PROSPERITAS VESTRA FINIS NOSTRA!

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Genetic Diagnostic Study among Pediatric Patients with Hereditary Pathology Who Received Genetic Counselling

THESIS SUMMARY

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CONTENTS

ABBREVIATIONS

AD – Autosomal dominant

AR – Autosomal recessive

array CGH – Array-based comparative genomic hybridisation

ASD – Autism spectrum disorder

CA – Cytogenetic analysis

CAPC – Child and Adolescent Psychiatry Clinic

CAs – Congenital anomalies

CES – Clinical exome sequencing

ChA – Chromosomal abnormalities

ChD – Chromosomal disorders

CNV – Copy Number Variation

FCC – First Children's Clinic

FISH – fluorescence in situ hybridisation

FRAXA – Fragile X syndrome

GC – Genetic counselling

IEMs – Inborn errors of metabolism

LMGV – Laboratory of Medical Genetics – Varna

MCA – Multiple congenital anomalies

MGC – Medical-genetic counselling

MLPA – Multiplex ligation-dependent probe amplification

ID – Intellectual disability

NGL – National Genetics Laboratory

NGS – Next-generation sequencing

DD – Developmental delay

PCR – Polymerase chain reaction

PHOC – Pediatric Haematology and Oncology Clinic

PICU – Pediatric Intensive Care Unit

RD – Rare Diseases

SCC – Second Children's Clinic

VUS – Variant of uncertain significance

WES – Whole exome sequencing

WGS – Whole genome sequencing

1. INTRODUCTION

Genetic (hereditary) diseases and congenital anomalies affect about 6% of live births and are among the leading causes of childhood morbidity and mortality (*Farnaes et al., 2018*). Studies show that about 20 – 35% of newborns and children up to 5-years deaths are due to genetically determined disorders (*Stevenson et al., 2005; Kochanek et al., 2012*). These diseases can cause lifelong mental, physical, hearing, or visual impairment for those who survive.

Hereditary diseases and congenital disabilities are associated with severe adverse effects for children, their families, health systems and society. Most genetic diseases have a low individual frequency, which is why they belong to the so-called *rare diseases* (RD) group, affecting between 6 and 8% of people in their lifetime (*Zurynski et al., 2017*). About 70 – 80% of RD have a genetic etiology, and a large proportion of them (50 – 75%) have a childhood onset (*Nguengang Wakap, 2020; Bick et al., 2019*).

Diagnosing a patient with a *rare* genetic disease is complicated because RD are characterised by various symptoms and disorders that vary significantly from one nosological entity to another. Regardless of his/her speciality, the physician often does not come across an analogous case in his practice, and sometimes the case remains unique. Most often, the diagnosis is made with a significant delay. Even when the diagnosis is on time, patients and their families experience significant difficulties obtaining more detailed information about the disease itself – risk for offspring, prognosis, a reference genetic laboratory in our country and abroad for its confirmation, treatment options and prenatal diagnosis. At this point, the medical geneticist plays an essential role in reaching an accurate etiological diagnosis. Thus, the experience and knowledge of a medical geneticist, in many cases, significantly shorten the diagnostic search. This is crucial for the prognosis and treatment of those affected and for the genetic counselling of families regarding reproductive planning and prenatal diagnosis. In most cases, preventing the advent of new patients is the only possibility for the prevention of genetic diseases. Despite significant progress in gene therapy, the number of successfully treatable genetic diseases is still minimal.

In recent years, there has been a rapid development of technologies for genomic analysis and a sharp increase in scientific discoveries related to the pathogenesis of diseases at the molecular level. Medical innovations will increasingly focus on providing care tailored to individual patterns of genetic predisposition. More and more clinicians will need the exact genetic cause of a given

disease to find the most effective and specific preventive and therapeutic approach to relieve symptoms or prevent complications.

This thesis focuses on a current health problem – genetic diseases and congenital anomalies in pediatric patients. The need for genetic counselling for at-risk individuals is emphasised, and the possibilities and limitations of the currently applied genetic tests are discussed. The present paper summarises the experience of the Laboratory of Medical Genetics at St. Marina University Hospital – Varna in the genetic counselling and research of childhood patients with suspected hereditary pathology for a period of ten years.

Hypothesis: The lack of sufficient studies on the effect of medical-genetic counselling centres' activity with pediatric patients in our country on the diagnostic possibilities and increasing demand for genetic services was a prerequisite for research on the subject of the present thesis.

2. AIM AND OBJECTIVES

2.1. Aim

Descriptive epidemiological assessment and study of the effect of the genetic counselling activity as an approach for the clinical-genetic diagnosis in persons with suspected hereditary pathology in childhood based on the experience of the University Genetic Counselling Unit for a period of ten years.

2.2. Tasks

1. To **differentiate** the contingent of patients aged 0 – 8 years, registered at the office for medical genetic counselling for a period of 10 years, to select patients with an unexplained/unproven disorder subject to diagnostic activity and present **characteristics** (descriptive-epidemiological and classification by referral indications).

2. To summarise and analyse the results of the conducted laboratory-genetic studies (within and outside the sectors of the university hospital structure) for evaluation of the diagnostic **laboratory contribution** in clarifying the aetiology of hereditary disorders in children.

3. To assess the **overall activity** of the MGC as the main structure for genetic assistance in the LMGV in the multidisciplinary diagnostic process of revealing the genetic aetiology of diseases and predispositions in childhood patients.

4. To analyse the role of the MGC's **active** *genetic diagnostic (laboratory and advisory*) activity in a selected group of patients with an *unexplained/unverified disease status* for evaluation of the genetic unit's participation and contribution.

5. To provide *guidelines for improving the approach* for conducting a clinical-genetic evaluation of a hereditary disease in children as a part of the overall multidisciplinary care for the patient in the clinical-laboratory practice.

3. MATERIAL AND METHODS

3.1. Material

3.1.1. Clinical material (patient contingent)

The current study addresses individuals under 18 years of age with suspected genetic or congenital pathology. It covered a ten years period, from January 2011 until December 2020 and was conducted at the Medical University – Varna and the Laboratory of Medical Genetics – Varna (LMGV).

For the indicated period, a total of **3 124** pediatric patients aged 0 to 18 years (average age of 5.9 years) were registered at the MGC for counselling and/or genetic analysis. Of them, 1 855 (59.4%) are male, and the remaining 1 269 (40.6%) are female (M:F = 1.5:1).

The studied patients/their biological samples have been assessed at the LMGV during their hospitalisation in clinics at St. Marina University Hospital –Varna, other hospital facilities in or outside the city or on an outpatient basis. *Patients hospitalised* at St. Marina University Hospital were referred by physicians from:

• First Children's Clinic (FCC) with Pediatric Intensive Care Unit (PICU);

• Second Children's clinic (SCC);

• Paediatric Haematology and Oncology Clinic (PHOC);

• Child and Adolescent Psychiatry Clinic (CAPC).

Outpatients were referred by:

• GPs;

• clinicians and specialists from outside hospital structures (mainly neonatology units) and pre-hospital care;

• on parents' own initiative.

The number of patients and residents of the Varna region prevails, but patients from other regions were counselled as well, or samples were made, mainly for patients from North-Eastern Bulgaria.

In order to present the activity of the genetic counsellor in working with pediatric patients and distinguish it from the laboratory work itself, a group of 968 individuals (31.0%) was selected from the total number of registered (3124). For them, an active diagnostic approach of the MGC (laboratory and counselling) was applied to establish a clinical and/or genetic diagnosis of a suspected or unexplained disorder with the participation of a specialist in medical genetics.

3.1.2. Biological material (samples)

Samples for cytogenetic analysis from lymphocyte cultures and molecular genetic analysis from peripheral blood (PCR, RT-PCR, FRAX screening, MLPA) were processed according to laboratory protocols.

Biochemical, enzymatic, and a large part of molecular genetic methods have been carried out at:

- National Genetics Laboratory (NGL, Laboratory of Clinical Genetics), the Obstetrics and Gynecology University Hospital "Mother's House" – Sofia;

- Genetic Medical Diagnostic Laboratory (GMDL) Genica – Sofia;

- Department of Medical Genetics – Sofia;

- Laboratory of genomic diagnostics – Sofia;

- Genetic laboratories abroad.

Biological material (24-hour urine, whole blood, blood on a filter blank) was collected from the medical and diagnostic structures at St. Marina University Hospital –Varna, other hospital facilities, in a home environment for outpatients and/or in the LMGV. Biological material (whole blood or isolated DNA), sent to scientific and diagnostic laboratories in and outside the country, is stored and transported according to the conditions for the respective sample, with the logistics and registration function being performed by the doctoral student and his associates.

3.2. Methods

In the list of applied methods, only those performed by the doctoral student and/or associates within their activity at LMGV have been presented. Data from genetic analyses carried out as part of the laboratory's routine activity have been used after informed consent by the children's parents for the use of information subject to confidentiality. The conduct of new for LMGV genetic analyses in a selected group of patients was funded by a scientific project (No. 18015) of the Science Fund of MU-Varna. The Research Ethics Committee authorised the prospective study at the Medical University - Varna with protocol No. 83/16.05.2019.

3.2.1 Clinical methods

Documentary method

The approach to collecting maximum information about clinical and paraclinical data on patients, as well as tracking the development of the diagnostic process, includes data collection through:

- *data processing* based on the available hospital software program (Gamma MultiLab), the available laboratory records and personal files of counselled patients stored in the LMGV;

- *medical history reports and results of laboratory analysis* of individuals from the region covered by the neonatological and paediatric units served by the LMGV and tested for chromosomal disease, metabolic or other monogenic disease or predisposition;

- individual *feedback contact* (by phone, email, on-site).

Genealogical method and family history

The activity is a non-directive and non-imperative communication to obtain family history and genealogical charts of all children referred for genetic counselling. The aim is to conduct a genealogical analysis of the enclosed relatives for at least three generations.

The doctoral student or associate physician-consultants at the office had personal encounters with parents/other relatives/guardians. Detailed information was collected on:

- family history of hereditary diseases/congenital anomalies;
- reproductive pathology;
- history of pregnancy and childbirth;
- neonatal status;
- developmental history – general health status, growth and developmental progress, behaviour, accompanying diseases, examinations conducted to date, and treatment.

Physical examination

A standard clinical examination was performed, or information from the referring specialist's records was used, including:

- general clinical status;
- anthropometric indicators (height, weight, growth rate, head circumference);
- pubertal development;
- search and description of facial and body dysmorphic disorders;
- photo and/or video recording of children with dysmorphism after signed informed consent from a parent/guardian and storing the materials in a database protected by an access code.
- assessment of mental development/behavioural deviations by a paediatrician/child neurologist/psychologist/psychiatrist.

3.2.2. Laboratory methods

For the purposes of the set tasks, various genetic research methods have been developed and rationally used over the years, both at local and national levels. In case of technological impossibility to carry out the necessary laboratory diagnostics at the LMGV, the relevant biological material or patient was referred to an accredited laboratory in Bulgaria or abroad.

Performed at LMGV:

Cytogenetic method

Cytogenetic analysis (CA) on biological material from peripheral blood is performed according to an established protocol and in accordance with the "Medical Genetics" Medical Standard. According to the protocol, CA was performed on 10 –12 metaphase plates, differentially stained with the GTG technique at a 400 – 550-band level of resolution. If mosaicism is suspected, the number of plates analysed increases to 100. The routinely used methodology for cytogenetic analysis does not enable the diagnosis of minor chromosomal rearrangements (resolution up to 5–10 Mb) and low-grade mosaicism.

Molecular-genetic (DNA) methods

• Isolation of DNA (pre-analytical phase)

Isolation of high-molecular-weight DNA from venous blood by the salt method or by a kit based on the interaction between silica gel membrane and DNA, following the manufacturer's instructions (QIAmp DNA Blood Mini Kit, Qiagen, Hilden, Germany or GeneJET Whole Blood Genomic DNA Purification Mini Kit, ThermoFisher). The quantity and quality of DNA acquired by both methods were measured with a NanoDrop 2000c (ThermoScientific), with target purity values of 260/280 ~ 1.8; 260/230 ~ 2.0.

• dTP-PCR (direct triplet-primed PCR) and melting curve analysis (screening for fragile X-chromosome syndrome, FRAXA)

An identification kit (The FastFrax FMR1 Screening Kit, Biofactory Pre Ltd) designed to distinguish pre- and full-mutation samples from normal FMR1 alleles (≥ 55 rpts) was used to identify a multiplied CGG repeat. The dTP-PCR assay was performed according to the manufacturer's instructions. Two border control DNA samples with 41 and 53 CGG repeats (NA20244, NA20230) (Coriell Cell Repositories) were used to establish cut-off temperatures.

Samples showing an increased CGG repeat were further confirmed by the FastFrax FMR1 Sizing Kit (The Biofactory Pre Ltd).

- **Multiplex ligase-dependent probe amplification (MLPA) reaction**

Samples were subjected to MLPA analysis according to the manufacturer's recommendations (SALSA MLPA Probemix P245 Microdeletion Syndromes-1A and P036 Subtelomers Mix 2B, MRC Holland). Kit P245-1A contains 50 specific probes for genes associated with 33 diseases, kit P036 2B – 41. Electrophoresis was performed using a GeXP Beckman Coulter genetic analyser with a standard 600 size (Sciex). Data were exported and analysed with Coffalyser specialised software. Ten reference DNA sequences were included to normalise the data.

- **Real-Time PCR**

Predisposition to celiac disease – the test method is Real-Time PCR on QuantStudio Dx apparatus, ThermoFisher with Genvinset HLA Celiac commercial kits, IVD and CE marked. There are three allelic groups of interest – DQB1*02, DQA1*05, and DQB1*03:02. With this method, we can determine 9 genotypes. Each genotype assesses the degree of genetic predisposition based on a 5-point scale and the magnitude of the risk.

Hereditary predisposition to thrombosis (thrombophilia) – using real-time polymerase chain reaction (RT-PCR) on an apparatus (QuantStudio Dx, Applied Biosystems, USA), DNA samples were genotyped for polymorphisms FV Leiden (G1691A), F II (G20210A), PAI 4G/5G and MTHFR (C677T), according to the manufacturer's protocol (Generi Biotech) IVD and CE marked.

Gilbert syndrome – to identify the two most common genotypes – the homozygous polymorphism of two different bases (TA) in the TATAA sequence of the promoter region of the UGT1A1 gene, commercial kits Gb GENETIC Gilbert, IVD and CE marked, QuantStudio Dx apparatus, ThermoFisher were used. The extra bases decrease the affinity of the TATAA region for the binding protein, leading to decreased gene expression. UGT1A1*1/*28 alleles corresponding to the corresponding (TA)6/(TA)7 number of repeats in the UGT1A gene were investigated. The study includes only the most common genetic variants leading to hereditary unconjugated hyperbilirubinemia – Gilbert's syndrome.

Conducted outside the LMGV

Selective Metabolic Screening/Enzyme Analysis; microarray analysis; Sanger sequencing, next-generation sequencing, Methylation-specific MLPA, Methylation-specific PCR; as well as part of the MLPA analyses, molecular genetic screening for FRAXA, for hereditary predispositions (celiac disease, thrombophilia, Gilbert's syndrome) in the initial years of the study period.

3.2.3. Dysmorphology databases and software online-based programs

Due to the low frequency and variety of dysmorphic syndromes and genetic conditions, *phenotype-based software and dysmorphology computer programs* are often used in clarifying the diagnosis of consulted paediatric patients. Some of the sources used in the practice of the doctoral student and associates are presented:

- OMIM (Online Mendelian Inheritance in Man), (John Hopkins University) (*www.ncbi.nlm.nih.gov/omim*);
- London Medical Databases/ Winter-Baraister Dysmomorphology Database (https://www.face2gene.com/lmd-library-london-medical-database-dysmorphology/);
- FACE2GENE *(https://www.face2gene.com/*) – next-generation phenotyping with facial recognition and analysis of additional clinical features. Provides access to London Medical Databases. The use of this program started after 2015.
- ORPHA.NET (*https://www.orpha.net/)* – with reliable information about rare diseases, orphan drugs, expert centres and networks, laboratories and diagnostic tests;
- Monarch Initiative (*https://monarchinitiative.org/analyze/phenotypes*).

3.2.4. Statistical data processing methods

The following statistical methods and software programs were used for statistical analysis and presentation of the results:

1. *Non-parametric* analyses – Chi-square test or Fisher's exact test;

2. *Regression* analysis – a trend model used to predict future values of a time series;

3. *Graphical* analysis – for visualising and illustrating certain regularities or dependencies through different types of diagrams; Microsoft Excel 2016 – a software program for digital data processing;

4. *Graph Pad Prism* 9 – a software program used for statistical data processing.

4. RESULTS

A retrospective analysis was conducted for a **descriptive epidemiological assessment** (number of patients, distribution by years, age, sex, indication for referral) and *diagnostic-research approach* (indication – detected pathology) of children's contingent of individuals registered in the Genetic Counselling Office.

For a 10-year period, the results of a) genetic analyses performed to confirm a referral clinical diagnosis and b) a panel of laboratory tests (made in or outside the LMGV) for an etiological account of hereditary diseases and predispositions were systematised. The diagnostic success rate of the performed genetic analyses in the childhood patients referred for a specific condition or with an underlying unspecified/unclear genetic condition was calculated, and the detected pathology by nosological units was presented.

