



Agnese Zariņa

ORCID 0000-0002-0242-5796

Genetic Factors Characterising the Clinical Course of Wilson's Disease

Summary of the Doctoral thesis
for obtaining a doctoral degree (*Ph.D.*)

Sector – the Basic Sciences of Medicine,
including Pharmacology

Sub-Sector – Medical Genetics

Content

Abbreviations.....	4
Introduction	6
Topicality of the Study	7
Novelty of the Thesis	8
Aim of the Study	8
Study Objectives	8
Hypothesis	9
1. Material and methods.....	10
1.1 Study Groups.....	10
1.2 Molecular Investigations	11
1.3 Birth Prevalence Detection of WD.....	16
1.4 Statistical Analysis of the Data.....	16
2. Results.....	18
2.1 Prevalence of Wilson’s Disease in Newborn Cohorts	18
2.2 Characteristics of Wilson’s Disease Patients	18
Gender and Age at Onset of First Symptoms.....	18
Description of First Symptoms in WD patients	19
2.3 Analysis of the <i>ATP7B</i> Gene.....	21
Analysis of the <i>ATP7B</i> Gene Exons (Coding Parts)	21
Analysis of the <i>ATP7B</i> Gene Promoter.....	26
<i>ATP7B</i> Gene Screening with MLPA.....	26
<i>ATP7B</i> Gene Assay with qPCR	27
Impact of <i>ATP7B</i> Gene Allelic Variants on the WD Phenotype.....	27
2.4 Analysis of the <i>ATOX1</i> Gene	28
2.5 Analysis of the <i>COMMD1</i> Gene	30
2.6 Analysis of the <i>CP</i> Gene	33
2.7 Analysis of Allelic Variants of the <i>HFE</i> Gene	36
3. Discussion.....	39
Conclusions	50
References	51
Publications and presentations.....	61
Acknowledgments	64

Abbreviations

ACMG	American College of Medical Genetics and Genomics
ATOX1	Antioxidant 1 copper chaperone
ATP7A	ATPase copper transporting alpha
ATP7B	ATPase copper transporting beta
BKUS	Children Clinical university Hospital
BLAST	Basic Local Alignment Search Tool
COMMD1	Copper metabolism domain containing 1
CP	Ceruloplasmin
del	Deletion
DNA	Deoxyribonucleic acid
HFE	Human homeostatic iron regulator protein
HGMD	Human Gene Mutation Database
HGVs	Human Genome Variation Society
IQR	Interquartile range
KRIT1	Ankyrin repeat containing
MAF	Minor allele frequency
MLPA	Multiplex ligation – dependent probe amplification
mRNA	Messenger ribonucleic acid
OMIM	Online Mendelian Inheritance in Man
P.Stradins	Pauls Stradins Clinical University Hospital
KUS	
PAGE	Polyacryl gel electrophoresis
PCR	Polymerase chain reaction
PCR Bi-	Bidirectional PCR (polymerase chain reaction) amplification of
PASA	specific alleles
qPCR	Quantitative polymerase chain reaction

RAKUS	Riga East Clinical University Hospital
RFLP	Restriction fragment length polymorphism analysis
rs	Reference SNP (single nucleotide polymorphism)
RSU SLMG	Rīga Stradiņš University Scientific Laboratory of Molecular Genetics
SD	Standard deviation
Taq	Thermus aquaticus
UTR	Untranslated region
WD	Wilson's disease

Introduction

Wilson's disease (WD) is an autosomal recessive disorder of copper metabolism caused by approximately 800 allelic variants of the gene *ATP7B* with prevalence of 1 per 30,000 individuals in Europe (HGMD, n.d.).

Clinical manifestations of WD are very different, mostly including hepatic pathology and neurological symptoms. WD can be symptomatic at any age, but predominantly the first symptoms appear between five and 35 years of age (Ferenci et al., 2003). Although the disease is monogenic (a single gene is involved in the pathogenesis), there are significant differences in clinical manifestation and age at onset of first symptoms in patients of the same genotype, even within a single family.

Although the disease is caused by various allelic variants of the *ATP7B* gene, studies are ongoing to explain the variability of Wilson's disease. There are several studies on the different effects of the *ATP7B* gene allelic variants on the function of the ATP7B protein. There have been various studies on the association of the genotype-phenotype within the *ATP7B* gene – several with a positive finding [3,(Gromadzka et al., 2005), several with a negative finding (Moller et al., 2011; Mukherjee et al., 2014).

The possible effect of other genes on the WD phenotype has been discussed as well, with the main focus on genes encoding proteins involved in copper metabolism, e.g., *ATOX1* and *COMMD1*, as well as genes containing pathogenic variants that can influence inflammation in liver cells, e.g. *HFE*. The ATOX1 protein is a copper chaperone (small corrector molecule) that delivers copper to the metal binding domains of ATP7A and ATP7B (ATPase), while the COMMD1 protein is involved in the regulation of copper homeostasis, and changes have been found in Bedlington terriers suffering from copper toxicosis. Both genes have been studied among WD patients in different

populations, but results are inconsistent [7,(Lovicu et al., 2006) 9, (Simon et al., 2008; Czlonkowska et al., 2018).

WD diagnosis is based on diagnostic guidelines adapted in 2001 (EASL, 2012), where reduced levels of ceruloplasmin in the blood are one of the main diagnostic criteria. Other reasons, such as allelic variants of the *CP* gene encoding ceruloplasmin, may also cause this change.

Several studies have found an association between allelic variants in the CP gene that, by altering blood levels of ceruloplasmin, may be associated with increased iron accumulation in the brain, affecting the clinical course of multifactorial diseases such as Parkinson's disease (Zhao et al., 2015).

Topicality of the Study

Wilson's disease is one of the rare genetic diseases that can be successfully treated. Often, clinical diagnosis is delayed until copper has already accumulated in the tissues and irreversibly damaged them. DNA diagnostics provide the opportunity to diagnose WD presymptomatically (more often with relatives of already-diagnosed patients through sequential testing/cascade screening of family members). Detecting this disease and initiating preventive therapy before the onset of liver or neurological dysfunction can save a person's life and prevent disability. In Latvia, DNA diagnostics of Wilson's disease have been performed since 2004, mainly based on the detection of the most common pathogenic allelic variant, H1069Q, which causes 35–45 % of cases in WD (Hedera, 2017). Direct sequencing of the *ATP7B* gene is required to detect other WD-causing allelic variants. The *ATP7B* gene has 21 exons (coding parts of the gene), the direct sequencing of which is a time-consuming and costly process, which is why molecular analysis of Wilson's disease patients in Latvia, identifying allelic "hot spots", would help develop the algorithm for Wilson's molecular diagnostics, diagnosing WD in other patients faster and cheaper.

If other genes were found to influence the clinical course of Wilson's disease, a more complete picture of the disease's pathogenesis would probably be obtained, leading to a better understanding of the disease's progression, paving the way for a more complete prognosis of disease progression.

Novelty of the Thesis

The present study first investigates allelic variants of *CP* gene among WD patients. Analysis of these variants in association with Parkinson's disease and atrial fibrillation is described in the literature before. For the first time, Wilson's disease prevalence in newborn cohorts in Latvia was determined.

Aim of the Study

The aim of the study was to investigate genetic factors characterising Wilson's disease and determine their association with clinical manifestations of the disease.

Study Objectives

- 1) To detect the most common Wilson's disease-causing *ATP7B* gene allelic variant H1069Q by PCR-BiPASA in patients with clinically confirmed or suspected Wilson's disease and to determine the incidence of this variant;
- 2) to perform direct sequencing of exons (coding regions), promoter region (non-coding part) and to perform *MLPA* (multiplex ligation – dependent probe amplification) of the *ATP7B* gene in patients with clinically confirmed or suspected Wilson's disease which is not molecularly confirmed, and to detect the most common allelic variants;
- 3) to determine the prevalence of Wilson's disease in Latvian neonatal cohorts;

- 4) to compare clinical data of Wilson's disease patients with allelic variants of the *ATP7B* gene;
- 5) to determine allelic variants of the *ATOX1*, *COMMD1*, *CP* and *HFE* genes among molecularly confirmed patients with Wilson's disease and to estimate their possible association with the WD phenotype.

Hypothesis

Wilson's disease is a monogenic genetic disorder caused by various pathogenic allelic variants of the *ATP7B* gene, but different clinical courses are influenced by various pathogenic allelic variants of the *ATP7B* gene as well as changes in other genes involved in copper and iron metabolism (*ATOX1*, *COMMD1*, *CP* and *HFE*).

1. Material and methods

1.1 Study Groups

The study was approved by the Central Ethics Committee of the Republic of Latvia and was conducted in accordance with international law, Latvian law, and the Helsinki and Taipei Declaration. Prior to commencement of the study, each patient became familiar with the “Information for the patient” and confirmed their consent by signing a “Confirmation of consent”. If the individual was a minor at the time of collection of their biological material, one or both biological parents had signed the informed consent form. Prior to 2016, the study was conducted within the framework of a study entitled “Polymorphisms of Gene Encoding Genes Involved in Xenobiotic Metabolism in the Population of Latvia and Their Role in Choosing Therapy for Liver-Metabolized Medicines” (courtesy of the Central Ethics Committee of the Republic of Latvia).

The study identified data from all patients whose biological samples were sent to the RSU Scientific Laboratory of Molecular genetics (SLMG) for WD DNA diagnostics. In a further study, patients were enrolled on the basis of Leipzig’s diagnostic criteria: 64 patients with confirmed WD (four points or more according to the criteria); 14 patients with clinically suspected WD (2–3 points according to Leipzig diagnostic criteria), and eight patients with WD detected by family screening.

Blood samples for DNA analysis were taken from peripheral blood at various medical facilities: various inpatient and outpatient units of Children’s Clinical University Hospital (BKUS), various inpatient and outpatient units of Riga East Clinical University Hospital (RAKUS) (mostly from the stationary “Infectology Centre of Latvia”) during the period from 2001 till 2019.

A control population was used to test the new allelic variants of the *ATP7B* gene: 93 individuals aged 39–82 with no clinical data on Wilson’s disease who had signed their confirmations of consent to unrestricted use of their biological material in various studies, and data about the incidence of *ATP7B* gene allelic variants from 42 individuals of various ages without known hepatic or neurological diseases from the RSU SLMG internal database based on analysis of clinical exome data (these individuals had also signed confirmations of consent to unrestricted use of their biological material in various studies).

Acquisition of Clinical Data

Disease history, clinical symptom characterisation, laboratory analysis and other diagnostic test results were obtained from physician-filled genetic examination submission forms, as well as from outpatient and inpatient cards of WD patients at RAKUS “Infectology Center of Latvia” inpatient unit and BKUS.

1.2 Molecular Investigations

All molecular investigations were carried out at the RSU SLMG from 2006 to 2018.

DNA Extraction from Peripheral Blood

From 2006 to 2015, DNA was extracted using the phenol-chloroform method. The method was adapted by the SLMG according to the DNA extraction method described by *John and colleagues* (John et al., 1991). Since 2015, DNA extraction has been performed using a commercial kit – the Innu Prep DNA mini kit (Analytic Jena, Germany) – using the manufacturer’s protocol.

Detection of the *ATP7B* Gene Allelic Variant H1069Q by PCR Bi-PASA

This method was adapted from Polakova et al., 2007 (Polakova et al., 2007).

All patients with clinically confirmed or suspected WD were subjected to the PCR Bi-PASA method to detect the most common WD-causing allelic variant in Europe – c.3207C> A (p.H1069Q).

Direct Sequencing of the *ATP7B* Gene

Exon sequencing of *ATP7B* gene exons and adjacent introns (\pm 20 nucleotides) was performed in all WD patients with clinically confirmed or suspected WD and not having the most common allele (p.H1069Q) in both gene alleles. For the sequencing of exons of the *ATP7B* gene and their adjacent introns (\pm 20 nucleotides), primers were selected by using the free-access software “Primer 3” (Primer3 Input (Version 0.4.0), n.d.). Exon sequencing was done by using adapted manufacturer’s protocol (Zarina et al., 2017). Sequencing electropherograms were analysed using “*Chromas*” version 2.4. The resulting sequences were compared with the available reference sequence from “*BLAST*” database – NG_008806.1; NM_000053.3 and NP_000044.2 (BLAST: Basic Local Alignment Search Tool, n.d.).

