct loading of the reaction products on an ALF (automated laser fluorescence DNA sequencer, GE Healthcare, Uppsala, Sweden) sequencing gel (fig. 6).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{manifold_sequencing}
\caption{The principle of manifold sequencing: i) binding of biotin-labeled PCR products to the streptavidin-coated support; ii) alkaline denaturation of comple-}
\end{figure}
mentary strands; iii) annealing of the fluorescein-labeled sequencing primer; iv) extension reactions in the presence of dideoxynucleotides; v) transfer of reaction products to the sequencing gel using the manifold support, the products being released from the supports by being denatured in a layer of formamide, previously added to the top of the gel. Reprinted from (Barbany et al. 1999), with permission from the publishers.

Study II

In the second study we wanted to estimate the carrier frequency of mutations causing WD in Sweden, by analysing the two most common mutations in 2640 anonymous blood samples. The genotyping was performed by minisequencing on a solid support (Syvänen et al. 1993). This method is based on primer extension of one out of two labeled nucleotides on single-stranded PCR products. When performing the PCR amplification, one primer is biotinylated. The biotinylated PCR product is bound to the streptavidin-coated solid support. During the ensuing washing, unbound product as well as dNTPs and primers are removed. Alkaline washing will generate single-stranded DNA. In the subsequent minisequencing reaction, a detection primer is hybridised immediately flanking the variable nucleotide position and is extended by a single radiolabeled nucleotide. The signals from the incorporated nucleotides in the two sequences are measured and the results are obtained as numeric values directly reflecting the relative amounts of the sequences in the sample (fig. 7). To be able to analyse such a large amount of samples, a pooling strategy with pooling of 10 samples for each sequencing reaction was used. Thus, one allele will constitute 5% of the total number of sequences analysed. We also estimated the allele frequencies of eight of the neutral polymorphisms found in study I by minisequencing a superpool of 2500 DNA samples. The allele frequencies in the pooled DNA sample was computed by comparing the ratio derived from the pool with the ratio derived from an heterozygous person, in which the alleles are present in a 1:1 ratio, according to the formula:

\[
\begin{align*}
    f_{\text{allele 1}} &= \frac{R_{\text{pool}} / R_{\text{Het}}}{1 + R_{\text{pool}} / R_{\text{Het}}} \\
    f_{\text{allele 2}} &= \frac{1}{1 + R_{\text{pool}} / R_{\text{Het}}}
\end{align*}
\]

The allele frequencies of the polymorphisms were also investigated by genotyping 20 randomly selected individual DNA samples as a control.
Figure 7. The principle of the solid-phase minisequencing method. A DNA fragment spanning the polymorphic site is amplified using one biotinylated and one unbiotinylated PCR primer. The biotinylated PCR product is captured in a streptavidin-coated microtitration well, and the unbiotinylated DNA strand is removed by alkaline denaturation. The nucleotides at the polymorphic site are identified in the immobilised DNA strand by two separate minisequencing reactions. In this reaction a detection-step primer annealing immediately adjacent to the mutation is elongated by a DNA polymerase with one single $^3$H-labeled dNTP complementary to the nucleotide at the polymorphic site. The detection-step primer is released after the reaction, and the incorporated label is measured in a scintillation counter. The result of the test is expressed as the ratio between the labels incorporated in the two reactions. Reprinted from (Syvänen et al. 1993), with permission from the publishers.
Study III

