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RĪGAS STRADIŅA
UNIVERSITĀTE

Daiga Bauze

**GENETIC ASPECTS
OF AUTISM SPECTRUM
DISORDERS**

Summary of the Doctoral Thesis
for obtaining the degree of a Doctor of Medicine
Speciality – Medical Genetics

Rīga, 2014



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The doctoral thesis was carried out in the Children's University Hospital, Children's Psychiatry and Medical Genetic Clinics and Latvian Biomedical Research and Study Centre

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ABBREVIATION

ADOS	Autism Diagnostic Observation Schedule
ALT	Alaninaminotraspherasis
AST	Aspartataminotraspherasis
ASD	Autism spectrum disorders
CI	Confidence interval
CNS	Central nervus system
CNV	Copy number variation
CYP	Cytochrome
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4 th Edition
FISH	Fluorescence in Situ Hybridization
GWAS	Genome-wide association study
ICD-10	World Health Organization's International Statistical Classification of Diseases 10 th Edition
IQ	Intelligence quotient
LOD	Logarithm of the odds
MAF	Minor allele frequency
OR	Odds ratio
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SD	Standard deviation
SNP	Single nucleotide polymorphism
VSIA BKUS	Children's University Hospital
χ^2	Chi-squared test

1. INTRODUCTION

One of the most common early child psychiatry and neurology disorders associated with autistic spectrum (ASD) is characterized by social-interaction-communication disorders and stereotyped patterns of behavior (ICD-10, DSM-IV). ASD starting at the age of three can cause the child severe disability if not diagnosed in a timely fashion.

DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th edition) and ICD-10 (World Health Organization's International Classification of Diseases, 10th edition) Classification of Diseases diagnostic ASD defines the basic criteria and divides them into five subtypes: Childhood Autism, Asperger Syndrome, Atypical Autism, Childhood Disintegrative Disorder, and Rett Syndrome (DSM-IV, ICD-10). In order to determine the diagnosis, and in order to objectively assess the patient's state of health, the standardized behavior and personality diagnostic scale is used as an additional tool (Lord et al., 2002).

Over the past decade, diagnostic technology has improved. Nevertheless, there has been a clear increase in the prevalence of ASD. These figures are from 35 to 60 per 10,000 children worldwide (Tantam and Girgis, 2008; Fombonne, 2008; Elsabbagh et al., 2012). There are no statistically significant differences between different ethnic, cultural and socioeconomic populations (Elsabbagh et al., 2012). In early studies, it was found that ASD is four times more common among boys than among girls, but by following the latest data from the epidemiological studies it was found that girls actually were diagnosed with ASD in excess of boys (3:1), and they actually experienced the process more severely than boys did (Myers and Johnson, 2007; Fombonne, 2008; Fombonne, 2009).

ASD is characterized by high heritability reviewed Following a recent prospective longitudinal studies, the data revealed that the risk of recurrence was 18.7% if a family had a child with ASD (Chakrabarti and Fombonne, 2001; Icasiano, 2004; Lauristen et al. 2005; Ozonoff, 2011).

The etiology of ASD is based on one of the first theories that believed that family and psychosocial factors may have been the cause of disorder development. This theory was challenged because ASD is a complex disorder whose etiology can simultaneously be involved in the environmental and genetic factors at the same time (Bettelheim 1967, Rutter and Schopler 1987, Courchesne et al., 2003; Gilberg, 2006; Gardener et al., 2011).

In ASD genetic research, unbalanced obligations for candidate genes were carried out in association studies and experimental studies in animal models, as well as in whole genome association studies (GWAS) , and in the whole genome CNV (Copy number variation) studies (Freitag et al., 2010). In 10–15% cases of ASD, this may have been one of the major early signs of monogenic diseases (Folstein and Rosen-Sheidley, 2001). It is accepted that all ASD also be categorized by etiological factors. A distinction in syndromic ASD, which occur in 15% of the population in the case of all ASD and 85% of cases of idiopathic non-syndromic ASD, is that the cause remains unknown (Gillberg and Coleman 1996; Lint and Persico, 2009). Next-generation sequencing technologies provide a broader analysis of the human genome. Exome sequencing is a rapid and economical method to detect rare *de novo* mutations in complex diseases. It is believed that ASD is a sporadic disorder and that it is the most common cause of *de novo* mutations (O’Roak et al., 2011).

One common belief is that ASD patients are characterized by a phenotype that distinguishes them from other individuals. One of the research aims is to determine the existence of not only behavioral, social-

communication, language and intelligence differences between ASD and healthy individuals, but also anthropometrical ones (Abrahams and Geschwind, 2008, Miles et al., 2008; Özgen et al. 2010).

ASD patients often have co-morbid disorders (Gillberg, 2006). It is known that 75–80% of ASD patients have varying degrees of mental retardation and 11–39% experience possible epileptic seizures (Gillberg, 2006; Tuchman, 2006). Forty-two percents of children with mental retardation have epileptic seizures, whereas ASD children without mental retardation have a frequency of seizures occurring in only 6-8% (Tuchman et al., 1991). Language development disorders in ASD patients may be associated with both mental retardation and an individual symptom unrelated to IQ level of development. Children with specific language disabilities in ASD have signs that may include the mild to moderate form, or may even be the only ASD leading symptom (Gillberg, 2006).

ASD is a complex disorder, which often tends to have more serious co-morbid conditions; therefore, the choice of treatment is complex. It is based on training with special teaching methods to integrate these children into society. In the treatment process a multi-disciplinary team is involved where the emphasis is put on language ability, communication and social skills development. In severe cases, medication is used (Correia et al., 2010). 31% of the ASD medication used was antipsychotic medication, antidepressants and mood stabilizers (Mayers, 2007, Mandell et al., 2008; Taylor et al., 2009; Correia et al., 2010). Risperidone is the only second-generation antipsychotic medication that is approved for use in pediatric patients (Jesner et al., 2008; Mandell et al., 2008; Stahl, 2008; ZVA, 2011).

Drug efficacy and reduction of the risk of future adverse effects would require individualized therapy using pharmacogenetic options (Woodcock and Lesko, 2009, Costa e Silva, 2013). First-and second-generation antipsychotic

medication by several cytochrome P450 metabolism markers, one of which is *CYP2D6*, provides antipsychotic drug metabolism in the liver; in fact, this gene polymorphism may be the most common cause of adverse events (Rodriguez-Antona et al., 2009; Correia et al., 2010).

Early diagnosis allows ASD to begin in early childhood socializing and integrating into society, providing an adequate non-treatment and drug therapy selection, thus reducing the possibility of the child disabilities. This is a topical study due to the fact that thus far there had not been an ASD, Anthropometric, Genetic and Pharmacogenetics study of this kind done in Latvia.

1.1. Aim of the Study

To find the development of the genetic risk factors in non-syndromic autism, as well as to examine the clinical data, anthropometric measurements, molecular genetics data analysis, the characteristic phenotype of the patients and to evaluate drug therapy options with the pharmacogenetics method.

1.2. Tasks of the Study

1. To create a well-described autism spectrum disorder sample study and obtain anthropometric measurements, as well as cytogenetic, biochemical and molecular investigations to be used as information specifically tailored for this study questionnaire.
2. To analyze the phenotypic, anthropometric parameters in autism spectrum disorder patients and to compare the results of measurements with the general population in order to discover the inherent distinctive features of the ailment.

3. To take chromosome 11q22.3, 11p13, 11p15.4 and 15q13.3-15.3-14 loci of genetic markers for autism spectrum disorder patients and the control group in order to find an association to autism spectrum disorders.
4. To determine cytochrome (CYP) P450 marker *CYP2D6* alleles *CYP2D6*4*, *CYP2D6*41* connection with the second generation antipsychotic medicine Risperidone on its effectiveness and side effects.
5. To identify a family with autism spectrum disorders. If their family tree data indicates monogenic diseases, then full exome sequencing shall be done in order to find potential autism spectrum disorder-causing candidate genes.

1.3. Hypothesis of the Study

1. Autism spectrum disorder patients may be of a great height, weight, head circumference and have other phenotypic features specific to these disorders in general and distinct from other populations. Using anthropometric measurement analysis, it could be created a phenotypic description of autism spectrum disorder characteristics.
2. According to the autism spectrum disorder candidate gene studies data, there was not one dominant chromosomal locus involved in the etiology of disorders. Chromosome 11p13, 11p15.4-15.3, 11q22.3, 15q13.3-14 locus polymorphisms may be associated with autism spectrum disorders.
3. By genotyping the markers P450 *CYP2D6*4* and *CYP2D6*41* alleles may determine their relationship to the second-generation psychotropic medication risperidone efficacy and side effects. This in turn, enabled an assessment of each individual ASD patient as applied in drug efficacy and risk to adverse reactions.
4. Autism spectrum disorders were sporadic, which may be involved in the etiology of spontaneous *de novo* mutations that were not related to a

specific type of inheritance. Using exome sequencing, it was possible to analyze a larger amount in order to identify potential autism spectrum disorder pathogen gene variants.

1.4. Scientific Novelty of the Study

This study summarizes an ASD patient representative sample in Latvia and makes a clinical and genetic evaluation of said sample. Phenotypic and anthropometric parameters are set up for the representative sample of ASD patients. This is the first study in Latvia in which ASD non-syndromic molecular genetic studies are conducted. A statistically significant association with SNP (single nucleotide polymorphisms) are revealed rs112112733 that are localized in chromosome 11q22.3 between existing genes *DDX10* and *EXPH5* that might be potential ASD candidate genes. Frequency analysis of ASD patients for pharmacogenetic markers of *CYP2D6*4*, *CYP2D6*41*, are performed. Risperidone one of the most frequently used medications as second-generation antipsychotics has its most common side effects and efficacy analyzed. Exome sequencing of an entire family with Asperger's syndrome, which corresponds to an autosomal dominate inheritance type can be found as a potential ASD candidate gene option *KCNH6*.

1.5. Practical Novelty of the Study

In this study, based on the literature review, there were recommendations made for early diagnosis of ASD in children under the age of three in pediatric, child psychiatry and child neurology practices. An algorithm was developed for ASD investigation in child psychiatry and medical genetics counseling.

1.6. Elaboration of the Study

The current study was carried out in the Children`s Psychiatric and Medical Genetics Clinics, University Children`s Hospital, Riga, Latvia. DNA extraction from the patients' biological material and molecular genetic studies were done at Latvian Biomedical Research and Development Centre, Riga, Latvia

The Riga Stradins University Committee of Medical Ethics approved the study.

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1.7. Outline of the Thesis

The thesis is composed on 150 pages in Latvian, following classical scheme. The work is structured in ten chapters: Introduction; Literature review; Subjects and Methods; Results; Discussion; Conclusions; Publications; Acknowledgements; References and Appendixes. Text of thesis is supplemented by 20 Tables; 10 Figures and 4 Appendixes. Reference list consist of 298 cited references.