Analysis of Pathogenicity of Allelic Variants

All of the detected allelic variants were screened in the “Wilson Disease Mutation Database” developed by the University of Alberta (active replenishment and maintenance of the database was discontinued in 2010) (University of Alberta, n.d.) and the “ClinVar” database of the National Center for Biotechnology Information (NCBI) (NCBI, n.d.). Guidelines for the interpretation of novel sequence variants from the American College of Medical Genetics and Genomics (ACMG) were used to test the pathogenicity of previously undescribed allelic variants (Richards et al., 2015). *Polyphen-2*,

SIFT, *Mutation Taster* and *Panther* software (Adzhubei et al., 2010) were used for criterion PP3, and previously undescribed variants were tested in the control group. In turn, primers from the previously described study were used for direct sequencing of the *ATP7B* gene promoter (5'UTR) region (Cullen, Prat and Cox, 2003). The *ATP7B* gene promoter was sequenced in all patients with clinically confirmed or suspected WD with no pathogenic allele variants in both gene alleles using PCR-BiPASA and *ATP7B* gene exons sequencing.

Direct Sequencing of the *ATOX1* gene

Gene sequencing was performed on all patients for whom WD was molecularly confirmed (two pathogenic allelic variants in the *ATP7B* gene were identified) to determine the possible association of *ATOX1* gene variants with the variable WD phenotype. For the sequencing of exons of the *ATOX1* gene and their adjacent introns (± 20 nucleotides), primers were selected by using the free-access software *Primer 3* (Primer3 Input (Version 0.4.0), n.d.). The resulting sequences were compared with the available reference sequence from “BLAST” – NC_000005.10, NM_004045.3 and NP_004036.1 (BLAST: Basic Local Alignment Search Tool, n.d.).

Direct Sequencing of the *COMMD1* gene

Gene sequencing was performed on all patients for whom WD was molecularly confirmed (two pathogenic allelic variants in the *ATP7B* gene were identified) to determine the possible association of *COMMD1* gene variants with the variable WD phenotype. Primers from previously published data were used for gene sequencing (Gupta et al., 2010). The resulting sequences were compared with the available reference sequence from “BLAST” – NC_000002.12, NM_152516.3, NP_689729.1 (BLAST: Basic Local Alignment Search Tool, n.d.).

Direct Sequencing of the *CP* gene

Gene sequencing was performed on all patients for whom WD was molecularly confirmed (two pathogenic allelic variants in the *ATP7B* gene were identified) to determine the possible association of *CP* gene promoter variants with WD clinical symptoms. The *CP* gene promoter region (not all of the gene) sequencing was selected based on the information in other studies concluding that changes in the *CP* gene promoter may be associated with altered levels of ceruloplasmin thus the *CP* gene changes might lead to WD like phenotype (Zhao et al. 2015). For the sequencing of the *CP* gene promoter, primers were selected by using the free-access software “Primer 3” (Primer3 Input (Version 0.4.0), n.d.), but the other two primers were selected from previously published information (Zhao et al., 2015). Four primers were used for analysis: outer – for the PCR product, internal (along with the external) for the sequencing reaction. The resulting sequences were compared with the available reference sequence from “BLAST” – NC_000003.12 (BLAST: Basic Local Alignment Search Tool, n.d.).

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was performed on patients with clinically confirmed or suspected WD that were not molecularly confirmed after *ATP7B* gene sequencing. The protocol for performing MLPA was adapted from the reagent manufacturer’s website (MRC-Holland, n.d.).

Quantitative PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) and the comparative CT method (“Delta Delta C (T) method”) according to recommendations by Livak et al. (Livak and Schmittgen, 2001) and *Qiagen* were performed in order to validate the possible duplications detected by MLPA. Primer sequences of interest were selected to amplify the region analysed by MLPA probes, while exon 2 of *ATP7B* was used as a reference region, with copy changes excluded

by MLPA and Sanger sequencing, along with the *KRIT1* gene fragment (*KRIT1* selected as a reference because it has very rare changes in the number of copies). The primer sequences were designed using the *NCBI Primer BLAST* free-access software (BLAST: Basic Local Alignment Search Tool, n.d.). For primer sequences, annealing temperature and product lengths, see the Table 1.1. Prior to conducting qPCR, all PCR conditions were optimised and the efficiency and equivalence of the amplification was tested using a *Standard curve* obtained at four ten-fold serial dilutions. In turn, the specificity of the reaction was verified using high-resolution melting (HRM) analysis.

The qPCR included the following: 1) samples suspected of altering copy number in the *ATP7B* gene from MLPA results; 2) samples from clinically healthy individuals for whom copy number changes in the *ATP7B* gene were excluded by MLPA; 3) samples without DNA – negative control samples. All reactions were performed at least four times (i.e. with four successive repetitions). The norm was considered to span “Delta Delta C (T)” values ranging from 0.7 to 1.3, which corresponds to two copies in the particular area of interest.

Table 1.1

Primer sequences, annealing temperatures and PCR product lengths of the *ATP7B* exons for qPCR

Ex. ¹	Primer 1 (5'–3')	Primer 2 (5'–3')	T. ² (°C)	L. ³ (bp)
16	AGTCCCCCAGACCTTCT CTG	CGTGGTCTGTCATAGC GTCA	60	101
18	TTTGCAGAGGTGCTGC CTT	CAATGGCCACACCCAT GTCT	63	136
19	GATTTGCTGGATGTGG TGGC	TGCAATGGGTATCCCA ACCA	63	111
2	GTGGTCACCCTCCAAC TGAG	GAAGTGCCTCGATAGC CCTC	61	190

¹ – exon; ² – primer annealing temperature; ³ – length of the *PCR* product.

Detection of Allelic Variants of the *HFE* gene

Analysis of the gene variants: C282Y (c.845G>A; p.Cys282Tyr) and H63D (c.187C>G; p.His63Asp) was performed on all patients for whom WD was molecularly confirmed (two pathogenic allelic variants in the *ATP7B* gene were identified) by using *PCR-RFLP* (Mura, Ragueneas and Ferec, 1999; Garry et al., 1997).

1.3 Birth Prevalence Detection of WD

In the approximate birth prevalence detection of WD in Latvia the previously reported approach was used (Reilly, Daly and Hutchinson, 1993). The number of patients (symptomatic and asymptomatic siblings detected in family screening) with WD was divided by the total number of births (the data were obtained from the Latvian Central Statistical Bureau) (LCSP, n.d.) during the period of time from 1964 till 2012. Patients born before 1964 were excluded from prevalence estimation since biochemical diagnostics of WD in Latvia have been available since 1980 only (data obtained by telephone contact with representatives of P. Stradins KUS Joint Laboratory). The preceding 16 birth years were included because 16 is the average age for WD expression (Coffey et al., 2013), so 1964 was taken as the initial point of reference (1980 – 16 = 1964).

1.4 Statistical Analysis of the Data

Statistical processing of the data was performed using *IBM SPSSv22.0* and *PLINK 1.07* software. Normal distribution of the data was checked using histograms and the *Shapiro-Wilk* test. Central tendencies of normally distributed data were characterised using mean values with standard deviations,

while data that did not correspond to normal distributions were characterized using median values and interquartile ranges.

ANOVA and *Chi-square*, or *Kruskal-Wallis* and *Fisher's exact* tests were used to assess the impact of patient genotypes on the phenotype, for continuous and categorical data, respectively. The frequency of the *CP* and *COMMD1* gene alleles was tested for Hardy-Weinberg equilibrium prior to further data analysis. The association of *CP* and *COMMD1* gene variants with the Wilson's disease phenotype and the *ATP7B* gene genotype were analysed by the allelic, dominant, recessive, and genotypic birth patterns (type). The significance level $\alpha = 0.05$ was used to test the hypotheses.

2. Results

2.1 Prevalence of Wilson's Disease in Newborn Cohorts

Prevalence in neonatal cohort included 59 patients with molecularly confirmed WD as well as eight patient relatives who were initially asymptomatic. Between 1964 and 2012, there were 1,463,083 live births in Latvia (LCSP, n.d.). Calculation: 67 patients/1,463,083 live births = 4.58 cases per 100,000 live births, or 1:21,800.

2.2 Characteristics of Wilson's Disease Patients

Clinical and genetic data from 64 patients with clinically and/or molecularly confirmed WD (Leipzig's diagnostic criteria of at least 4 points) were used in the study. Data from the first case of WD in a given family were used in the study, and relatives were excluded. The following clinical and laboratory data in WD patients were analyzed: type of first symptoms; age at onset of first symptoms; serum ceruloplasmin (g/dl); 24 hour urinary copper (μg) – the first measurement was taken into account.

Gender and Age at Onset of First Symptoms

Male to female ratio was 1.13:1 (34 males and 30 females). The age at onset of first symptoms of WD was between 3 and 54 years, with a mean age of 24.00 years (SD = 11.55 years), although most patients were in the 25–30 age group. The distribution of WD patients by age is shown in Figure 2.1.

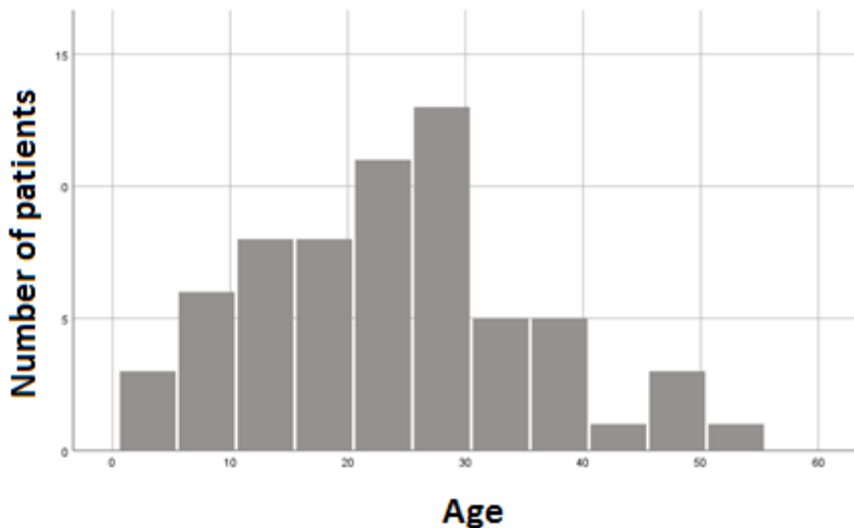


Figure 2.1. **Distribution of patients by age at the onset of the first WD symptoms**

Description of First Symptoms in WD patients

All patients were divided into three groups according to the first type of WD clinical symptoms: 1) hepatological symptoms; 2) neurological and/or psychiatric symptoms; 3) mixed (hepatologic and neurological and/or psychiatric symptoms). WD characteristic symptom – the Kayser-Fleischer ring was not included in the analysis due to the lack of data.

Liver disease as the first manifestation of WD among patients was observed as: chronic hepatitis, liver cirrhosis, or acute liver failure (fulminant hepatitis). The most common complaints with liver disease were: abdominal pain, bloating, nausea, jaundice, nasal bleeding, increased abdominal volume, fatigue, weakness and/or itching. Neurological symptoms manifested as: tremors of the hands and/or body, ataxia, cognitive impairment and speech disorders. A combination of chronic hepatitis or liver cirrhosis with hand and/or body tremor, intellectual disability, dysarthria and dysphagia has been reported

under mixed symptoms. Two of the patients also had a history of less-frequent first manifestations which have been described in the literature as possible signs of WD: one had amenorrhea, another had a late pregnancy termination, and both had liver cirrhosis at the time of diagnosis. For distribution of the first WD symptoms, see Table 2.1.

Table 2.1

Distribution of the first WD symptoms in Latvian patients

First symptoms of WD	Symptoms and/or nosological form of a disease	Number of patients (absolute numbers)	Total number of patients
Liver disease	Chronic hepatitis	27	42
	Liver cirrhosis	13	
	Acute liver failure	2	
Neurological and/or psychiatric symptoms	Hand and/or body tremor	12	17
	Dysarthria + hand and/or body tremor	3	
	Paranoid-hallucinatory syndrome	1	
	Ataxia + cognitive impairment	1	
Mixed symptoms	Chronic hepatitis + hand and/or body tremor	1	5
	Liver cirrhosis + hand and/or body tremor	2	
	Liver cirrhosis + intellectual disability	1	
	Liver cirrhosis + dysarthria, dysphagia	1	

The patients' first clinical symptoms (by symptom group) were compared with: blood levels of ceruloplasmin; 24 hour urinary copper (μg); gender; age of onset. For results see Table 2.2.

No association of the first symptoms with changes in laboratory parameters was found. Neurological symptoms (both alone and in combination with hepatologic symptoms) were observed later, but no statistically significant difference was observed ($p = 0.066$). Comparison of clinical symptoms

between the sexes revealed the tendency for men to have a higher incidence of neurological symptoms, while women displayed a higher incidence of hepatological symptoms, but there was no statistically significant difference in the data.