4.1. Descriptive epidemiological characteristics of the patients included in the study

4.1.1. Number of patients by year

For the study period (2011 – 2020), medical-genetic counselling and/or genetic tests were carried out at the Laboratory of Medical Genetics - Varna for 3 124 children of a total of **6 745** (46.3%) individuals registered for the period. The total number (6 745) does not include individuals registered in the office for genetic screening during pregnancy (a total of 29 527 women) and patients (including children) with *cytogenetic tests of bone marrow cultures* (a total of 2 459 analyses). The distribution by year of the number of children managed at the MGC and/or genetic analysis shows a tendency towards a significant increase (Fig. 1).

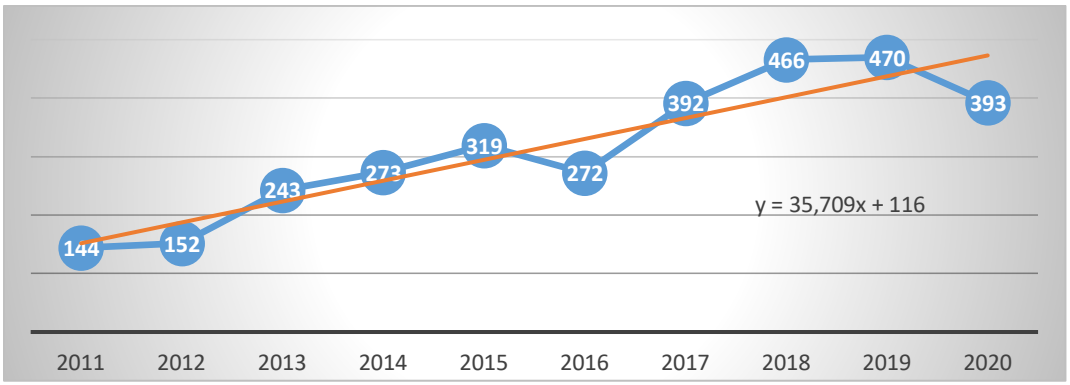

Figure 1. Trend pattern of paediatric patients managed at the MGC and/or genetic analysis for the analysed period

4.1.2. Age-sex characteristics

The age of the individuals included in the study ranged from 0 to 18 years (mean age of 5.9 years). The percentage distribution of the analysed contingent by age shows a predominance of those between 0 and 6 years old – 60.6% (n = 1 926) (Fig. 2).

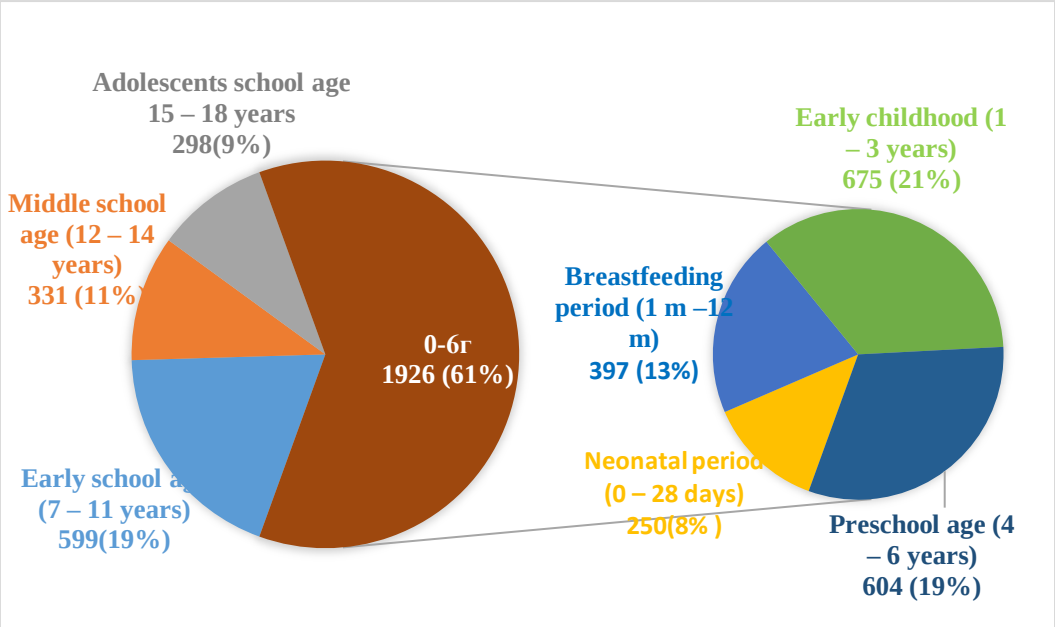

Figure 2. Percentage distribution by age

The distribution by gender shows that 1 855 (59.4%) of the individuals are male, and the remaining 1 269 (40.6%) are female (M:F =1.5:1).

4.1.3. Referral sources

The majority of patients (82.1%) were referred to MGC and/or for genetic analysis by pediatric structures of the University Hospital – mostly from the First Children's Clinic (Endocrinology and Metabolic Diseases, Nephrology and Cardiology) with a total of 1330 (42.6%) referred (including 118 from the Children's Intensive Care Unit), followed by the Second Children's Clinic (gastroenterology, neurology and pulmonology) – a total of 1125 (36%), the Paediatric Haematology and Oncology Clinic (2.6 %) and the Child and Adolescent Psychiatry Clinic (1%) (Fig. 3). Outpatients were referred from neonatology departments in Varna and other cities and from outpatient specialists (mainly paediatricians), as well as patients who sought genetic counselling independently at the initiative of relatives.

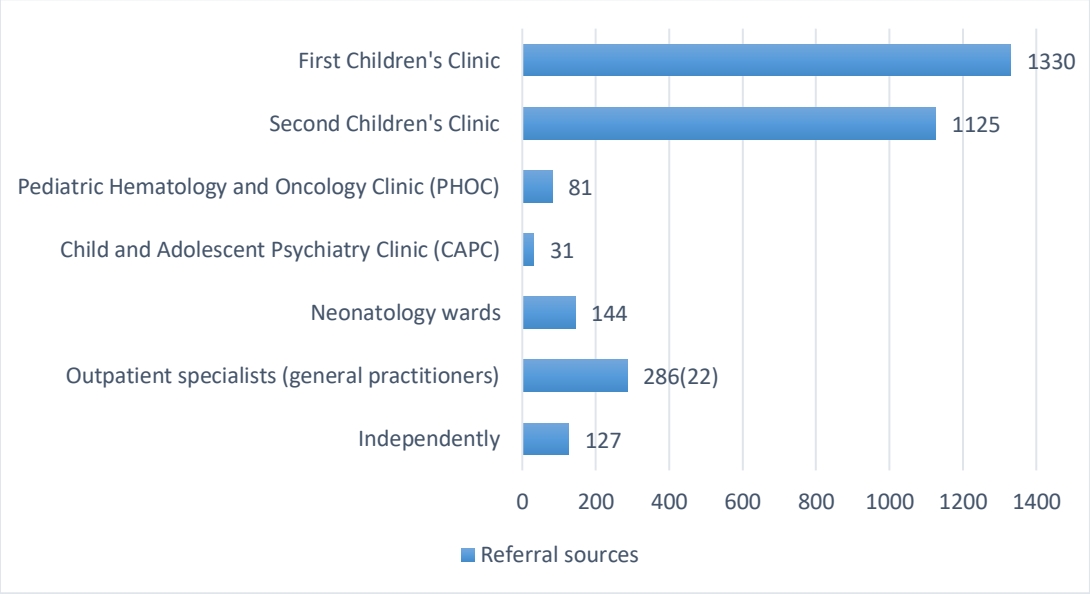

Figure 3. Distribution by referral sources

4.1.4. By types of indications

According to the clinical indication for referral to MGC and/or genetic analysis, the studied contingent of patients can conditionally be divided into **6** main groups:

• **Group I** – with a suspected chromosomal disease – **971 (31.1%)**;

• **Group II** – with a suspected monogenic (non-metabolic) disease – **517 (16.5%);**

• **Group III** – with a suspected hereditary metabolic disease (IEM) – **148 (4.7%);**

• **Group IV** – with a leading clinical phenotype of single or multiple congenital anomalies/unclear dysmorphic syndrome, with or without intellectual disability (ID) – **745 (23.8%);**

• **Group V** – with a leading clinical phenotype of an developmental delay, ID, behavioural disorders – autism spectrum disorders (ASD) – **473 (15.1%);**

• **Group VI** – Others (*tested for hereditary predispositions, including pharmaco-genetic defects*) – **270 (8.6%).**

According to the degree of clinical probability, groups **I, II** and **IV** can be presented in subgroups.

Group I was subdivided into 3 subgroups:

a) probable *autosomal* chromosomal disease – **189 children** (6.0%);

b) probable *gonosomal* chromosomal disease, sexual development disorders – **217** (6.9%);

c) growth deviations (short/tall height) – **565 children** (18.1%), without concomitant symptoms/anomalies.

The number of patients in group I is the largest, increasing significantly compared to the number of individuals with *short/tall height* indications (Fig. 4) in the period after 2014.

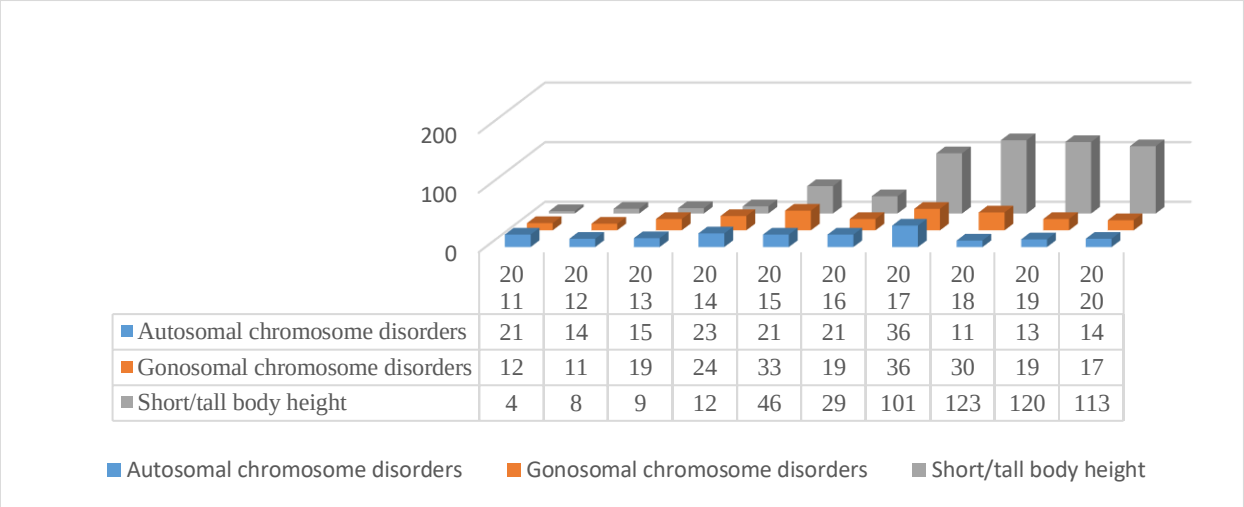

Figure 4. Distribution of group I patients by age

In **group II** – probable monogenic disease, three subgroups of children were distinguished:

a) with a clinical diagnosis of known and relatively more common *monogenic conditions* (cystic fibrosis and beta-thalassemia) – 201 (6.4%);

b) with leading neurological/neuromuscular symptoms and suspected monogenic disease (including mitochondrial disease) – 94 (3.0%);

c) with suspicion of another monogenic disease/syndrome (Marfan's syndrome, Noonan's syndrome, Osteogenesis imperfecta, Achondroplasia, Renal polycystosis, etc.), including *hereditary cancer syndrome* – 222 (7.1%).

Group IV – indications of single/multiple CAs/dysmorphic syndrome of unclear origin – three subgroups:

a) children with single congenital anomalies – 220 (7.0%) (Fig. 5);

b) children with MCA/ dysmorphic syndrome of unclear origin with or without ID – 453 (14.5%);

c) children with suspected disease related to impaired imprinting – 72 (2.3%).

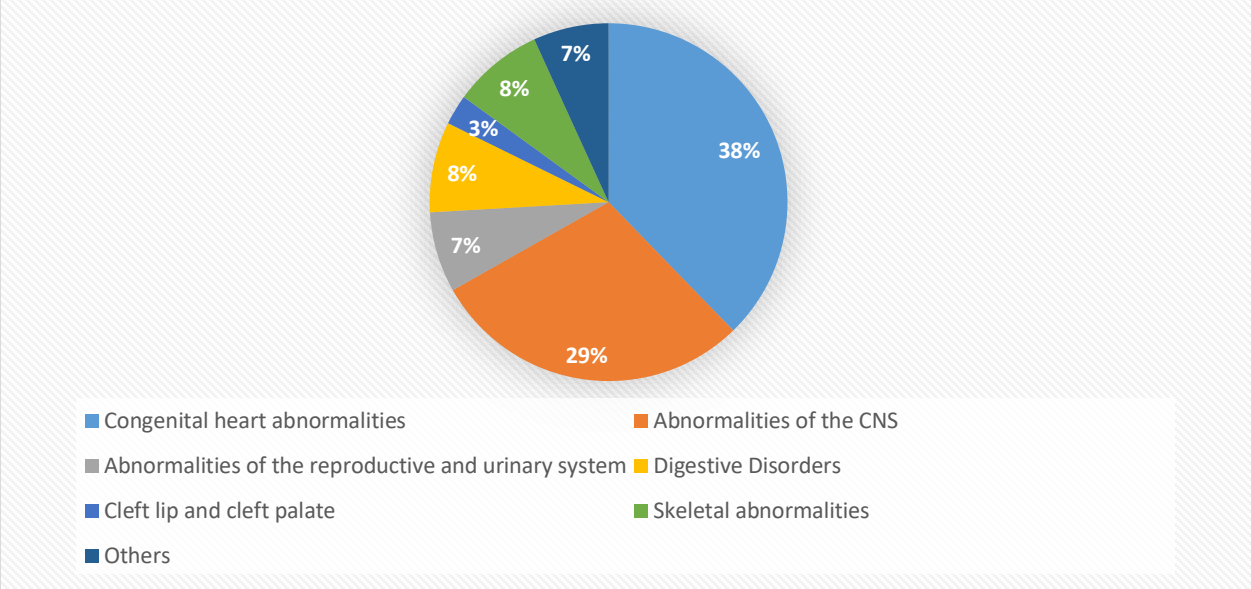

Figure 5. Distribution of types of single congenital anomalies among group IV patients

In **group V**, the patients were predominantly boys, with their percentage being statistically significantly higher ($p < 0.00001$) than that of girls in this group, compared to the gender ratio of

all referred patients for the analysed period. Most group V individuals were referred for cytogenetic analysis and/or molecular genetic screening/diagnosis for fragile X syndrome.

4.1.5. Characteristics of a selected group of patients for active diagnostic (laboratory and counselling) activity

From the total number of childhood patients included in the study (3 124), a **group of 968 individuals** (31.0%) was selected, in which an **active diagnostic** approach (laboratory and counselling) was applied to establish a clinical and/or genetic diagnosis of suspected or unexplained disorder involving a medical geneticist.

The selected group of patients subject to **diagnostic activity** was subdivided according to the *leading phenotype* of the indication for the referral:

• **A** –growth and/or sexual development disorder – 55 (5.7%);

• **B** – suspicion of a monogenic (non-metabolic) disease/syndrome, including *hereditary cancer syndrome* – 186 (19.2%);

• **C** – probable hereditary metabolic disease – 105 (10.8%);

• **D** – single or multiple CAs/dysmorphic syndrome of unclear origin, with or without ID – 469 (48.5%);

• **E** – developmental delay/ID/ASD– 153 (15.8%).

This specific group of patients did not include:

a) individuals suspected of having a **recognisable** chromosomal syndrome (e.g. trisomy 21, etc.);

b) individuals suspected of a **known** monogenic disease (cystic fibrosis, Wilson's disease, beta-thalassemia);

c) individuals with suspected hereditary **predisposition** (Gilbert's syndrome, celiac disease, thrombophilia, glucose-6-phosphate dehydrogenase pharmacogenetic defect, etc.

d) referred **biological material** without the personal involvement of a genetic counsellor, even though the individuals were registered in the office for Medical-genetic counselling as patients undergoing genetic analysis to validate clinical diagnosis from a referral unit.

The selected group of patients (968) included 572 boys and 386 girls (ratio 1.5:1), with the majority of persons aged 0 – 6 years (68.3%).

When comparing the ratio between the number of patients in the first and the last two years of the reporting period for the general contingent (1:2.9) and a selected (1:1.8) group of patients, the application of the non-parametric Chi-square test did not show a statistically significant difference ($p = 0.212$). However, a statistically significant difference is observed with increasing dynamics of the selected group of patients, despite the general decline in the last year 2020 (Fig. 6). Following the trend model for the reporting ten-year period, we can assume that after 2 years the number of children in need of active genetic counselling will be 135. After 5 years – 152, i.e. the trend is gradually increasing.