2. SUBJECTS AND METHODS

This study was conducted from 2006 to 2013 in four phases.

In Phase One of the Study, from 2006 to 2013 ASD patients were selected in the Children`s University Hospital Children`s Psychiatry and Medical Genetics clinics. In ASD patient selection, where used the ASD diagnostic criteria, standardized behavior and personality scales for the ADOS test in selection. In this were compared ASD anthropometric measurements as a

percentile of the general population compared to the IQ level and frequency of seizures among ASD patients by gender in order to find possible ASD characteristic phenotypic features.

In Phase Two of the Study, from 2010 to 2011 the Latvian Biomedical Research and Study Centre isolated the DNA of ASD patients' biological material. Latvian Biomedical Research and Study Centre conducted a case-control association study in which it analyzed four rare allele variants (rs11212733, rs1394119 which was localized in 11q22.3, which was localized in 11p15.4-15.3 locus, rs2421826, which was localized in chromosome 11p13 locus, and rs1454985, which localized locus 15q13.3-14) for ASD patients and control groups in order to determine the potential SNP associations with ASD.

In Phase Three of the Study, from 2011 to 2012, the Latvian Biomedical Research and Study Centre did analysis on the second-generation antipsychotic medicine Risperidone for ASD patients. The marker cytochrome groups (CYP) P450 *CYP2D6* were chosen. The study analyzed the two alleles of the *CYP2D6* gene: *CYP2D6*4* (rs3892097) and *CYP2D6*41* (rs28371725). A case-control association study of allele frequency of ASD patient and control groups was performed. A certain degree of Risperidone was metabolized and analyzed and compared to ASD patients' most frequent adverse reactions and their relation to *CYP2D6* haplotype when using the medication Risperidone.

In Phase Four of the Study, from 2011 to 2013, in all ASD subjects, a family was found in which the ASD family tree was according to the inherited monogenic inheritance pattern and this would identify genes that cause disease in exome sequencing. In 2011, the Beijing Genomics Institute in China did exome full sequencing on three family members. From 2012 to 2013 using these exome sequencing results in the selected eight gene variants they were able to use the Sanger sequencing method for all family members.

2.1. Subjects

2.1.1. ASD patients

Since 2006, 196 patients have been selected for this research in consultation with Children's University Hospital Children's Psychiatric and Medical Genetic Clinics. Initially, patients were selected as per ICD-10 diagnostic criteria and Standard behavioral and personality scales: THE CHAT (*The Checklist for Autism in Toddlers*, the first 18 month test), (Baron-Cohen et al., 1992); AQ (*Autism Spectrum Quotient* – autistic spectrum disorder questionnaire for children aged four to 11 years of age), (Baron-Cohen et al., 2006); Cambridge University behavioral and personality questions for children up to four years of age; Cambridge University social and communicative development issues; CASD (*Childhood Asperger Syndrome Test* – childhood Asperger Syndrome Test), (Scott et al., 2002; Williams et al., 2006).

In the event of a positive result, each patient with a suspected possible case of ASD, was sent to the clinical psychologist who then performed a specialized, standardized diagnosis using the ADOS (*Autism Diagnostic Observation Schedule*) test, rendering for each patient the proper diagnostic model based on age, in order to confirm ASD (Lord et al., 2002).

Since language development disorders and varying degrees of mental retardation is the most common level of ASD co-morbid disorders for each patient the speech therapist assessed the expressive and receptive language skills. In addition, a clinical psychologist examined intelligence in determining IQ level on the Woodcock-Johnson Intelligence Scale for children (Woodcock, 2003), used the Munich functional development of diagnostic tests (Hellbrugge et al., 1994), Wechsler test (Wechsler, 1974), that were modified according to each child's age group. In this way, 173 patients were identified as having diagnosed ASD.

From the research group certain patients were excluded with other psychiatric disorders, which did not match the ASD diagnostic criteria.

To be more of a homogeneous research group and to exclude syndromic autism, each patient subgroup was examined by a physician geneticist. When necessary, the indications for ASD patients were sent for biochemical, genetic, cytogenetic, molecular investigations to exclude frequent monogenic syndromes, congenital metabolic diseases and chromosomal syndromes.

When there were appropriate indications, the Children's University Hospital Medical Genetics Clinic conducted the following standard tests:

- 1) In the Cytogenetic laboratory: standard karyotype analysis of the 15q11.13 deletion or duplication, 22q11.2 deletion, chromosomal regions subtelomere analysis with FISH (fluorescent in situ hybridization method) was conducted;
- 2) In the DNA laboratory: Analysis of the *FMRI* gene (fragile X chromosome syndrome), *MeCP2* gene analysis (Rett syndrome), and DNA methylation analysis of the 15q11-13 locus (Angelman and Prader-Willy syndrome exclusion) were done;
- 3) In the Genetic Biochemistry laboratory: the range of organic acids, mucopolysaccharides qualitative and quantitative analysis of urinary oligosaccharides, amino acid spectrum of the blood and urine were analyzed.

Each ASD patient's parents or guardians became acquainted with and then signed the RSU Ethical Commission approved consent form allowing their children to participate in this research as well as the use of their biological material (blood samples) for further genetic research.

2.1.2. ASD patient questionnaire

The data on patients registered with ASD was specially created for this study questionnaire that included the most comprehensive picture of the patient's early development, the onset of sickness and its development as well as family status.

The questionnaire contained the following information on each patient:

- 1) Family history and Family tree going back three generations;
- 2) History of pregnancy;
- 3) The newborn's process of development and psycho-motor development of the child up to one year of age;
- 4) The child's psycho-motor development up to three to four years of age;
- 5) Anthropometric measurements were taken;
- 6) Epileptic seizures were assessed;
- 7) All patients underwent:
 - EEG (electroencephalography) to exclude epileptic activity;
 - Hearing test, to exclude poor hearing.
- 8) Patients with a history of unexplained seizures and developmental regression, language loss, MRI (magnetic resonance imaging) and/or CT (computed tomography) underwent tests to rule out organic CNS damage.
- 9) All patients underwent the following analyses:
 - Children's University Hospital Clinical laboratory: a complete blood count (red blood cells, white blood cells and platelets, hemoglobin, erythrocyte sedimentation rate as per the Westgrain method

- Children's University Hospital Clinical laboratory blood biochemical indicators: aspartataminotransferase (ASD), alaninaminotransferase (ALT), lactate, uric acid, blood glucose, homocysteine. In case of necessity–folic acid, vitamin B₁₂ in the blood serum.
- 10) For patients who were objectively assessed and revealed indications for genetic investigation had their findings sent to Children's University Hospital Medical genetics clinic, for further investigation and based on the clinical, genetic tests that had been made.
 - 11) In the patients with suspicion of possible genetic abnormalities in addition to:
 - EhoKG (echocardiography) to exclude a possible congenital heart disease;
 - USG (ultrasonography) of the abdominal cavity to rule out congenital abnormalities in the liver, the kidneys, the urinary passages;
 - The advice of an oculist to exclude visual impairment.
 - 12) The patient biological material (a blood sample) with the written consent of a parent or guardian to allow the biological material to be used for further research purposes, which were taken to Latvian Biomedical Research and Study Centre, where the DNA samples were extracted, stored and analyzed.
 - 13) A photograph of the patient (only with the written consent of parent or guardian for data collection).

Since the study was retrospective and prospective and was conducted in four successive phases in the period from 2006 to 2013, the number of patients in each research phase was different (Fig. 2.1).

In Phase One of the Study, by going through Children's university Hospital Children's Psychiatry and Medical Genetics Clinic Medical Records and advisory data for the period from 2006 to 2013, 196 patients were evaluated who were suspected of having ASD. Of these patients, 173 were diagnosed with ASD and selected for further study. In order to have a homogeneous group in the study, another 23 patients were excluded with confirmed monogenic or chromosomal abnormalities, which made up the syndromic ASD group. Of the remaining 150 patients, non-syndromic ASD phenotypic measurements and anthropometric data were analyzed.

In Phase Two of the Study, the study group was formed from the existing 150 non-syndromic ASD patients. A sample of 95 patients was taken during the period from 2010 to 2011 with the written consent of a parent or guardian, biological material was taken for further genetic research

In Phase Three of the Study, the study group was formed from the existing 150 non-syndromic ASD patients. A sample of 113 patients was taken during 2011 to 2012 on which a pharmacogenetical marker analysis was performed.

30 patients of this group with ASD aged four to 15 (average = 7.16 years, SD = 2.39), had medication administered using the second generation anti-psychotic risperidone. The effectiveness and side effects of this medication were analyzed.

In Phase Four of the Study, from 2011 to 2013 the already-existing ASD research group selected a family with Asperger syndrome that adhered to the autosomal dominant inheritance type criterion. Full exome sequencing was carried out on three family members and sequencing as per the Sanger method was carried out on all five family members.

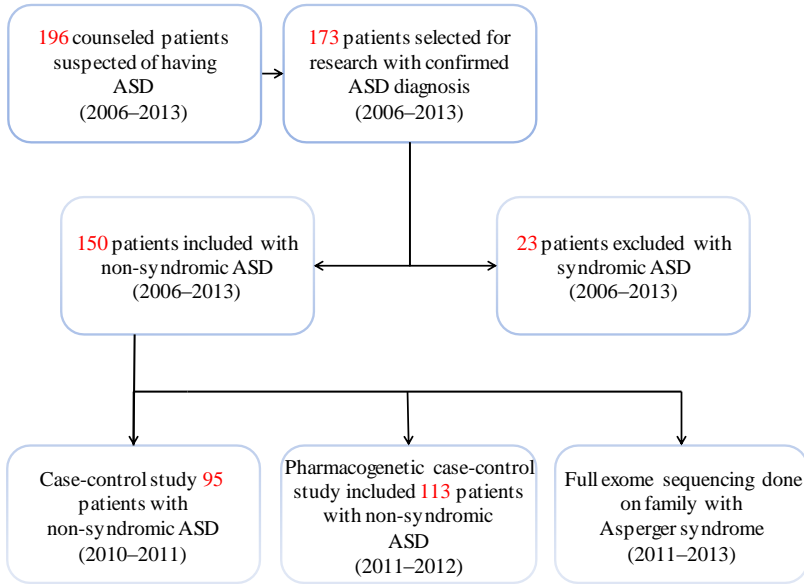


Fig. 2.1. Patient selection algorithm

2.1.3. A Family with Asperger Syndrome

In order to identify possible ASD candidate genes, from the research group, as per the autosomal dominant inheritance type, selected a family with Asperger syndrome in which three family members had full exome sequencing done.

The chosen family had the following criteria: the child and one of his parents had had ASD in order to correspond to the autosomal dominant inheritance type; in addition, the family had to have had Asperger syndrome for two generations. (Fig. 2.2.).