Table 2.2

Relationship between first WD symptoms and other laboratory and clinical findings

Clinical and biochemical parameters	Hepatological (n = 42)	Neurological and/or psychiatric (n = 17)	Mixed (n = 5)	p-value
Serum ceruloplasmin, g/dL; median (<i>IQR</i>)	0.11 (0.08–0.13)	0.13 (0.08–0.15)	0.08 (0.07–0.15)	0.562
24-hour urinary copper, µg; median (<i>IQR</i> *)	197 (136–373)	163 (96–268)	374 (365–384)	0.370
Males : females; absolute numbers	19 : 23 (p = 0.09)	12 : 5 (p = 0.081)	3 : 2 (p = 0.192)	-
Age at onset, years; average (± SD**)	20.21 (± 9.10)	29.82 (± 13.25)	36.00 (± 8.75)	0.066

**IQR* – interquartile range; **SD – standard deviation

2.3 Analysis of the *ATP7B* Gene

Analysis of the *ATP7B* Gene Exons (Coding Parts)

WD was molecularly confirmed in 51 patients, as well as their seven first-degree relatives, 37 of whom were homozygous and 14 compound heterozygous; in five patients, the pathogenic allelic variant was found in a heterozygous state, while no pathogenic variants were found in eight patients. For genotypes of patients and their relatives, see Table 2.3.

Table 2.3

Genotypes of the *ATP7B* gene in Wilson's disease patients in Latvia and their relatives

Genotype according to the HGVS nomenclature*	Number of patients	Number of relatives	Total number
c.[3207C>A];[3207C>A]; p.[His1069Gln];[His1069Gln]	37	4	41
c.[3207C>A];[c.2304dupC]; p.[His1069Gln];[p.Met769Hisfs*26]	3	1	4
c.[213_214del];[c.1934T>G]; p.[Val73GlufsX4];[p.Met645Arg]	1	0	1
c.[3207C>A];[c.213_214del]; p.[His1069Gln];[p.Val73GlufsX4]	1	0	1
c.[3207C>A];[c.2293G>A]; p.[His1069Gln];[p.Asp765Asn]	1	0	1
c.[3207C>A];[c.2305A>G]; p.[His1069Gln];[p.Met769Val]	1	0	1
c.[3207C>A];[c.3402delC]; p.[His1069Gln];[p.Ala1135GlnfsX13]	1	0	1
c.[3207C>A];[c.3472_3482del]; p.[His1069Gln];[p.Gly1158Phefs*2]	1	2	3
c.[3207C>A];[c.3800A>G]; p.[His1069Gln];[p.Asn1267Gly]	1	0	1
c.[3207C>A];[c.3971A>C]; p.[His1069Gln];[p.Asn1324Thr]	1	0	1
c.[3207C>A]; [c.4106C>T]; p.[His1069Gln];[p.Ser1369Leu]	1	0	1
c.[2304dupC];[2605G>T]; p.[Met769Hisfs*26];[Gly869*]	1	0	0
c.[3649_3654del];[2817G>T]; p.[Val1217_Leu1218del];[p.Trp939Cys]	1	0	0
c.[3106G>A];[=]; p.[Val1036Ile];[=]	1	0	1
c.[3207C>A];[=]; p.[His1069Gln];[=]	4	1	5
c.[=];[=]; p. [=];[=]	8	0	8

*reference sequence: NM_000053.3 and NP_000044.2.

One patient was found to have compound heterozygous with two pathogenic allelic variants of the *ATP7B* gene (p.Met769Hisfs * 26; Gly869 *), one resulting in a protein amino acid frame shift and the other a terminating

variant resulting in premature stop codon formation. The genotype found is the only one containing two pathogenic allelic variants, one of which is not a non-synonymous amino acid change and therefore it was not used in further statistical analysis of the data. However, when analysing the clinical parameters of this patient, the patient first developed symptoms of WD at a relatively late age of 32, had a very low level of ceruloplasmin at first measurement of 0.043 g/dl (normal > 0.20 g/dl), and 2122 µg (normal < 70 µg) copper in 24-hour urine after receiving D-penicillamine; patient was diagnosed with decompensated liver cirrhosis.

A total of 23 allelic variants were identified among WD patients, 15 of which were identified as disease-causing. Of all the *ATP7B* gene alleles in patients, 83.59 % were identified as pathogenic or likely pathogenic, while 16.41 % remained unidentified. Analysis of allele frequencies revealed that only three allelic variants were present in more than one individual: c.3207C>A (p.His1069Gln); c.2304dupC (p.Met769Hisfs*26) and c.213_214del (p.Val73GlufsX4). For the percentage of alleles, see Table 2.4.

Allelic variants in the *ATP7B* gene were found in 12 (out of 21) exons. The most common allelic variants were found in three exons: 14, 8 and 2, accounting for 76.56 % of the detected variants. For variant c.3620A>G (p.His1207Arg), the pathogenicity described above was considered controversial – it is described as both pathogenic (Abdelghaffar et al., 2008) and benign (Loudianos et al. 1999), but according to the ACMG guidelines the variant is being classified as benign by the following criteria (P – evidence of pathogenicity; B – evidence of benign impact):

- 1) PP2 (supporting evidence of pathogenicity) – missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease;

2) BA1 (stand alone evidence of benign impact) – allele frequency is > 5 % in general population; the frequency of the current allelic variant in *GnomAD* database is 0.148 (GnomAD, n.d.);

3) BP4 (supporting evidence of benign impact) – Benign computational verdict because 5 benign predictions from computational prediction tools – *DANN*, *EIGEN*, *MutationAssessor*, *PrimateAI* and *REVEL* (Varsom, n.d.);

4) BP6 (supporting evidence of benign impact) – Reputable source recently reports variant as benign – the particular variant is described as benign in databases “*ClinVar*” (BLAST: Basic Local Alignment Search Tool, n.d.) and “*UniProt*” (Blastp on UNIPROTKB_HUMAN, n.d.).

Table 2.4

Percentage distribution of *ATP7B* gene pathogenic allelic variants

Allelic variant*	<i>ATP7B</i> exon	Number of alleles	% of alleles
c.3207C>A (p.His1069Gln)	14	89	69.53
c.2304dupC (p.Met769Hisfs*26)	8	4	3.13
c.213_214del (p.Val73GlufsX4)	2	2	1.56
c.1934T>G (p.Met645Arg)	6	1	0.78
c.3106G>A (p.Val1036Ile)	14	1	0.78
c.2293G>A (p.Asp765Asn)	8	1	0.78
c.2305A>G (p.Met769Val)	8	1	0.78
c.3402delC (p.Ala1135GlnfsX13)	15	1	0.78
c.3472_3482del (p.Gly1158Phefs*2)	16	1	0.78
c.3800A>G (p.Asn1267Gly)	18	1	0.78
c.3971A>C (p.Asn1324Thr)	19	1	0.78
c.4106C>T (p.Ser1369Leu)	20	1	0.78
c.2605G>T (p.Gly869*)	11	1	0.78
c.3649_3654del (p.Val1217_Leu1218del)	17	1	0.78
c.2817G>T (p.Trp939Cys)	12	1	0.78
Unidentified	-	21	16.41

*Reference sequence: NM_000053.3 and NP_000044.2.

For the locations of allelic variants (in association of the protein structure) identified in patients in the *ATP7B* gene, see Figure 2.2.

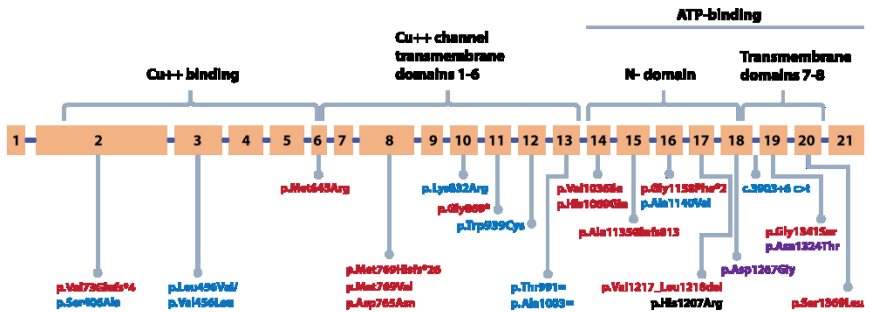


Figure 2.2. Location of allelic variants of the *ATP7B* gene in association of the protein structure

Pathogenic variants marked in red; non-pathogenic in blue; variant with controversially described pathogenicity in black; new, previously undescribed pathogenic variants – with purple

Most allelic variants have been previously described in literature, but two of the variants found – c.3800A>G (p.Asp1267Gly) in exon 18 and c.3971A>C (p.Asn1324Thr) in exon 19 – have not been previously described. Guidelines for the interpretation of novel sequence variants of ACMG were used to test the pathogenicity of both of the above allelic variants (Richards et al., 2015). Four pathogenicity prediction tools or programs were used along with the pathogenicity criteria: *Polyphen-2*, *SIFT*, *Mutation Taster* and *Panther* software, which identified both as likely pathogenic and pathogenic (see Table 2.5.)

Both novel variants were also tested in the control group. An unspecified variant of exon 18 was found in one control allele (with a minor allele frequency of 0.005) that did not differ from the allele frequency in the

patient group ($p > 0.05$). In contrast, the allele of exon 19 was not found in any alleles of the control groups. For results of the pathogenicity test for allelic variants, see Tables 2.6. and 2.7.

Table 2.5

Results of pathogenicity prediction for two novel variants

Allelic variant (according to the HGVS nomenclature)		Allelic variants pathogenicity prediction tools			
Reference: NM_000053.3	Reference: NP_000044.2	<i>Polyphen-2</i>	<i>Mutation Taster</i>	<i>SIFT</i>	<i>Panther software</i>
		Prognosis	Prognosis	Prognosis	Prognosis
c.3800A>G	p.Asp1267Gly	Probably damaging	Disease causing	Damaging	Probably damaging
c.3971A>C	p.Asn1324Thr	Probably damaging	Disease causing	Damaging	Probably damaging

Analysis of the *ATP7B* Gene Promoter

Analysis of the *ATP7B* gene promoter revealed five allelic variants: rs28362532, rs9563084, rs145371060, rs749629764 and rs1055659322, none of which are pathogenic.

***ATP7B* Gene Screening with MLPA**

The MLPA was prepared on all WD patients, which did not have two pathogenic variants on the *ATP7B* gene. The MLPA results showed suspected alterations (duplications) in the number of copies of the *ATP7B* gene in one patient (coefficient of relative fluorescent signal = 1.31), as well as changes in the number of copies of copies 18 and 19 in another patient (coefficient of relative fluorescent signal = 1.34). The results obtained were tested using qPCR.

***ATP7B* Gene Assay with qPCR**

No changes in the number of *ATP7B* gene exon 16, 18 and 19 copies were confirmed by qPCR in the two patients mentioned in the previous paragraph.

Impact of *ATP7B* Gene Allelic Variants on the WD Phenotype

To evaluate the effect of allelic variants of the *ATP7B* gene on the phenotype, the variants were divided into two groups: “mild” (M) or missense (non-synonymous single nucleotide substitution) and “severe” (S), which included both nonsense (with STOP codon creation) and frame-shift variants. Both types were compared with clinical manifestations and laboratory findings of WD. For detailed results, see Table 2.8.

Table 2.6

Pathogenicity characterisation of novel allelic variants of the *ATP7B* gene (c.3800A>G; p.Asp1267Gly) according to ACMG guidelines

Evidence of pathogenicity (note)	Explanations for the evidence of pathogenicity	
PS4 (proof is not fulfilled)	Frequency in WD patient population	Frequency in control group
	0.0078	0.0037
	p = 0.5163; OR = 2.2990	
PM1 (proof is fulfilled)	The variant alters the protein metal-binding domain (“Blastp on UNIPROTKB_HUMAN” n.d.)	
PM2 (proof is fulfilled)	The specific variant was not found in the “1000 genome project” database (“Chr13: 52.51M-52.51M - 1000 Genomes Browser” n.d.)	
PM5 (proof is fulfilled)	Pathogenic variants found in WD database (“Home Page” n.d.): c.3800A>C (p.Asp1267Aal) and c.3800A>T (p.Asp1267Val)	
PP3 (proof is fulfilled)	Four pathogenicity tools describe the variant as likely pathogenic or pathogenic (see Table 4.5)	
PP4 (proof is fulfilled)	The patient has clinical symptoms characteristic of WD and the pathogenic variant p.H1069Q has also been detected in the other allele	
Conclusion: 3 moderate proofs (PM1 + PM2 + PM5) + 2 supporting (PP3 + PP4) = <i>likely pathogenic</i>		

Table 2.7

Pathogenicity characterisation of novel allelic variants of the *ATP7B* gene (c.3971A>C; p.Asn1324Thr) according to ACMG guidelines

Evidence of pathogenicity	Explanations for the evidence of pathogenicity	
PS4 (proof is not fulfilled)	Frequency in WD patient population	Frequency in control group
	0.0078	0.0000
	p = 0.2038; OR = undefined	
PM1 (proof is fulfilled)	The variant alters the transmembrane domain of the protein (“Blastp on UNIPROTKB_HUMAN” n.d.)	
PM2 (proof is fulfilled)	The specific variant was not found in the “Exome Aggregation Consortium “database, but there is another variant in the same position – c.3971A>G (p.Asn1324Ser) – rs760285767, with a minor allele frequency of 0.000008 (“Reference SNP (RefSNP) Cluster Report: Rs760285767” n.d.)	
PP3 (proof is fulfilled)	Four pathogenicity tools describe the variant as likely pathogenic or disease causing (see Table 4.5)	
PP4 (proof is fulfilled)	The patient has clinical symptoms characteristic of WD and the pathogenic variant p.H1069Q has also been detected in the other allele	
Conclusion: 2 moderate proofs (PM1 + PM2) + 2 supporting (PP3 + PP4) = <i>likely pathogenic</i>		

A statistically significant difference was found between the *ATP7B* gene genotype and blood ceruloplasmin levels when comparing single nucleotide substitution to the terminating (nonsense) or frame-shift variants – the blood level of ceruloplasmin was more significantly reduced when the nonsense variant was combined with the frame-shift allele.