In the third study, 12 patients with four different mutational settings were compared concerning psychopathological parameters. DNA sequencing as described earlier was used for genotyping. The clinical assessment comprised CPRS (Comprehensive Psychopathological Rating Scale) expert rating and the Karolinska Scales of Personality (KSP). CPRS comprises 65 items (40 reported items and 25 observed items) of psychopathological symptoms (Åsberg et al. 1978). One item is a global measurement of illness and one item takes into account the assumed validity of the rating performed, graded 1–3, where 1 means poor and 3 represents good validity. All 65 items of psychopathological symptoms are colloquially formulated, and staged from 0 to 3 by half point steps, where 0 indicates absence of the particular symptom. A rating of 1 is a description that could apply to a pathological deviation from the individuals own norm, but might equally well be considered a normal variation in a group of people. A rating of 2 clearly indicates pathological symptoms and 3 indicates the most severe degree. Three subscales from CPRS have been used in the present study; a 10-item Brief Anxiety Scale (BSA) (Tyrer et al. 1984), a 10-item depression rating scale – the Montgomery-Åsberg Depression Rating Scale (MADRS) (Montgomery and Åsberg 1979), and an 8-items Obsessive Compulsive Symptoms Scale (OCD) (Lindström and Lindström 1996). KSP is a self-reported inventory comprising 135 questions grouped into 15 scales (Schalling et al. 1987). The 15 scales can be grouped in the following way: 1. Impulsivity, sensation seeking and social withdrawal scales: Impulsiveness, i.e. acting on the spur of the moment, non-planning; Monotony Avoidance, i.e. avoiding routine, need for change and action; Detachment, i.e. avoiding involvement with others, withdrawn. 2. Psychopathy versus conformity scales: Socialization, i.e. positive childhood experiences, good school and family adjustment; Social Desirability, i.e. socially conforming, friendly and helpful. 3. Anxiety-related scales, a) Nervous tension and distress: Somatic Anxiety, i.e. autonomic disturbances, restlessness, panicky; Muscular Tension, i.e. tense and stiff, b) Cognitive-social anxiety: Psychic Anxiety, i.e. worrying, anticipating, lacking self-confidence, sensitive; Psychasthenia, i.e. easily fatigued, feeling uneasy when urged to speed up and when facing new tasks; Inhibition of aggression, i.e. non-assertive, sad rather than angry when scolded, cannot speak up. 4. Hostility-related scales: Suspicion, i.e. suspicious, distrustful, trusting people’s motives; Guilt, i.e. remorseful, ashamed of bad thoughts, 5. Aggressivity-related scales: Indirect Aggression, i.e. sulking, slamming doors when angry; Verbal Aggression, i.e. getting into arguments, telling people off when annoyed; Irritability, i.e. irritable, lacking patience. The scales have been demonstrated to have long-term stability (Gustavsson 1997).
The raw scores of KSP were transformed into T-score (Mean = 50 and SD = 10) on basis of random sample of normal controls (200 men and 200 women).

Study IV

In the fourth study, PET with $^{61}$Cu was used to investigate copper uptake in liver in male subjects; four patients with WD, two of them recently diagnosed and without treatment, three controls heterozygous for mutations in $ATP7B$, two controls with non-Wilsonian liver cirrhosis, and four controls without $ATP7B$ mutations. $^{61}$Cu was prepared by irradiating nickel foils with 6 MeV deuterons at the MC17 cyclotron of Uppsala University PET Center (Tolmachev et al. 1998) and the copper was subsequently separated using a liquid extraction technique with dithizone in carbon tetrachloride, chloroform and methylene chloride. Hydrochloric acid was used for the re-extraction of copper. The final solution was diluted 1 in 10 in saline and a final amount of about 40 MBq was injected intravenously. The dose level in our patient studies was about 2.5 mSv, which is an acceptable level for diagnostic purposes.

PET measurements were performed with the General Electric Medical Systems (GEMS) 4096. A standardised supine position was selected so as the liver was situated in the field of view. Emission scans were performed for the first 60 minutes (10 x 1 minute followed by 10 x 5 minutes), ensued by transmission and emission scans at 2.5, 4.5, 6.5 and 8.5 hours following tracer injection.

To calculate the transport rate of the tracer from plasma into the liver, blood samples were taken at the same times as the emission scans were performed and the plasma radioactivity concentrations were measured in a well-type crystal detector cross-calibrated with the camera.

Before reconstruction, the files were attenuation corrected, using the transmission scan, corrected for scattered radiation, and filtered with a 4.2-mm Hanning filter. The matrix size was 128 x 128, and the pixel size was 2.0 mm.

For the basic analysis, we used the semiquantitative approach based on the calculation of a distribution value, for which the term standardised uptake value (SUV) was introduced by Strauss and Conti: SUV = tissue concentration (MBq/g)/(injected dose [MBq]/body weight [g]). Three circular regions of interest (ROIs), altogether about 100 cm$^2$, were placed in the lateral, central and medial portion of the liver upon visual inspection of the radioactivity images. The average time-activity curve for these ROIs was used for the subsequent analysis.