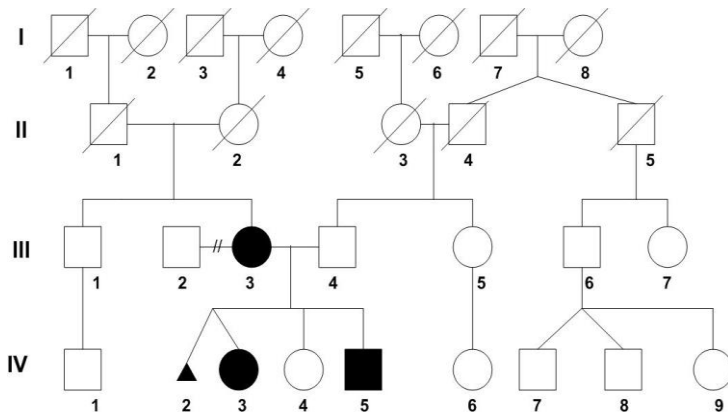


Fig. 2.2. Family history pedigree structure

IV-3. The patient was an 11-year-old girl with behavioral disorders. Social adaptation and communication disorders manifested themselves in the form of difficulty in contacting with her peers. She had emotional disorders, and at times, aggressive behavior towards other family members. The girl was peculiar, with repetitive hand movements, as well as having a specific interest in dinosaurs, a language disability, limited facial mimicry, clumsiness, communication problems with peers and family members, and had some stereotyped rituals. When diagnosed with Asperger's syndrome, IQ was normal (> 70).

IV-4. The patient was an eight-year-old girl. She was born from the second pregnancy of her mother and had no abnormal developments. At seven years of age the girl began to experience behavioral disorders. The girl became aggressive towards her younger brother and older sister. Obsessive-compulsive disorder complicated her condition. She lost interest in learning, had sleep disturbances, difficulty adapting to school, as well as repetitive hand

movements. The girl was investigated but ASD was not diagnosed. IQ was normal (> 70)

IV-5. The patient was a seven-year-old boy. At the age of five the boy began to have affective mood disorders, depressive disorders, repetitive hand movements and increased sensory perception. The boy began to have communication and socialization problems both at school and at home and he became indifferent to his peers. He was diagnosed with Asperger's syndrome. IQ was normal (> 70).

III-3 (mother). The patient was a 45-year-old woman, who from an early age as a child at school had communication problems, socialization problems and a specific interest in dragons and dinosaurs. During adolescence she had a depressive disorder. According to the clinical psychologist diagnosis, she had Asperger syndrome

III-4 (father). The patient was a 45 year-old man. Mental Health disorders were not detected.

2.1.4. Control group

The control group consisted of 190 randomly selected, potentially healthy, mutually unrelated people from the genome database at the Latvian Biomedical Research and Study Centre. These test subjects did not have a history of ASD or other psychiatric disorders in the family and the gender relations control group was matched with that of the study group.

2.2. Methods

2.2.1. Anthropometric measurements for ASD patients

Anthropometric measurements were taken for all ASD patients. The chief measurements were taken for height, weight and head circumference.

Each patient's ASD measurements were evaluated as per the Latvian children physical development assessment standards (Krumina et al., 2007) and the International Standard percentile curves (Jones, 2006). ASD compared anthropometric measurement percentiles to that of the general population percentile readings.

As the most frequent ASD co-morbid disorders, mental retardation and a patient's history of epileptic seizures and language impediments were analyzed. The frequency of these ailments was compared by gender for ASD patients.

2.2.2. DNA extraction from ASD patients and control group participants

For ASD patients and the control group participants, DNA was isolated from a blood sample at the Latvian Biomedical Research and Study Center using the standard chloroform – phenol method (Sambrook et al., 1989), as per the Latvian State Population genomic database standards.

2.2.3. ASD association research in SNP selection

SNP were selected on the basis of publications and ASD extensive genome association studies, which analyzed ASD potentially important loci. For the SNP selection criteria, the assessed publication study group size was selected, which consisted of representatives from the different ethnic populations as well as non-syndromic ASD subphenotypes (language disorders, mental retardation) and previously associated ASD with SNP most significant LOD values (Liu et al., 2008; Cho et al., 2011). There were two extensive, yet unrelated genome-wide studies (Liu et al., 2008; Cho et al., 2011). In the first study, 1397 families with ASD from North America and Europe had 5371 SNP analyzed (Liu et al., 2008). Conversely, in the second study, 42 Korean families

with ASD had 3022 SNP analyzed (Cho et al., 2011). In the light of this research, the samples of four potentially meaningful SNP were involved in the development of ASD. The selected SNPs had a higher association with ASD and in evaluating the p-values, they were less than 0.0001. Genotyping was selected for the following markers: rs11212733, which was localized in chromosome 11q22.3 locus ($p = 9.76 \times 10^{-6}$), (Cho et al., 2011); rs1394119 ($p = 0.00004$), which was localized locus 11p15.4-15.3; rs2421826 ($p = 0.0003$), which was localized on chromosome 11p13; and rs1454985 ($p = 0.00001$), which was localized locus 15q13.3-14 (Cho et al., 2011; Liu et al., 2008).

2.2.4. ASD association for research in selecting SNP genotyping

A case-control association study for SNP genotyping in the Latvian Biomedical Research and Study Centre using TaqMan reagent principles was carried out. Genotyping with RT-PCR/HRM (real-time polymerase chain reaction with high-resolution melting analysis) in the ViiATM7 Real-Time PCR System (Applied Biosystems, Carlsbad, USA) and GeneAmp® PCR System 9700 system (Applied Biosystems) (ABI 7500 Real-Time PCR System, Applied Biosystems, Carlsbad, USA) was implemented according to the manufacturer's recommendations. The results obtained were analyzed manually with the 7500 Real Time PCR System Software.

2.2.5. Pharmacogenetical SNP selection

In ASD patient pharmacotherapy Risperidone, one of the most frequently selected medications, is a second-generation antipsychotic, which is approved by the International Pediatric Association and the Latvian State Agency of Medicines (SAM) as an authorized medical treatment for children

from five to 17 years of age (Mandell et al., 2008; Stahl, 2008; Taylor et al., 2009; ZVA, 2011).

Risperidone is metabolized in the liver. The metabolic process is an essential group of cytochrome (CYP) P450 CYP2D6 marker, which is extensively hydrolyzed in the liver as 9-hydroxy-risperidone. CYP2D6 primarily provides 50% of drug metabolism (Ingelman-Sundberg et al., 2007; Rodriguez-Antona et al., 2009).

Risperidone for both metabolites play a role in the effectiveness of treatment and the development of side effects. The *CYP2D6* gene is highly polymorphic. Based on publications, databases, and commercially offered tests, the study selected two alleles of this gene: *CYP2D6*4* (rs3892097), *CYP2D6*41* (rs28371725) (Ingelman-Sundberg et al., 2007; Rodriguez-Antona et al., 2009; Anderson, 2010; Correia et al., 2010; ZVA, 2011; cgcggenetics, 2011).

2.2.6. Pharmacogenetical SNP genotyping

For the markers *CYP2D6*4* (rs3892097) and *CYP2D6*41* (rs28371725) the genotyping was carried out at the Latvian Biomedical Research and Study Centre using TaqMan reagents; genotyped using real-time PCR performed with the ViiATM7 Real-Time PCR System (Applied Biosystems, Carlsbad, USA) and the GeneAmp ® PCR System 9700 System (Applied Biosystems) (ABI 7500 Real-Time PCR system, Applied Biosystem, Carlsbad, USA), as recommended by the manufacturer.

The results obtained were analyzed by 7500 Real Time PCR System Software.

2.2.7. Risperidone therapy assessment criteria

The efficacy assessment of risperidone therapy was conducted according to the following ASD behavioural symptoms: self-injury, aggression, destructive behaviour, sleep disturbances, stereotypic movements and rituals, and increased irritability. Language, communication and sociability skills were also assessed. The efficacy was examined to determine whether the treatment effects were beneficial, ineffective or adverse. The risperidone efficacy was evaluated from one month to two years after the start of pharmacotherapy.

Risperidone adverse reactions were classified as more common ($\geq 1/10$), common ($\geq 1/100$ to $< 1/10$), uncommon ($\geq 1/1000$ to $< 1/100$), rare ($\geq 1/10\ 000$ to $< 1/1000$) and very rare ($< 1/10\ 000$) as detailed by the manufacturer and State Agency of Medicines Registry (VZA, 2011). More common risperidone adverse reactions were prolactin elevation, weight gain, increased appetite, tachycardia, sedation and extrapyramidal symptoms. Uncommon adverse reactions were prolongation of the corrected QT interval, anaemia, thrombocytopenia, dizziness, dyskinesia, lethargy and rash.

The safety of risperidone therapy was evaluated by the following physical measures: prolactin level, aspartate transaminase (AST) and alanine aminotransferase (ALT) levels, electrocardiographic parameters and observing symptoms of neurological adverse effects, including extrapyramidal movements.

2.2.8. Full Exome sequencing for a family with Aspergera syndrome

A family diagnosed with Asperger's syndrome in two generations of full exome sequencing with an affected child (IV-3) as well as both parents (III-3, III-4) was examined by the Beijing Genomics Institute in China (Beijing Genomics Institute) using the Illumina HiSeq 2000 system paired-end sequencing method.

2.2.9. Exome sequencing data analysis

Exome sequencing results of the analysis carried out in cooperation with the Latvian Biomedical Research and Study Centre professionals (Fig. 2.3.); further analysis of selected SNP met the alternative allele's dominant type inheritance model.

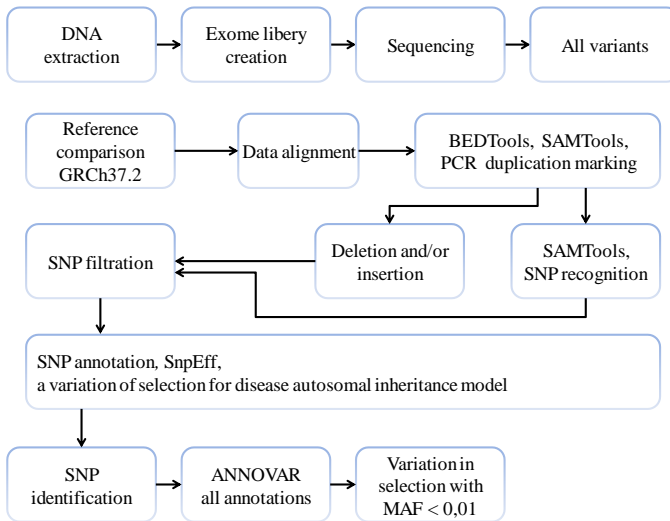


Fig. 2.3. Exome Sequencing data analysis algorithm

Selected SNP, the frequencies of which 1000Genome database was ≤ 0.01 , was not found in the database dbsnp132 (1000genome, 2013).