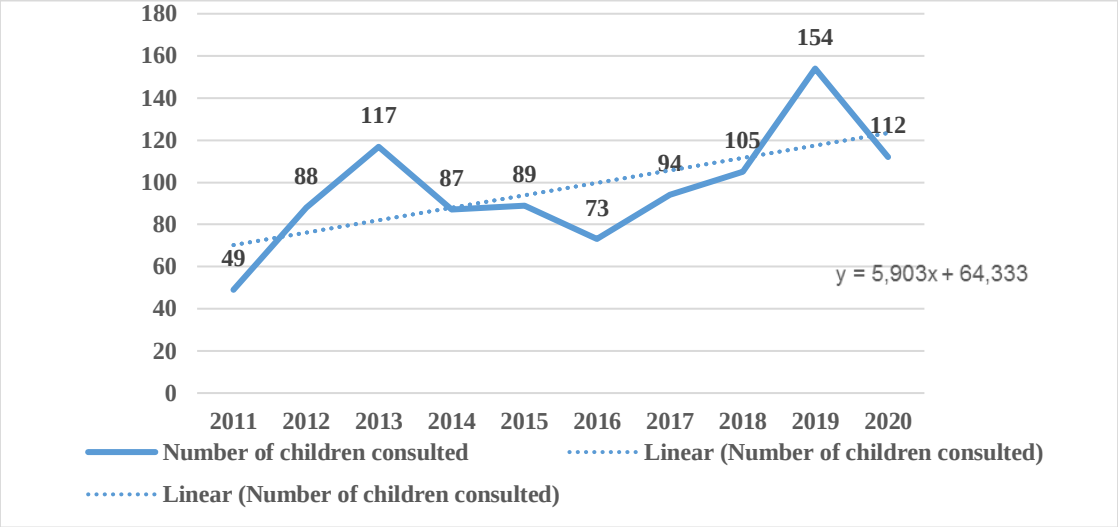

Figure 6. Trend model of children from the selected group who have been managed at the MGC by year

Leading in referrals of the selected contingent of patients for **diagnostic activity** (laboratory and counselling) are the Children's Clinical Units of the hospital – 73.8% of those referred, with a certain predominance of the FCC with PICU (Fig. 7).

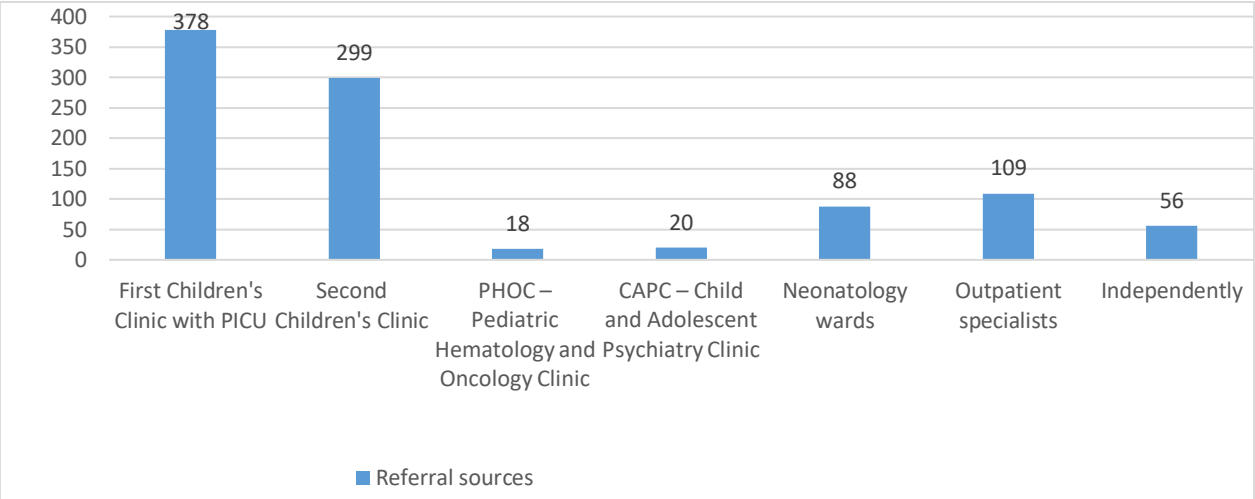

Figure 7. Breakdown by referral sources

4.2. Presentation and analysis of the results of laboratory tests by type of analysis and MGC's genetic diagnoses of the children's contingent

A total of 3 124 childhood patients were registered in the MGC during the 10-year period study period. The majority had a definitive clinical referral for a particular disease or predisposition (cytogenetic, monogenic) and a specified reference for laboratory service/counselling on the interpretation of the results or family medical history. In the remaining (968) cases, the patients were registered in the MGC for *diagnostic activity* (laboratory and counselling) due to an unclear/unspecified disease. We reported and analysed their results as a separate category.

According to patients' referral indications and diagnostic options, different types of genetic/metabolic tests were conducted. In several cases, mainly with an unclear clinical diagnosis, more than one type of analysis was performed.

4.2.1. Conventional cytogenetic analysis (CA) of lymphocyte cultures

CA was performed on the lymphocyte cultures of **2 026** patients for the indicated period. This constitutes **the highest share (64.9%)** of the entire children's contingent registered in the Laboratory of Medical Genetics – Varna during the studied period. Six hundred sixty-seven patients (32.9%) subject to active diagnostic genetic counselling were also included in the CA

analysis of lymphocyte cultures. The number of conducted cytogenetic analyses increased over the years, corresponding to the increase in the number of registered children's contingent (Fig. 8).

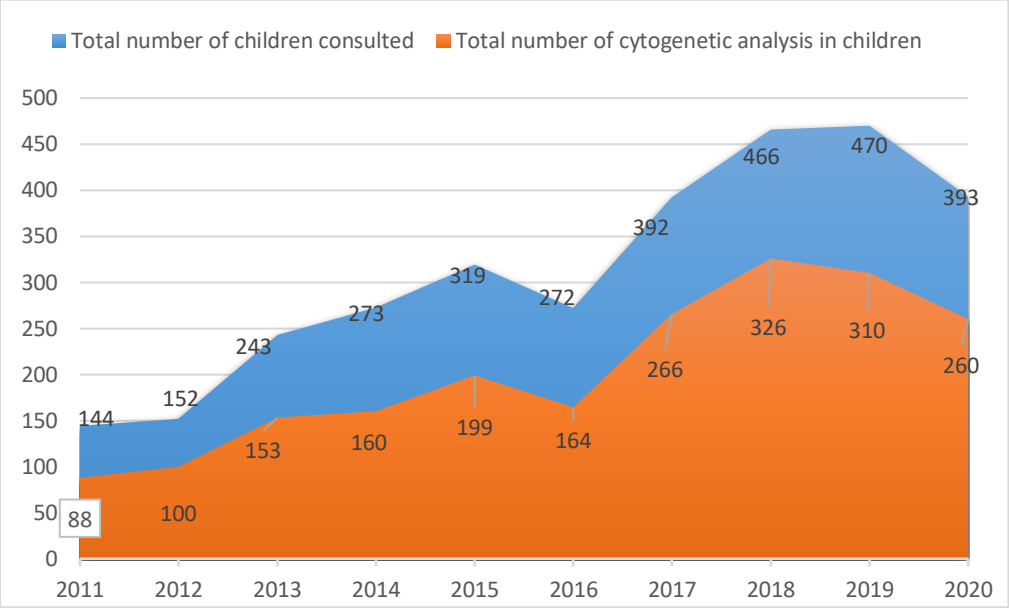

Figure 8. Distribution of patients by year: total number of children counselled and tested with CA

Cytogenetic abnormalities were found in a total of 281 (13.9%) of all 2 026 patients examined over 10 years.

The distribution of the detected chromosomal pathology by gender shows an equal number, but the proportion of detected disorders in the examined girls (18.4%) is statistically significantly higher than that in the examined boys (11.1%) (χ^2 =1 5.716; $p < 0.05$), due to the prevalence of boys in group V (Fig. 9).

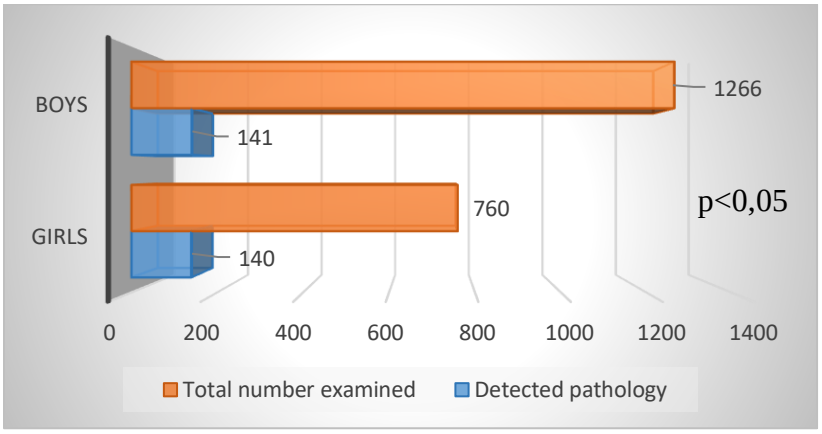

Figure 9. Distribution by gender of the number of individuals with detected cytogenetic pathology

The distribution by indication for CA referral and detected pathology by groups is presented in Table 1. Patients are mainly from I, IV and V groups.

Table 1. Distribution of patients who underwent CA by indication and % detected pathology

Indications	**Number (%) examined patients**	**Number (%) of detected pathology**	**Share (in%) of the detected pathology**
Group I (971 patients)			
a) probable *autosomal* ChD	189 (9.3%)	157 (83,1%)	55.9%
b) probable *gonosomal* ChD, other disorder in sexual development	217 (10,7%)	28 (12.9%)	9.9%
c) impaired *growth* (short/tall height)	565 (27.9%)	25(4.4%)	8.9 %
Group IV (594 patients)			
a) *single* congenital anomalies	132 (6.5%)	8 (6.1%)	2,8%
b) *multiple* congenital anomalies/unknown dysmorphic syndrome	462 (22.8%)	41 (8.9%)	14.6%
Group V (461 patients)			
***DD*/ID and/or ASD**	461(22,8%)	22 (4.8%)	7.9%
TOTAL	**2026 (100%)**	**281**	**100%**

In comparing the percentage of detected pathology among the groups of referred children, the expected highest detection percentage is in group Ia – 157 confirmed out of 189 referred (83.1%). Interestingly, in the subgroup of referred children with isolated CAs (IVa), the percentage of detected pathology is relatively high – 6.1% of the subgroup (2.8% of pathology).

With regard to the distribution of the detected chromosomal pathology by groups in terms of the number of examined persons, a statistically significantly higher percentage of detection was found in group I compared to the other two groups ($\chi^2 = 96.561$, $p < 0.00001$) (Fig. 10).

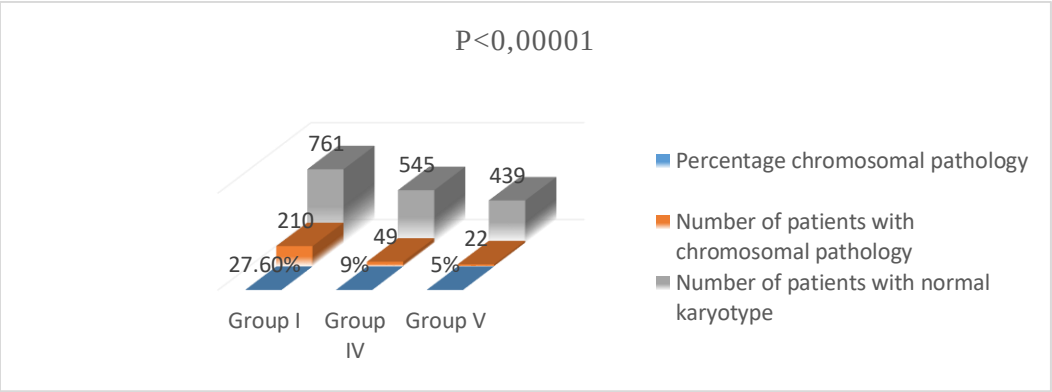

Figure 10. Distribution of chromosomal pathology in 281 patients by groups of indications

The distribution by type of cytogenetic findings (Fig. 11) shows a predominance of autosomal (80.1%) over sex-chromosomal pathology, as well as numerical aneuploidy (60.1%) over structural (including syndromes with chromosomal fragility) and combined chromosomal disorders.

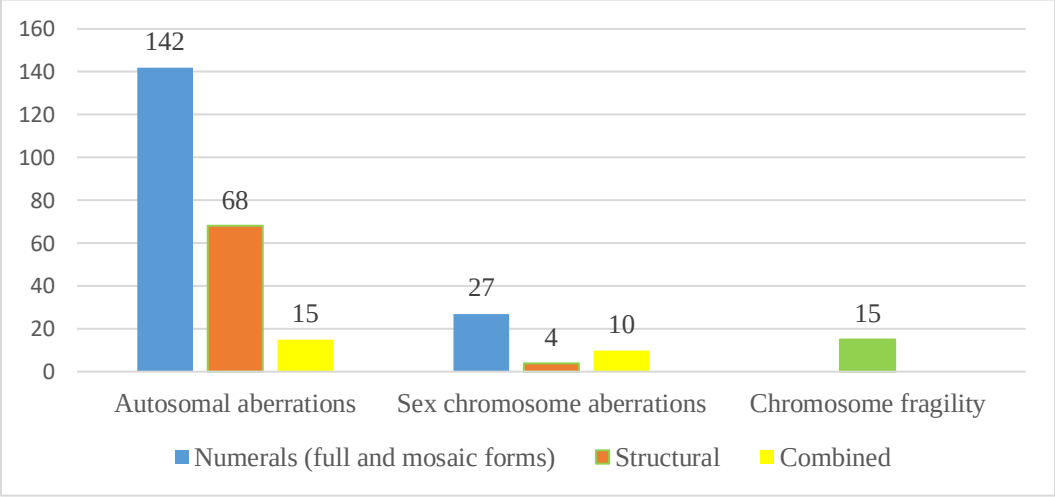

Figure 11. Distribution of pathologies by type of chromosomal disorder

The leading etiological cytogenetic diagnosis is represented by an **autosomal numerical disorder** (aneuploidy), found in 149 patients, constituting 53.0% of the detected pathology (Fig. 12).

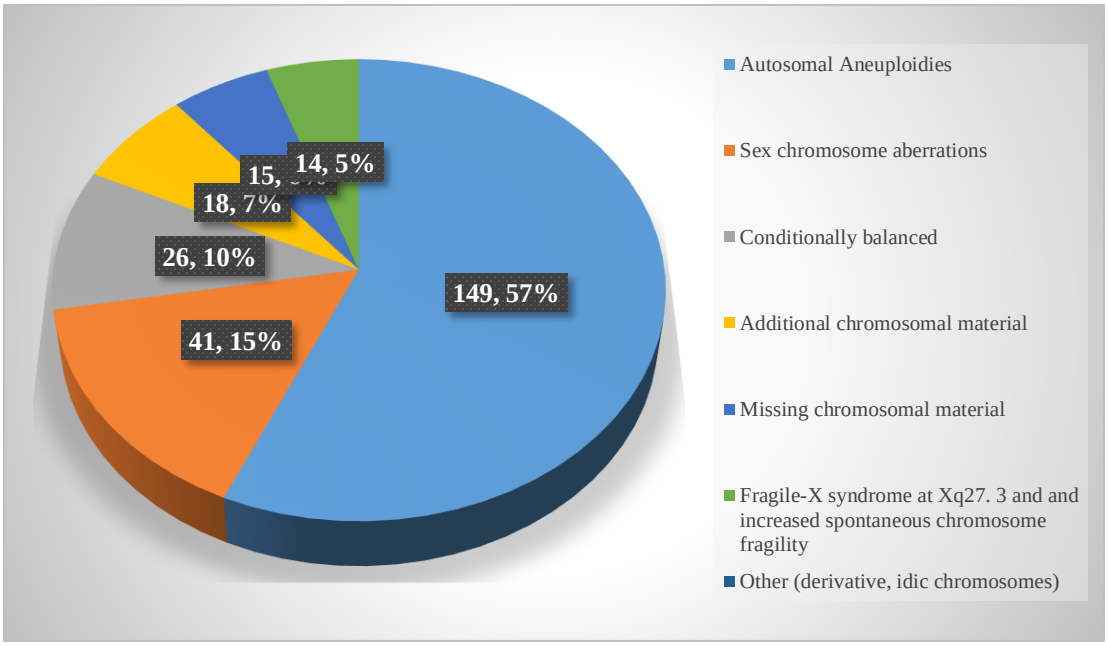

Figure 12. Revealed chromosomal pathology in children for the period 2011 – 2020

The majority of diagnosed autosomal aneuploidies – 140 (49.8% of all pathologies) is associated with trisomy 21 (63 girls and 77 boys, ratio 1:1.2) in various forms and variations: 130 with free trisomy, 5 of variant translocations, 4 with mosaic trisomy and one complex aneuploidy with extra 21 and X chromosomes.

Abnormal structural autosomal anomalies were found in 76 cases (27.0% of the revealed pathology). B*alanced chromosomal rearrangement* was found in 26 individuals (9.3% of pathologies or 1.2% of the cytogenetically examined): Robertsonian translocation in 4 cases (1.4%); reciprocal translocation in 12 (4.2%); inversion in 10 (3.5%). In the remaining 50 cases (17.8% of the pathologies), an *unbalanced rearrangement* was detected: missing genetic material in 18 patients (6.4%) –2 of them with a ring chromosome, 16 - with a deletion; additional genetic material in 8 cases (2.8%) – duplication or unspecified origin. We conditionally attribute 10 cases (3.5%) to this subsection with a marker chromosome (numerical aberrations with additional material of uncertain clinical significance and categorisation). Derivative chromosomes were described in 11 children (3.9%), isodicentric – in 2, and combined inversion and isodicentric disorder in 1 case.