To analyse the kinetics of liver $^{61}$Cu uptake, time-activity curves for the liver ROI were compared with plasma kinetics of total $^{61}$Cu. The liver cop-
per influx rate constant (Ki) was calculated using the multiple time graphical analysis (Patlak and Blasberg 1985) using plasma radioactivity as reference. It could be shown that the method effectively generates a linear description of the increase of $^{61}$Cu liver/plasma ratio from 5 minutes of real time after injection. The slope (i.e. Ki) was determined by linear regression analysis using least square regression analysis from data collected 5 to 60 minutes of real time after tracer injection.

Student’s independent $t$-test was used to calculate $p$-values between Ki values of the different groups.

Study V

In the fifth study, cancer incidence was retrospectively studied in two patient cohorts from the UK (one in Cambridge between 1955 and 1987 and one in London between 1988 and 2000; $n = 310$) as well as from one in Sweden (between 1966 and 2002; $n = 53$).

The incidence figures of the Swedish WD patients were compared with Swedish cancer incidence statistics from the Swedish Board of Health and Welfare (http://www.socialstyrelsen.se/Statistik/statistik_amne/Cancer).
Results and discussion

Study I

By sequencing WD patients from 24 WD families in Sweden, a newly developed method for handling sequencing reactions was used for the first time in sequencing a large monogenetic disease gene in 1996. The year before, the tumour suppressor gene p53 had been sequenced in 316 breast cancer samples with the same method, first using RNA to cDNA conversion and four PCR amplifications in each patient sample (Bergh et al. 1995). In that study, only four patients had to be excluded due to an incomplete p53 sequence.

Of the 48 alleles examined in our study, mutations were found in 44, giving an accuracy of 92%. In the mid 1990s, sequencing was still quite labourious and screening for mutations with e.g. SSCP was almost always done prior to sequencing. The first major mutational surveys in WD patients were done in 1995 in Italy and Canada (Figus et al. 1995; Thomas et al. 1995), using SSCP as screening. In those studies around 50% of putative mutations were found. A much more sensitive method, denaturing high-performance liquid chromatography (DHPLC) has subsequently been developed, showing a sensitivity approaching 100% (Nickerson et al. 2000). A study investigating five Sardinian WD families with DHPLC showed a complete success rate, except for a polymorphism that could not be found, probably because it was situated only 13 bp from one of the primer sequences (Weirich et al. 2002).

As in our study, Figus et al. and Thomas et al. also found that about one third of the mutations in patients from Northern and Eastern Europe was a C to A transversion at position 3207. This results in a change of amino acid 1069 histidine to glutamine (H1069Q), located in the conserved SEHPL domain, which is important for ATP-dependent phosphorylation (Tsivkovskii et al. 2003). This mutation was found in 13% of the Mediterranean population (Figus et al. 1995), excluding Sardinia. In Sardinia a high rate of consanguinity has resulted in the highest world-wide incidence of WD, around 1 in 7,000. In that population a specific 15-nt deletion in the 5' UTR accounts for 6 out of 10 mutated alleles (Loudianos et al. 1999). H1069Q is the most prevalent mutation in the European population with highest numbers in Central Europe; 70% in Poland and around 60% in Austria and Eastern Germany (Caca et al. 2001; Riordan and Williams 2001).
Except for H1069Q, only two mutations were found in more than two families in our study. T977M had previously been described and was then believed to be a polymorphism (Thomas et al. 1995), but we could not find any other sequence changes in our patients. It has later been shown that this mutation makes ATP7B completely without activity in a yeast complementation assay (Forbes and Cox 1998). This mutation has now been found in five families in Sweden including one unpublished, in three of which the patients were homozygous. R1319X was found in three families, all compound heterozygous. Genotype phenotype correlations were hard to demonstrate, as the number of patients is small, but a trend towards a later age at disease onset was seen in H1069Q. Several studies have been published concerning this mutation and its phenotype. Most of the studies have found a trend towards late disease onset and more neuropsychiatric problems than hepatic symptoms have been reported, but some studies have not been able to corroborate these findings (Riordan and Williams 2001; Stapelbroek et al. 2004). We have ourselves encountered some exceptions concerning the belief that H1069Q should be a benign mutation. During the last decade we have seen a boy who was homozygous for H1069Q presenting with hepatic symptoms at age 10 and one woman aged 20 presenting with fulminant hepatic failure requiring liver transplantation. One of the patients in our study was a 28 years old woman with rapidly progressing hepatic failure in the post partum period, who could be successfully diagnosed with WD in 24 hours by performing manifold sequencing that could prove her being homozygous for H1069Q. This illustrates the capacity using manifold sequencing to sequence a large gene in a comparatively short time in order to confirm a suspicion of a specific disease. One main advantage with manifold sequencing, apart from easy parallel handling of many sequencing reactions, is that the risk of sample contamination or mix-up is minimised, which is imperative when dealing with patient samples. Since our study was done, sequencing techniques have rapidly evolved, particularly with the advent of capillary electrophoresis methods (Kan et al. 2004), making it possible to sequence large amounts of DNA in a short time. This made it possible to finish the human genome project in 2003 and to continue to sequence new species’ genomes. However, the manifold sequencing method is a most useful method when it comes to sequencing a given gene.