2.2.10. Potential candidate gene selection

The creation of a SNP list by localization was compared with the already existing ASD databases. From these databases, potential ASD candidate genes were selected. The exclusion criteria were as follows:

- 1) Only the *Homo sapiens* models were selected in order to find the potential disease-causing candidate genes in people;
- 2) Where CNS gene manifestations occurred, SNP should be excluded, which did not manifest themselves in the CNS;
- 3) Whereas the aim of the study was to find possible non-syndromic ASD candidate genes, the established SNP list should exclude previously described monogenic diseases SNP from the database (omim, 2013).

In order to choose potential ASD candidate genes, the following evidence-based databases were used: AutDB (autDB, 2013), OMIM (omim, 2013), Ensembl (ensembl, 2013), GeneCards (genecards, 2013), EMBL-EBI (ebi, 2013).

2.2.11. Candidate gene sequencing and analysis

Eight different variants of sequencing were selected (*ARPP21*, *KCNH6*, *KCNJ10*, *KIAA051*, *LRFN2*, *PCDHA9-10*, *MPDZ*, *PLD5*). As per the Sanger method all family members with Asperger syndrome (III-3, III-4, IV-3, IV-4, IV-5) and using autosomal dominant heredity type model, five potentially significant genes (*KCNJ10*, *ARPP21*, *PLD5*, *KCNH6*, *MPDZ*) were analyzed.

In order to determine the potential SNP pathogenicity, the polymorphism site is determined in the transcripts that encode proteins using the Ensemble (ensemble, 2013) and Mutalyzer (mutalyzer, 2013) databases.

2.3. Statistical Data Analysis

The data was analyzed using descriptive and analytical statistical methods.

Descriptive statistics were used containing percentages, mean, Standard deviation and median value. Anthropometric measurements were taken (height, weight, and head circumference) as a percentile of the percentage of patients between ASD and gender in the general population. An inter-comparison between the genders of ASD patients of co-morbide disorders event frequency (mental retardation, epileptic seizures, and language impediments) was also conducted

Analytical statistical hypothesis testing, comparing the investigational ASD group gender, in order to verify the anthropometric measurements and frequency of concomitant diseases associated with ASD, the Fisher's Exact test was used (Fisher Exact). It has been stated that the p-values for the statistical significance threshold shall be less than 0.05. The odds ratio (OR), which is a probability of the event into one of the groups regarding the same event probability of the second group was calculated with 95% confidence interval (CI).

The SPSS 19 Windows software was used for the processing of statistical data (SPSS Inc., Chicago, IL, USA).

The PLINK 1.06 software was used for the analysis of the case control ASD and the selected SNP association research analysis (Purcell et al., 2007). To compare the ASD and control group data for statistical processing, the Standard Chi-squared test (χ^2) with the Bonferoni correction or multiple testing corrections were used. All of the analyzed markers were determined by or were a deviation of the Hardy-Weinberg Equilibrium. The Haplotype analysis was implemented using the Standard Chi-squared (χ^2) test. Alleles frequency and

95% confidence interval was determined using the standard Chi-squared (χ^2) test as well. A statistically significant association exists if $p < 0.05$. The covariates used for linear regression analysis were: ASD patient age, gender, IQ level and epileptic seizures in a patient's history.

ASD and pharmacogenetics marker study used descriptive statistics percentages, analysis of allele frequencies of patients and the control group using Fisher's Exact Test. A statistically significant association was considered when $p < 0.05$. The second-generation antipsychotics risperidone adverse drug reactions and therapeutic correction frequency of patients were evaluated. The ASD performed haplotype analysis of patients on the control group. Linear regression analysis was also used to determine the relationship between the dose of risperidone and the selected genetic markers. The covariates were the child's age, gender, dose of risperidone, IQ levels, AST, ALT indicators. Statistical analyses were performed using PLINK version 1.06 software (Purcell et al., 2007).

Exome sequencing data analysis was executed with the freely accessible GATK (gatk, 2013) and ANNOVAR (annovar, 2013) software programs.

3. RESULTS

3.1. ASD patient anthropometric and clinical description

The first phase of the study was carried out by Children's University Hospital Children's Psychiatry and Medical Genetics clinics. The ASD patients were analyzed according to their anthropometric measurement indicators and the frequency of ASD co-morbid diseases.

According to the ICD-10 diagnostic criteria, ADOS test results selected 173 patients with ASD. In order to differentiate syndromic autism

from non-syndromic, ASD patients had genetic investigations conducted. As a result, there were 23 (13.29%) monogenic, chromosomal and inherited metabolic disorders whose initial manifestation in the patient had ASD with the development of regression.

Data on the number of detected genetic abnormalities and their percentage distribution were collected on Table 3.1. These patients were excluded from further study.

The study analyzed 150 ASD patients whose mean age was 8.1 (SD = 3.15) years. From the 150 patients in the investigated group, there were 121 ASD patients (80.66%) who were boys, whose mean age was 7.9 (SD = 2.82) years and 29 (19.33%) who were girls with a mean age of 8.4 (SD = 4.24) years.

Excluding the syndromic ASD sample of patients, the further study group consisted of 150 non-syndromic ASD patients. ASD patient anthropometric characteristics and frequency of co-morbid disorders averages were described and analyzed.

When analyzed by ASD subtypes, the median age of patients in the childhood autism group (n = 58) was 7.6 (SD = 2.54) years, in the Asperger syndrome group (n = 4), the mean age of children was 7.7 (SD = 3.34) years and in the ASD group of patients (n = 88), the mean age of children was 8.3 (SD = 3.46) years.

According to the distribution of diagnoses for all study patients that included ASD, childhood autism group had 58 (38.66%) patients; the Asperger's syndrome group had four (2.66%) patients, but the ASD group, had 88 (58.66%) patients.

Table 3.1

Syndromic autism number of patients and percentages for the selected patient group

Genetic Pathology	Cases	Percentage
Complex Chromosomal change 46,XY,t(1;2;17)(q23;p21;q25.3)	1	4.35%
Complex Chromosomal change 46,XY,der(1)(1pter→1q25:13q21→13q32:7p21→7pter), der(7)(13qter→13q21:7q31→7p21:7q32→7qter), der(13)(13pter→13q21:1q25→1qter)	1	4.35%
15q11-13 duplication (MIM #608636)	2	8.69%
15q11-13 deletion Angelman syndrome (MIM #105830)	3	13.04%
Kabuki syndrome (MIM #147920)	1	4.35%
22q11.2 deletion DiGeorge syndrome (MIM #188400)	2	8.69%
Fragile X chromosome syndrome FMR1 gene mutation (MIM #300624)	6	20.09%
Rett syndrome MECP2 gene mutation (MIM #312750)	2	8.69%
α -1-mannosidose (MIM #609458)	1	4.35%
Cx26 genetic mutation (sensoneural hearing loss) (MIM #220290)	2	8.69%
Tubercular sclerosis (MIM #191100)	2	8.69%

By analyzing the gender percentages between all ASD subtypes, it was observed that childhood autistic boys were found in 81.03%, of cases and

18.96% for girls; for boys Asperger syndrome was present in 75%, and 25% for girls; boys with ASD were found to have 80.68% while the girls had 19.32% in all cases of ASD subtypes. Statistically significant differences between the gender groups in the case of ASD were found ($p = 0,485$).

By analyzing the ASD patients' anthropometric measurements percentile indicators, the norm was considered to be between the fifth and the ninety-fifth percentile. The results, which were less than 5%, were considered small for their age whereas, anything above 95% was considered large for their age (Jones, 2006; Krumina et al., 2007). The ASD patient and general population height, weight, head circumference were analyzed in terms of percentile.

ASD patients were compared to each patient's anthropometric measurements percentile distribution relative to the distribution in the general population. Height, weight and head circumference were compared, the results are shown in Fig. 3.1.

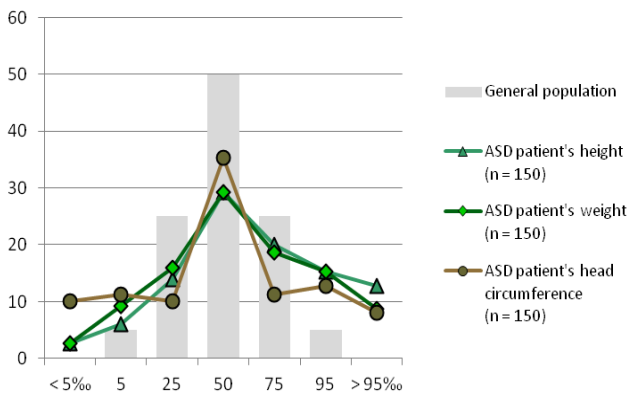


Fig. 3.1. ASD patient and general population anthropometric measurements in terms of percentile

As the most common co-morbid disorders for ASD patients were the degree of mental retardation and epileptic seizure frequency. It was found that

of all ASD patients 29 had normal intelligence (IQ >70) or (18.66%) of patients, 77 (51.33%) patients had mild mental retardation (IQ 50–69), 34 (22.66%) patients had moderate (IQ 35–49) mental retardation, and ten (6.66%) had severe (IQ 24–30) mental retardation.

When comparing IQ between the genders no statistically significant differences were found.

By analyzing the medical record data on the frequency of epileptic seizures included in the study, a history of all ASD subtype patients reported that 135 (90%) patients, reported seizures, eight (5.33%) patients had febrile seizures in early childhood, two (1.33%) of the patients revealed a number of epileptic seizures in their lifetime, but five (3.33%) patients were diagnosed with epilepsy. When analyzing by gender, epileptic seizures were found in boys of the ASD group in eight patients (6.61%) while in girls seizures were observed in seven patients (24.13%). When compared by gender, epileptic seizures had a statistically significant higher incidence in girls of the ASD group ($p = 0.0106$). Comparing the frequency of seizures in ASD patients and the degree of mental retardation, a statistically significant difference was observed. In the mild mental retardation group, the epileptic seizures were less frequent ($p = 0.057$); whereas, in the severe mental retardation group there was a more prevalent occurrence ($p = 0.009$).

By analyzing language development disorders, it was found that 148 (98.66%) patients were diagnosed with both expressive and receptive language development disorders. In fact, only two (1.33%) patients in the ASD group had no language disorders observed. When the data was analyzed by gender distribution, the boys of the ASD group had language disorders in 119 patients (98.34%). In girls of the ASD group language disorders were found in all 29 patients (100%).

3.2. The selected SNP association analysis

In the second research phase of the study 95 patients of the ASD group were studied. This phase was derived from the patients' biological material and whose parents had given their consent to allow the use of the child's biological material and the analysis of 190 randomly selected, relatively healthy control representatives. The genotyping was conducted on four genetic markers: rs11212733, rs1394119, rs2421826 and rs1454985.

In the analysis of genotype distribution of the Hardy–Weinberg Equilibrium, it was discovered that the allele distribution in ASD patients and the control group were in balance with the exception of the rs1394119 polymorphism ($p < 0.05$). Therefore, this polymorphism was excluded from further study. The results were presented in Table 3.2.