While comparing the presence of the H1069Q allele variant in the genotype with other variants, a statistically significant difference was found between the more reduced level of ceruloplasmin in the blood and the non-H1069Q allele variant in the genotype (see Table 2.9).

2.4 Analysis of the *ATOX1* Gene

During analysis of the coding regions of the *ATOX1* gene and adjacent non-coding regions (exon-intron boundaries), four patients were found to be

carriers of a single allelic variant – rs571657964 (NM_004045.3: c.7-15delC) in the heterozygous state (MAF = 0.023 in WD patients; MAF = 0.012 in the Estonian population (NCBI, n.d.). This allelic variant is located in the non-coding part of the gene, intron 2, 15 nucleotides downstream of exon 2. (BLAST: Basic Local Alignment Search Tool, n.d.) By analysing the variant using free-access software *Human Splicing Finder* (Human Splicing Finder - Version 3.1, n.d.), where it is possible to find the possible effect of allelic variants on splicing, the conclusion was made that this allelic variant has no effect on splicing. Taking all of the above into consideration, the variant was excluded from further statistical analysis because it most likely has no effect on *ATOX1* gene expression.

Table 2.8

Relationship of allelic variants of the *ATP7B* gene to clinical symptoms and laboratory findings of WD

Clinical and biochemical parameters	<i>ATP7B</i> genotype*		p-value
	M/M*	M/S** or S/S***	
Total frequency; absolute numbers (%)	42 (82.35 %)	9 (17.65 %)	-
Serum ceruloplasmin, g/dL (median (<i>IQR</i>))	0.11 (0.08–0.14)	0.068 (0.02–0.10)	0.015
24-hour urinary copper, µg (median (<i>IQR</i> *))	264 (160–389)	156 (94–438)	0.332
Age at onset (years ± SD)	24.24 (± 11.20)	18.11 ± 7.36)	0.101
Hepatological symptoms, absolute numbers (%)	30 (58.82 %)	8 (15.69 %)	0.571
Neurological symptoms, absolute numbers (%)	7 (13.73 %)	1 (1.96 %)	0.417
Mixed symptoms; absolute numbers (%)	5 (9.80 %)	0 (0 %)	0.206

*M/M – missense/missense; **M/S – missense/nonsense or frame-shift; ***S/S – nonsense or frame-shift/nonsense or frame-shift

Table 2.9

Relationship of allele variants of the *ATP7B* gene (depending on the presence of H1069Q) to WD clinical symptoms and laboratory findings

Clinical and biochemical parameters	<i>ATP7B</i> genotype*		p-value
	H1069Q/H1069Q (n = 36)	H1069Q/other variant or other variant/other variant (n = 3)	
Total frequency; absolute numbers (%)	36 (70.59 %)	12 (23.53 %)	-
Serum ceruloplasmin, g/dL (median (<i>IQR</i>))	0.107 (0.080–0.135)	0.080 (0.070–0.110)	0.003
24-hour urinary copper, µg (median (<i>IQR</i> *))	268 (161–470)	188 (125–185)	0.263
Age at onset (years ± SD)	24.889 (± 10.980)	19.000 (± 9.502)	0.064
Hepatological symptoms, absolute numbers (%)	26 (68.42 %)	12 (31.58 %)	0.488
Neurological symptoms, absolute numbers (%)	7 (87.50 %)	1 (12.50 %)	0.569
Mixed symptoms; absolute numbers (%)	3 (60.00 %)	2 (40.00 %)	0.654

2.5 Analysis of the *COMMD1* Gene

By analysing the coding parts of the *COMMD1* gene, three allelic variants were identified: rs569267407, rs55677935 and rs9096. The frequency of allelic variants of the *COMMD1* gene found in this study was compared with the *GnomAD* database. (*GnomAD*, n.d.) For results, see Table 2.10.

Table 2.10

Variants of the COMMD1 gene found in WD patients

Variant	NM_152516.3	NP_689729.1	Location in <i>COMMD1</i>	MAF*	MAF**	p-value
rs569267407	c. -68_67deITT	-	Intron 1	0.0088	0.0020	0.1469
rs55677935	c.358C>T	p.Arg120Trp	Exon 2	0.0440	0.0215	0.1553
rs9096	c.492C>T	p.Asp164=	Exon 3	0.0968	0.1263	0.3247

*MAF – minor allele frequency in the current study; **MAF – minor allele frequency in the *GnomAD* – in European (non-Finnish) population (*GnomAD*, n.d.)

Frequencies of *COMMD1* variants did not significantly differ from the frequencies listed in the *GnomAD* database. Since the variant rs569267407 is located in the non-coding part of the gene, its potential effect on gene splicing was analysed (using *Human Splicing Finder* software) (*Human Splicing Finder* - Version 3.1, n.d.). Since impact was not predicted, this variant was not further analysed. The two remaining variants were tested for Hardy-Weinberg equilibrium. As all variants had p-values of > 0.005 , their distribution corresponds to the Hardy-Weinberg equilibrium (see Table 2.11 for p-values and genotype distributions).

Frequency of alleles or genotypes (by different inheritance models) was compared to the first clinical symptoms of WD patients (taking into account only those patients with molecularly confirmed WD, $n = 49$) and *ATP7B* gene genotype (taking into account all patients with clinically confirmed or suspected WD, $n = 62$). For results, see Table 2.12.

Table 2.11

**Correspondence of allelic variants of the *COMMD1* gene
to Hardy-Weinberg equilibrium in WD patients**

Variant	Allele	Genotype	p-value
rs55677935	C/T	1/3/58 (TT/TC/CC)	0.0803
rs9096	T/C	1/10/51 (CC/CT/TT)	0.4444

No statistically significant differences were found between allele/genotype frequencies and first clinical manifestations of WD. During comparison of the incidence of both variants (in the dominant inheritance model) and the age at onset of symptoms, no differences were observed (rs9096 – p = 0.112; rs55677935 – p = 0.146).

Table 2.12

**Relation of the allelic variants of the *COMMD1* gene
to the WD phenotype and the *ATP7B* genotype**

Variant	A1 *	A2 **	Inheritance model	Neurological symptoms n = 7	Hepatological symptoms n = 42	p- value
rs55677935	C	T	Genotype (TT/TC/CC)	0/0/7	1/2/39	0.9999
	C	T	Allelic (T/C)	0/14	4/80	0.9999
	C	T	Dominant (TT + TC/CC)	0/7	3/39	0.9999
	C	T	Recessive (TT/TC + CC)	0/7	1/41	0.9999
rs9096	T	C	Genotype (CC/CT/TT)	0/1/6	1/6/35	0.9999
	T	C	Allelic (C/T)	1/13	8/76	0.9999
	T	C	Dominant (CC+CT/TT)	1/6	7/35	0.9999
	T	C	Recessive (CC/CT + TT)	0/7	1/41	0.9999

Table 2.12 continued

Variant	A1 *	A2 **	Inheritance model	Non-WD*** n = 13	WD**** n = 49	p- value
rs55677935	T	C	Genotype (TT/TC/CC)	0/1/12	1/2/46	0.6202
	T	C	Allelic (T/C)	1/25	4/94	0.9999
	T	C	Dominant (TT + TC/CC)	1/12	3/46	0.9999
	T	C	Recessive (TT/TC + CC)	0/13	1/48	0.9999
rs9096	C	T	Genotype (CC/CT/TT)	0/3/10	1/7/41	0.5510
	C	T	Allelic (C/T)	3/23	9/89	0.7142
	C	T	Dominant (CC + CT/TT)	3/10	8/41	0.6847
	C	T	Recessive (CC/CT + TT)	0/13	1/48	0.9999

* – allele 1; ** – allele 2; ***Non-WD – patients with at least one unidentified allelic variant in the *ATP7B* gene; ****WD – patients with two allelic variants in the *ATP7B* gene

2.6 Analysis of the *CP* Gene

Seven allelic variants were analysed by focusing the non-coding portions (promoter) of the *CP* gene: rs66508328, rs67870152, rs16861642, rs73020328, rs73956813, rs66703613 and rs11708215. All variants were tested for Hardy-Weinberg equilibrium. Since all variants had p-values of > 0.005, their distribution corresponds to the Hardy-Weinberg equilibrium (see Table 2.13 for p-values and genotype distributions). As the first six of these variants are inherited together, and no differences have been found between their allele

frequencies (see Table 2.13 for allele frequencies), the one that differed from the other variants – rs66508328 and rs11708215 – was chosen for analysis.

Table 2.13

Characterisation of allelic variants of the *CP* gene promoter identified in Wilson’s disease patients

Variant	Alleles	MAF*	MAF**	p-value	Genotypes	p-value
rs66508328	G > A	0.0902	0.1068	0.5500	2/7/52 (AA/AG/GG)	0.0608
rs11708215	A > G	0.175	0.2078	0.3771	2/17/41 (GG/GA/AA)	0.9999
rs67870152	C > T	0.0902	0.1069	0.5541	2/7/52 (TT/TC/CC)	0.0608
rs16861642	G > A	0.0902	0.1071	0.5485	2/7/52 (AA/AG/GG)	0.0608
rs73020328	C > T	0.0902	0.1072	0.5430	2/7/52 (TT/TC/CC)	0.0608
rs73166855	G > A	0.0902	0.1070	0.5485	2/7/52 (AA/AG/GG)	0.0608
rs66953613	T > G	0.0902	0.1069	0.5485	2/7/52 (GG/GT/TT)	0.0608

*MAF – minor allele frequency in the current study; **MAF – minor allele frequency in the “*GnomAD*” – in European (non-Finnish) population (GnomAD, n.d.)

None of the variants among WD patients differed with statistical significance from those described in the “GnomAD” database. Frequencies of alleles and genotypes (according to different inheritance patterns) were compared with the first symptoms of WD patients (considering only those patients with molecularly confirmed WD) and the genotype of the *ATP7B* gene. See the comparison in Table 2.14.

Table 2.14

Association of *CP* gene promoter allelic variants with WD phenotype and *ATP7B* genotype

Variant	A1 *	A2 **	Inheritance model	Neurological symptoms n = 8	Hepato- logical symptoms n = 41	p- value
rs 66508328	A	G	Genotype (AA/AG/GG)	0/1/7	0/6/35	0.9999
	A	G	Allelic (A/G)	1/15	6/76	0.9999
	A	G	Dominant (AA + AG/GG)	1/7	6/35	0.9999
	A	G	Recessive (AA/AG + GG)	0/8	0/41	0.9999
rs 11708215	G	A	Genotype (GG/GA/AA)	0/2/6	0/15/25	0.6938
	G	A	Allelic (G/A)	2/14	15/65	0.4998
	G	A	Dominant (GG + GA/AA)	2/6	15/25	0.7290
	G	A	Recessive (GG/GA + AA)	0/8	0/40	0.6938
					NonWD*** n = 12	WD**** n = 49
rs 66508328	A	G	Genotype (AA/AG/GG)	2/0/10	0/7/42	0.0289
	A	G	Allelic (A/G)	4/20	7/91	0.2240
	A	G	Dominant (AA + AG/GG)	2/10	7/42	0.9999
	A	G	Recessive (AA/AG + GG)	2/10	0/49	0.0361
rs 11708215	G	A	Genotype (GG/GA/AA)	2/0/10	0/17/31	0.0016
	G	A	Allelic (G/A)	4/20	17/79	0.9999
	G	A	Dominant (GG + GA/AA)	2/10	17/31	0.3059
	G	A	Recessive (GG/GA + AA)	2/10	0/48	0.0373

* – allele 1; ** – allele 2; ***NonWD – patients with at least one unidentified allelic variant in the *ATP7B* gene; ****WD – patients with two allelic variants in the *ATP7B* gene

No statistically significant differences were found between the allele frequencies and the first clinical manifestations of WD. Statistically significant differences were found between the two variants analysed and the *ATP7B* genotype – rs66508328 variant AA and rs11708215 variant GG genotype (both by recessive inheritance model) were more common in patients with non-WD – WD cases with unconfirmed *ATP7B* gene genotype.