Study II

One way of elucidating the frequency of a given monogenetic disease is by estimating the carrier frequency of the disease gene in the normal population and using the Hardy–Weinberg equation for calculating an approximate prevalence. To be able to use such a calculation, some requirements have to be met. The studied genotype must be randomly distributed in the popula-
There should be no significant nonrandom mating (inbreeding), a low degree of de novo mutations and a low degree of selection and migration. The Swedish population is not believed to have the same pattern of bottleneck genotypes as for instance the Finnish (Peltonen et al. 1999), so a fairly random distribution of the gene-pool could be envisaged in Sweden. Furthermore, the Swedish population has been stable with a relatively low degree of immigration until the late 60s. Inbreeding in our WD patients is rare and de novo mutations have not been frequently reported in the WD scientific literature, but this is largely unknown, as most of the parents of analysed patients have not been investigated for carriership.

In study II we thus estimated the frequency of WD in the Swedish population. We found four alleles with the sequence variant C3207A (the most frequent disease causing mutation in Europe) in 5280 alleles in a normal Swedish population sample, i.e. 1 in 1320 alleles giving a carrier frequency of that mutation in 1 in 660 normal people in Sweden. The second most common disease causing allele C2930T was only found in one allele. For calculation of the overall rate of carriership of any WD-causing mutation, the proportion of C3207A in WD patients must be known. In study I we found about one in three mutated alleles being C3207A, which is in good accordance with other studies, e.g. Olivarez et al. who have performed a similar carrier study (Olivarez et al. 2001). They used one in three when obtaining a WD prevalence of 1/55,000. Using one in three we arrive at a Swedish prevalence of 1/193,600. Due to the skewed age profile in our material, with a preponderance for older patients with C3207A, we have calculated the allele frequency of C3207A in patients born in the 1960s. These were selected because most of the patients born in that decade ought to have been diagnosed by now and patients born earlier are more likely to have died without being diagnosed. Using these figures we get an allele frequency of close to one in four for C3207A. Using the Hardy–Weinberg calculation, a frequency of WD in the Swedish population would then be 1/110,000 with a 95% confidence interval of 1/20,000 to 1/1,200,000. The mean number of newborns each year in Sweden is around 110,000, which gives a mean incidence in the Swedish population of around one new case of WD each year. This is in good accordance with our observations of patients with WD in Sweden. The Swedish population thus seems to have a lower frequency of WD than what has been reported from other parts of the world. A prevalence of 1 in 30,000 is widely accepted, based on studies in East Germany (Bachmann et al. 1979) and Japan (Saito 1981). The discrepancy between the frequency figures in Sweden and those worldwide could be explained in three different ways. First there might be a true difference attributed to the Swedish population being different in the number of WD mutation carriers. It could also be due to the relatively small numbers in our study. The number of C3207A mutations found could be too low (or too high). In order to get a clue about this risk, we also analysed the second most common Swedish
mutation (C2930T), seen in approximately one out of seven of our WD patient alleles. This mutation was only found once in our material (leading to a deduced frequency of 1/570,000), at least strengthening the contention that the WD frequency is not higher than 1/110,000. The third explanation would be that the worldwide frequency is overestimated and that it should be more close to 1/100,000 with local areas of higher prevalences like Sardinia (Giaghaddu et al. 1985) and Japan (Saito 1981). Other prevalence studies show a somewhat lower number than 1/30,000. In an Irish study, a birth incidence rate of 17 per million live births was found, giving a gene frequency of 4.1 ‰ (Reilly et al. 1993). Whether allowing for a maximal degree of consanguinity or not, the frequency of WD in Ireland would be 1/60,000 to 1/80,000. As mentioned above, Olivarez et al. in 2001 found a prevalence of about 1/55,000. When Cauza et al. investigated the geographic distribution of all WD patients in Austria, they found only half of the patients that should have been diagnosed calculating from known prevalence figures presented above (Cauza et al. 2000). This either means that a large proportion of patients is not diagnosed or that the prevalence figures are too high.