With the analysis of selected markers, it was found that SNP rs11212733 was statistically significant when associated with ASD ($p = 0.008$, $p_{\text{adjusted}} = 0.024$). In turn, rs2421826 ($p = 0.456$, $p_{\text{adjusted}} = 1.0$) and rs1454985 ($p = 0.291$, $p_{\text{adjusted}} = 1.0$) polymorphisms had no statistically significant association with ASD.

By analyzing haplotypes rs11212733/rs2421826, which are localized in the 11th chromosome, the haplotype frequency was seen to be most frequent in the ASD patient group in A/A ($p = 0.122$), but in the control group it was T/A ($p = 0.012$). This difference between groups was statistically significant.

In the ASD linear regression analysis, in order to determine the association of ASD with analyzed SNP polymorphism, as covariates were used: the ASD patient age, gender, IQ levels and a history of epileptic seizures. In the analysis, a statistically significant association was not detected and the results were not reflected.

Table 3.2

Results of Case-Control Association Study

SNP	Chromosomal localization	MAF cases	MAF controls	χ^2	P value	P adjusted	OR	95%CI
rs11212733 g.12041239 T>A	11q22.3	0,552	0,432	6,982	0,008	0,024	1,625	1,13–2,31
rs2421826 g.35170605 G>A	11p13	0,352	0,432	0,554	0,456	1,0	1,154	0,79–1,68
rs1454985 g.4205023 T>C	15q13.3– q14	0,447	0,399	1,118	0,291	1,0	1,217	0,84–1,75

SNP - single nucleotide polymorphism

MAF - minor allele frequency

χ^2 – Chi squared test

P_{adjusted} – Bonferroni correction for three markers applied

CI – Confidence interval

OR – Odds ratio

3.3. Analysis of Pharmacogenetic Marker CYP2D6

The objective of pharmacogenetic testing here was to determine the frequency of *CYP2D6**4, *CYP2D6**41 alleles in ASD patients and control groups, and examine their role in prognosis of the effectiveness of the selected therapy, using the second generation antipsychotic risperidone.

In evaluating the effectiveness of drug-induced therapy, taking into account that the research was conducted in 2011 and 113 ASD patients were recruited, the cytochrome group's P450 metabolism level was set for the 113 and for 190 control group members. Analyzing both of these groups, no

statistically significant differences were noted. Allele *CYP2D6*4 T* frequency among the patients was 0.191, in the control group – 0.188 ($p = 0.933$, OR = 1.019), allele *CYP2D6*4I T* frequency among the patients was 0.113, in the control group – 0.053 ($p = 0.012$, OR = 2.257).

Analyzing the frequency of the *CYP2D6*4I/CYP2D6*4* (rs28371725/rs3892097) haplotypes between ASD patients ($n = 113$) and the control group ($n = 190$), there were no statistically significant differences. The most frequent haplotype in both groups was *CYP2D6*4I/CYP2D6*4* (rs28371725/rs3892097). C/C frequency in the patients' group was 0.729 ($p = 0.428$), but the *CYP2D6*4I/CYP2D6*4* (rs28371725/rs3892097) TT haplotype remained undetected in both control and patient groups.

Eighty-three patients in the ASD group did not receive pharmacotherapy, because educational and behavioural intervention methods were used for relieving ASD core symptoms. Thirty patients from this group received pharmacotherapy with risperidone. These patients comprised 23 boys and seven girls aged between four and 15 years (mean age 7.16 years, SD = 2.39).

In order to analyze the connection between polymorphism and therapy effectiveness, the patients on risperidone, were divided into two groups, those with effective therapy ($n = 28$), and ineffective ($n = 2$).

Taking into account that the ineffective risperidone therapy was concluded in two patients only, statistical methods in comparing data were not utilized, but – in analyzing the genotypes in the *CYP2D6*4I* allele in the effective risperidone therapy group, CC (genotype) was most frequently observed (85.7%). In the ineffective risperidone therapy group, the genotype TT in *CYP2D6*4I* was not found; one patient was recorded with CT, another – with CC. In analyzing genotypes in the *CYP2D6*4* allele in the effective risperidone group, the most frequent genotype was CC (78.6%), while in the

ineffective group, genotypes TT and CC did not appear, but genotype CC was discovered in two patients.

The ASD patient group set up for further research, which used risperidone in monotherapy, was tested for side-effects. Drug-induced therapy with risperidone was suspended after side-effects were discovered, and a switch made to psychotropic medication authorized for use in child psychiatry.

Of the 30 ASD cases that received risperidone monotherapy, seven patients (23.33%) had their therapy changed due to adverse drug reactions. Twenty-three ASD patients (76.66%) did not experience adverse reactions to risperidone. The most common adverse reactions recorded were elevated prolactin level (for five patients, 16.6%) and weight gain (for one patient, 3.33%). ASD patients were given an electrocardiogram and only one patient (3.33%) was found to have an abnormal heart rhythm. No extrapyramidal adverse effects or other neurological symptoms were observed.

Comparing the *CYP2D6*41/CYP2D6*4* (rs28371725/rs3892097) CT haplotype in effective and ineffective risperidone groups, its connection to ASD disorders and the effectiveness of pharmacotherapy was not determined, therefore this cannot be utilized in therapy prognosis for our group.

In analyzing the *CYP2D6*4* allele in the risperidone side-effects group, frequency of the T allele was 0.07143, while T in the group without side-effects was at 0.1522 ($p = 0.4365$, OR = 0.4286, 95%CI = 0.0481–3.189). Another allele – *CYP2D6*41*, in the risperidone side-effect group had T frequency at 0.07143, while the group without side-effects, T was at 0.1304 ($p = 0.5471$, OR = 0.5128, 95%CI = 0.0564–4.663). In analyzing haplotype *CYP2D6*41/CYP2D6*4* (rs28371725/rs3892097) C/T in those with and without risperidone side-effects, no significant connections were discovered ($p = 0.431$).

The average dosage of risperidone that patients used was 0.6 mg/dn (SD = 0.56). All those who took risperidone during monotherapy, both the liver biomarkers (AST, ALT) were normal. For these patients, a linear regression analysis examined risperidone dosage, AST and ALT indicators in connection with *CYP2D6*4*, *CYP2D6*41* alleles, with a patient's gender, age and weight as covariates. The analysis did not confirm an association between these parameters (data not shown).

3.4. Exome Sequencing Analysis in Family with Asperger Syndrome

The family with Asperger Syndrome (in two generations) – two parents and one affected child, underwent full exome sequencing and analysis of data which was compared versus hg19 human genome. Samples III-3, III-4, IV-3 overlapped the reference genome exome by 91.63%, 91.66% and 91.78%. (Fig. 3.5.).

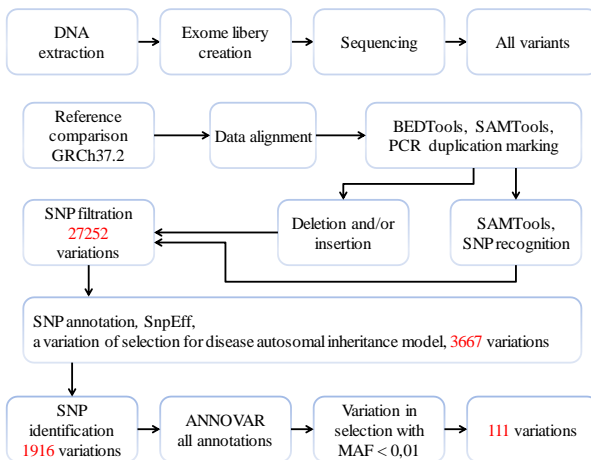


Fig. 3.5. Algorithm of Exome Data Analysis

As a result of SNP screening and annotation, according to the ailment's hereditary dominant, potential ASD SNP variants were selected in 111 genes.

SNP in these genes induced a change of amino acids, i.e. *missense* mutation. Further ahead in the process, the annotated SNP list was compared with existing, fact-based data bases in order to select the potentially most significant SNP, expressed in CNS, and possibly linked to ASD.

As a result, eight gene variants were chosen that, based on research data, expressed in CNS. Of them, *KCNJ10* and *PCDHA9-10* are noted in the ASD data base as potential candidate–genes.

In the continued process, the eight were given referential order, coding nucleotide sequences, change of amino acids in protein and frequency of allele change in the exome sequencing project for all populations (annovar, 2013). For data, see Table 3.3.

Gene sequencing was conducted for the rest of the family, based on the Sanger method. The autosomal dominant inheritance model was chosen, taking into account the family's anamnesis data, that the mother is ill. As a result, five SNP theoretically significant genes were detected in the family with Asperger Syndrome: *KCNJ10*, *ARPP21*, *PLD5*, *KCNH6*, and *MPDZ*. To determine possible pathogenicity of remaining SNP, polymorphism's place was determined in transcripts that code protein using the Ensembl (ensembl, 2013) and Mutalyzer (mutalyzer, 2013) data bases. The theoretically key changes were analyzed in direct links to the SIFT and Polyphen data bases which found that a possible change of the pathogen could occur in the genes *LRFN2*, *KIAA0513* and *KCNH6* (Table 3.4.). Three genes – *KIAA051*, *LRFN2*, and *PCDHA9-10* were eliminated as these variants were observed also in healthy family members – the father and a child. Following a number of screening stages, mutation in gene *KCNH6* was discovered, a probable cause of the progression of Asperger Syndrome.

Table 3.3.

Annotation for potential ASD candidate-gene variants

Gene/SNP	Coding amino acid dynamics (referential order, coding nucleotide sequences, change of amino acid in protein)	ESP5400_ALL¹ MAF
<i>ARPP21</i> (rs151173813)	NM_001267617:c.G1726A:p.A576T	0.003439
<i>KCNH6</i> (rs138601922)	NM_030779:c.A161G:p.Y54C	0.003997
<i>KCNJ10</i> (rs115466046)	NM_002241:c.G53A:p.R18Q	0.01227
<i>KIAA051</i> (rs139487660)	NM_014732:c.G37A:p.D13N	0.002138
<i>LRFN2</i> (no rs)	NM_020737:c.G983A:p.R328H	0.000093
<i>PCDHA9-10</i> (rs79247475)	NM_018898:c.C2558G:p.P853R	0.003067
<i>MPDZ</i> (no rs)	NM_001261406:c.A4525G:p.I1509V	0.000104
<i>PLD5</i> (rs140243407)	NM_001195812:c.G13A:p.A5T	0.000327

¹ ESP5400_ALL — MAF in Exome Sequencing Project dataset (5,400 exomes) for all populations (annovar ,2013)

Table 3.4.