In 42 cases of structural chromosomal aberrations was possible to investigate the parents and siblings and establish the disorder's origin. Family history was found in 19 patients (45.2%).

The application of chromosomal investigation in search for a structural defect – cytogenetic markers in monogenic disorders led to the discovery of fragility at the Xq 27.3 locus in 10 (3.5%) individuals and increased spontaneous chromosomal fragility in 5 (1.7%). These patients were referred for molecular genetic analysis to confirm/exclude monogenic disease. As a result, a diagnosis of Fragile X syndrome (5 boys), Fanconi anaemia (2 children) and Nijmegen breakage syndrome (1 child) was made.

Numerical and structural anomalies of the sex chromosomes were found in 41 individuals (14.5% of the pathologies), mainly affecting the X chromosome. The gender ratio showed a statistically significant three times more frequent detection of these disorders in girls (n = 31) compared to boys ($p < 0.00001$, Fisher's exact test).

The most common finding is the confirmed clinical diagnosis of Turner syndrome with classic monosomy X in 7 children (2.4% of the pathologies) and mosaic variants with numerical and/or structural changes on X (including isochromosome, deletion, ring chromosome) in 16 girls (5.6 %). Age at diagnosis ranged from 2 months to 17 years (mean age 12 years). In 5 patients (1.7%) – 2 with phenotypic female sex and 3 with phenotypic male sex, monosomy X and a second cell line containing a Y chromosome – cytogenetic variants of mixed gonadal dysgenesis were detected. In another 2 (with phenotypic male and female) – karyotype 46,XX/46,XY was detected. Polysomy X was found in 2 girls (0.2% of the examined girls) and polysomy Y – in 2 boys (0.1% of the examined boys).

Classic Klinefelter syndrome with karyotype 47,XXY was confirmed in 4 boys (1.4% of the total pathology) aged 9, 14, 15 and 17 (mean age 14 years).

A wide discrepancy between phenotypic and genotypic sex was found in 3 patients (1.0%) with karyotype 46,XY in the phenotypic female sex. Two of these cases involved 17-year-old girls diagnosed with complete androgen insensitivity syndrome (CAIS) and Swyer syndrome, and the third case involved a newborn with a clinical diagnosis of Denys-Drash syndrome.

4.2.2. DNA analysis for selective screening of monogenic diseases, hereditary predispositions to diseases, pharmacogenetic defects

• DNA analysis for known monogenic diseases

Cystic Fibrosis (CF)

Molecular genetic analysis for mutations in the CFTR gene was performed in a total of 175 patients during the 10-year reporting period. Almost all of them were tested at the NGL. Based on the identified genotype, the disease was confirmed in 21 (12.0%): 13 (7.4%) homozygotes and 8 (4.6%) compound heterozygotes. *The age* of the diagnosed persons varies from 3 months to 16 years (average 3.1 years), and in the great majority, 71.4% (n = 15), the diagnosis was made prior to 1 year of age.

In another 20 (11.4%) patients, only one pathological variant of the cystic fibrosis gene was detected. All of them were registered in the MGC due to the impossibility of complete diagnostic coverage of the CFTR gene (in years, the standard diagnostic coverage varies between 70 and 97%).

The distribution of patients *by genotype* (verified) showed a predominance of delF508 homozygotes, followed by compound heterozygotes with the presence of delF508, other less common compound heterozygotes and homozygotes (Fig. 13). The most frequently detected mutation in the CFTR gene was delF508, found in 16 of 21 (76.2%) confirmed patients.

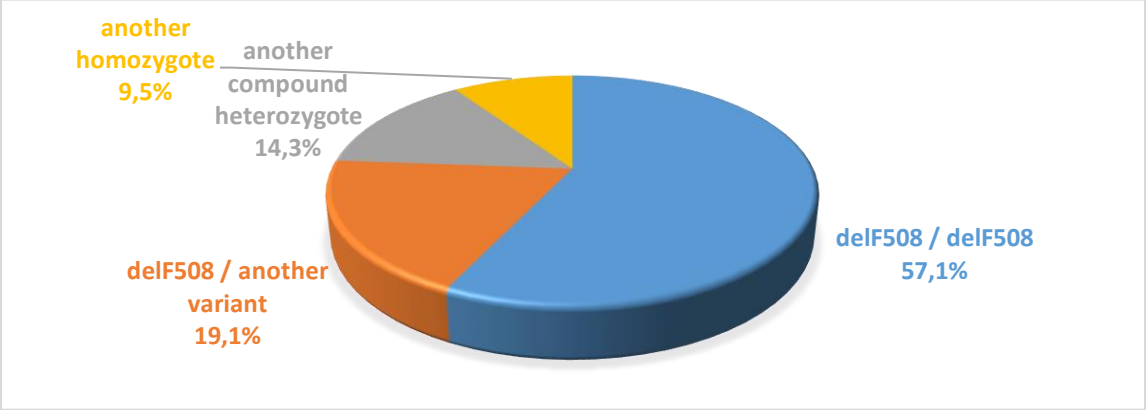

Figure 13. Distribution of diagnosed CF cases by genotype

Wilson's disease

Molecular genetic analysis for a mutation in the ATP7B gene for Wilson's disease was performed in 26 patients, and the disease was confirmed in 6 (23.0%) of those examined. In another 4 (15.3%), heterozygous *carriage* of a pathogenic variant in the ATP7B gene was detected, but no second pathogenic allele was detected at a gene coverage of 95%. The most common mutation found in

both homo- and heterozygous genotypes was H1069Q, except for one child (compound heterozygous for two other mutations – pArg616Gln/p.Thr1092Met).

Beta-thalassemia

For DNA analysis of the β-globin gene, were referred 24 children with clinical suspicion or a positive family history of beta-thalassemia – 10 girls and 14 boys with a mean age of 3.1 years. Pathogenic variants in a homozygous genotype, confirming the disease, were found in 6 (25.0%) of them at an average age of 2.1 years. In another 14 (58.3%) individuals, a carrier genotype was established – 10 of them were detected due to anaemic syndrome and the remaining 4 due to positive family history. The most frequently revealed pathogenic variant (in 10 children) in a homo- or heterozygous state was codon 39 (C > T).

• DNA analysis for hereditary predispositions to diseases

Celiac disease

Molecular genetic analysis of a panel of predisposing celiac allele groups HLA-DQB1*02, DQA1*05, and DQB1*03:02 was performed in 142 children (64 girls and 78 boys, ratio 1:1.2). From the beginning of 2018, it was possible to carry out the analysis at the LMGV for one-third – 49 (34.5%) patients. In 94 (66.2%) individuals – 46 girls and 48 boys, a genotype associated with high or very high risk for celiac disease was found.

Gilbert syndrome

Molecular genetic analysis for Gilbert syndrome was performed in 46 patients (11 girls and 35 boys), with a mean age of 12.6 years, with a primary indication of unexplained elevated unconjugated bilirubin. The LMGV analysis started in 2019 and, in two years, covered half of the patients (23) of all those tested for the analysed period. Homozygous carrier of the pathogenic predisposing variant UGT1A1*28 was found in 34 (73.9%) of the examined – 7 girls and 27 boys (ratio 1:3.8), with an average age was 13.6 years. Another 7 children (15.2%) were found to be heterozygous carriers.

Thrombophilia

For the study period, 31 childhood patients were referred for molecular genetic testing for hereditary predisposition to thrombosis due to vascular incidents (deep venous thrombosis, other

vascular thromboses, stroke). After 2016, the majority (67.7%) were examined at the LMGV when the DNA analysis for thrombophilia was launched. High risk was found in 6 of the individuals (19.3%), based on an established heterozygous genotype for the R506Q mutant variant in the Factor V gene (Factor V Leiden), with one child also being a heterozygous carrier of a predisposing polymorphic variant G20210A in factor II (prothrombin) gene.

• DNA analysis for pharmacogenetic defects

Glucose-6-phosphate dehydrogenase deficiency

Due to suspicion of an X-linked recessively transmitted defect in the enzyme glucose-6-phosphate dehydrogenase, a screening test of blood spots on a filter blank (G-6-PDH-colorimetric spot test) was performed to determine the activity of G6PD in 22 patients (17 boys and 5 girls in a ratio of 3.4:1); in 5 (22.7%) boys, evidence of G6FD deficiency was found, leading to an abnormal reaction to some medications, foods and infectious agents.

Pharmacogenetic defects in the treatment with thiopurines and corticosteroids

Genotyping (Real-time PCR) for the most common allelic variants of the TPMT gene, encoding the enzyme thiopurine methyltransferase, associated with the metabolism of thiopurines, was performed in 14 children requiring treatment with drugs from this group. A normal genotype was found in all tested (homozygous wild type). In 7 childhood patients, specific polymorphisms in the NR3C1 and NALП1 genes affecting the response to glucocorticoid treatment were genotyped. In 3 children (42.8%), a genotype associated with the lack of response to treatment with glucocorticoids was found.

4.2.3. Results of the diagnostic (laboratory and counselling) *activity* in a *selected* group of patients with genetically unspecified/unproven disease

Most patients subject to diagnostic *activity* (laboratory and counselling) were referred to the MGC office due to an unclear cause of the clinical disorder and possible genetic aetiology. These patients underwent one or more genetic/metabolic tests, depending on the laboratory, technical and financial possibilities for the period.

In 91.2% (n = 883) of a total of 968 consulted, at least one type of laboratory genetic/metabolic analysis (mainly CA) was conducted (Fig. 14).

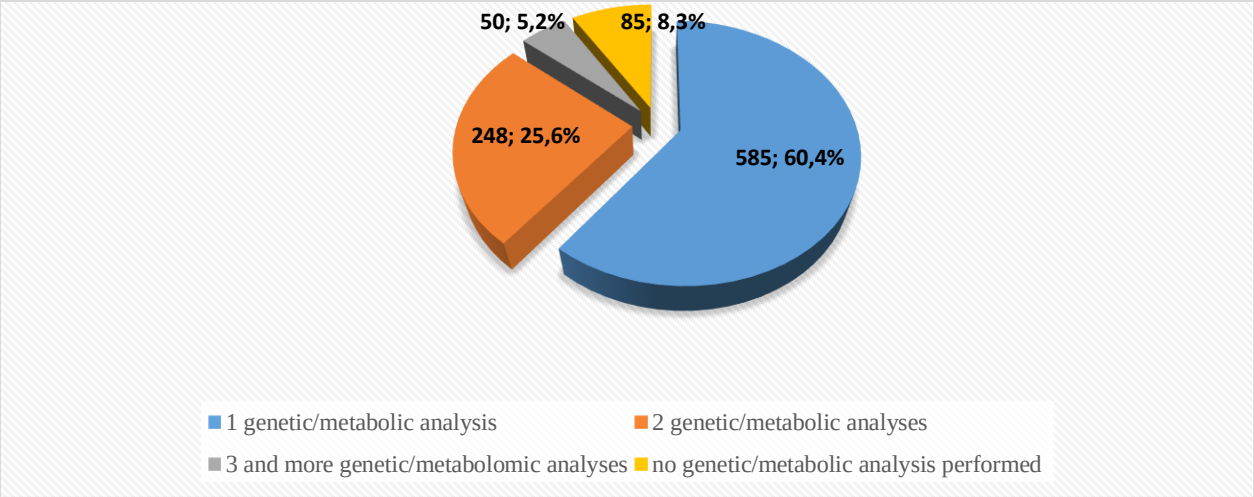

Figure 14. Distribution of consulted patients by number of performed analyses

An important non-laboratory method used in the diagnostic activity is the software products for a genetic database by clinical phenotype, as part of the work of the genetic consultant, especially in individuals with dysmorphism, with or without retardation in the NPD/MR.

4.2.3.1. Diagnostic activity at the Laboratory of Medical Genetics - Varna

Conventional cytogenetic analysis (CA) of lymphocyte cultures

We present data from conventional cytogenetic analysis on lymphocyte cultures only in actively consulted patients with an unspecified/unclear diagnosis. These are 667 persons (68.9% of a total of 968 *actively counselled* or 32.9% of a total of 2 026 *cytogenetically examined*) for the study period. The full description of established diagnoses, including identifiable chromosomal aneuploidies, is presented in the "Results of conventional cytogenetic analysis " section (4.2.1.).

It includes primarily individuals from group D– 412, followed by those examined from group E– 136, and group A – 55. A pathological result was found in 50 (7.5%) of the examined. Figure 15 shows the distribution of the selected patients with performed CA by groups of indication and % detected pathology.

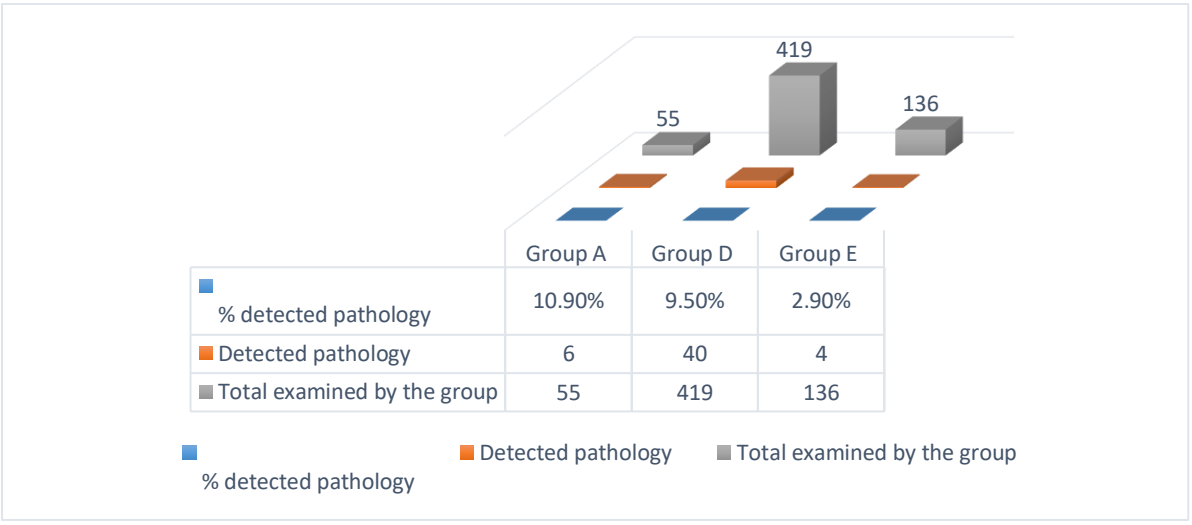

Figure 15. Distribution of patients with performed CA by indication group and % detected pathology

The results show a predominance of autosomal (82.0%) over gonosomal pathology and structural chromosomal abnormalities (66.0%) compared to numerical ones. All established structural aberrations fall under the unbalanced category.

Molecular genetic analysis for FRAXA syndrome

Molecular genetic analysis for FRAXA syndrome was conducted on 105 children aged 2 to 17 years (mean age of 6.5 years). Of them, 94 (89.5%) were male, and the remaining 11 (10.5%) – were female (M:F =8.5:1). Almost all – 97 (92.4%) were patients with a referral indication from group E, and the remaining 8 – from group D.

A positive result for FRAXA syndrome was revealed in **6 children** (5.7%) out of 105 males. Four patients were diagnosed in the LMGV (validated in the National Genetic Laboratory), and two were diagnosed in an external laboratory.

The samples of 82 persons (78.1% of those examined) were tested in the LMGV and 23 – in external laboratories (NGL and GMDL Genica). In Varna, molecular genetic **screening for a Fragile X syndrome** became possible after winning a *Scientific Project (No. 18015)* to the Science Fund of MU-Varna (contention session 2018) to cover retrospectively and prospectively a selected group of 52 children – 45 (86.5%) boys and 7 (13.5%) girls with intellectual disabilities and/or ASD.

The screening results of these patients are presented by number, sex, and indication (Fig. 16). All the selected patients had a delayed NPD, including a delay in speech development.

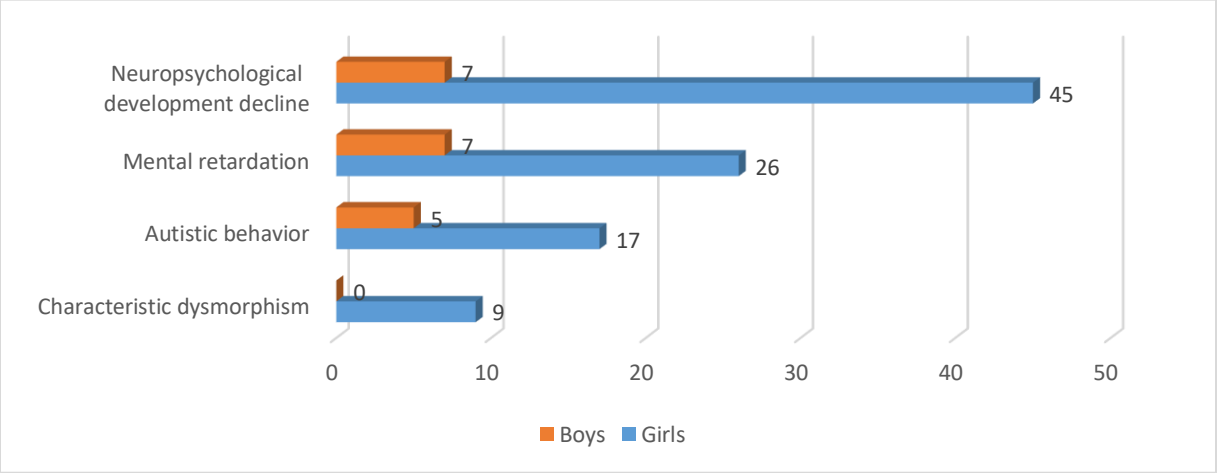

Figure 16. Clinical presentation of the selected group of patients based on leading clinical criteria for inclusion in the study

Molecular genetic analysis revealed that the samples of 3 cases (5.7%) reported amplification of the DNA segment in the syndrome-targeted region. These patients were boys aged 8 (case 1), 9 (case 2), and 11 (case 3) years. Their clinical characteristics are summarised in Table 2. Further examination of samples from the mothers of Patient 1 and Patient 2 also showed amplification of the DNA region. Confirmatory genetic analysis at the NGL showed that all three boys had a full mutation, and both mothers had a premutation of the pathological allele.