In study II a new way of investigating haplotypes in large samples by pooling 10 samples and then performing solid phase minisequencing were performed. A discrimination factor of 5% are then needed and we could show that this was feasible without any risk of missing a mutation by doing titration curves where a cut-off of around 1% for C2930T and 3% for C3207A was found. The method was also used to analyse the ratios of eight intragenic polymorphisms and the results were compared by minisequencing 20 random individual DNA samples from the original sample cohort. In six cases the similarity was excellent but lower in the remaining two, as could be expected by pure chance.

Study III

Genotype phenotype correlations have been focused on age at disease onset and whether hepatic or neuropsychiatric symptoms were present at onset. Kamilla Portala et al. have thoroughly investigated different psychopathological variables and personality traits in our WD population (Portala et al. 2001a; Portala et al. 2001b). In study III we investigated four groups from that population, each with three patients, three groups being homozygous and one compound heterozygous for mutations in the WD gene.

Concerning psychopathological symptoms, the patients homozygous for W779X had the lowest scores on the total CPRS, mainly due to less pronounced reported CPRS items and a low variability as compared to patients homozygous for T977M, H1069Q mutations respectively, or those compound heterozygous for H1069Q/R1319X. The three patients with
H1069Q/R1319X mutations had the highest scores on the total CPRS as well as on reported and observed items.

The patients who were homozygous for the W779X mutation had the highest scores on the KSP Psychopathy vs. conformity related scales; Social desirability and Socialization scales as well as the lowest scores on the Impulsivity, sensation seeking and social withdrawal scales; Impulsiveness, Monotony Avoidance and Detachment scales. The patients homozygous for the T977M mutation also had relatively high scores on the Psychopathy vs. conformity related scales whereas patients with H1069Q/R1319X mutations had the lowest scores on these scales, below those of healthy volunteers.

Our results support the notion that patients with WD resulting from the homozygous stop codon W779X in exon 8 and the T977M mutation in exon 13 respectively seem to present with a different phenotype as compared to patients with the other mutations. Patients who were homozygous for the W779X mutation but also patients with the T977M mutation tended to differ the most as concerns psychopathology and personality traits from patients with the other mutations investigated. All patients with the W779X and T977M mutations also had undetectable caeruloplasmin levels, consistent with previous findings, where significantly lower caeruloplasmin levels were noticed in patients with frameshift and nonsense mutations as compared with ordinary missense mutations (Panagiotakaki et al. 2004). This further supports the notion that W779X and T977M mutations also are functionally more severe concerning the parameters studied in our trial.

Patients with WD express an abnormal metabolism of neurotransmitters, particularly noradrenaline, dopamine and serotonin, as judged by neuroimaging findings (Nyberg et al. 1982; Hawkins et al. 1987; Oertel et al. 1992; Westermark et al. 1995; Jeon et al. 1998; Eggers et al. 2003; Hesse et al. 2003) and biochemical findings at autopsy of a young girl with WD (Nyberg et al. 1982). According to our results, the KSP scales that mainly differ in the patients for the APT7B gene mutations include Psychopathy vs. conformity related scales as well as Impulsivity, sensation seeking and social withdrawal scales. Socialization, often found to be extremely low in patients with antisocial personality disorder, has been linked to deficiencies in the serotonergic systems (Virkkunen et al. 1994). Socialization has also been linked to deficiencies in the glucose metabolism (Farde et al. 1997), and a reduced glucose metabolism in all brain regions except the thalamus has been demonstrated in WD patients (Hawkins et al. 1987). The withdrawal scale, i.e. Detachment has been found to correlate negatively with D2 receptor density and dopamine transporter binding in healthy humans (Farde et al. 1997; Lakso et al. 2000).