Potential candidate gene version of pathogenicity test

Gene	SNP identification	SNP chromosomal position	Transcripts	Possible SNP pathogenicity	
				SIFT ¹	Polyphen ¹
<i>KCNJ10</i>	rs115466046	NC_000001.10:g.160012270 C>T	NM_002241.4(KCNJ10_i001): c.53G>A, p.(Arg18Gln)	Benign 0.34	Benign 0.098
			NM_001195811.1(PLD5_i001):c.451G>A, p.(Ala151Thr)	Benign 0.33	Possible pathogene 0.749
			NM_001195812.1(PLD5_i001): c.13G>A, p.(Ala5Thr)	ND	ND
<i>PLD5</i>	rs140243407	NC_000001.10:g.242383388 C>T	NM_152666.2(PLD5_i001): c.637G>A, p.(Ala213Thr)	Benign 0.26	Possible pathogen 0.84
			NM_152666.1(PLD5_i001): c.361G>A, p.(Ala121Thr)	Benign 0.18	Possible pathogen 0.84
<i>ARPP21</i>	rs151173813	NC_000003.11:g.35780947 G>A	NM_001267617.1(ARPP21_i001):c.1726G >p.(Ala576Thr)	Benign 0.46	Benign 0.023
			NM_001267619.1(ARPP21_i001):c.1786G >A,p(Ala596Thr)	Benign 0.48	Benign 0.037
<i>PCDHA</i>	rs79247475 ^a	NC_000005.9:g.140362125 C>G	NM_016300.4(ARPP21_i001): c.1783G>A,p.(Ala595Thr)	Benign 0.29	Benign 0.007
			NM_018905.2(PCDHA2_i001): c.2513C>G, p.(Pro838Arg)	ND	ND

Continuation of Table 3.4.

Gene	SNP identification	SNP chromosomal position	Transcripts	Possible SNP pathogenicity	
				SIFT ¹	Polyphen ¹
<i>LRFN2</i>	TMP_ESP_6_40399870	NC_000006.11:g.40399870 C>T	NM_020737.1(LRFN2_i001): c.983G>A, p.(Arg328His)	Pathogen 0.01	Pathogen 0.981
<i>MPDZ</i>	TMP_ESP_9_13126523	NC_000009.11:g.13126523 T>C	NM_001261406.1(MPDZ_i001): c.4525A>G,p.(Ile1509Val)	Benign 1	Benign 0.001
			NM_001261407.1(MPDZ_i001): c.4525A>G,p.(Ile1509Val)	Benign 1	Benign 0.001
<i>KIAA0513</i>	rs139487660	NC_000016.9:85100714 G>A	NM_003829.4(MPDZ_i001): c.4624A>G, p.(Ile1542Val)	Benign 1	Benign 0.001
			NM_003829.3(MPDZ_i001): c.4624A>G, p.(Ile1542Val)	Benign 1	Benign 0.001
<i>KCNH6</i>	rs138601922	NC_000017.10:g.61601584 A>G	NP_055547.1: c.37G>A,p.Asp13Asn	Pathogen 0.03	Pathogen 0.991
			NM_030779.2(KCNH6_i001): c.161A>G,p.(Tyr54Cys)	Pathogen 0	Pathogen 0.974
			NM_173092.1(KCNH6_i001): c.161A>G, p.(Tyr54Cys)	Pathogen 0	Pathogen 0.991

¹ data from the Ensembl database (ensembl, 2013)

ND – no data

^a as per the data of the *NCBI* data base. It is possible that SNP is found in the gene *PCDHA* paralogs, data for this SNP is likely to be seen as artifacts

4. DISCUSSION

At the conducted study, the involved group of ADS patients does not represent all Latvian ASD population. The study data do not reflect information about all ASD cases in Latvia but they allow judging the general ASD trends. Patients with syndromic autism were excluded from the study and further ASD patients with non-syndromic or idiopathic autism were analyzed. A phenotype and genotype-focused data base was set up, anthropometric parameters were analyzed, as were the association between polymorphism and ASD. The choice of non-medication and medication therapy was evaluated, efficiency and most common adverse reactions of the second-generation antipsychotics risperidone were determined in association with the selected pharmacogenetic marker. Accordingly to autosomal dominant inheritance model, a family with Asperger's syndrome in two generations was chosen and complete exome sequencing for three family members was performed to find the possible candidate genes of ASD that might be involved in the ASD etiology.

These data are unique because it is the first ASD study in Latvia.

4.1. Anthropometric and Clinical Characterization of ASD Patients

In order to describe the possible ASD phenotype, anthropometric parameters were analyzed and the clinical symptoms of ASD; also sought were possible differences in comparison with the standard population. In this research phase, a clearly characterized and recorded sample group was assembled, used later in further genetic research. Information was accumulated about the severity of ASD's clinical manifestations and influential factors – of

considerable importance in resolving social-psychological and education issues in Latvia in the future.

In seeking ASD's phenotypical symptoms, anthropometric and clinical parameters were analyzed via the ICD-10 diagnostic criteria, rating scales and the ADOS test. IQ of the ASD patients was also measured, also, anamnesis data in medical records, the age bracket of the initial epileptic seizure was analyzed, seizure type and rate of reoccurrence.

For a more homogenous ASD group, excluded were those diagnosed with syndromic autism; the research showed that 13.29% of ASD patients are in this category. The frequency of syndromic autism recorded in our research coincides with similar research conducted in other countries, reminding that this was the first study in Latvia (Gilberg and Coleman, 1996; Muhle et al., 2004; Lintas and Persico, 2009). ASD quite often is the most characteristic symptom in patients suffering from chromosomal or monogenic illness. Chromosomal microscopic aberrations, deletion, inversion and translocations are seen in about 5% (Jacquemont et al., 2006; Sebat et al., 2007; Marshall et al., 2008; Christian et al., 2008). In our research as well, 4.35% of cases showed aberrations as the ASD ethiological factor. In turn, in monogenic diseases, as in other populaces, boys most readily have Fragile X syndrome, girls – Rett syndrome (Muhle et al., 2004, Lintas and Persico, 2009).

Height, weight and head circumference were selected as the principal anthropometric measurements. They were made in all ASD subgroups and analysis conducted on their percentile % versus standard population percentile %.

In analyzing the ASD total height, weight and head circumference percentile percentage in relation to standard population, it became clear that ASD patient measurements have higher non-standard values (< 5‰ and > 95‰), also, lower value by 50‰, compared to standard population,

particularly head circumference, which could be defined as “small” or “too large for one's age.” If the statistics indicate almost symmetrical division around the central axis (50‰), then, in examining the parameters separately for the boys and girls groups, a certain asymmetry was noted, particularly in height and weight, and furthermore, especially among the girls (increased, 75‰). This increase is not apparent among the boys, despite the asymmetrical height/weight gauge. At the same time, head circumference index is symmetrical (50‰); also observed – smaller head girth (< 5‰) and larger (> 95‰), compared to the standard population parameter. The same division holds true for the girls' group. Still, high results differ in the common tendency (5‰). It cannot be said that this is a characteristic tendency in the ASD small girls sampled population (n = 29).

It is not unusual for ASD patients to have larger head circumference (Sacco et al., 2007; Miles et al., 2008), that is clearly confirmed by the data gathered in this research study, even though asymmetry is seen in division of gender. We see that percentage-wise that there are more boys whose head-size matches 95‰, which can be considered the norm's upper boundary line. For girls, it is lower in comparison, even though girls have more percentage-wise, both > 95‰, and < 5‰.

Compared to the results of an anthropometric research study conducted in Latvia (2010) to evaluate the risk of obesity in children (7–8 years of age only) just starting school (1st grade), conclusions can be drawn that the obesity tendency remains intact. Comparing gender, however, the tendency is more prevalent in boys – which is marked in our research as well, where boys (9.1%) percentage-wise, show a greater tendency for weight indicators above 95‰ than do girls (6.9%) (Velika et al., 2011). Weight gain could be caused by eating disorders, characteristic of ADS patients, or as a side-effect of drug-induced therapy. ASD patients mostly have a bland diet, usually

carbohydrates only. Furthermore, in cases of severe depression and behavioral disorders in drug-induced therapy, ASD patients are administered conventional and second generation antipsychotics – both of which name weight gain as a side-effect.

Intellectual disability or mental retardation is a common ASD co-morbid disorder. The research disclosed that 80.66% of ASD patients in Latvia are diagnosed with various level intellectual disabilities and in only 18.66% of cases are the intellect deemed normal. The data gathered showed intellectual disability as one of the most common complications of ASD; its frequency in various populations can be measures between 50–70% (Gillberg, 2006; Sacco et al., 2007). Comparing gender in this regard, no significant difference was noted, still, there was a tendency in the girls' group of minor and severe intellectual disability, compared to the boys. This is in tune with previous research study data concerning more serious ASD activity among girls population (Fombonne, 2009).

The frequency of epileptic seizures in the population as a whole is seen in 0.1–5% of cases. In ASD patients on the hand is seen in – 8–30% of cases (Danielsson et al., 2005; Tuchman and Cuccaro, 2011). Those who suffer from intellectual disability the re-occurrence rate for epileptic seizures were 37% (Gillberg and Coleman, 1996; Tuchman and Cuccaro, 2011). Our research found that only 5.33% of patients with ASD experience such seizures. One hypothesis here could be that the frequency of seizure is closely connected to a child's age (Spence and Schneider, 2009). Epilepsy is marked by two onset spikes: up to age five and adolescence (Folstein and Rosen-Sheidley, 2001; Spence and Schneider, 2009), but the greatest risk zone for the onset of epilepsy in ASD patients is specifically adolescence (Spence and Schneider, 2009). In our research, the average age is eight years, which matches the period that has rare cases of epileptic seizure. Another hypothesis

is that the epilepsy as a co-morbid state is more commonly present in patients with different monogenic and chromosomal pathologic conditions. To make the research group more homogenous, excluded were those patients diagnosed with a genetic pathology. Possibly, the presented hypotheses here could verify why the frequency of epileptic seizures was less than in studies conducted in other populations.

Forty-two percent of ASD patients with intellectual disability are in turn diagnosed with epilepsy (Folstein and Rosen-Sheidley, 2001). Seizures, as the research showed, were more prevalent in patients with high degree of intellectual disability than in those with normal IQ ($p = 0.009$), which backs up the hypothesis that a high degree of intellectual disability can be more readily linked to epileptic seizures. One explanation for this could be early CNS defect, intrauterine, perinatal or postnatal infection, hypoxia or other environmental factors, that could be the cause for stunted growth after the age of one. A second hypothesis, that coincides with the acknowledged data, that patients with intellectual disability, are at greater risk that epileptic seizures could ensue in regression of intellectual development (Tuchman, 2006). ASD's clinical manifestation occurs in children up to age two, which is in alignment with the risk period for recurrent epileptic seizures, and in this period also – mental development regression is observed in ASD patients. This means that if a patient's medical history mentions as little as just one epileptic seizure, one can surmise that this can induce regression of a child's mental development, which in turn could provoke recurrent epileptic seizure development, thereby provoking even higher degree of intellectual disability. Each and every epileptic seizure could trigger lower IQ levels in ASD patients. The younger the child is facing the onset of epileptic seizures the greater the risk of more severe intellectual disability. These seizures were registered in greater number for girls with a high degree of intellectual disability ($p = 0.0106$).