Table 2. Clinical manifestations in patients with a complete mutation in the FMR1 gene

Clinical phenotype	**Case 1**	**Case 2**	**Case 3**
Age (years)	**8**	**9**	**11**
Intellectual disability (degree)	**+ (moderately)**	**+ (lightly)**	**+ (lightly)**
Developmental delay	+	+	+
Autism/autistic behaviour	+	-	+
Hyperactivity	+	+	+
Stereotyped movements	+	-	+
Facial dysmorphism	+	-	-
Macroorchidism	+	-	+

Increased joint extensibility	+	+	-
Obesity	-	-	+

MLPA analysis for microdeletion/microduplication syndromes

Multiplex ligase-dependent probe amplification (MLPA) for common microdeletion/microduplication syndromes and/or subtelomeric aberrations was performed on 86 children (39 girls and 47 boys). All examined individuals had referral indications from group D (single or multiple CAs/unclear dysmorphic syndrome, with or without MR – 68.6% (n = 59) and group E (developmental delay/ID and/or ASD) – 31 .4% (n =27).

Pathological findings were diagnosed in a total of 13.9% (12 individuals – 9 boys and 3 girls). In 5 (5.8%) cases with an unclear cause of MCA and/or ID and/or ASD, MLPA analysis helped to establish a genetic diagnosis. In another 5cases – the diagnosis was a clinically suspected syndrome; in two patients, the analysis confirmed/specified a cytogenetic finding.

The analysis of the samples of 54 patients (62.8% of those examined) was performed in the LMGV. For 32 (37.2%) patients, the analysis was performed in external laboratories (NGL and GMDL Genica). In the LMGV, MLPA screening for microdeletion/microduplication syndromes became possible after winning the *Scientific Project* (N18015) to the Science Fund of MU-Varna to cover prospectively and retrospectively a selected group of 54 children registered in the office for genetic counselling.

As a result of the applied algorithm of selective MLPA analysis, 5 (9.3%) individuals with microdeletions/microduplications associated with a phenotype overlapping with the patient's clinical picture were revealed (Table 3). The average age of the diagnosed patients was 1.7 years. These findings were validated by microarray analysis performed in an external laboratory.

Table 3. Distribution of patients with detected pathology by indication and subtype, MLPA analysis and results

Patient (gender, age)	**Indication**	**≥ 1 genetic analysis performed**	**Result**

Boy, 2 months	group D Prader-Willi syndrome	Common microdeletion/microduplication syndromes	**Deletion in region 15q11.2**
Boy, 1 year	group D Wolf-Hirschhorn syndrome	Common microdeletion/microduplication syndromes	**Deletion in region 4p16.3**
Boy, 7 months	group D DiGeorge syndrome	Common microdeletion/microduplication syndromes	**Deletion in region 22q11.21**
Boy, 6 years	group E	Subtelomeric screening	**Deletion in region 1q44**
Boy, 8 months	group D	- Common microdeletion/microduplication syndromes - Subtelomeric screening	**Norm** **Duplication region 14q11.2**

Abnormalities from MLPA screening were found in another 5 individuals (duplication in regions 15q11.2 and 12p13.33; deletion in regions 22q11.21 and two in 22q13.33), but microarray analysis in the patients and/or MLPA in the parents did not allow their categorisation as pathologic findings. In three of the patients, the study confirmed the clinically suspected diagnosis (Prader-Willi syndrome, Wolf-Hirschhorn syndrome and DiGeorge syndrome), and in two, it helped to establish an unexpected pathology (1q44 del in combination with 1q42.13-q44dup, and another case of 14q11.2 dup).

4.2.3.2. Diagnostic activity outside the Laboratory of Medical Genetics Varna

Microarray analysis for molecular karyotyping

During the study period, microarray analysis was conducted on 44 children patients – 4.5% of the counselled individuals. In all of them, it was applied as a second or third diagnostic/validation method after CA and in a few cases after MLPA. The analysis by year shows that 74% were carried out in the last 3 years. They were performed at the Department of Medical Genetics – Sofia, the Laboratory of Genomic Diagnostics – Sofia or abroad. In 11 (25%) of the examined, the cause was clarification/verification of a pathological result of performed *cytogenetic or MLPA analysis*. In the remaining 33 examined (all with a normal karyotype), the reason was multiple congenital

anomalies/unclear dysmorphic syndrome, with or without a DD. Pathology was detected in 8 patients (24.2%) aged between 1 month and 12 years, an average of 4.3 years (Table 4).

Table 4. Detected pathogenic variants in patients with unclear cause of MCA/MR

Molecular karyotyping result	**Age**
arr[hg19] 7q11.23(72,404,312-73,771,491)x1	6 m
arr[hg19] 15q21.3q22.2(57309619x2, 57414087_62947243x1, 62986766x2)	10 y
arr[hg19] Xq25q26.3(123,464,057-134,293,066)x3	1 y
arr[hg19] 8p23.2 (2,625,441-4,118,569)x3	1 y
arr[hg19] 5p13.2 (37,479,936-37,738,033)x3	5 y
UPD(20)mat.arr[hg19]20p13p11.21(377,22625,124,088)x2htz,20p11.21q13.32(25,130,395-57,086,518)x2hmz,20q13.32q13.33(57,086,507-62,751,454)x2	5 y
arr[hg19] 16p11.229238831x2, 29500252_30240067x3, 30279427x2	2 y
arr[hg19] 17q25.1q25.3(73815062_76174585x3)	11 y

Other molecular genetic analyses for monogenic diseases

Of essential importance for the proof/validation of monogenic disorders are studies using the methods of gene-panel sequencing (Sanger, next-generation sequencing), less often – methylation-specific PCR, MLPA, etc., performed mainly in the National Genetics Laboratory, GMDL Genica, Genomic Diagnostic Laboratory, as well as in laboratories abroad.

Analyses were conducted in 167 out of 968 (17.3%) selected individuals subject to genetic diagnostic activity. The follow-up by years of the conducted **molecular genetic analyses** for this group shows an emphasis (52.1%) in the last 3 years (Fig. 17). The increase in the number of these analyses in the last three years compared to the first three of the analysed period is 5-fold, statistically significant ($p < 0.00001$).

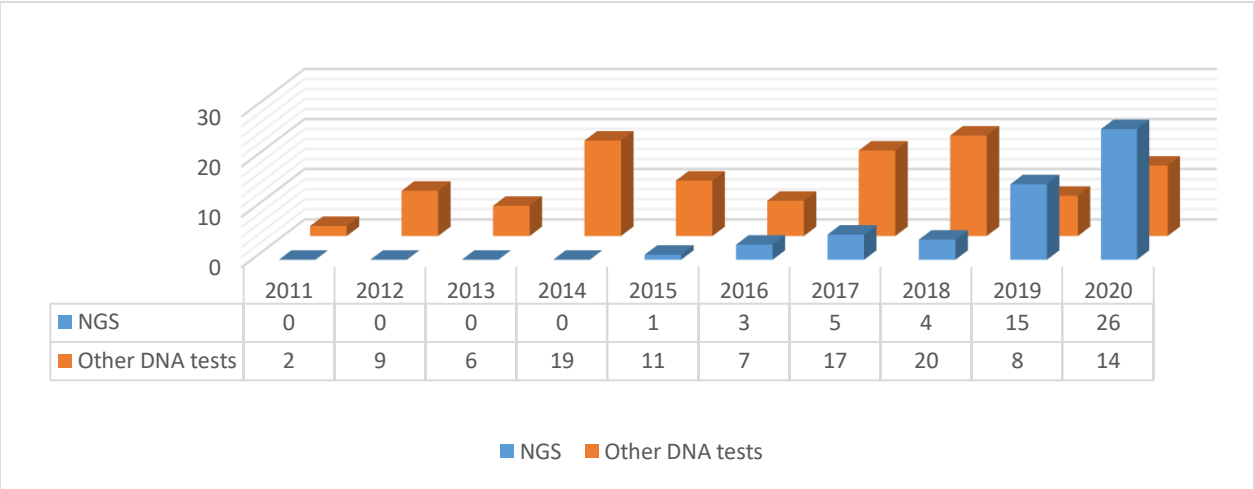

Figure 17. Distribution by year of conducted NGS and other DNA analyses among consulted individuals

A genetic diagnosis was confirmed/revealed in **89** studied patients **(53.3%)**, at a mean age of 5.4 years. The highest percentage (51.4%) of the examined individuals was from **Group B** (suspected monogenic (non-metabolic) disease, including *hereditary cancer syndrome*), and the highest share of established diagnoses was in this group as well (Table 5).

Table 5. Distribution of patients with DNA analysis performed and pathology revealed by referral groups

Group/indication	DNA analysis performed		Pathogenic variant detected		
	Number of children	% of the group	Number	% of those tested	% of total pathology
Group A (55 children in total) growth and/or sexual development disorder	5	9%	1	20%	1.1%
Group B (186 children in total) suspected monogenic (non-metabolic) disease	86	46.8%	54	62.8%	60.7%
Group C (105 children in total) probable hereditary metabolic disease	12	11.4%	7	58.3%	7.9%
Group D (469 children in total) single and multiple CAs/	58	12.3%	25	43.1%	28.1%

dysmorphic syndrome of unclear origin, with or without ID					
Group E (123 children in total)	**6**	**4.9%**	**2**	**33.3%**	**2.2%**

Targeted exome sequencing led to diagnostic clarification in 21 of 37 (56.7%) patients in whom it was administered; whole-exome or clinical exome sequencing aided diagnostic clarification in 5 of 17 (29.4%) patients with referrals.

Molecular genetic studies, which made it possible to diagnose 88 patients in childhood, are associated with discovering **rare genetic diseases** (Table 6). The relatively large number of diagnosed patients with leading neurological/neuromuscular symptoms (including mitochondrial and some IEM) is striking – 29 (32.6% of the pathology).

Table 6. Verified rare diseases/syndromes by molecular genetic methods for 10 years

With neurological symptomatology	**Dysmorphic/malformative syndromes**	**Hereditary metabolic diseases**	**Diseases associated with impaired imprinting**
Spinal muscular atrophy type 1 (4 individuals)	Hutchinson-Gilford progeria syndrome	Glycogenosis 1A	Silver–Russell syndrome (7 individuals)
Cerebellar ataxia (2 individuals)	Beare-Stevenson cutis gyrate syndrome	Niemann-Pick disease (3 individuals)	Beckwith-Wiedemann syndrome (5 individuals)
Sarcoglycanopathy (LGMD2C) (2 individuals)	Kabuki syndrome	Dent's disease	UPD 20 mat
Dravet syndrome	LEOPARD syndrome	Lowe syndrome	**Other**
Pontocerebellar hypoplasia type 1B (2 cases)	Bardet-Biedl syndrome	Zellweger syndrome	Juvenile nephronophthisis
Congenital cataract with facial dysmorphism and neuropathy (CCFDN)(4 cases)	Fanconi anaemia (2 individuals)	Alpha-1-antitrypsin deficiency	Complete Androgen Insensitivity Syndrome (CAIS)
Congenital myasthenia gravis (2 cases)	Nijmegen breakage syndrome	Canavan disease	Andersen-Tawil syndrome
Rett syndrome (2 cases)	Cartilage-hair hypoplasia syndrome (2 individuals)	Mucopolysaccharidosis type 4	Diamond-Blackfan anaemia
Duchenne muscular dystrophy (2 individuals)	Cornelia de Lange syndrome		Congenital hyperinsulinism (ABCC8 gene)

Hypomyelinating leukodystrophy (H-ABC syndrome) (2 individuals)	Cardio-facio-cutaneous syndrome	**Hereditary cancer syndromes**	Familial hemophagocytic lymphohistiocytosis
	Noonan syndrome (5 individuals)	Tuberous sclerosis (2 individuals)	Autosomal dominant cryopyrin-associated periodic syndrome
Mitochondrial diseases (mtDNA mutation)	Mowat-Wilson syndrome	Neurofibromatosis type 1 (3 individuals)	Albright's hereditary osteodystrophy
MELAS syndrome	Greig cephalopolysyndactyly syndrome	Peutz-Jeghers syndrome	Arima syndrome
Leigh syndrome	Osteoporosis – pseudoglioma syndrome	Von Hippel-Lindau syndrome	Autosomal dominant polycystic kidney disease (2 individuals)
	Jansen de Vries syndrome		Retinitis pigmentosa (AP)
			Achondroplasia

The distribution of established diagnoses by pattern of inheritance/mechanism of occurrence shows a predominance of autosomal recessive conditions (41.6% of the pathology), followed by autosomal dominant – 33.7%, and X-related (7.9%) and mitochondrial diseases (2, 2%). Conditions due to impaired imprinting were found in 7.8% of those examined (14.6% of pathology). Only 6 (20%) of the cases of autosomal dominant pathology were an inherited pathogenic variant, and in the remaining 24 – a sporadically occurring condition. There was a positive family history in 7 patients with established autosomal recessive diseases (cerebral ataxia, Pontocerebellar hypoplasia type 1B, Cartilage-hair hypoplasia syndrome, CCFDN). The diagnosis was established due to an affected second child in the family.

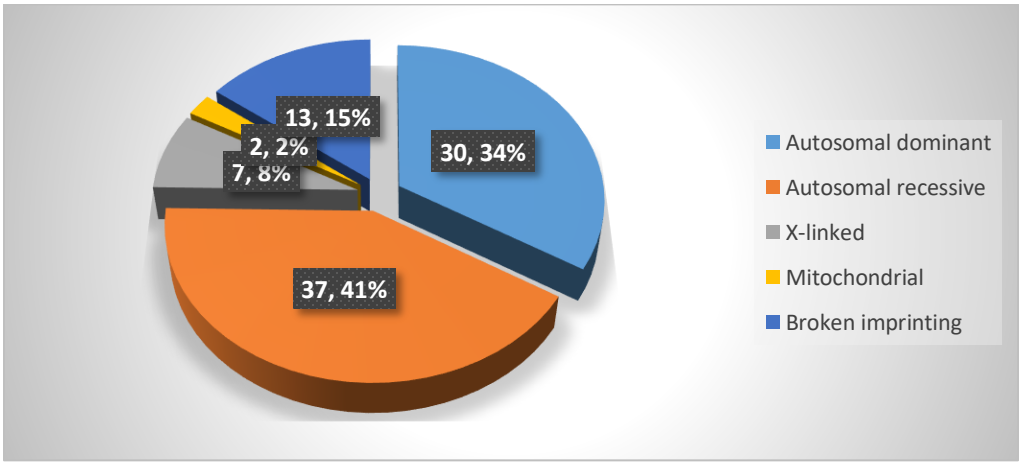

Figure 18. Distribution of diagnosed conditions by mode of inheritance

Selective metabolic screening/enzyme assay for IEM

Selective metabolic/enzymatic analysis was conducted in 126 patients (13.0% of the actively consulted) for the study period, aged from 4 days to 15 years (mean age 3.1 years), the majority – 73.0% aged 0 – 3 years. This screening was performed by sending blood and urine samples to the NGL–Sofia metabolomics department.

Metabolic abnormalities were found in 26 (20.6%) individuals, and a specific metabolic disease was diagnosed in 14 of 126 (11.1%) examined patients, with a mean age of 3.6 years (Table 7). Half of the verified cases (7) concern lysosomal disease. All established hereditary metabolic diseases (except mitochondrial Leigh syndrome) were classified as monogenic disorders with autosomal recessive inheritance.

Table 7. Diagnosed metabolic diseases

Diagnosed metabolic disease	Diagnostic method	Age of diagnosis
Alkaptonuria	Metabolic screening	2 months
Alkaptonuria	Metabolic screening	6 months
GM1 gangliosidosis	Metabolic/enzyme screening	6 months
Leucinosis (MSUD)	Metabolic screening	1 month
Metachromatic leukodystrophy	Enzyme screening	2 years
Mucolopysaccharidosis type VI (Maroteaux-Lamy)	Enzyme screening	10 years
Niemann-Pick type C	Enzyme screening	9 years
Niemann-Pick type A/B	Enzyme screening DNA analysis	10 years
Niemann-Pick type A/B	Enzyme screening DNA analysis	15 years
Синдром на Zellweger ***Zellweger syndrome***	Metabolic screening DNA analysis	3 years
Propionic acidemia	Metabolic screening	1 year
GM1 gangliosidosis	Metabolic/enzyme screening	8 months
Canavan disease	Enzyme screening DNA analysis	1 year
Leigh синдром	Metabolic screening DNA analysis	11 months

4.2.3.3. Results of the diagnostic counselling activity in a selected group of patients with genetically unspecified disease – software online-based programs assisting genetics practice

For the reporting 10-year period, the activity of the genetic counselling office for 968 patients was associated with laboratory-diagnostic and with collaborative clinical-diagnostic evaluation to clarify syndromic or single (non-syndromic) genetic disorders in 118 (10.8%) and 41 (4.2%) patients.