This is the first time that genotypes of a gene that is not known to be directly involved in neurotransmission in the central nervous system could be implicated in giving diverse phenotypic psychopathological manifestations.
Study IV
When applying $^{61}$Cu PET of the liver, the four patients with WD showed significantly slower uptake of copper than the three heterozygotes ($p = 0.01$) and four normal controls ($p = 0.02$) respectively. However, compared with two patients with alcoholic liver cirrhosis, only a trend towards lower copper uptake was seen ($p = 0.14$). Our data corroborate the findings by Bush et al. performed already in 1955 and later studies by among others Osborn and Walshe during the 1960s (Bush et al. 1955; Osborn and Walshe 1969). In contrast to results obtained in some of their studies, we did not find any differences in copper uptake between heterozygotes and normal individuals. When comparing, it should be remembered though that their studies largely were performed over 2 to 24 hours, whereas our investigation covered the very early copper uptake phase occurring already within the first hour following the i.v. administration of $^{61}$Cu and using an evaluation method that allowed for a separate calculation of copper uptake from that of further intracellular copper handling and excretion. In a study from the mid 1970s, two patients, two probable heterozygotes and one presumably normal control individual were investigated with a double labelling technique also involving $^{51}$Cr together with standard $^{64}$Cu measurements (Günther et al. 1975). As in our study, focus was on the early hepatic uptake of copper and the results during the first hour were quite similar to those obtained by us.

That the rate of copper uptake into the liver is diminished in patients with WD, thus seems to be an unrefutable fact. It is reasonable to suggest that the lowered copper uptake could reflect a downregulation of Ctr1, but the physiological importance of that is currently unknown. An interesting question is raised by the fact that we and others have found a somewhat diminished copper uptake in some patients with (preferentially alcoholic) cirrhosis of the liver. Some studies indicate that patients with alcoholic liver cirrhosis have increased levels of hepatic copper (Zarski et al. 1985; Rodriguez-Moreno et al. 1997). If this is true, it could strengthen the contention that the amount of intracellular copper in the hepatocytes is the key factor for explaining the slower copper uptake. Another possibility is that the cirrhosis per se could lead to a reduced blood flow and consequently to diminished uptake over time. This is however unlikely concerning the low degree of fibrosis in the two of our patients that had been investigated – fibrosis scores (Ishak et al. 1995) were 1/6 and 3/6 respectively. Moreover, Osborn and Walshe conclude in their study in Lancet 1969 that they were not able to find any direct correlation between impaired uptake of copper and either the structural or functional abnormality in the liver (Osborn and Walshe 1969). They explain their findings of reduced uptake with a progressive saturation of binding sites for copper. Studies during the last decade on Ctr1, the prime membrane transporter regarding copper uptake, have shown that elevated intracellular copper levels stimulate the endocytosis and degradation of Ctr1.
(Petris et al. 2003; Guo et al. 2004). A reduced capacity of Ctr1 might then explain the reduced hepatic uptake in our and previous studies. Since no consistent differences were detected between previously untreated or treated WD patients in our study, it is possible that the hepatocyte copper levels necessary to inhibit Ctr1 function would not have to be elevated to extreme levels.

The results of our study imply that $^{61}$Cu liver PET might be used as an additional rapid and little invasive diagnostic technique in WD.

Study V

In our combined cohort of 363 patients, gathered in Uppsala, Cambridge and London, nine patients had developed aggressive intra-abdominal malignancies. Only two of these were classified as hepatocellular carcinomas (HCC). Patients with another metabolic disorder leading to cirrhosis of the liver, familial haemochromatosis (FH), have an increased risk of developing HCC. Earlier studies have described up to 200-fold increases, but later studies have shown lower figures (Kowdley 2004). Interestingly, no occurrence of HCC have been published in the 46 (Shang et al. 2006) described families with acaeruloplasminemia, a disorder characterised by diabetes mellitus, retinal degeneration and neurological symptoms. Acaeruloplasminemia leads to iron accumulation in most organs, including the liver, but usually does not produce any hepatic symptoms and does not result in liver cirrhosis (Gitlin 1998).