In analyzing speech impediments, expressive and receptive problems were noted in both the girls and boys ASD groups, and they are connected to degree of disability. Speech proficiency is one of the keys to a child's intellectual development as it facilitates socialization, communication, and cognition processes. Any snag in development of speech results in a delay, and could be one of the factors in the progression of intellectual disability. Of utmost importance therefore is introducing speech and language skills as soon as possible.

The team working with an ASD child must include a speech therapist, an ergo therapist, a special education instructor, a psychologist and a child psychiatrist. In planning each child's program, a comprehensive analysis of problem areas, also resources must be conducted. Crucial are the level of speech development and individual traits, examined by the speech therapist. Also important – the psychologist's conclusion on intellectual skills that – in cases of ASD, can run the gamut from severe mental retardation to the brilliance of a genius.

Diagnosis of ASD in Latvia often comes much too late, and adequate ambulatory care is not available in many locations that would provide a multi-disciplinary approach for early intervention in cases of ASD. The average age of a child diagnosed with one of the ASD sub-types is eight, which means that precious time has been lost for any positive results from drug-induced therapy, both in speech development, training of the special instructor, socialization and social integration. In other words, as abnormalities appear due to non-diagnosis, ASD patients run the risk of facing a higher degree of intellectual disability and a crippling future.

Also important for the child's well-being is to involve the family in the process, or rather – educate the family so that the child's integration unfolds as smoothly as possible. Therefore, in any therapeutic aspect, the ASD patient's

family must be an active component – nixing passively observing the specialist(s) and waiting for results. In fact, therapy should be conducted within the family fold.

4.2. The selected SNP Association Analysis

In ASD the etiology is meaningful for the seventh chromosome, in which there were uneven obligations, candidate genes, and CNV studies of ASD have found candidate genes (IMGSAC, 1998; Trikalinos et al., 2006; Alacorn et al., 2002; Alacorn et al., 2005; Schellenberg et al., 2006; Stankiewicz and Lupski, 2010, Freitag et al., 2010). Less studied were the 11th and the 15th chromosomal association with ASD (Szatmar et al., 2007, Liu et al., 2008; Cho et al., 2011). Significant were the 11p15.4-p15.3 and 15q13.3-q14 loci (Spence et al., 2006; Duvall et al., 2007). Therefore, these loci were selected for this study. Four SNPs were analyzed that may have been involved in the etiology of ASD. SNP selection based on literature data, the 11th and 15th chromosomes may play a role in the etiology of ASD. On the basis of whole genome association studies, which analyzed ASD subphenotype included 976 families from the Autism Genome Project, which found in ASD a significant association with 11th rs2421826 chromosome 11p13 (LOD = 2.55, $p = 0.0003$), 11p14-p15.3 rs1394119 (LOD = 3.40, $p = 0.00004$), and 15q13.3-q14 rs1454985 (LOD = 4.01, $p = 0.00001$) loci (Liu et al., 2008)

In turn, 2011 Cho with his colleagues in an association study, which analyzed 3022 SNPs, selected the most significant 30 SNPs, of which, statistically significant association with ASD were rs11212733 ($p = 9.76 \times 10^{-6}$), which is localized in 11q22.3 locus (Cho et al., 2011). Therefore, in this study they selected for the four SNP, to analyze whether these SNP played a role in the development of ASD in the ASD patient group.

In this study, an SNP association with rs2421826, rs1394119 and rs1454985 was not found with ASD. One of the reasons could have been the small sample size of the ASD patient group. Another might have been the homogeneity of the ASD group taking into account that all patients with syndromic autism were excluded.

The analyzed SNP rs11212733, which was localized between 11q22.3 chromosome exofillina 5 (*EXPH5*) gene 5' and DEAD (Asp-Glu-Ala-Asp) box polypeptide 10 (*DDX10*) for gene 3' regions, there were statistically meaningful association with ASD ($\chi^2 = 6.982$, $p_{\text{adjusted}} = 0.033$, OR = 1.625, 95% CI 1.13–2.31).

EXPH5 are protein-coded genes, which are localized in the 11th chromosome long arm 22.3 locus. It is known that this gene functions associated with Rab27, which regulates non-neuronal cell exocytosis, such as catalytic pellets (lytic granules), the secretion of cyto-toxic T cells, pancreatic β -cells produce insulin, and the released histamine-containing granules of mast cells. Rab27 is also responsible for the transport of melanosomes in melanocytes (Kondo et al., 2006). *EXPH5* not involved in neurological processes, and it is possible that it has no significant role in the development of ASD.

DDX10 gene is localized in the 11th long arm of chromosomes 22–23 locus and encodes RNA helicase involved in numerous cellular processes related to RNA secondary structural changes. This gene involved in the initiation of translation is used, the cell nucleus and mitochondrial splicing, and ribosome splaisosome creation. Although it is not known which RNA molecules affect the helicase, reduced *DDX10* gene function may lead to regulatory problems on the RNA level (Savitsky et al., 1996). In ASD etiology there are other groups of *DDX* genes involved. In the Autism database as potential ASD candidate genes *DDX11* and *DDX35* genes are mentioned

(autDB 2013). *DDX11* gene, which is localized in chromosome 12p11 locus, is associated with neuroglial cell differentiation disorders in ASD patients (Hu et al., 2006). *DDX35* gene, which is localized in chromosome Xp22.11 locus and is encoded by RNA helicase, did not find association with ASD (Pinto et al., 2010). *DDX10* gene could also be a possible candidate gene, which would need to carry out further studies for the development of ASD.

When analyzing the possible haplotype association with ASD, the rs11212733/rs2421826 T/A haplotype frequency control and ASD groups of patients ($p = 0.012$) statistically differed. rs11212733/rs2421826 haplotype T/A were seen in the control group. This indicates that this haplotype may be protective. Hypothetically, one would assume that this haplotype may be associated with a lower risk of developing ASD.

Linear regression analysis was used to examine ASD patients associated with SNP and diagnosis intelligence (normal, mild, moderate and severe mental retardation), seizures existence (yes/no in the medical history) was not a statistically significant association ($p < 0.05$). Possible involvement could be influenced by a patient's gender, age, head circumference, IQ and a history of epileptic seizures; these figures were used as covariate. In this case however, there were no statistically significant results ($p < 0.05$).

4.3. Pharmacogenetic marker CYP2D6 analysis

The third phase of the study was to evaluate the second-generation antipsychotic drug risperidone pharmacotherapy efficacy and tolerability in patients with ASD. For ASD patients in severe cases, medication was used for treatment: if a patient has pronounced hyperactivity and attention-deficit syndrome, aggression, epileptic seizures, sleep disorders, mood disorders as well as obsessive-compulsive disorders and has symptoms similar to schizophrenia (Mandell et al., 2008). In the treatment of this disorder

psychotropic medications are primarily used. Atypical or second-generation antipsychotics, antidepressants, long-acting mood stabilizers and psychostimulants are frequently used. Therapeutic options are directly related to the prevailing syndrome. However, the choice of drug therapy is problematic because often the medication may have a paradoxical, or inadequate response (Gillberg, 2006). Pharmacotherapy of ASD patients are selected individually. Evidence-based data indicate that risperidone, methylphenidate and separate use of SSRIs in the treatment of ASD behavioral disorders (Taylor et al., 2009). One of the most frequently recommended second-generation antipsychotic medications is risperidone (Correia et al., 2010). Note, however, that the drug can cause serious side effects: metabolic disorders, such as weight gain, hyperprolactinemia and large doses may have serious neurological, extra-pyramidal side effects (West and Waldrop, 2006; Jesner et al., 2007). There is no study and data on drug efficacy and tolerability in children under five years of age. Consequently, the choice of drug and dose should be adjusted individually according to the clinical course (Taylor et al., 2009).

Given that psychiatric patients in practice, due to the uneven distribution of clinical course and different drug metabolism, there are possible differences in response to psychotropic medication influences; indeed, it is often impossible to use of antipsychotic treatment regimens for all patients equally. Although rare, clinical practice of personalized medicine can be used, which is based on determining the level of drug metabolizing before drug treatment. In psychiatric practice, patients have heterogeneous clinical courses and different individual responses to psychoactive drugs. Each patient's response to treatment is different; depending on way that patient metabolizes the drug. Using personalized medicine opportunities by identifying each patient psychotropic drug metabolizing degree, it possible choose the most appropriate medications and dosages (Woodcock and Lesko, 2009). Psychotic disorders

may be caused simultaneously by several factors, both environmental as well as genetic, thus adversely affecting normal neurotransmission in the brain. Genotyping of the fixing of genetic variation in encoding proteins involved in neuro-transmission, as well as psychotropic metabolized drugs (Costa e Silva, 2013). Thus, application of pharmacogenetics psychiatric practice has the potential to increase the clinical efficacy and reduce side effects (Woodcock and Lesko, 2009). At the moment, pharmacogenetic tests are being offered for psychotropic medications, by which it is possible to identify an individual patient psychotropic medications and dosages (cggenetics, 2011).

On individual differences in drug metabolism the primary culprits are the CYP450 enzymes and oxidative catalytic reactions (Anderson, 2010). One of the most important genes involved in metabolized second-generation antipsychotics, including risperidone providing metabolism enzyme regulation was the *CYP2D6* gene, which was very polymorphic (Ingelman-Sundberg et al., 2007).

By using the existing allele list, our study did achieve desired results. The bio-transformation of risperidone in the liver ensured that several P450 markers would be active at the same time. In this research, only one *CYP2D6* was analyzed. However, the antipsychotic metabolized medication meaning was not only for *CYP2D6*, but also for *CYP3A* and *CYP1A2* (Rodriguez-Antona et al., 2009). This could be the underlying reason as to why our research results turned out negative. Even though *CYP2D6* was seen as the most significant, it could be possible that all involved markers and their interaction carry an overall more important meaning than the isolated marker analysis for that analysis might not produce expected results. For the pharmacogenetic markers there was no basis found neither in the identification of risperidone efficacy nor in its side effects. Taking into account that the *CYP2D6* gene is a polymorph it is possible that the rest of the gene alleles that are involved in risperidone metabolism

should be analyzed. This is due to the fact that *CYP2D6*4*, *CYP2D6*41* provided little information as per our study results.

In our study the most frequent changes in treatment was due to hyperprolactinemia. The most common side effect of risperidone was found to be hyperprolactinemia, the cause of which is likely to be associated with ASD neurotransmitter defects, but not primarily with the metabolism of xenobiotics. Dopamine inhibits prolactin release when stimulated D2 receptors are stimulated. On the other hand, serotonin promotes prolactin release 5HT2 receptors are stimulated (Stahl, 2008). If D2 receptors are blocked, dopamine can no longer inhibit the release of prolactin, thereby prolactin levels are elevated. However, if 5HT2A receptors are inhibited simultaneously serotonin can no longer stimulate prolactin release. By blocking the D2 receptors hyperprolactinemia is mitigated (Stahl, 2008). If the ASD case neurotransmitter regulation mechanism becomes compromised, it means that it is possible that ASD may provoke hyperprolactinemia, irrespective of the treatment with risperidone. Medicine side effects may be related to ASD patient's metabolism, serotonin and dopamine synthesis, due to the fact that side effects were observed in patients with varying degrees of mental retardation. This means that the more severe the ASD course, the higher the risk of developing side effects and that the treatment will be ineffective, regardless of the medication group and metabolic levels. Hypothetically, one can conclude that hyperprolactinemia is not associated with the *CYP2D6*4*, *CYP2D6*41*, 5HT2 metabolism. The hypothesis, based on publications that neurotransmitter imbalance in ASD may cause hyperprolactinemia (Stahl, 2008).