The main task was establishing a symptom complex with a single etiopathogenesis in multiple anomalies based on a specific phenotype. In some cases, the diagnosis was based/supported by a specific finding from imaging studies (Tuberous sclerosis, Joubert syndrome, Allagile syndrome, etc.), specific skin manifestations (Neurofibromatosis type1, Congenital ichthyosis, Epidermolysis bullosa, Incontinentio pigmenti, etc.), a characteristic combination of congenital anomalies/dysmorphic marks (Cornelia de Lange syndrome, Holt-Oram syndrome, Trichorhinophalangeal syndrome, Thanatophoric dysplasia, Oral-facial-digital syndrome, etc.), meeting specific diagnostic criteria (Marfan syndrome, Noonan syndrome, Bardet-Biedl syndrome, etc.), distinctive neurological status, electromyography data (Friedreich's ataxia, Dejerine-Sottas syndrome, etc.), or teratogenic effects (Fetal alcohol syndrome, Valproate embryopathy, etc.), positive family history.

An essential part of diagnosing children with suspected genetic disease was the use of **software online-based programs and dysmorphology databases**. They signified to be an indispensable tool in the diagnostic search, especially in the group of children with a leading clinical picture of multiple CAs/unclear dysmorphic syndrome, with or without MR (group D). We report the contribution of online-based software programs to the clinical diagnosis of children with a specific phenotype.

Frequently used in the practice of the doctoral student and medical genetics specialists are OMIM, ORPHA.NET, London Medical Database, Face2Gene, as well as specialised dysmorphological literature references such as "Smith's Recognisable Patterns of Human Malformation" and others. Since 2015 the use of ***Face2Gene*** has been almost a mandatory part of the diagnostic process when counselling children with facial dysmorphism (but not only) and serves simultaneously as a registry

of dysmorphic patients. Due to the fact that the application requires patient's photos, in some cases, the refusal of relatives to photograph the child did not allow us to use it.

Facial dysmorphism was described in 53.7% (n = 520) of the counselled individuals over the 10-year period. The use of the program started in 2016, and until the end of 2020, the photographs of 214 patients were uploaded, with a description of detected small and large anomalies, other clinical manifestations and anthropometric indicators. In 71 (33.2%) of the cases, a genetic or clinical diagnosis was confirmed, and in 51 (71.8% of 71) of them, the diagnosis was in the first 10 suggested by the program (32 – 45.1% of them on the first place).

The use of *Face2Gene* instigated the establishment of rare diagnoses such as Mowat-Wilson syndrome (Fig. 18), Coffin-Siris syndrome (Fig. 19), Kabuki syndrome, Coffin-Lowry syndrome (Fig. 20), Greig cephalopolysyndactyly syndrome, Wiedemann-Steiner syndrome etc., some of which are genetically confirmed. In other more well-known and recognisable syndromes, such as Bardet-Biedl syndrome, Cornelia de Lange syndrome, Cardio-facio-cutaneous syndrome, Noonan syndrome, Trichorhinophalangeal syndrome, Silver–Russel syndrome, DiGeorge syndrome, Wolf-Hirschhorn syndrome and others, the program confirmed the clinical opinion.

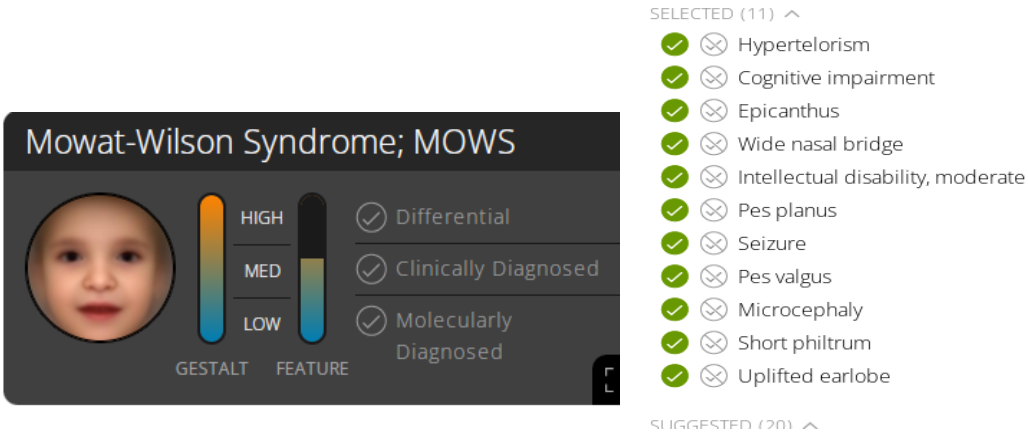

Figure 19. Analysis of a patient with an unclear genetic syndrome with the Face2Gene program and a subsequently confirmed genetic diagnosis of Mowat-Wilson syndrome

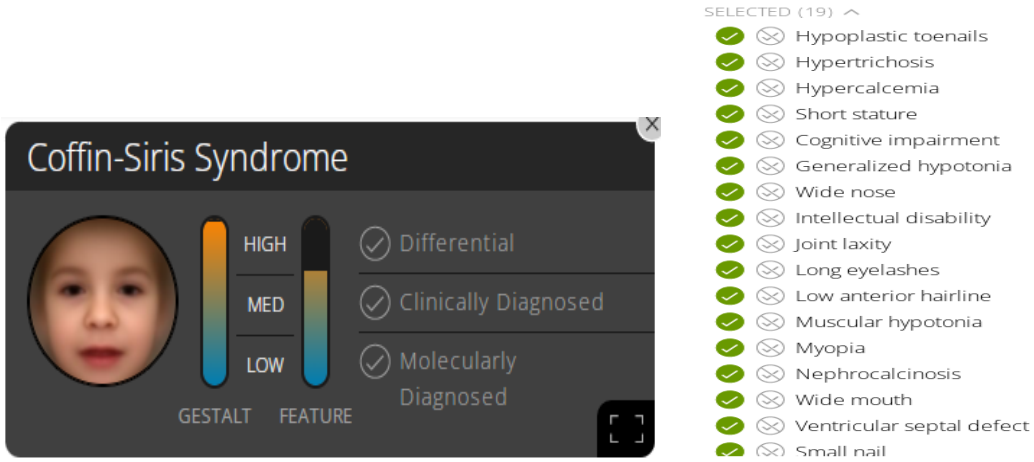

Figure 20. Analysis of a patient with an unclear genetic syndrome with the Face2Gene program and the subsequent clinical diagnosis of Coffin-Siris syndrome

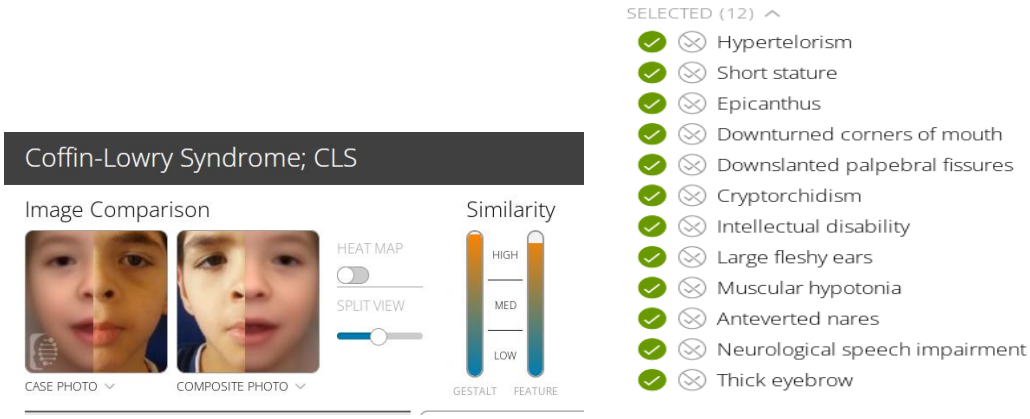

Figure 21. Analysis of a patient with an unclear genetic syndrome with the Face2Gene program and an accepted clinical diagnosis of Coffin-Lowry syndrome

4.2.4. Summary of results

From the overall activity for the covered paediatric contingent of patients

As a result of the multidisciplinary approach to diagnosing patients in childhood, a genetic and clinical diagnosis was established in 696 patients (22.3%) out of 3124 individuals registered for a 10-year period (Table 8). These are:

- 537 (17.2% of all consulted) patients have laboratory (genetically and metabolically) verified causes of the disease;

- 118 (3.8%) have clinical diagnoses (syndromes);

- 41 (1.3%) have a single (non-syndromic) congenital anomaly.

Table 8. Confirmed and/or established diagnoses by category of genetic classification of paediatric patients registered in the MGC during the reporting period

Category of the genetic disorder		**Diagnosed individuals in total from all registered in MGC**		**Diagnosed individuals as a result of MGC activity**	
		Number	% of pathology	Number	% of pathology
Chromosomal diseases	Microscopically visible imbalanced disorders	240	34.5%	50	7.2%
	Micro (deletions/duplications)	18	2.6%	18	2.6%
Monogenic diseases	Autosomal recessive *	79 (+ 39*)	11.3% (17.0%*)	46	6.6%
	Autosomal dominant	30	4.3%	30	4.3%
	X-linked	13	1.9%	13	1.9%
	Mitochondrial	2	0.3%	2	0.3%
	Imprinting diseases	13	1.9%	13	1.9%
Hereditary predispositions - monogenic and multifactorial	Celiac disease	94	13.5%		
	Gilbert syndrome	34	4.9%		
	Thrombophilia	6	0.9%		
	Single (non-syndromic) congenital anomalies	41	5.9%	41	5.9%
	Glucose-6-phosphate dehydrogenase deficiency	5	0.7%		
	Pharmacogenetic defects treatment (thiopurines, steroid)	3	0.4%		
Clinical diagnoses		118	16.9%	118	16.9%
TOTAL		**696 (+ 39)**	**100%**	**331**	**47.6%**

**number of detected heterozygotes tested for suspected cystic fibrosis, beta-thalassemia and Wilson's disease*

The summarised data from the laboratory genetic tests performed on childhood patients for the study period (2011 – 2020) are presented in Table 9.

Table 9. Genetic/metabolic studies and detected pathology by type of analysis in paediatric patients registered in the Medical Center for the study period

Type of analysis performed	Number of individuals examined	Number of persons with detected pathology	% detected pathology
Cytogenetic analysis *	2026	281	13.9 %
Molecular genetic analysis for cystic fibrosis	175	21 homozygotes (21 - heterozygotes)	12.0%
Molecular genetic analysis for Wilson's disease	26	6 homozygotes (4 - heterozygotes)	23.0%
Molecular genetic analysis for beta-thalassemia	24	6 – homozygotes (14-heterozygotes)	25.0%
Molecular genetic analysis for Gilbert syndrome	46	34	73.9%
Molecular genetic analysis for celiac disease susceptibility	142	94 with high or very high risk	66.2%
Molecular genetic analysis for thrombophilia predisposition	31	6 (FV and/or F II heterozygotes)	19.4%
Molecular genetic analysis for pharmacogenetic deficiency in thiopurine and corticosteroid treatment	21	3 - lack of response to corticosteroids	14.3%
Test for G6FD deficiency (G-6-PDH-colorimetric spot test)	22	5	22.7%
Molecular genetic analysis/screening for FRAXA syndrome	105	6	5.7%
MLPA for microdeletion/microduplication syndromes	86	12	13.9%
Microarray analysis (aCGH)	44	19	43.2%
Other molecular genetic analyzes (PCR, MLPA, Sanger sequencing)	113	63	55.8%
Next Generation Sequencing (NGS)	54	26	481%
Selective metabolic screening/enzyme assay for inborn errors of metabolism	126	14	11.1%

** This category also includes the pathological results found in 50 (7.5%) of 667 selected, actively consulted patients with an unclear diagnosis and 39 patients with the autosomal recessive disorder (cystic fibrosis, beta-thalassemia and Wilson's disease) with a heterozygous genotype of a pathogenic allele*

Only homozygotes were included in the cases of detected pathology of autosomal recessive disorders, but the number of detected heterozygotes is also presented. The conditional addition of

39 patients with the autosomal recessive disease (cystic fibrosis, beta-thalassemia, and Wilson's disease) with a clinical phenotype and a laboratory-detected heterozygous genotype in the absence of a second pathogenic variant (shown in Table 9) would change the diagnostic detectability of the study of 735 individuals (23.5%).

From the diagnostic activity (laboratory and counselling) of a selected group of patients

The contribution of the *genetic diagnostic activity* of the Medical Center for establishing the genetic and/or clinical diagnosis in patients with an unclear/unproven clinical disorder is related to the identification of 331 individuals (34.2% of the selected group of 968 patients or 10.6% out of all 3124 persons registered). These are patients with the following established diagnoses (Fig. 22):

- 172 (17.7%) laboratory diagnoses: cyto-/molecular-genetic; metabolic/enzymatic;
- 118 (10.8%) clinical diagnoses of a disease/syndrome with a specific phenotype;
- 41 (4.2%) single (non-syndromic) congenital anomalies.

In **65.8%**, it was not possible to establish a specific clinical or genetic diagnosis. This includes registered healthy siblings, along with missing information due to impossible or unachieved proband/family follow-up and clarifying diagnostic testing beyond the scope of the present study.

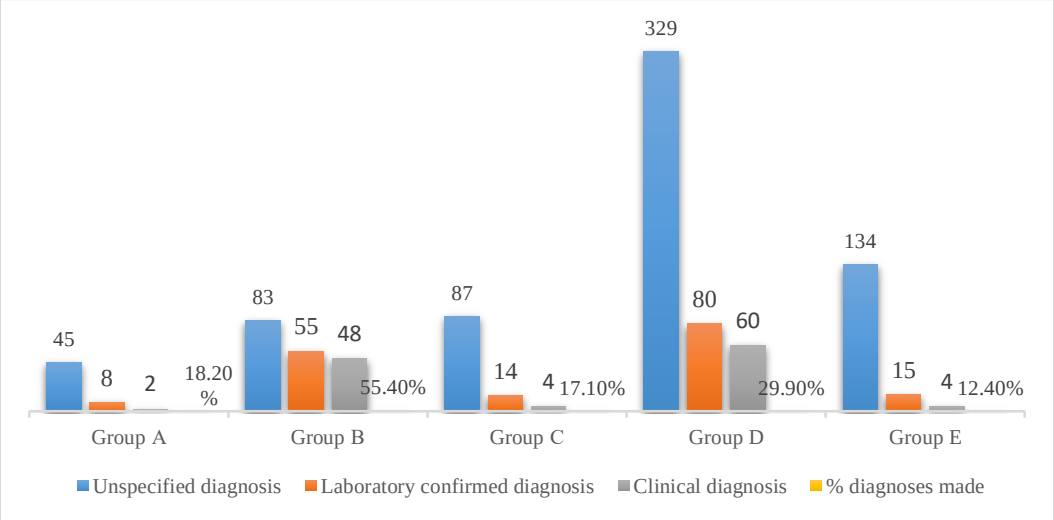

Figure 22. Distribution of counselled patients' referral groups and established/set diagnoses

There is a clear trend of increasing demand and success rate of the *diagnostic activity (laboratory and counselling)* for genetic services for childhood patients. For the period 2011 – 2015, 430

children were consulted, with established diagnoses in 56 (13.0%). For the period 2016 – 2020, 538 individuals were consulted, and a diagnosis was established in 116 (21.6%) (Fig. 23). The application of the non-parametric test – Chi-square showed a statistically significant difference (χ^2 = 11.923; p = 0.0005).

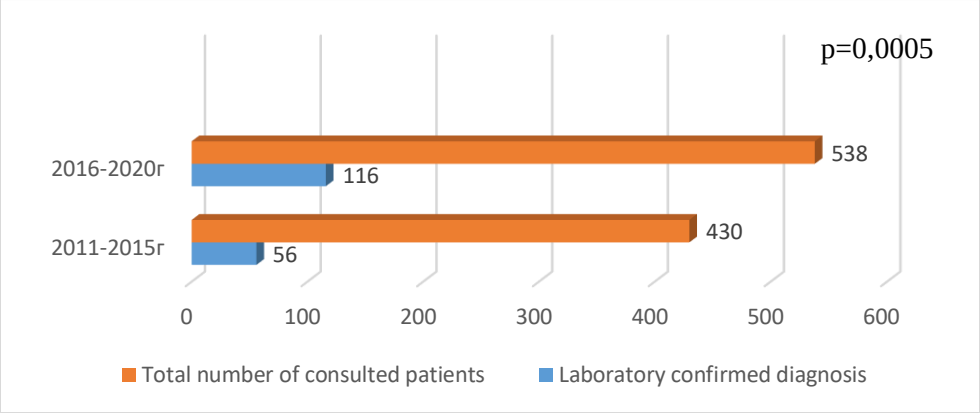

Figure 23. Distribution of counselled patients by established diagnoses for the first and second half of the analysed period

Conventional cytogenetic analysis on lymphocyte cultures revealed 50 individuals (7.5% of 667 examined) with single or predominantly multiple CAs/unclear dysmorphic syndrome, with or without ID.