2.7 Analysis of Allelic Variants of the *HFE* Gene

Two pathogenic variants were analysed in the *HFE* gene: C282Y and H63D. See Table 2.15 for the distribution of genotypes.

Table 2.15

Distribution of *HFE* genotypes in WD patients according to the first symptoms of the disease

Genotype according to <i>HFE</i> allelic variants	First symptoms of WD (n = 61)			
	Hepatological	Neurological	Mixed	Total
C282Y/C282Y	1	0	0	1
C282Y/H63D	1	- 0	0	1
C282Y/N	3	- 0	0	3
H63D/N	17	1	1	19
N/N	20	13	4	37

N – wild-type allelic variant in the *HFE* gene, considering C282Y and H63D variants

While analysing the most common pathogenic variants of the *HFE* gene in WD patients, two patients were found to have *HFE* gene genotypes that molecularly confirm the diagnosis of hereditary hemochromatosis. One of the patients was a man diagnosed with WD at the age of 35 who had chronic hepatitis, while the other patient was a woman diagnosed with WD at the age of

25 with signs of liver cirrhosis. Both patients had extremely low serum ceruloplasmin – 0.054 g/L in the male and 0.089 g/L in the female (normal range > 0.2 g/L).

Table 2.16

Age and serum ceruloplasmin levels of WD patients in relation to *HFE* genotype

Sex	<i>HFE</i> genotype	Parameter	Age at onset	Serum ceruloplasmin (g/L)
Males	N/N (n = 12)	Average	21.67	0.11
		Standard deviation (SD)	10.72	0.05
	Pathogenic allelic variant/N (n = 7)	Average	23.71	0.11
		Standard deviation (SD)	15.41	0.04
	Pathogenic allelic variant/ Pathogenic allelic variant (n = 1)	Average	35.00	0.05
		Standard deviation (SD)	-	-
		p-value	0.596	0.512
Females	N/N (n = 13)	Average	22.31	0.09
		Standard deviation (SD)	9.30	0.05
	Pathogenic allelic variant/N (n = 11)	Average	17.82	0.12
		Standard deviation (SD)	7.73	0.04
	Pathogenic allelic variant/ Pathogenic allelic variant (n = 1)	Average	24.00	0.09
		Standard deviation (SD)	-	-
		p-value	0.421	0.367

N – wild-type allelic variant of the *HFE* gene, considering C282Y and H63D variants

When comparing clinical and biochemical data with *HFE* gene genotypes, no statistically significant differences were found (see Table 2.16 for the results).

3. Discussion

The aim of this study was to investigate genetic factors of WD, characterising the spectrum and frequency of disease-causing allelic variants in Latvia, their correlation with the clinical manifestations of the disease, as well as to identify other possible genetic factors affecting Wilson's disease. The results show that WD in Latvia is relatively common, caused by various pathogenic allelic variants in the *ATP7B* gene, but most of the phenotype/genotype association is not observed, suggesting that variability between WD clinical symptoms and laboratory findings is affected by allelic variants in other genes, but the hypothesis was not approved in the study.

The prevalence of Wilson's disease in neonatal cohorts in Latvia is approximately 1:21,800 for live births. Note that the prevalence estimate is approximate, since the youngest patient in this study was born in 2012. Taking into account that the average age at onset of first symptoms of WD is 16 years (Coffey et al., 2013), children born with WD during this period are potentially asymptomatic and undiagnosed, suggesting that the prevalence may be even higher. In literature, prevalence of WD varies in different populations. It ranges from 1:7,000 to 1:100,000, but the most frequently cited WD prevalence is 1:30,000 (Czlonkowska et al., 2018). Over the years, the prevalence of WD has, for the most part, increased, which can be explained by the availability of genetic tests for adults today and the improved education of clinical physicians in genetic diseases, Wilson's disease among them (Czlonkowska et al., 2018). In Latvia, WD prevalence is more common than 1:30,000, which indicates that WD diagnostics in Latvia are at a good level. In contrast, in a 2013 study by Coffey (UK) the number of carriers of two pathogenic allelic variants of WD was set at 1:7000 (Coffey et al., 2013). In that study, WD pathogenic variants were tested in 1,000 healthy individuals by sequencing the *ATP7B* gene. WD

patients in Latvia were diagnosed initially based on clinical symptoms or only molecularly between asymptomatic relatives of patients; therefore, inconsistencies in frequency could be caused by: 1) insufficient recognition of WD by clinical symptoms; 2) incomplete penetrance of WD pathogenic allelic variants; 3) different age at onset of WD symptoms (Czlonkowska, Gromadzka and Chabik 2009; Czlonkowska, Rodo and Gromadzka, 2008).

Wilson's disease was most common in Latvian patients between the ages of 25 and 30, with a mean age of 24.00 (SD = 11.55) – the youngest patient at the time of diagnosis being three years old and the oldest being 54 years old. The age distribution of Latvian patients is similar to that described in literature – in most cases, the first symptoms of WD appear between the age of five and 35 years. The youngest described patient with impaired liver function due to Wilson's disease was eight months old (Abuduxikuer et al., 2015). Approximately 3 % of patients develop symptoms after the fourth decade (Ferenci et al., 2007), while older patients develop their first symptoms by the eighth decade (Ala et al., 2005). Wilson's disease can manifest at any age, and can be considered in any patient with an uncertain liver or neurological disease.

Mostly, WD can be diagnosed symptomatically and clinically, but molecular diagnostics can be extremely important in detecting asymptomatic WD patients, especially among relatives, so it is important to detect the most common allelic variants in specific populations to facilitate WD diagnosis. The most common pathogenic allelic variant of the *ATP7B* gene in Europe is H1069Q (c.3207C>A, p.His1069Gln), which varies in frequency from 15 % in France to 72 % in Poland (Gomes and Dedoussis, 2016). In this study, the most common allelic variant was also c.3207C>A (p.His1069Gln), found in 69.53 % of alleles, which corresponds to the frequency described above in most European populations, including Poland (72 %). *Kucinskis* reported a very high incidence of this variant in Lithuania – 92.3 % (Kucinskis et al., 2008) – but

the study included only 13 patients and may have rendered imprecise results. Other pathogenic variants were much less common in the study, with only three variants occurring in more than one allele: exons 2, 8, and 16 of the *ATP7B* gene (c.2304dupC (p.Met769Hisfs*26); c.213_214del (p.Val73Gluff)*4) and c.3472_3482del (p.Gly1158Phefs*2)), the latter of which was found in three siblings. All of the above findings indicate great allelic heterogeneity in WD patients in Latvia. Allelic variants in the *ATP7B* gene were found in 12 of 21 exons. The most common allelic variants were found in three exons: 14, 8 and 2 – accounting for 76.56 % of the detected variants. These data suggest that the aforementioned three exons should be tested first when analysing new WD cases in Latvia. Given the high degree of allele heterogeneity; however, short of finding the most common variant (H1069Q), it would be more cost effective and rational to sequence all other exons in the gene.

Most of the variants found in the current study were described before in prior studies. For example, the variant in exon 16 (c.3472_3482del; p.Gly1158Phefs*2) is described in only two populations, Poles (Gromadzka et al., 2005) and Lithuanians (Kucinskas et al., 2008).

Two variants found in this study were novel: c.3800A>G (p.Asp1267Gly) and c.3971A>C (p.Asn1324Thr). By using the ACMG Interpretation Guidelines for New Sequence Variations (Richards et al., 2015), it was demonstrated that both variants are “most likely pathogenic” and were therefore included in further analysis. For variant c.3620A>G (p.His1207Arg), the pathogenicity described before was considered controversial – it was described as both pathogenic (Abdelghaffar et al., 2008) and benign (Loudianos et al., 1999), but in August 2017 the database “ClinVar” was updated, and this variant was declared be benign/most likely benign (BLAST: Basic Local Alignment Search Tool, n.d.); it was therefore not included in further data processing and analysis.

83.59 % of all patients' *ATP7B* gene alleles were identified as pathogenic or likely pathogenic, but 16.41 % of alleles remained unidentified, so the study included non-coding region-promoter analysis of the *ATP7B* gene, and as possible large (multiple-exon) duplications or deletions were searched using MLPA method.

Unfortunately, the latter two methods did not improve the frequency of the identified alleles. The reasons for undetected allelic variants could be various: 1) possible allelic variants deep in introns or 3'UTR (3' untranslated region) that were not included in this study; 2) WD is caused by some unusual mutation mechanism, such as chromosomal translocations or other structural aberrations of chromosomes that include the *ATP7B* gene; 3) "Allelic dropout" – presence of benign allelic variants at primer binding sites that do not allow for the amplification of a particular gene copy, so that only one copy of the gene (inherited from one parent) can be read by direct sequencing (Stevens et al. 2017); 4) WD-like phenotype could be caused by changes in other genes; 5) other diseases clinically interpreted as Wilson's disease. However, the first two of the aforementioned explanations are highly unlikely, as Coffey identified 98 % of alleles in clinically confirmed WD patients in their study and concluded that "the likelihood that WD is caused by allelic variants in other genes is very low" (Coffey et al., 2013).

In other studies, the frequencies of detected alleles vary: Beom H. Lee reported 84.6 % of detected alleles in a large study of Korean WD patients (using both direct sequencing and MLPA) (Lee et al., 2011); another study identified 68.5 % of alleles (Okada et al., 2000). In a Turkish study, the frequency of detected alleles was 71.875 % (Simsek Papur, Akman and Terzioglu, 2015). Futher, in Denmark, 100 % (Moller et al., 2011), in China, 97.1 % (Hua et al., 2016a), and in India only 77 % of alleles were identified – including analysis of the *ATP7B* gene promoter and MLPA (Mukherjee et al.,

2014). Differences in detected allele frequencies suggest possible differences between populations.

Conflicting findings have been reported in previously published studies attempting to establish correlations between phenotype and genotype (Stapelbroek et al., 2004; De Bem et al., 2013; Chappuis et al., 2007) (Vrabelova et al., 2005). In a meta-analysis study of results from 11 different centres, variant c.3207C>A (p.His1060Gln) was associated with later and predominantly neurological symptoms (Stapelbroek et al., 2004). In other studies, no statistically significant association between the c.3207C>A (p.His1069Gln) variant and clinical symptoms was found (De Bem et al., 2013; Chappuis et al., 2007; Vrabelova et al., 2005). The results of this study show a tendency for neurological and mixed symptoms (neurological and/or psychiatric in combination with hepatologic) to appear later than hepatological symptoms alone; this is consistent with other studies (Stapelbroek et al., 2004), although the finding is not statistically significant. In this study, there was no statistically significant association between certain WD-causing allelic variants and clinical symptoms or age at onset of disease.

In another study, *Gromadzka* classify *ATP7B* variants into two groups: “severe” (including frame-shift and *nonsense* variants) and “mild” (including non-synonymous or *missense* variants) (Gromadzka et al., 2005). In that study, the authors found a strong association between the genetic variant type and the WD phenotype, concluding that “severe” allelic variants cause clinically more severe symptoms and earlier WD expression than “mild” variants. The current study also looked at the relationship between the different allelic variants. The data showed a tendency for the “severe” variants to be associated with earlier WD expression, but the data were not statistically significant ($p = 0.101$). A statistically significant difference was observed between the presence of “severe” variants in the genotype and serum ceruloplasmin – if there was at

least one frame shift or *nonsense* variant in the genotype, the level of serum ceruloplasmin was lower than in the homozygous genotype with only the “mild” variants. These data partly coincide with the *Gromadzka* study, although it should be noted that, in the present study, the two genotype groups were numerically very different (42 patients versus 9 patients). A similar study was also carried out by *Cocos* reporting the association of frame shift variants with earlier WD onset (Cocos et al., 2014).

Due to the important interaction of the *ATOX1* protein with *ATP7B*, there have been several studies looking for possible changes within the *ATOX1* gene in patients with Wilson’s disease, but with negative results elsewhere (Simon et al., 2008; Lee et al., 2011; Czlonkowska et al., 2018). In this study, no changes were found in the *ATOX1* gene that would affect protein activity; therefore, it has not been demonstrated that changes in the *ATOX1* gene could modify the WD phenotype.

Looking for changes in other genes that could affect clinical manifestations of WD, the researchers focused on the *COMMD1* gene, which has been shown to be pathogenic to copper toxicosis in Bedlington Terriers, which is clinically similar to Wilson’s disease in humans (van De Sluis et al., 2002). There have been several studies analysing the possible effects of allelic variants of this gene in humans. *Stuehler* found that substitution of the nucleotide at position 492 from T to C (p.Asn164=) is associated with earlier (by about 10 years) manifestation of clinical symptoms of WD. Since this is a silent variant (i.e., one that does not change the amino acid in the protein), the authors report the possible effects of this variant on the structure, stability and processing of mRNA, which may influence further gene expression. The authors of this study also mention that the variant found was a marker for some other changes that could affect copper metabolism (Stuehler et al., 2004). The present study also found the aforementioned variant among Latvian WD

patients. An analysis of its possible association with the clinical manifestations of the disease did not reveal any association with the hypothesis mentioned above.