It might be necessary to select additional markers of treatment efficacy evaluation. It is possible that the studied group capacity is too small to be able to assess the importance of pharmacogenetic markers. However, from the point

of view of personalized medicine, in each individual case, the marker should be informative because of the commercial test case; the result is applied to each case individually rather than on the whole for large research groups. In this study, the selected pharmacogenetic markers for *CYP2D6*4*, *CYP2D6*41* were not associated with risperidone efficacy and adverse reactions. Haplotype analysis an association with *CYP2D6*4/CYP2D6*41*(rs28371725/rs3892097) C/T ($p = 0.4$) was not found with risperidone dosages and genetic markers therefore, they cannot be used for risperidone pharmacotherapy prognoses.

In the study, the cytochrome groups (CYP) P450 marker *CYP2D6* the changed allele *CYP2D6*4*, *CYP2D6*41* frequency did not differ in the ASD patient and control group. For ASD patients in the linear regression analysis when examining the relationship between the SNP and biochemical indicators of AST, ALT, no significant association ($p < 0.05$) was found. Potential liabilities could be affected by the patients' gender, age, IQ and a history of epileptic seizures. These figures were used as covariates in linear regression. Nevertheless, in this case there still were no significant results ($p < 0.05$).

4.4. Exome Sequencing Analysis for Family with Asperger Syndrome

ASD has multiple factors, and its hereditary mechanics remain unclear. One hypothesis has it that in the progression of ASD, CNV plays a significant role, also point mutations (Sebat et al., 2007; Marshall et al., 2008; Glessner et al., 2009; Pinto et al., 2010; Levy et al., 2011; Sanders et al., 2011). Not just one gene is involved in ASD's etiology but combinations of several gene variations are possible simultaneously, in tandem with the environmental factors. Typical of ASD is a wide array of clinical symptoms; from extremely severe low-functioning to high-functioning, with the pertaining to social behavior, communication also speech and learning disorders. In the grouping of ASD disorders, childhood autism and Asperger syndrome have well-defined

diagnostic criteria, and are, in fact, the opposite of all ASDs. In research today, the focus is on the genetic probe of all ASDs, but published studies contain limited data on genetic probing of Asperger syndrome.

Rarely does a child psychiatrist come across Asperger syndrome cases with children in their early stage of development because the symptoms are usually mild. Later in life, as the school years begin, the disorder (in the adolescent) can be accompanied by other mental dysfunctions, like obsession-compulsion, schizotypal personality disorder, affective disorder, etc., which only in retrospect, according to personal medical history, can one surmise that the patient was experiencing abnormalities of social functioning, emotion and communication already in childhood, that could relate to symptoms of Asperger syndrome.

Asperger's syndrome is more characteristic of young males than females. However, considering data from epidemiological research studies of recent years, prevalence indexes are growing, thanks to upgraded diagnostic technologies (Gillberg, 2006; Fombonne, 2009).

For full exome sequencing a family with two generations of Asperger syndrome was selected, moreover, the disorder was diagnosed for the mother and both of her children – a boy and a girl. Even though Asperger syndrome can be passed down both autosomally or linked to X chromosome (Betancur, 2011), still, per family tree, for the particular family chosen, the most plausible was the autosomal dominant inheritance type. Analyzing exome sequencing data, singled out were pathogenic changes in five genes: *KCNJ10*, *ARPP21*, *PLD5*, *KCNH6* and *MPDZ*. To rate its potential pathogenicity, sequencing variants were tested in a direct link with the SIFT (sift, 2013) and PolyPhen (polyphen, 2013) data bases, also Ensembl (ensembl, 2013) and Mutalyzer (mutalyzer, 2013) data bases. Delved into as well – published studies about the coded protein possible role in these genes in ASD pathogenesis.

KCNJ10 (*potassium inwardly-rectifying channel, subfamily J, member 10*) gene – positioned in the 1q23.2 locus, regulates synaptic activity between neurons. K channel regulator, connected to ASD (Sicca et al., 2011) and epilepsy (Buono et al., 2004). *KCNJ10* has one transcript. Identified change of nucleotide found in the exon, and induces amino acids arginine-to-glutamine transfer. The frequency of changes of nucleotides in all populations in the exome sequencing project is 0.01227 (ensembl, 2013). In conducting impact simulation for nucleotide change variant, utilizing PolyPhen and SIFT programs, polymorphism is not noted as potentially pathogenic; therefore it was discarded from our research as disease-causing.

ARPP21 (*21-kD cAMP-regulated phosphoprotein*) gene – localized in the 3p24.3 locus and encoded protein which was the calmodulin (CaM; 114180) signal regulators, whose main purpose was in neurotransmitter system regulation. It regulates CaM-dependent kinase (CaMKI) and protein phosphatase-2B (PP2B) (Rakhilin et al., 2004). The Gene manifested itself at nucleus caudate, putamen, nucleus accumbens, cerebellar cortex and the neocortex areas (Brene et al., 1994). *ARPP21* gene had 37 transcripts. The identified nucleotide replacement in the encoded part had five transcripts and could introduce alanin replacement with treonine. The given nucleotide replacement was not described having to do with the pathology and the total population exome sequencing project had a frequency of 0.003439 (ensembl, 2013). Additionally, in doing the simulation of the influence of nucleotide replacement by using PolyPhen and SIFT software programs, it was found that polymorphism was most probably not a pathogen.

PLD5 (*Phospholipase D family, member 5*) gene – localized in the 1q43 locus and encoded in proteins, which regulate axonal formation and the neurotransmitter glutamate receptory metabotropic signal system (Dhami and Ferguson, 2006; Kanaho et al., 2009). The genome in wide ranging association

studies discovered that one of the polymorphisms (rs2196826) was involved with ASD that did not have language impairment (Anney et al., 2010). *PLD5* gene had eight transcripts. In our study, the identified nucleotide replacement encoded part was found in four of eight transcripts and resulted in amino acid changes. The transcripts mentioned encoded inactive enzyme is forms and therefore, the identified change was most likely not pathogen. This agrees with SIFT program simulation data. The identified SNP (rs140243407) was described both in the 1000Genome data base as well as the total population exome sequencing project whose frequency was 0.000327(ensembl, 2013).

MPDZ (*multiple PDZ domain protein*) gene – localized in the 9p23 locus, the encoded protein is provided by protein interactions in relation to the serotonin 5-HT-2C receptor (Ullmer et al., 1998). *MPDZ* gene has 21 transcripts, nucleotide change encoding part of the ten-localized gene transcription, which leads to replacement of isoleucine to valine. Nucleotide substitution was not described in relation to pathology and the entire population exome sequencing project whose frequency was 0.000104 (Ensemble, 2013). In the variant nucleotide change impact simulation using PolyPhen and SIFT software, polymorphism was not recognized as a pathogen.

KCNH6 (*potassium voltage-gated channel, subfamily H (eag-related), member 6*) gene – localized in the 17q23.3 locus. Its encoded protein had to do with ERG (*Ether-A-Go-Go-related gene 2*), which was defined by behavior. The gene was manifested in the CNS, particularly in the olfactory bulbs, cortex, hippocampus, hypothalamus, and cerebellar regions (Papa et al., 2003). *KCNH6* gene has six transcripts. Our study identified a nucleotide change in two transcripts in their encoded part and can introduce replacement of the amino acid tyrosine to cytosine. In the case of nucleotide change impact simulation using PolyPhen and SIFT software, polymorphism was recognized as a pathogen. For the aforementioned nucleotide replacement,

functional pathology studies have not yet been carried out nor have they been described in relation to pathology. Since the entire population exome sequencing project had a frequency of 0.003997 (ensemble, 2013), as well as modulating the pathogenicity of both programs, it was found of the pathogen in our family this change was more likely to lead to Asperger's syndrome. A marker was identified for a chromosome in the 17q11 -21 locus, which had a substantial connection to ASD (Younan et al., 2003; Stone et al., 2004; Cantor et al., 2005), but thus far has not been described as having *KCNH6* gene association with ASD.

What was accomplished here was the knowledge that *KCNH6* could be one possible pathogenic variant for families with Asperger syndrome. To test this, analysis of this variant with other families suffering from the syndrome is necessary, to determine whether the variant has meaning for the syndrome's etiology and/or whether the syndrome could be inherited as a dominant from one generation to the next. Evaluation of pathogenesis requires further functional research studies.

Taking into account that ASD diagnostics in Latvia has arrived late, based on international predecessors, recommendations have been drawn up for Latvia's general practitioners, pediatricians, child neurologists and psychiatrists pertaining to children up to three years of age. Early diagnoses, as well as appropriate choice of therapy, stand to reduce the number of disabled children and facilitate integration into the community.

5. CONCLUSIONS

1. According to the anthropometric measurements taken and phenotypic features the mutual comparison of both presents a well-described sample of autistic spectrum disorder, which includes data for this particular study on the questionnaire. It was found that the varying degrees of mental retardation rate were 80.66%, the frequency of seizures was 5.33%, and the percentage of language disorders was 98.66%. Epileptic seizures were more frequent in patients with severe mental retardation ($p = 0.009$).
2. As per the anthropometric measurements taken, ASD patients had a noticeably larger head circumference and greater body weight in comparison to the general population.
3. A statistically significant correlation was found between autism spectrum disorders and the SNP rs11212733 ($p = 0.008$), which was localized in the 11q22.3 locus between *DDX10* and *EXPH5* genes. These genes could quite possibly be autistic spectrum disorder candidate genes.
4. In this study, cytochrome group (CYP) P450 *CYP2D6* marker alleles *CYP2D6*4* T, *CYP2D6*41* T frequency was not different for autistic spectrum disorder patients when compared to the control group. Also, in autism spectrum disorder medication used in the treatment of second-generation antipsychotic, a relationship was not found between risperidone adverse drug reactions and cytochrome group (CYP) P450 *CYP2D6* marker alleles of *CYP2D6*4* T, *CYP2D6*41* T, (studied markers were not informative).
5. One family with Asperger's syndrome had a full exome sequencing done using the autosomal inheritance dominant model type. Several potential

candidate genes were selected from which, (as per the Sanger sequencing model) the pathogen *KCNH6* gene variant was identified, which could possibly be an ASD candidate gene.

6. PUBLICATION

6.1. Publication on the study research topic

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6.2. Abstracts

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