Molecular genetic screening for FRAXA syndrome revealed 6 children (5.7% of 105 selected patients) with a DD, autistic spectrum behaviour, and rarely with characteristic dysmorphism.

MLPA analysis for frequent microdeletion/microduplication syndromes and/or subtelomeric aberrations revealed, confirmed or clarified 12 individuals (13.9% of 86 selected children), referred for multiple congenital anomalies or DD.

Molecular karyotype microarray analysis of 44 individuals revealed 8 patients (24.2% of 33 with a normal cytogenetic result) and helped clarify a chromosomal or MLPA finding in another 11 patients with multiple congenital anomalies, with or without DD/ID.

Molecular genetic studies, mainly DNA sequencing, were performed on 167 individuals (17.1% of those actively consulted). A genetic diagnosis was detected or confirmed in 89 (53.3%) individuals, mean age of 5.4 years, all cases with rare genetic diseases. The highest detection rate (60.7%) was in patients from group B – suspected monogenic (non-metabolic) disease, including hereditary

cancer syndrome, followed by patients from group D (28.1%) – single and multiple CAs/unclear dysmorphic syndrome, with or without ID. Neurological/neuromuscular symptoms were predominant (32.6% of the pathology). By the mechanism of occurrence and mode of inheritance, autosomal recessive conditions prevailed (41.6%), followed by autosomal dominant (33.7%) and X-linked (7.9 %). An advantage of the methods was the diagnosis of mitochondrial diseases (2.2% of pathologies), as well as those due to impaired imprinting (14.6% of pathologies).

Selective metabolic/enzymatic analysis was conducted in 126 patients (13.0% of those actively consulted), mostly (73.0%) in neonatal and early childhood ages 0 – 3 years. The metabolic disease was diagnosed in 14 (11.1% of the studied patients), and another 12 irrelevant metabolic abnormalities were found.

Regarding the *financing* of the performed laboratory cytogenetic and metabolic/enzymatic tests for the consulted patients: 92% (733 out of a total of 793 tests performed) of the tests were covered by state subsidy (within hospitalisation). This share is significantly smaller in the case of molecular genetic studies – the percentage of state-subsidised patients (within a hospitalisation period or financed by the Children's Treatment Fund at the Ministry of Health) was 16.7% (67 out of 402 conducted), and another 47.8% (192 out of 402) were examined as part of the research project – either at home or abroad. State-of-the-art high-resolution genomic analyses by next-generation sequencing (NGS) were primarily privately funded – 63.0 % (34 out of 54 individuals surveyed). These facts are established by the analysis of performed tests by years. The analyses reveal a 5-fold *statistically significant* increase of patients undergoing molecular genetic studies, mainly DNA sequencing, for the last three years compared to the first three years of the reporting period (see Figure 27). The vast majority of them – microarray analysis (74%), were conducted in the last 3 years.

5. DISCUSSION

5.1. Discussion of the results of descriptive epidemiological characteristics of the studied contingent

Descriptive epidemiological characteristics of all registered patients

The number of conducted MGCs and/or referred samples/childhood patients to LMGV for analysis has been increasing over the years. The increase has been almost three times greater in the last two years compared to the first two of the analysed period (Fig. 1). The trend is in correlation to the increase in the number and types of analyses available to patients and the popularisation of the speciality. Many authors report growing demand for genetic services worldwide (*Riesgraf et al., 2015; Stoll et al., 2018; Jacobs et al., 2020; Jenkins et al., 2021*). Schmidtke et al. reported a 70% increase in genetic counselling in Germany in 2019 compared to 2009 (*Schmidtke et al., 2020*).

Regarding the referral sources for genetic services for the children's contingent, it is logical that the significantly larger share of patients (82%) are referred by the Children's Clinics of the University Hospital, where the medical-genetic office is based. A study by Diamonstein et al. among physicians in Texas showed that physicians working at University Hospitals were twice as likely to refer patients to GC compared to other specialists (*Diamonstein et al., 2018*).

About 9.1% of the individuals in our study were referred for MGC/genetic tests by outpatient specialists, mostly paediatricians with a speciality as endocrinologists, gastroenterologists, neurologists, etc. General practitioners are also included in this referral group, but the share of those referred by them is minimal (0.7%). An explanation may be that they refer patients with a possible genetic disease to specialists, according to the leading clinical manifestation or for hospitalisation. Thus, indirectly, the patients come to the LMGV's attention. Physicians' insufficient awareness of genetic diseases and research is an essential referral factor. A study by *Klitzman et al.* among 220 internists in the United States indicated that over 80% needed training regarding genetic testing and indications for referral to a genetic specialist (*Klitzman et al., 2013*). In another study, again in the United States, the main barriers to referrals for genetic counselling/testing was physicians' uncertainty about when it is needed and patient's cost concerns (*Truong et al., 2021*). In our country, the Genetic Counselling service is not covered by the National

Health Insurance Fund. Thus, outpatient specialists and general practitioners cannot provide a free referral for MGC.

In 4% of the cases, the parents/relatives independently decide to carry out MGC and/or genetic testing, most often on the occasion of a positive family history of genetic pathology. A slightly lower share (1.7%) of self-referred individuals is reported by *Kovacheva et al.* in a study of the counselling activity of the Medical Genetics Center in Pleven with pediatric patients for three years –1994 –1996 (*Kovacheva et al., 1998*). The number of self-referred patients for genetic counselling/testing will likely increase over the years, given the widespread availability of information on the internet about rare diseases, including symptoms, diagnostic options, and the increasing accessibility of genetic services. According to Bleyer et al., some parents of children with undiagnosed rare diseases seek ways to reach an accurate diagnosis themselves due to the lack of interest, incompetence or overwork of their paediatricians (*Bleyer et al., 2020*).

Categorising pediatric patients by indication for MGC and/or genetic analysis is complex, given the enormous clinical heterogeneity of childhood hereditary conditions and organisational-diagnostic algorithms. Therefore, the division into 6 main groups is rather conditional.

The largest number of individuals is in **group I** – 971 (31.1%) – *probable chromosomal disease*. This group includes patients suspected of a recognisable autosomal or gonosomal (including sexual development disorder) chromosomal disease and a large number of individuals with growth abnormalities (mainly short stature – subgroup Ic) without accompanying symptoms/anomalies. Since 2014, they have been referred mainly by paediatric endocrinologists to a hospital for CA due to a requirement of the modern consensus guidelines for initial diagnosis in children with short/tall body height (Fig. 4)

Group II includes patients with a probable *monogenic (non-metabolic) disease*, including *hereditary cancer syndrome* – 517 (16.5%), based on specific clinical phenotype, meeting certain diagnostic criteria, laboratory and imaging results, and positive family history. One *subgroup* consists of individuals with a clinical diagnosis/suspicion of relatively more familiar and known monogenic diseases – *cystic fibrosis and beta-thalassemia*. They are mainly referred by paediatric clinics for assistance in carrying out genetic analyses. The *second subgroup* is individuals with leading *neurological/neuromuscular symptoms* and suspected monogenic disease (including mitochondrial disease). The collaboration with children's clinical neurologists and pre-hospital

specialists is extremely good. They often seek our opinion and assistance in cases of suspected genetic pathology (patients referred by them are categorised into the other groups of indications as well – suspected IEM, CNS anomalies, MCA/unclear dysmorphic syndrome, NPD disorders). A *third subgroup* included patients with a clinical diagnosis of an *identifiable monogenic disease/syndrome*. The variety is huge: connective tissue and skeletal diseases (Achondroplasia, Osteogenesis imperfecta, Marfan syndrome, Ehlers-Danlos syndrome, etc.); cardiovascular diseases (Hypertrophic cardiomyopathy, Andersen-Tawil syndrome, Holt-Oram syndrome), excretory system diseases (autosomal dominant renal polycystosis, Alport syndrome), vision (Retinitis pigmentosa, Familial blepharophimosis), hearing (Congenital deafness), skin (Congenital ichthyosis, Epidermolysis bullosa, Incontinentio pigmenti), haematological diseases (Fanconi anaemia, Haemophilia), hereditary cancer syndromes and predispositions (Neurofibromatosis type 1, Tuberous sclerosis, Peutz-Jeghers syndrome) as well as relatively well-known malformations monogenic syndromes such as Noonan syndrome, Bardet-Biedl syndrome, Cornelia de Lange syndrome and others. All this diversity of referral diagnoses shows the extreme clinical heterogeneity of childhood genetic diseases affecting different organs and systems.

Expectedly large – 745 (23.8%) is the proportion of referred **group IV** patients with a leading clinical picture of single or multiple congenital anomalies/unclear dysmorphic syndrome, with or without ID. Excluding the subgroup of those with apparently single congenital disabilities, most of the remaining patients have an unclear combination of multiple anomalies or dysmorphism. The collaboration with children's endocrinologists at the hospital is very good. In addition to being the main referring specialists for patients with rare diseases/syndromes, the endocrinologists are also excellent specialists in their clinical diagnosis, treatment and follow-up. Since 2016, an Expert Center for Rare Endocrine Diseases has operated at St. Marina University Hospital – Varna, part of the European Network for Rare Endocrine Diseases (Endo ERN). The centre has a multidisciplinary team and is specialised and certified in diagnosing, treating, and following patients with sexual differentiation disorders and congenital hyperinsulinism. In addition to these diseases, the team also specialises in other rare diseases, such as Turner, Noonan, Silver-Russel, Prader-Willi syndrome and others. With the centre launch, the number of persons with rare diseases referred to MGC/genetic testing increased in the last three years of the reporting period.

In our study, 473 (15.1%) of all referred patients fall into **group V** – individuals with a leading clinical phenotype of a neuropsychiatric disorder (delay, ID, behavioural disorders – autism spectrum disorders). These are predominantly boys (83%), with most of these patients being referred for cytogenetic analysis and/or molecular genetic screening/diagnosis for fragile X syndrome. Impairment in NPD/ID and/or autism spectrum disorder are common indications for paediatric patients' referral for MGC/genetic testing (*Pletcher et al., 2007*). Their number may be underestimated because not enough individuals with these problems still have access to genetic services (*Savatt and Myers, 2021*).

Descriptive epidemiological characteristics of the selected group of patients

In this group, the emphasis is on counselling patients. It involves the active participation of a genetic counsellor in determining the genetic diagnosis of an unclear or unconfirmed disorder/syndrome. The number of conducted MGCs has increased over the years, correspondingly to the increase in the general group. However, according to the trend model (Fig. 6), a smooth increase in this number has been predicted, probably due to the pandemic in the last year of the study period. Nevertheless, the expectations are for increasing demand and provision of genetic services (especially NGS technologies) (*Lalonde et al., 2020*), providing precise and detailed genotype-phenotype correlation analysis through genetic counselling.

Indications for referral in the selected group of individuals overlap to a large extent with the indications of the general group. However, the number of patients in group A is significantly smaller compared to the corresponding group I. For growth disorder (short/tall body height without accompanying symptoms/anomalies) or a disorder in sexual development, only 55 of the total registered 565 individuals with height deviations and 406 persons with suspected chromosomal disease were **actively** counselled. The explanation lies in the criteria for forming a selected group of patients, which did not include those with identifiable chromosomal diseases and growth disorders, referred mainly for cytogenetic analysis.

The most frequent indication for genetic counselling is the suspicion of unclear dysmorphic syndrome/MCA with or without MR – almost 50% of all referrals. The trend of a constant increase in the number of patients in this group is expected to be maintained due to the surge of options for molecular karyotyping analysis or other methods with high-resolution capabilities.

5.2. Discussion of the results of conducted genetic and metabolic studies

5.2.1. Conventional cytogenetic analysis

Cytogenetic analysis is a primary method for diagnosing diseases and conditions related to chromosome disorders and is one of the main activities of the Laboratory of Medical Genetics – Varna. A clear trend towards an increase in the number of conducted studies is observed (Fig. 8). Considering the constant development and introduction of modern innovative technologies, the expectation is that the trend will change without being reversed.

Regarding the detected cytogenetic pathology of 13.9% (281 of 2026 examined), the rate is similar but slightly lower compared to similar studies among this age group ranging from 16.6 to 17.6% (*Navsaria et al., 1993; Rauch et al., 2006; Grozdanova et al., 2017*) and significantly lower than other pathology reports – from 39 to 50.6% (*Thillainathan et al., 2015; Uwineza et al., 2016*). The disparity may be due to the patient selection criteria in the cited studies, which included childhood individuals with a very likely chromosomal disease, in contrast to the patients studied by us, who were not narrowly selected. The above explains the high percentage of detected pathology in patients from referral groups Ia and Ib (probable autosomal and gonosomal ChD) – a total of 65.8% (Table 1), compared to other groups with a lower plausibility of ChD.

The most frequently diagnosed chromosomal disorder in our study was trisomy 21 (Down syndrome) – 50% of the detected chromosomal pathology. The same proportion of T21 was reported by Grozdanova et al. in a 10-year study among cytogenetically examined Bulgarian children (*Grozdanova et al., 2017*), as well as by other authors (*Dayakar et al., 2010; Mao et al., 2015; Thillainathan et al., 2015*). In other cytogenetic studies among paediatric patients with MCA/MR, this rate was higher and ranged from 62 to 78% (*Khalid et al., 2018; El-Attar et al., 2021; Mohammed et al., 2011; Uwineza et al., 2016*).

Cytogenetic analysis has established itself as an essential first step in the general genetic testing plan in the diagnostic algorithm for conditions associated with sexual development disorders. It is noteworthy that the relatively small number of cases with sex chromosomes aberrations identified by us – 41 (14.5%), compared to the autosomal ones (Fig. 11). This low frequency, also reported by other authors (*Mao et al., 2015, Uwineza et al., 2016*), is probably due to the fact that such patients are less phenotypically manifested, compared to those with autosomal disorders; usually,

they seek counselling at a later stage on account of delayed puberty or even infertility. The gender ratio showed a statistically significant three times more frequent detection of these disorders in girls compared to boys, a ratio also reported by Mao et al. in 183 diagnosed paediatric patients with sex chromosome abnormalities (*Mao et al., 2015*).

Our data confirm the results of studies showing that, despite the high population frequency of Klinefelter syndrome (1:500 – 1:1000 males), only 25% of the cases are diagnosed, mainly in elderly persons, because of infertility and less often during puberty (*Bonomi et al., 2017*). The most frequently detected gonosomal chromosomal disease in our study was Turner's syndrome – 23 girls (8.2%); a frequency comparable to that reported in the literature – from 2% to 20% among cytogenetically examined paediatric patients (*Mao et al., 2015; Uwineza et al., 2016; Thillainathan et al., 2015*). Early diagnosis is crucial for timely treatment with recombinant growth hormone to improve final height and sex hormone replacement therapy, as well as for comprehensive follow-up (*Gravholt et al., 2019*). Rankova et al. reported their observations about treatment with recombinant growth hormone in girls with Turner syndrome: the problem is still insufficient recognition of the condition by general practitioners and paediatricians and, in most cases, late referral to endocrinologists for treatment (*Rankova et al., 2021*).

Structural autosomal chromosomal disorders were found in 76 individuals (27.0% of pathologies). Included in this number are 40 (14.2%) cases with proven clinical significance, 26 (9.3%) with unclear impact of conditionally balanced rearrangement, and 10 (3.5%) cases with unclear/controversial phenotypic impact of marker chromosomes. According to literature data, our values are close to those reported by Mao et al. (26.7%) and higher than other studies reporting a frequency of structural autosomal rearrangements of 10% (*El-Attar et al., 2021*), 18 .5 – 19% (*Duarte et al., 2004; Khalid et al., 2018*) to 21.4-23% (*Mohammed et al., 2011; Dayakar et al., 2010*).

5.2.2. Results of DNA analysis for monogenic diseases, hereditary predispositions to diseases, pharmacogenetic defects

Cystic fibrosis is one of the most common, severe autosomal recessive diseases for patients of the white race. Studies for Bulgaria show that 1/33 are carriers of a pathogenic variant in the CFTR gene, and the frequency of those affected is about 1:3600 (*Savov, 2011*). Our results show that the most frequently detected variant in the CFTR gene in the confirmed individuals is delF508 (76.2%)

(Fig. 13). A similar but slightly higher percentage (83%) was reported by Savov, with no age limit (*Savov, 2011*). A study by Petrova et al. showed a lower percentage of delF508 (55%) among 140 studied Bulgarian patients, at the expense of those revealed by NGS in search for rare variants (*Petrova et al., 2019*). In 2019, an Expert centre on cystic fibrosis at St. Marina University Hospital – Varna was created. An increase in the coverage of CF patients from North-eastern Bulgaria is expected. The centre functions at the Second Children's Clinic at St. Marina University Hospital – Varna.

Counselling and the organisation of patients' testing for *Wilson's disease* are some of the priorities of the LMGV (launched in the distant 1997), motivated by the possibility of conducting effective therapy. For the study period 2011 – 2020, the diagnosis was confirmed in 23% of the examined children's patients. The *H1069Q* mutation was found in 90% of the established homo- and heterozygotes. This is the most frequently detected pathogenic variant among Bulgarian patients (*Todorov et al., 2005; Mihaylova et al., 2012*), as well as in patients from Central and Eastern Europe (*Medici et al., 2019*).