In another study in 2010, *Gupta* reported the effect of another variant c.521C>T (p.Thr174Met) on the WD phenotype – they associate this variant with more elevated urine copper levels as well as increased cell apoptosis in WD patients (*Gupta et al.*, 2010). In the present study, no such variant of the *COMMD1* gene was found in any of the WD patients in Latvia.

There have been several studies on the effects of *COMMD1* gene variants on WD, but unfortunately no association has been confirmed in these studies (*Lovicu et al.*, 2006; *Weiss et al.*, 2006).

Several studies have been conducted on the effects of *HFE* gene variants on the development of WD symptoms. Heterozygote genotype of the most common pathogenic variants (C282Y and H63D) with hereditary haemochromatosis is rarely associated with hepatic injury due only to iron accumulation; some studies have suggested that elevated liver iron levels may influence the course of other liver diseases (*Bulaj et al.*, 1996).

The effect of heterozygote genotypes of *HFE* gene (according to the pathogenic variants) on WD pathway is controversial: *Hafkemeyer* described a patient with WD and heterozygote (in most common variants) genotype in the *HFE* gene that showed iron accumulation in the liver (*Hafkemeyer et al.*, 1994), but *Erhardt et al.* found no significant association between iron and *HFE* gene pathogenic variants (*Erhardt et al.*, 2002). *Sorbello* have demonstrated the effect of pathogenic variants of the *HFE* gene on extra iron accumulation in WD patients, which influences the development of liver disease. The same study concluded that adjusting the dose of medication in WD treatment to prevent additional iron accumulation was only effective in the wild-type variant of *HFE* (*Sorbello et al.*, 2010).

Unfortunately, available clinical data were insufficient in order to evaluate iron metabolism in the present study, but no statistically significant differences were found in the effect of the two most common variants of the *HFE* gene on the early onset of WD symptoms and serum ceruloplasmin levels, which does not support the hypothesis that *HFE* variants affect the development of inflammation of the liver.

The study data are only indicative of the tendency, but in order to further analyse hepatic inflammation changes in various *HFE* gene genotypes, it would be necessary to expand the study patient population and to select additional clinical and biochemical markers.

In this study, 83.59 % of *ATP7B* gene alleles were identified from WD patients with a clinically confirmed WD diagnosis, but 16.41 % remained unidentified. Some possible explanations have already been mentioned in the discussion above, but another explanation could be the fact that WD patients with a clinically confirmed WD diagnosis may not have WD at all, but Wilson's disease-like disease. Clinical and laboratory diagnostic criteria for WD were developed in 2001 and have not changed since then. Several studies on the revision of guidelines and criteria have been published (Hedera, 2017) but the diagnostic framework has remained unchanged at the European level (EASL Clinical Practice Guidelines: Wilson's Disease, n.d.). Decreased serum ceruloplasmin and neurological symptoms are mentioned as two criteria in the diagnosis of WD, although in the current study normal serum ceruloplasmin level was not observed in any of the patients. In WD, the level of serum ceruloplasmin is reduced due to copper accumulation, but this can also occur through other causes such as aceruloplasminaemia and malabsorption (EASL Clinical Practice Guidelines: Wilson's Disease, n.d.). Determination of serum ceruloplasmin levels is recommended as a first step in the diagnosis of WD (Roberts and Schilsky, 2008). It does serve as a screening method, but even

with very low levels of serum ceruloplasmin is not enough to confirm the diagnosis. Serum ceruloplasmin levels < 20 mg/dL are useful in WD diagnostics, but the overall predictive value for the test diagnosis is very low at 5.9 % (Cauza et al., 1997). Ceruloplasmin is the major copper-binding protein in plasma (> 90 % bound to copper). It exists in two forms, as copper-bound holoceruloplasmin and as non-copper-containing apoceruloplasmin (Hellman and Gitlin, 2002). Almost always, it is measured using antibody-based radioimmunoassays that are unable to discriminate between the two isoforms of ceruloplasmin, leading to an overestimation of copper-binding protein levels. The level of serum ceruloplasmin in the blood can also be determined enzymatically, by measuring its copper-dependent oxidase activity. This test is capable of distinguishing apoceruloplasmin from holoceruloplasmin but is not widely used. Very low serum ceruloplasmin (< 5 mg/dL) is highly associated with WD, but such low rates may also be found in other diseases, such as aceruloplasminaemia caused by pathogenic allelic variants in the *CP* gene. Neurological symptoms in case of aceruloplasminaemia may mimic WD, but this is actually due to iron accumulation (Hedera, 2017). In such cases, first-degree relative serum ceruloplasmin (also reduced in heterozygous carriers of the pathogenic allelic variant) should be checked for confirmation of aceruloplasminaemia, as well as the usual findings of aceruloplasminaemia include finding of reduced serum iron, elevated ferritin, diabetes, and evidence of the iron accumulation in the brain by magnetic resonance imaging (Hellman and Gitlin, 2002). Taking into account the aforementioned lack of symptoms and laboratory abnormalities in WD patients without a molecularly confirmed diagnosis, the diagnosis of aceruloplasminaemia is questionable, but alterations or allelic variants in the *CP* gene have been described in various studies: both Parkinson's disease (Zhao et al., 2015) and atrial fibrillation (Adamsson Eryd et al., 2014; Arenas de Larriva et al., 2017), which concluded that changes in the

promoter (non-coding part) of the *CP* gene may be associated with altered levels of serum ceruloplasmin. In the present study, it was found that the AA genotype of the rs66508328 in the *CP* gene promoter and the GG genotype of the rs11708215 variant (both by recessive birth pattern) were more common in patients without a WD-confirmed *ATP7B* gene genotype. This could suggest that the above variants in the *CP* gene may affect gene expression, leading to reduced levels of serum ceruloplasmin, which in turn leads to increased iron accumulation in the brain, leading to Parkinson's-like symptoms which are also characteristic of Wilson's disease. Of course, these data are advisory, suggesting a possible direction for further development of research by increasing the size of the patient population, as the patient groups for the present study relatively small and, although the differences were statistically significant, given the size of the small groups, the data may include random and accidental findings. *Zhao* also published data on these variants of the *CP* gene promoter and concluded that rare variants of the variants are more common in Parkinson's disease patients and are associated with an earlier onset of the disease (*Zhao et al.*, 2015).

3.1. Limitations of the study

Most analyses in the study have been conducted with a relatively small group of patients – 64. The small group of patients has several explanations:

- 1) Wilson's disease belongs to the group of rare diseases with the most frequently mentioned prevalence – 1:30,000. Taking into account this prevalence and the average population of Latvia (1.92 million) [31], there should be around 65 patients in Latvia.
- 2) Patients with Wilson's disease in Latvia were mostly referred to hepatologists – it is likely that a larger group of patients would be covered by an involvement of wider range of specialists.

- 3) There was no great response from first-degree relatives of patients – perhaps the group of patients to be analysed could be larger.
- 4) Given that patient samples were collected over a relatively long period, all patient samples were no longer available for all molecular analyses.

Given the small number of patients, the data in this study are of advisable character that should preferably be tested in a larger population, for example, in interstate studies.

3.2. Clinical recommendations

1. All patients with clinically confirmed or suspected Wilson's disease should initially be screened for the most frequent allelic variant, but in case of negative result – the exons of the *ATP7B* gene should be sequenced. If the diagnosis is not molecularly confirmed, it is recommended that the differential diagnosis of the disease be reviewed;
2. Genetic screening of relatives (especially first degree) is recommended for Wilson's disease patients, especially those who are molecularly confirmed;
3. It is recommended that patients with Wilson's disease, especially those with predominant neurological symptomology who are not molecularly confirmed, be diagnosed with other possible causes of the symptoms and biochemical changes.

Conclusions

1. The most common WD-causing allele in the *ATP7B* gene in Latvian WD patients was c.3207C>A (p.His1069Gln) – 69.53 % alleles, which corresponds to the European average;
2. The most common pathogenic allele variants of the *ATP7B* gene are found in three exons: 14, 8 and 2, but high allelic heterogeneity of the *ATP7B* gene is observed among Latvian patients, indicating that all *ATP7B* gene exons are rationally to be sequenced, but direct sequencing of *ATP7B* gene promoter region does not increase the number of pathogenic alleles detected; no significant deletions or duplications of *ATP7B* gene are detected in patients with Wilson's disease in Latvia, indicating that MLPA does not crucially improve the number of detected pathogenic alleles;
3. The prevalence of Wilson's disease in Latvian neonatal cohorts is 1:21,800 for live births, which corresponds to the European average;
4. There is no association between pathogenic allelic variants of the *ATP7B* gene and clinical manifestations in patients with Wilson's disease in Latvia, indicating a lack of phenotype-genotype association, which rejects the hypothesis;
5. Genotypes GG of the rs66508328 variant and AA of the rs11708215 in the *CP* gene promoter region are more common in Wilson's disease patients with non-molecularly confirmed genotype in the *ATP7B* gene and neurological symptoms suggesting the possible association of *CP* gene promoter variants with characteristic neurological symptoms and biochemical changes; the lack of association of *ATOX1*, *COMMD1* and *HFE* gene variants with the Wilson's disease phenotype suggests a minor role for these genes in Wilson's disease pathogenesis, which rejects the hypothesis.

References

- Abdelghaffar, T. Y., Elsayed, S. M., Elsobky, E., Bochow, B., Buttner, J. and Schmidt, H. 2008. Mutational Analysis of ATP7B Gene in Egyptian Children with Wilson Disease: 12 Novel Mutations. *Journal of Human Genetics*. 53(8), 681-87. DOI: 10.1007/s10038-008-0298-7
- Abuduxikuer, K., Li, T. L., Qiu, Y.L., Wang, N. L. and Wang, J. S. 2015. Wilson Disease with Hepatic Presentation in an Eight-Month-Old Boy. *World Journal of Gastroenterology*. 21(29), 8894-8902. DOI: 10.3748/wjg.v21.i29.8981
- Adamsson, E., S., Sjogren, M., Smith, J. G., Nilsson, P. M., Melander, O., Hedblad, B. and Engstrom, G. 2014. Ceruloplasmin and Atrial Fibrillation: Evidence of Causality from a Population-Based Mendelian Randomization Study. *Journal of Internal Medicine*. 275(2), 164-71. DOI: 10.1111/joim.12144
- Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., Kondrashov, A. S. and Sunyaev, S. R. 2010. A Method and Server for Predicting Damaging Missense Mutations. *Nature Methods*. 7(4), 248-49. DOI: 10.1038/nmeth0410-248
- Ala, A., Borjigin, J., Rochwarger, A. and Schilsky, M. 2005. Wilson Disease in Septuagenarian Siblings: Raising the Bar for Diagnosis. *Hepatology*. 41(3), 668-70. DOI: 10.1002/hep.20601
- Bem, R. S. D., Raskin, S., Muzzillo, D., Deguti, M. M., Cancado, E. L. R., Araujo, T. F., Nakhle, M. C., Barbosa, E. R., Munhoz, R. P., and Teive, H. G. 2013. Wilson's Disease in Southern Brazil: Genotype-Phenotype Correlation and Description of Two Novel Mutations in ATP7B Gene. *Arquivos de Neuro-Psiquiatria*. 71(8), 503-7. DOI: 10.1590/0004-

282X20130078

- BLAST: Basic Local Alignment Search Tool. US National Library of Medicine. Available from: <https://blast.ncbi.nlm.nih.gov/Blast.cgi> [viewed 21.10.2018].
- Blastp on UNIPROTKB_HUMAN. [Database]. Available from: <https://www.uniprot.org/blast/uniprot/B20181015AAFB7E4D2F1D05654627429E83DA5CCE008F81L?alignment=1> [viewed 16.10.2018.].
- Bulaj, Z. J., Griffen, L. M., Lynn B. J., Corwin Q. E. and Kushner, J. P. 1996. Clinical and Biochemical Abnormalities in People Heterozygous for Hemochromatosis. *New England Journal of Medicine*. 335(24), 1799-1805. DOI:10.1056/NEJM199612123352403.
- Cauza, E., Maier-Dobersberger, T., Polli, C., Kaserer, K., Kramer, L. and Ferenci, P. 1997. Screening for Wilson's Disease in Patients with Liver Diseases by Serum Ceruloplasmin. *Journal of Hepatology*. 27(2), 358-362. DOI: 10.1016/S0168-8278(97)80182-1
- Chappuis, P., Callebert, J., Quignon, V., Woimant, G. and Laplanche, J.L. 2007. Late Neurological Presentations of Wilson Disease Patients in French Population and Identification of 8 Novel Mutations in the ATP7B Gene. *Journal of Trace Elements in Medicine and Biology*. 21(1), 37-42. DOI: 10.1016/j.jtemb.2006.11.002
- Cocos, R., Sendroiu, A., Schipor, S., Bohiltea, L. C., Sendroiu, I. and Raicu, F. 2014. Genotype-Phenotype Correlations in a Mountain Population Community with High Prevalence of Wilson's Disease: Genetic and Clinical Homogeneity. *PLoS One*. 9(6), e98520. DOI:10.1371/journal.pone.0098520
- Coffey, A. J., Durkie, M., Hague, S., McLay, K. et al. 2013. A Genetic Study of Wilson's Disease in the United Kingdom. *Brain*. 136(5), 1476-1487. DOI:10.1093/brain/awt035