Only 17 cases (including the two of our own) with WD and HCC are found in the scientific literature (Savas et al. 2006) and one case published in December last year (Aydinli et al. 2006). This is in contrast to the high HCC risk in LEC rats, which is the best-studied animal model of WD. The LEC rat has a 900 bp deletion of the coding region at the 3’ end of the rat homologue of $ATP7B$ (Wu et al. 1994). The LEC rat shows a hepatic morbidity similar to WD, but a very high risk of progression to HCC (Masuda et al. 1988). Our two patients with HCC both had negative hepatitis serologies, otherwise a known risk factor for HCC. Both patients had liver cirrhosis at autopsy, also a risk factor for HCC. The histopathological findings in our seven patients that did not have HCC showed two cholangiocarcinomas and in the remaining five, widespread intra-abdominal cancers without known primary site, but with somewhat similar histopathology; multifocal, disseminated small, poorly differentiated adenocarcinomas. An additional two patients are described in our study, but not included in the calculations. They were two siblings living in the USA, both being diagnosed at a late age; the brother at 56 years of age and his sister presymptomatically at age 63. The brother died of unclassified “liver cancer” at 73 years of age and his sister had cholangiocarcinoma at age 85. The remaining nine patients in our study
were diagnosed with malignant disease between 28 and 62 years of age (median age 42 years). These patients had been on WD medication for a time of 11 to 39 years with a median of 20 years. Including the female American patient, at least three patients had cholangiocarcinomas. Malignancies arising from the bile ducts have been considered rare in FH patients, but were found in 7 out of 20 patients with FH (all being homozygous for C282Y – cysteine being substituted by tyrosine at residue 282 in HFE, the hereditary haemochromatosis protein) in a French study (Morcos et al. 2001). Four were hepatocholangiocarcinomas and three cholangiocarcinomas. Sporadic cases of cholangiocarcinomas in WD have been published (Kosminkova et al. 1995; Merle et al. 2007). In contrast to the LEC rat, Atp7b−/− mice do not show HCC but rather after nine months of age changes in the bile duct morphology consistent with cholangiocarcinoma (Huster et al. 2006). In a study by Schilsky et al., 54 LEC rats were examined and all developed cholangiofibrosis at 27 weeks of age. No animal had any sign of cholangiocarcinoma though and only two were excluded because of HCC (Schilsky et al. 1998). This is in contrast to previous Japanese studies showing a much larger proportion of HCC in LEC rats. The authors speculate that this discrepancy could be due to differing environmental or dietary factors or that it might be caused by different LEC rat lineages with another linked mutation in one of the lineages. Meng et al. showed that LEC rats transfected with human ATP7B largely were rescued from the hepatic abnormalities seen in LEC rats, including the cholangiofibrosis and high rate of HCC, implying the direct relationship between hepatic pathology and impaired ATP7B and raised copper levels of the liver (Meng et al. 2004). The transgenes also had significant reductions in liver iron levels. The hepatic injuries in the transgene animals became severe with increasing levels of copper, but iron was not frequently deposited in their liver implying that iron deposition does not directly cause hepatitis but could contribute to the copper-induced injury of the liver.

Our results raise the question whether not patients with WD, at least after more than 10 years of disease, should be closely monitored with e.g. echohepatography at regular intervals in order to diagnose any neoplastic changes early at a treatable stage. The EuroWilson patient register (www.eurowilson.org), supported by the European Union Research Framework Programmes, might become useful for follow-up of the risk of cancer development in patients with WD.
Conclusions

- By using manifold sequencing, a high proportion of mutations in the Wilson’s disease gene can be found.
- A trend towards later age at disease onset and more patients with neuropsychiatric symptoms were seen in patients homozygous for the most prevalent mutation in Central and Northern Europe – H1069Q.
- The deduced prevalence of Wilson’s disease in Sweden is 1 in 110,000.
- A pooling strategy is a feasible way of estimating SNP frequencies.
- Psychopathology and personality traits in patients with Wilson’s disease might correlate with genotype.
- $^{61}$Cu PET of the liver shows that the copper uptake is diminished in patients with Wilson’s disease, possibly reflecting increased hepatic copper content.
- $^{61}$Cu PET of the liver could be a valuable accessory investigative mode in patients with presumed Wilson’s disease.
- Patients with Wilson’s disease seem to have an increased risk of developing aggressive intra-abdominal malignancies.
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References


Cumings JN (1948) The copper and iron content of brain and liver in the normal and in hepatolenticular degeneration. Brain 71:410-415