- Cullen, L. M., Prat, L. and Cox, D.W. 2003. Genetic Variation in the Promoter and 5' UTR of the Copper Transporter, ATP7B, in Patients with Wilson Disease. *Clinical Genetics*. 64(5), 429-432. DOI: 10.1034/j.1399-0004.2003.00160.x
- Czlonkowska, A., Gromadzka, G., Büttner, J. and Chabik, G. 2010. Clinical Features of Hemolysis, Elevated Liver Enzymes, and Low Platelet Count Syndrome in Undiagnosed Wilson Disease: Report of Two Cases. *Archives of Gynecology and Obstetrics*. 281(1), 129-134. DOI: 10.1007/s00404-009-1080-6
- Czlonkowska, A., Gromadzka, G. and Chabik, G. 2009. Monozygotic Female Twins Discordant for Phenotype of Wilson's Disease. *Movement Disorders*. 24(7), 1066-1069. DOI: 10.1002/mds.22474
- Czlonkowska, A., Litwin, T., Dusek, P., Ferenci, P., Lutsenko, S., Medici, V., Rybakowski, J. K., Weiss, K. H. and Schilsky, M. L. 2018. Wilson Disease. *Nature Reviews Disease Primers*. 4(1), 21. DOI: 10.1038/s41572-018-0018-3
- Czlonkowska, A., Rodo, M. and Gromadzka, G. 2008. Late Onset Wilson's Disease: Therapeutic Implications. *Movement Disorders*. 23(6), 896-898. DOI: 10.1002/mds.21985
- EASL Clinical Practice Guidelines: Wilson's Disease. N.d. Available from: <https://www.easl.eu/medias/cpg/Wilsons-Disease/English-report.pdf> [viewed 29.10.2017.].
- EASL (European Association for the Study of the Liver). 2012. EASL Clinical Practice Guidelines. *Journal of Hepatology*. 56(3), 671-685. DOI: 10.1016/j.jhep.2011.11.007
- Erhardt, A., Hoffmann, A., Hefter, H. and Haussinger, D. 2002. HFE Gene Mutations and Iron Metabolism in Wilson's Disease. *Liver International*. 22(6), 474-478. DOI: 10.1034/j.1600-0676.2002.01732.x

- Ferenci, P., Caca, K., Loudianos, G., Mieli-Vergani, G., Tanner, S., Sternlieb, I., Schilsky, M., Cox, D. and Berr, F. 2003. Diagnosis and Phenotypic Classification of Wilson Disease. *Liver International*. 23(3), 139-142. DOI:10.1034/j.1600-0676.2003.00824.x
- Ferenci, P., Czlonkowska, A., Merle, U., Ferenc, S., Gromadzka, G., Yurdaydin, C., Vogel, W., Bruha, R., Schmidt, H.T. and Stremmel, W. 2007. Late-Onset Wilson's Disease. *Gastroenterology*. 132(4), 1294-1298. DOI:10.1053/j.gastro.2007.02.057
- Garry, P. J., Montoya, G. D., Baumgartner, R. N., Liang, H. C., Williams, T. M. and Brodie, S. G. 1997. Impact of HLA-H Mutations on Iron Stores in Healthy Elderly Men and Women. *Blood Cells, Molecules and Diseases*. 23(2), 277-287. DOI:10.1006/bcmd.1997.0144
- GnomAD. [N.d.] Available from: <https://gnomad.broadinstitute.org/> [viewed 08.04.2019.].
- Gomes, A. and Dedoussis, G. V. 2016. Geographic Distribution of ATP7B Mutations in Wilson Disease. *Annals of Human Biology*. 43(1), 1-8. DOI:10.3109/03014460.2015.1051492
- Gromadzka, G., Schmidt, H. H. J., Genschel, J., Bochow, B. et al. 2005. Frameshift and Nonsense Mutations in the Gene for ATPase7B Are Associated with Severe Impairment of Copper Metabolism and with an Early Clinical Manifestation of Wilson's Disease. *Clinical Genetics*. 68(6), 524-532. DOI:10.1111/j.1399-0004.2005.00528.x
- Gupta, A., Chattopadhyay, I., Mukherjee, S., Sengupta, M., Das, Sh. K. and Kunal Ray. 2010. A Novel COMMD1 Mutation Thr174Met Associated with Elevated Urinary Copper and Signs of Enhanced Apoptotic Cell Death in a Wilson Disease Patient. *Behavioral and Brain Functions: BBF*. 6(6), 33. DOI: <https://doi.org/10.1186/1744-9081-6-33>

- Hafkemeyer, P., Schupp, M., Storch, M., Gerok, W. and Häussinger, D. 1994. Excessive Iron Storage in a Patient with Wilson's Disease. *The Clinical Investigator*. 72(2), 134-136. DOI: <https://doi.org/10.1007/bf00184590>
- Hedera, P. 2017. Update on the Clinical Management of Wilson's Disease. *The Application of Clinical Genetics*. 10, 9-19. DOI: 10.2147/TACG.S79121
- Hellman, N. E. and Gitlin, J. D. 2002. C Eruloplasmin Metabolism and Function. *Annual Review of Nutrition*. 22(1), 439-458.
DOI:10.1146/annurev.nutr.22.012502.114457
- HGMD. [N.d.] Available from: <http://www.hgmd.cf.ac.uk/ac/index.php>
[viewed 20.10.2018.].
- University of Alberta. [N.d.] Wilson disease mutation database. [Home page]. Available from:
<http://www.wilsondisease.med.ualberta.ca/search3.asp?a=a> [viewed 16.10.2018.]
- Hua, R., Fang Hua, Yonggeng Jiao, Yu Pan, Xu Yang, Shanshan Peng, and Junqi Niu. 2016. Mutational Analysis of ATP7B in Chinese Wilson Disease Patients. *American Journal of Translational Research*. 8(6), 2851-2861. Available from:
<http://www.ncbi.nlm.nih.gov/pubmed/27398169>
- Human Splicing Finder - Version 3.1. [N.d.] Available from:
<http://www.umd.be/HSF3/> [viewed 18.10.2018.].
- John, S. W., Weitzner, G., Rozen, R. and Scriver, C. R. 1991. A Rapid Procedure for Extracting Genomic DNA from Leukocytes. *Nucleic Acids Research*. 19(2), 408. <http://www.ncbi.nlm.nih.gov/pubmed/2014181>
- Kucinskas, L., Jeroch, J., Vitkauskiene, A., Sakalauskas, R., Petrenkiene, V., Kucinskas, V., Naginiene, R., Schmidt, H. and Kupcinskas, L. 2008. High Frequency of the c.3207C>A (p.H1069Q) Mutation in ATP7B Gene of Lithuanian Patients with Hepatic Presentation of Wilson's

Disease. *World Journal of Gastroenterology*. 14(38), 5876-5879.

<https://doi.org/10.3748/wjg.14.5876>

Larriva, A. P., de, Norby, F. L., Chen, L. Y., Soliman, E. Z., Hoogeveen, R. C., Arking, D. A., Loehr, L. R. and Alonso, A. 2017. Circulating Ceruloplasmin, Ceruloplasmin-Associated Genes, and the Incidence of Atrial Fibrillation in the Atherosclerosis Risk in Communities Study. *International Journal of Cardiology*. 241(August), 223-228.

DOI:10.1016/j.ijcard.2017.04.005

LCSP. N.d. IDG010. Dzīvi un nedzīvi dzimušo skaits pēc dzimuma. Centrālās Statistikas Pārvaldes Datubāze. Pieejams no:

http://data1.csb.gov.lv/pxweb/lv/iedz/iedz__dzimst/IDG010.px/table/tableViewLayout1/?rxid=f259e646-b6c5-497f-bc7b-4d7e6317cea6
[sk.14.10.2018.].

Lee, B. H., Kim, J. H., Lee, S. Y., Jin, H. Y. et al. 2011. Distinct Clinical Courses According to Presenting Phenotypes and Their Correlations to ATP7B Mutations in a Large Wilson's Disease Cohort. *Liver International*. 31(6), 831-839. DOI:10.1111/j.1478-3231.2011.02503.x

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods*. 25(4), 402-408. DOI:10.1006/METH.2001.1262

Loudianos, G., Dessi, V., Lovicu, M., Angius, A. et al. 1999. Mutation Analysis in Patients of Mediterranean Descent with Wilson Disease: Identification of 19 Novel Mutations. *Journal of Medical Genetics*. 36(11), 833-836. Available from:

<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1734255&tool=pmcentrez&rendertype=abstract>

Lovicu, M., Dessi, V., Lepori, M. B., Zappu, A. et al. 2006. The Canine Copper Toxicosis Gene MURR1 Is Not Implicated in the Pathogenesis of Wilson

Disease. *Journal of Gastroenterology*. 41(6), 582-587. DOI:
10.1007/s00535-006-1807-0

Moller, L. B., Horn, N., Dysgaard Jeppesen, T., Vissing, J., Wibrand, F.,
Jennum, P. and Ott, P. 2011a. Clinical Presentation and Mutations in
Danish Patients with Wilson Disease. *European Journal of Human
Genetics : EJHG*. 19(9), 935-941.
DOI:<https://doi.org/10.1038/ejhg.2011.80>

MRC-Holland. [N.d.] Available from:

<http://www.mrcholland.com/WebForms/WebFormMain.aspx> [viewed
14.10.2018.].

Mukherjee, Shashwata, Shruti Dutta, Sulagna Majumdar, Tamoghna Biswas,
Preeti Jaiswal, Mainak Sengupta, Abhisek Bhattacharya, et al. 2014.
Genetic Defects in Indian Wilson Disease Patients and Genotype-
Phenotype Correlation. *Parkinsonism & Related Disorders*. 20(1), 75-81.
DOI:10.1016/j.parkreldis.2013.09.021

Mura, C., Raguenes, O. and Ferec, C. 1999. HFE Mutations Analysis in 711
Hemochromatosis Probands: Evidence for S65C Implication in Mild
Form of Hemochromatosis. *Blood*. 93(8), 2502-2505.
DOI:10.1182/blood.v93.8.2502.408k27_2502_2505

NCBI. [N.d.] Chr13: 52.51M-52.51M : 1000 Genomes Browser. Available
from:

[https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/?gts=rs10575
16740](https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/?gts=rs1057516740) [viewed 16.10.2018.].

Okada, T., Shiono, Y., Hayashi, H., Satoh, H. et al. 2000. Mutational Analysis
OfATP7Band Genotype-Phenotype Correlation in Japanese with
Wilson's Disease. *Human Mutation*. 15(5), 454-462.
DOI:10.1002/(SICI)1098-1004(200005)15:5<454::AID-
HUMU7>3.0.CO;2-J

- Polakova, H., Katrincsakova, B., Minarik, G., Ferakova, E., Ficek, A., Baldovic, M. and Kadasi, L. 2007. Detection of His1069Gln Mutation in Wilson Disease by Bidirectional PCR Amplification of Specific Alleles (BI-PASA) Test. *General Physiology and Biophysics*. 26(2), 91-96.
- Primer3 Input (Version 0.4.0). [N.d.] Available from:
<http://bioinfo.ut.ee/primer3-0.4.0/> [viewed 19.10.2018.].
- Reference SNP (RefSNP) Cluster Report: Rs760285767. [N.d.] Available from:
https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=760285767
[viewed 16.10.2018.].
- Reilly, M., Daly, L. and Hutchinson, M. 1993. An Epidemiological Study of Wilson's Disease in the Republic of Ireland. *Journal of Neurology, Neurosurgery, and Psychiatry*. 56(3), 298-300.
<http://www.ncbi.nlm.nih.gov/pubmed/8459248>
- Richards, S., Aziz, N., Bale, S., Bick, D. et al. 2015. Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 17(5), 405-423. DOI: <https://doi.org/10.1038/gim.2015.30>.
- Roberts, E. A. and Schilsky, M. L. 2008. Diagnosis and Treatment of Wilson Disease: An Update. *Hepatology*. 47(6), 2089-2111.
<https://doi.org/10.1002/hep.22261>
- NCBI. [N.d.] Rs571657964 RefSNP Report – DbSNP. Available from:
<https://www.ncbi.nlm.nih.gov/snp/rs571657964> [viewed 25.02.2019.].
- Simon, I., Schaefer, M., Reichert, J. and Stremmel, W. 2008. Analysis of the Human Atox 1 Homologue in Wilson Patients. *World Journal of Gastroenterology*. 14(15), 2383-2387. DOI: 10.3748/WJG.14.2383
- Simsek, P., Ozlenen, Sezin Asik Akman, and Orhan Terzioglu. 2015. Clinical and Genetic Analysis of Pediatric Patients with Wilson Disease. *The*

Turkish Journal of Gastroenterology. 26(5), 397-403.

<https://doi.org/10.5152/tjg.2015.0097>

Sluis, B. van De, Rothuizen, J., Pearson, P. L., van Oost, B. A. and Wijmenga, C. 2002. Identification of a New Copper Metabolism Gene by Positional Cloning in a Purebred Dog Population. *Human Molecular Genetics*.

11(2), 165-173. <http://www.ncbi.nlm.nih.gov/pubmed/11809725>

Sorbello, O., Sini, M., Civolani, A. and Demelia, L. 2010. HFE Gene Mutations and Wilson's Disease in Sardinia. *Digestive and Liver Disease*. 42(3),

216-219. <https://doi.org/10.1016/j.dld.2009.06.012>

Stapelbroek, J. M., Bollen, C. W., Ploos Van Amstel, J., Van Erpecum, K. J., Hattum, J. V., Van Den Berg, L. H., Klomp, L. W. J. and Houwen, R. H. J. 2004. The H1069Q Mutation in ATP7B Is Associated with Late and Neurologic Presentation in Wilson Disease: Results of a Meta-Analysis. *Journal of Hepatology*. 41(5), 758-763.

<https://doi.org/10.1016/j.jhep.2004.07.017>

Stevens, A. J., Taylor, M. G., Pearce, F. G. and Kennedy, M. A. 2017. Allelic Dropout During Polymerase Chain Reaction Due to G-Quadruplex Structures and DNA Methylation Is Widespread at Imprinted Human Loci. *G3 (Bethesda, Md.)*. 7(3), 1019-1025.

<https://doi.org/10.1534/g3.116.038687>

Stuehler, B., Reichert, J., Stremmel, W. And Schaefer, M. 2004. Analysis of the Human Homologue of the Canine Copper Toxicosis Gene MURR1 in Wilson Disease Patients. *Journal of Molecular Medicine*. 82(9), 629-634.

<https://doi.org/10.1007/s00109-004-0557-9>

Varsom. [N.d.] The Human Genomics Community. Rs7334118 SNV | Hg19. Available from: <https://varsome.com/variant/hg19/rs7334118> [viewed 21.02.2020.].

Vrabelova, S., Letocha, O., Borsky, M. and Kozak, L. 2005. Mutation Analysis

of the ATP7B Gene and Genotype/Phenotype Correlation in 227 Patients with Wilson Disease. *Molecular Genetics and Metabolism*. 86(1-2), 277-285. <https://doi.org/10.1016/j.ymgme.2005.05.004>

Weiss, K. H., Merle, U., Schaefer, M., Ferenci, P., Fullekrug, J. and Stremmel, W. 2006. Copper Toxicosis Gene MURR1 Is Not Changed in Wilson Disease Patients with Normal Blood Ceruloplasmin Levels. *World Journal of Gastroenterology*. 12(14), 2239-2242. <https://doi.org/10.3748/WJG.V12.I14.2239>

Zarina, A., Tolmane, I., Kreile, M., Chernushenko et al. 2017. Genetic Variation Spectrum in ATP7B Gene Identified in Latvian Patients with Wilson Disease. *Molecular Genetics and Genomic Medicine*. 5(4), 405-409. DOI:<https://doi.org/10.1002/mgg3.297>.

Zhao, N., Xiao, J., Zheng, Z., Fei, G., Zhang, F., Jin, L. and Zhong, C. 2015. Single-Nucleotide Polymorphisms and Haplotypes of Non-Coding Area in the CP Gene Are Correlated with Parkinson's Disease. *Neuroscience Bulletin*. 31(2), 245-256. <https://doi.org/10.1007/s12264-014-1512-6>

Publications and presentations

Published articles (3)

1. **Zarina, A.**, Tolmane, I., Kreile, M., Chernushenko, A., Cernevska, G., Pukite, I., Micule, I., Krumina, Z., Krumina, A., Rozentale, B., Piekuse, L. 2017. Genetic variation spectrum in *ATP7B* gene identified in Latvian patients with Wilson disease. *Molecular genetics & genomic medicine*, 5(4), 405–409.
2. Krumina, A., Pliss, L., Zarina, G., Puzuka, A., **Zarina, A.**, Lace, B., Elferts, D., Khrunin, A., Limborska, S., Klovins, J., Gailite, L. 2018. Population genetics of Latvians in the context of admixture between North-Eastern European ethnic groups. *Proceedings of the Latvian Academy of Sciences. Sect.B.* 72(3), 131–151.
3. **Zarina, A.**, Tolmane, I., Krumina, Z., Tutane, A. I., Gailite, L. 2019. Association of Variants in the *CP*, *ATOX1* and *COMMD1* Genes with Wilson Disease Symptoms in Latvia. *Balkan J Med Genet.* 22(2), 37–42.

Abstracts and participation in international congresses and conferences (8)

1. **Zarina A.**, Tutane A., Rots D., Kreile M., Tolmane I., Cernevska G., Pukite I., Krumina Z., Gailite L. 2018. *Is it sufficient with direct sequencing of ATP7B gene coding regions to diagnose Wilson disease?* - Environmental and Experimental Biology.16, 279. Abstract of the 7th Baltic Genetics Congress.
2. **Zarina A.**, Piekuse L., Kreile M., Tolmane I., Sondore V., Cernevska G., Pukite I., Krumina Z. 2016. Is COMMD1 gene a modifier locus of Wilson disease? *European Journal of Human Genetics*. ESHG Conference, Abstracts. 24, Suppl.1, 93.
3. **Zarina A.**, Piekuse L., Kreile M., Tolmane I., Keiss J., Cernevska G., Pukite I., Krumina Z., Krumina A. 2015. *Not typical North-European ATP7B gene mutation in Latvian patients with Wilson disease*. *European Journal of Human Genetics*. ESHG Conference, Abstracts. 23, Suppl.1, 144.
4. **Zarina A.**, Piekuse L., Kreile M., Steinberga Z., Tolmane I., Keiss J., Chernusenko A., Sondore V., Cernevska G., Krumina Z., Krumina A. 2014. *Mutations in genes HFE, SERPINA1, CFTR in Wilson's disease*

- patients in Latvia*. European Journal of Human Genetics. ESHG Conference, Abstracts. 22, Suppl.1, 397.
5. **Zarina A.**, Piekuse L., Kreile M., Tolmane I., Keiss J., Micule I., Eglite I., Kaze I., Cernevska G., Krumina A. 2013. *Inheritance of Wilson's disease and its clinical appearance in four unrelated families in Latvia*. RSU Collection of Scientific Papers, VI Latvian Gastroenterology Congress with International participation, Abstracts. Suppl.2, 48.
 6. **Zarina A.**, Piekuse L., Kreile M., Krumina A., Lace B., Keiss J., Sondore V., Cernevska G. 2011. *Mutation spectrum of Wilson disease in Latvia*. IV International Congress of Molecular Medicine, Istanbul, Turkey, Abstracts. 115.
 7. **Zarina A.**, Krumina A., Lace B., Keiss J., Sondore V., Cernevska G. 2009. *High frequency of Wilson Disease and new mutations found in Latvia*. 4th Baltic Sea Region Conference in Medical Sciences, Warsaw, Abstracts. 86.
 8. Krumina A., Keiss J., Sondore V., Cernevska G., **Zarina A.**, Lace B. 2008. *Clinical evaluation and molecular-genetic diagnosis of Wilson disease*. The American Society of Human Genetics, 58th Annual Meeting, Philadelphia, USA. Abstracts.156.

Abstracts and participation in local congresses and conferences (7)

1. **Zariņa A.**, Kreile M., Tolmane I., Černušenko A., Čerņevska G., Puķīte I., Krūmiņa Z., Gailīte L. 2018. *Vilsons slimība Latvijā: 17 gadu pieredze molekulārajā diagnostikā*. RSU Zinātniskā konference. Rīga, 242.
2. **Zariņa A.**, Tolmane I., Černušenko A., Puķīte I., Čerņevska G., Krūmiņa Z., Kreile M., Piekuse L. 2017. *ATOX1 gēna variantu ietekme uz Vilsons slimības klīnisko gaitu*. RSU Zinātniskā konference. Rīga, 292.
3. **Zariņa A.**, Piekuse L., Kreile M., Tolmane I., Čerņevska G., Puķīte I., Krūmiņa Z., Keiņš J. 2016. *Vilsons slimības pirmās klīniskās izpausmes bērniem vecumā līdz 18 gadiem Latvijas populācijā*. RSU Zinātniskā konference. Rīga, 162.
4. **Zariņa A.**, Piekuse L., Kreile M., Tolmane I., Keiņš J., Černušenko A., Čerņevska G., Puķīte I., Krūmiņa Z., Krūmiņa A. 2015. *Kritēriji Vilsons slimības klīniskajai un molekulārajai diagnostikai*. RSU Zinātniskā konference. Rīga. 173.
5. **Zariņa A.**, Piekuse L., Kreile M., Tolmane I., Keiņš J., Sondore V., Černušenko A., Čerņevska G., Krūmiņa Z., Krūmiņa A. 2014. *HFE*

- gēna mutāciju C282Y un H63D saistība ar Vilsona slimību Latvijas pacientiem.* RSU Zinātniskā konference. Rīga. 146.
6. **Zariņa A.**, Kreile M., Piekuse L., Čerņevska G., Keišs J., Mičule I., Krūmiņa A. 2010. *Vilsona slimības izraisošās mutācijas H1069Q biežums bērniem līdz 18 gadu vecumam Latvijas populācijā.* RSU Zinātniskā konference, Rīga. 240.
 7. **Zariņa A.** 2008. *Vilsona slimības fenotipiski genotipiskā struktūra Latvijā.* RSU 57. Medicīnas nozares studentu zinātniskā konference, Rīga. 21.-22.

Acknowledgments

I would like to express my gratitude all colleagues, peers, friends and relatives who have participated in the creation of the PhD Thesis.

First, I would like to express my sincere gratitude to both supervisors – assoc. prof. Ieva Tolmane and assist. prof. Zita Krūmiņa for tireless examination of the PhD thesis, giving advices and making corrections. I would especially like to thank Linda Gailīte, the scientific consultant of the doctoral thesis – the head of the RSU Molecular Genetics Scientific Laboratory – for the consultations, recommendations, corrections and unbelievable support.

I would like to say a special thankyou to all the participants and organizers of the first discussion session of the PhD thesis, which allowed to make significant corrections in the PhD thesis. Special thankyou to prof. Māra Pilmane.

Thank you to all colleagues of RSU Molecular Genetics Scientific Laboratory – both current and former. I would especially like to thank the first head of the laboratory – Baiba Lāce, who provided the first support in starting my career as a scientist; prof. Astrīda Krūmiņa for controlling and supervising the first steps in science; Ieva Mičule – for creating the first interest in the field of genetics; Dmitrijs Rots – for help in developing the research design and valuable advice in writing the PhD thesis itself; Madara Kreile – for advices and support.

I'm very thankful to the colleagues of the RSU Department of Biology and Microbiology – both current and former. Special thank you to the head of the department – prof. Juta Kroiča for her support in

difficult times and valuable advice. I would like to thank all the colleagues of the department about the emotional support provided, valuable advice and non-refusal of help, especially – prof. Edvīns Miklaševičs; assoc. prof. Zanda Daneberga, assoc. prof. Erika Nagle; assist. prof. Agrita Puzuka.

I'm also very grateful to the team of the Laboratory of Molecular Genetics of the RSU Institute of Oncology for their help in sequencing.

I would like express my appreciation to all the patients for agreeing to participate in the study, as well as the doctors of various clinics for referring patient samples for molecular studies, especially – prof. Jāzeps Keišs; prof. Ludmila Vīksna; MD Gunta Čerņevska; MD Ieva Puķīte; MD Aleksandrs Černušenko; MD Pauls Aldiņš; MD Gita Tauriņa, MD Ieva Grīnfeldē and others.

Thank you to everyone who has participated in the formatting of the PhD thesis, especially to Inga Znotiņa for help in formatting references.

I would also like to thank the employees of the RSU Department of Doctoral Studies for their responsiveness in the writing and further development of the PhD thesis.

Thank you to all my family members (especially my parents) and friends for the emotional support provided.