Fleischer B (1903) Zwei weitere Fälle von grünliche Verfärbung der Kornea. Klin Monatsbl Augenheilkd 41:489-491


Homén EA (1890) Eine eigenthümliche Familienkrankheit, unter der Form einer progressiven Dementia, mit besonderem anatomischen Befund. Zentralbl Neurol 9:514-518

Homén EA (1892) Eine eigenthümliche bei drei Geschwistern auftretende typische Krankheit unter der Form einer progressiven Dementia, in Verbindung mit ausgedehnten Gefässveränderungen (wohl Lues hereditaria tarda). Arch Psychiatr 24:191-229


Montgomery SA, Åsberg M (1979) A new depression scale designed to be sensitive to change. Br J Psychiatry 134:382-389


Ormerod JA (1890) Cirrhosis of the liver in a boy, with obscure and fatal nervous symptoms. St Bart Hosp Rep 26:57-68


Rumpel A (1913) Über das Wesen und die Bedeutung der Leberveränderungen und der Pigmentierungen bei den damit verbundenen Fällen von Pseudosklerose, zugleich ein Beitrag zur Lehre von der Pseudosklerose (Westphal-Strümpell). Dtsch Z Nervenheilkd 49:54-73


Siemerling E, Oloff H (1922) Pseudosklerose (Westphal-Strümpell) mit Cornealring (Kayser-Fleischer) und doppelseitiger Scheinkatarakt, die nur bei seitlicher Beleuchtung sichtbar ist und die dem nach Verletzung durch Kupfersplitter entstehenden Katarakt ähnlich ist. Klin Wochenshr i:1087-1089


Strümpell A (1898) Ueber die Westphal'sche Pseudosklerose und über diffuse Hirnsklerose, insbesondere bei Kindern. Dtsch Z Nervenheilkd 12:115-149


chemistries, glucose metabolism, and diurnal activity rhythms in al-
coholic, violent offenders, fire setters, and healthy volunteers. Arch
Gen Psychiatry 51:20-27

Völsch M (1911) Beitrag zur Lehre von der Pseudosklerose (Westphal-
Strümpell). Dtsch Z Nervenheilkd 42:335-352

Waldenström E, Lagerkvist A, Dahlman T, Westermark K, Landegren U
(1996) Efficient detection of mutations in Wilson disease by mani-
fold sequencing. Genomics 37:303-309


Semin Liver Dis 4:252-263

Walshe JM, Yealland M (1995) Not Wilson's disease: a review of misdiag-
nosed cases. QJM 88:55-59

(2002) Rapid identification of Wilson's disease carriers by denatur-

Westermark K, Tedroff J, Thuomas KA, Hartvig P, Långström B, Anders-
disease studied with magnetic resonance imaging and with positron
emission tomography using dopaminergic markers. Mov Disord
10:596-603

Westphal CFO (1883) Ueber eine dem Bilde der cerebrospinalen grauen
Degeneration ähnliche Erkrankung des centralen Nervensystems
ohne anatomischen Befund, nebst einigen Bemerkungen über para-
doxe Contraction. Arch Psychiatr Nervenkr 14:87-134, 767-769

Wilson SAK (1912) Progressive lenticular degeneration: a familial nervous
disease associated with cirrhosis of the liver. Brain 34:295-509

Wu J, Forbes JR, Chen HS, Cox DW (1994) The LEC rat has a deletion in
the copper transporting ATPase gene homologous to the Wilson dis-
ease gene. Nat Genet 7:541-545

Yamaguchi Y, Heiny ME, Gitlin JD (1993) Isolation and characterization of
a human liver cDNA as a candidate gene for Wilson disease. Bio-
chem Biophys Res Commun 197:271-277

Yang XL, Miura N, Kawarada Y, Terada K, Petrukhin K, Gilliam T, Sugi-
yama T (1997) Two forms of Wilson disease protein produced by al-
ternative splicing are localized in distinct cellular compartments.
Biochem J 326 (Pt 3):897-902

Zarski JP, Arnaud J, Dumolard L, Favier A, Rachail M (1985) Oligo-
eléments (zinc, cuivre, manganèse) dans la cirrhose alcoolique: in-
fluence de l'alcoolisme chronique. Gastroenterol Clin Biol 9:664-
669
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