The number of counselled and examined childhood patients with suspicion for *beta-thalassemia* for the study period is relatively small – 24, despite the high frequency for Bulgaria of this otherwise rare disease (carrier of the gene for beta-thalassemia in the Bulgarian population is 2.4 – 2.5%). Pathogenic variants – homo- and heterozygotes, were revealed in 20 (83.3%) of the 24 individuals who underwent genetic analysis for beta-thalassemia. This is probably due to the precise selection based on the clinical picture, laboratory tests, data from electrophoresis and family history of the referred individuals. However, first-degree relatives needing genetic testing/MGC remain out of sight for the clinicians, which has important implications for reproductive risk in the family.

A total of 219 individuals referred to their paediatricians were examined for hereditary predispositions to disorders with multifactorial aetiology (Gilbert's syndrome, celiac disease and thrombophilia).

Gilbert's syndrome is a benign condition, the most commonly inherited disorder of bilirubin metabolism, characterised by recurrent episodes of jaundice. The data show that half (50%) of the tests for Gilbert syndrome for the reporting period are from the last 2 years, which is probably also related to the launch of molecular genetic analysis in LMGV in 2019 (*Georgieva et al., 2021*). The

high percentage of genetically confirmed individuals (73.9%) can be explained by the high frequency of this condition affecting 5 – 10% of the population (*Wagner et al., 2018*), as well as the well-selected individuals for the study.

Celiac disease (gluten enteropathy) is also a common multifactorial condition, manifesting as a chronic multisystem immune-mediated disease upon consumption of gluten-containing food products in genetically predisposed individuals. It has been found to affect around 1% of the world's population (*Ludvigsson et al., 2019*). Studies show that the greater part of the affected population develops symptoms before age 10 (*Lebwohl et al., 2021*). Class II human leukocyte antigen (HLA) genes known as HLA-DQ2 and DQ8 are the best studied and primarily involved in the genetic predisposition. The high percentage of detected patients at high risk for celiac disease in our study (66.2% of those examined) is related to the well-selected individuals for the study, based on characteristic symptoms, positive immunological markers and family history. Genetic analysis for celiac disease predisposition started in 2018 at the LMGV. A campaign with the possibility of free genetic tests made it possible to popularise the condition (*Georgieva et al., 2018*).

Testing for *hereditary thrombophilia* in paediatric patients with vascular events is recommended, especially for high-risk secondary thromboprophylaxis (*van Ommen, 2017*). For the analysed 10-year period, 31 patients were examined, and in 6 (19.3%), all with venous thrombosis, a high risk for thrombophilia (heterozygous genotype for the R506Q polymorphism in the Factor V gene) was found.

5.2.3. Discussion of the diagnostic activity results (laboratory and counselling) in a selected group of patients with genetically unspecified diseases

5.2.3.1. Laboratory activity performed at LMGV

Cytogenetic analysis (see 5.2.1.)

Molecular genetic analysis for FRAXA syndrome

Fragile X syndrome is the most commonly inherited cause of ID and the leading monogenic cause of autism (*Lozano et al., 2016*). As clinical symptoms are often nonspecific, testing for a mutation

in the FMR1 gene is usually part of the routine genetic evaluation in cases of children, mainly boys, with developmental delay, ID and/or behavioural problems (*Spector et al., 2021*).

The frequency of patients with proven FRAXA syndrome found in our study – 5.7% (6 out of 105 studied) is in line with the literature data reported for individuals with developmental delay: 2 – 9% (*Ciaccio et al., 2017; Rzońca et al., 2016; Dean et al., 2019*). Similar results were also reported by L. Angelova in a population-genetic study of 290 school-age Bulgarian patients with ID, using a cytogenetic method (*Angelova, 1994*). The author reported a 5.9% incidence of the syndrome (5 boys and 1 sibling girl) in a clinically selected group of 76 boys and 25 girls. A higher percentage (12.5%) of patients with FRAXA syndrome diagnosed by molecular genetic analysis was described by Todorov et al., but in a selected group of 32 boys with a clinical diagnosis of Fragile X syndrome (*Todorov et al., 2010*).

The intensive research and development of a therapy for this syndrome raise the question of popularising the screening approach for boys with developmental delay/ID, mainly speech delay, outside the broader clinical spectrum. Both sexes show a wide range of learning disabilities in the context of normal, borderline IQ or mild to severe intellectual deficit (*Ciaccio et al., 2017*). In our study, all three diagnosed boys had developmental delay, especially speech, emerging as a pathognomonic feature of the syndrome, together with mild (case 2, 3) to moderate ID (case 1) and hyperactivity. Autistic behaviour, attention deficit (case 1 and 3), aggression and autoaggression (case 3) were also observed. Autism spectrum disorders, assessed by some authors (*Ciaccio et al., 2017; Kaufmann et al., 2017*) as a comorbidity of the disease, are found in 30 – 50% of males and 25% of females with this syndrome.

In the era of FRAXA molecular diagnostics, the syndrome is still difficult to recognise and diagnose, possibly due to the lack of an obvious phenotype at birth and the presence of discrete manifestations during the prepubertal period. Results from various studies show a mean age of diagnosis between 3 and 6.3 years (*Rzońca et al., 2016; Cotter et al., 2016; Bailey et al., 2009*). The mean age at diagnosis in our study was 9.3 years, older than in other studies, and this may be partly explained by the lack of molecular diagnostics for FRAXA performed routinely and reimbursed by the state. Diagnosis delays reduce the chance of early intervention and access to family support programs.

MLPA analysis for microdeletion/duplication syndromes

Copy number variations (CNVs), also known as microdeletions and microduplications, represent a common cause of DD/ID/MCA disorder (*Ceroni et al., 2018*). One of the molecular genetic methods used to prove such disorders is MLPA – a fast, sensitive, specific and reliable screening method suitable for routine diagnosis.

The established rate of detected diagnoses by MLPA in our patients – 13.9% (12 diagnosed out of a total of 86 examined over the 10-year period) correlates with other studies ranging from 5 to 23% (*Damnjanovic et al., 2015; Santa María et al., 2016; Ceroni et al., 2018; Miclea et al., 2021*), but the results are highly dependent on the selection criteria, the number of patients, number and types of probes used in MLPA kits. The selective MLPA screening carried out in the LMGV in prospectively and retrospectively selected 54 childhood individuals with an unclear reason for DD/MCA/ID and established a genetic diagnosis in 5 of them (9.3%), with an average age of 1.6 years (*Stoyanova et al., 2020*).

The extreme clinical and genetic heterogeneity of microdeletion syndromes makes them a diagnostic challenge, both for recognising the specific phenotype and for the application of adequate genetic testing. Optimum results could be achieved by initially targeting a specific nosological entity or group of conditions with similar pathogenesis and known molecular aetiology. As part of the multidisciplinary team, the genetic counsellor is expected to facilitate the effective implementation of genetic analysis (*Patch et al., 2018*). For example, these cases are confirmed with MLPA analysis Prader Willi syndrome, Wolf Hirschhorn syndrome and DiGeorge syndrome. The detected deletion in 1q44 and duplication in the 14q11.2 region are findings unrelated to known common microdeletion/microduplication syndromes. The overlap of the clinical picture in our patients and those reported in the literature provides grounds to accept the rearrangement as a probable pathogenetic cause (*www.orpha.net; Shelby et al., 2021*).

The microarray technique is a suitable method for confirming/rejecting detected deviations and specifying the involved gene region in a deleted/duplicated region (genotype-phenotype correlation), with the possibility of whole genome analysis. All five cases of MLPA disorders identified by us were validated by an independent technique (mainly microarray analysis).

5.2.3.2. Laboratory activity performed outside the LMGV structures

Microarray analysis

Microarray-based methods allow whole-genome screening combined with high resolution, which is why for the last several years, they have been recommended as the first diagnostic test in patients with unexplained developmental delay, ID, autism or MCA, instead of standard karyotyping (*Miller et al., 2010*).

In our study, this type of analysis was applied as a second or even third diagnostic (validation) method after conventional CA and, in several cases, after MLPA analysis. A microchip-based analysis was performed on only 44 (4.5% of the 968 actively consulted individuals). Pathogenic variants were found in 19 children (43.2% of the examined), including both newly discovered and validating cases.

Among the individuals with an unclear cause of MCA/dysmorphism/ ID (total 33), pathogenic variants were found in 8 (24.2%). This incidence is consistent with that reported in the literature, ranging from 13% to about 27% among children with unexplained ID with or without MCA/dysmorphism (*Perez et al., 2017; Lee, 2018; Ceroni et al., 2018; Pinheiro et al., 2020; Liu et al., 2022, Perovic et al., 2022*). For Bulgaria, studies among children with ID and congenital anomalies using array CGH were conducted by *Hadzydekova (2011), Stoeva (2012), Mihailova (2017)*, with a reported frequency of microstructural chromosomal aberrations ranging from 13.2% to 17.3%.

Due to its high diagnostic efficiency and the increasingly easy access to microarray analysis, it should also be applied in our country as the method of first choice in patients with unexplained ID/MCA.

Other molecular genetic studies

Because not all current molecular genetic analyses (besides MLPA for microdeletion/microduplication syndromes and DNA analysis for FRAXA screening) of many rare conditions are performed in the LMGV, the counselling activity is applied along with active collaboration with external laboratories and sending samples.

The number of examined patients in this category – 167 out of 968 (17.3%) is relatively small. For 65 individuals, the analyses were carried out thanks to a state subsidy or a foreign research project. Contact with the relevant laboratories/researchers was conducted by a genetic consultant – the doctoral student and associates. As a result of such collaboration, patients with suspected RAS-opathies, imprinting diseases, autosomal dominant polycystic kidney disease, rare and ultra-rare diseases such as Beare-Stevenson cutis gyrate syndrome, Hutchinson-Gilford progeria, Cartilage-hair hypoplasia, Dent's disease, Lowe syndrome, etc. have been examined and diagnosed.

Not surprisingly, the majority (> 50%) of these molecular genetic studies were conducted in the last 3 years of the reporting period, with a fivefold increase in the number of patients covered compared to the first 3 reporting years (Fig. 17). The increased application next-generation sequencing (NGS) – 45 investigated (> 80% share) for the last 3 years is particularly evident. Although they were developed and introduced about 15 years ago, their use by Bulgarian patients remains limited; self-financing is still common for patients' families.

The advantage of NGS technologies, characterised by speed, higher sensitivity, resolution and comparably lower costs compared to classical Sanger sequencing of individual genes, is indisputable (*Kulski et al., 2016*). A study by Stark et al. among 80 selected individuals with suspected monogenic pathology aged 0 – 2 years reported a significantly higher diagnostic success rate (57.5%) when WES was performed compared to classical or targeted sequencing or methylation analysis (13.7%) (*Stark et al., 2016*). However, each clinical case is individual and requires knowledge and experience in the advantages and disadvantages of the first and second-generation sequencing and indications for the application of these high-tech options (WGS, WES, CES, etc.). In this regard, good collaboration with laboratory genetics specialists is critical.

Achieving a definitive genetic diagnosis in patients with rare/ultra-rare genetic diseases can be a lengthy and difficult process; patients often undergo the so-called 'diagnostic odyssey' (*Macnamara et al., 2019*). With the application of NGS technologies, there is a high probability of significantly shortening this process (*Abou et al., 2016*). Still, both in our country and in highly developed countries, the application of high-resolution analyses in patients with suspected genetic disease is not a routinely applied approach (*Strande et Berg, 2016*). This is due to not only financial but also analytical limitations. There are difficulties in interpreting a result with bioinformatics data for

variants of uncertain significance (VUS), reporting of incidental or secondary findings when performing WES or WGS, and repeated DNA sequences that cannot be reliably detected.

The high percentage of revealed/confirmed pathology (53.3%) in 89 out of 167 examined can be explained by the good processing of the clinical phenotype of the patients and the correct selection of the most appropriate genetic method. In cases of unclear diagnosis, the choice approach involves whole-exome (WES) or clinical exome (CES) sequencing. In our study, the detection rate from the application of these analyses amounted to 29.4%, which corresponds to the literature data, despite the incomparably smaller number of patients in our study. A large number of studies using WES or WGS in childhood patients with a suspected genetic disease and different clinical manifestations have reported diagnostic success rates of 25% to 49% (*Stark et al., 2016; Tan et al., 2017; Costain et al., 2020; Zhang et al., 2021*). In Bulgaria, research on NGS in patients of different ages with rare diseases and suspected monogenic disease was conducted by Vazharova and reported a high percentage of detected genetic causes – 48% (21 out of 43 examined) (*Vazharova, 2016*).

Of interest is the significant number of genetically diagnosed patients with rare and ultra-rare dysmorphic syndromes (23.6%) and imprinting diseases (14.6%) (Table 6). Some malformation syndromes are suspected and diagnosed already in the neonatal period (Beare-Stevenson cutis gyrate syndrome, Nijmegen breakage syndrome, etc.) or in infancy and early childhood (Fanconi anaemia, Cartilage-hair hypoplasia syndrome, Greig cephalopolysyndactyly syndrome) due to specific phenotype, laboratory/imaging studies. Other patients received a clear genetic diagnosis at 12 – 13 years of age (Kabuki syndrome, Mowat-Wilson syndrome, Osteoporosis – pseudoglioma syndrome) or later - at 16 – 17 years of age (LEOPARD syndrome, Cardio-facio-cutaneous syndrome). Achieving a definitive genetic diagnosis (if possible) in such patients is often a lengthy and difficult task due to a nonspecific phenotype or a wide differential diagnosis among hundreds of rare genetic disorders.

A significant contribution of modern molecular genetic methods (Methylation-Specific PCR, MLPA, etc.) is the diagnosis of 13 patients (14.6% of the pathology) with imprinting disorders. The majority of confirmed cases are the result of collaboration with a genetic centre abroad. The excellency of the paediatric endocrinologists from the Varna Expert Center for Rare Diseases should be noted for the very well clinically evaluated and selected patients.

Selective metabolic/enzymatic analysis

Selective metabolic screening and/or enzyme analysis was performed in 126 childhood patients due to suspicion of a disease from the Inborn Errors of Metabolism group. Although almost all inborn errors of metabolism belong to the group of rare or ultra-rare diseases, their number exceeds 1600 according to the most complete database Inborn Errors of Metabolism Knowledgebase (www.IEMbase.org). Hence, their overall frequency is high – 1/500 newborns.

14 out of 126 examined (11.1%) were detected and confirmed as individuals with IEM as a result of a selective metabolic/enzyme analysis conducted in our study (Table 7). Considering that some of them are treatable diseases, their early diagnosis is essential. A study by *Hoytema van Konijnenburg et al*. shows that the number of treatable metabolic diseases manifesting mainly with developmental delay/MR is about 116 and has increased by 1/3 in the last eight years (*Hoytema van Konijnenburg et al., 2021*). The inclusion of such diseases in massive neonatal screening programs in different countries aims at timely diagnosis and treatment. In European countries, the number of investigated IEM varies between 0 and 30 (*Loeber et al., 2021*). In Bulgaria, only 2 diseases are covered – phenylketonuria and congenital adrenal hyperplasia, and there is currently a debate about its expansion through a new program.

5.2.3.3. Discussion of the results of dysmorphology programs in the genetic counselling activity

Over the 10-year period, *facial* dysmorphism was reported in more than half of the actively consulted individuals (**520; 53.7%**). Apart from the laboratory approaches for etiological clarification, a database of specialised dysmorphology programs also assists the genetic counsellor. An important first step in genetic counselling is a detailed collection of information about the family history, personal history of the child, a physical examination and a description of the abnormalities, including their photo documentation. Patient imaging is organised into files with the patient's name and suspected or confirmed diagnosis. Photography (with parental consent) is integral to the counselling process for patients with dysmorphisnm. This makes it possible to share the photos with other specialists at home and abroad (in cases of pronounced dysmorphism) for diagnostic clarification, as well as the use of online-based dysmorphology applications for the analysis of facial dysmorphisms such as *Face2Gene*.

The application of *Face2Gene* in our practice started after 2015 and became a mandatory stage in counselling patients (not only in childhood) with dysmorphism. In addition to placing a photo to search for facial overlap/resemblance with dysmorphic conditions, the application allows the input of the patient's established anomalies and other clinical manifestations, as well as anthropometric indicators. *Face2Gene* also serves as a registry of dysmorphic patients by entering names, date of birth, date of visit, and confirmed genetic or clinical diagnosis.

In most cases, Face2Gene did not lead us to a specific diagnosis. However, in 33.2% of the cases, a genetic or clinical diagnosis was confirmed: 71 out of 214 patient profiles were used in the database for 2016 – 2020. For the most part – 71.8% (51 of these 71) of the diagnoses fall into the first ten program proposals. Our application's 71.8% success rate was lower than that of Gurovich et al., which amounted to a 91% "*top-10 accuracy*" in detecting over 215 different genetic syndromes (*Gurovich et al., 2019*). The lower detectability in our patients may be related to the fact that some of the established diagnoses are rare microstructural disorders or ultra-rare dysmorphic syndromes for which there is insufficient data in the application. However, as Dolgin commented, *Face2Gene* is a publicly available app for healthcare professionals to diagnose rare genetic diseases by simultaneously uploading patients' photos and information, supplementing the data and increasing the app's accuracy (*Dolgin, 2019*).

It is estimated that about 30 – 40% of the rare diseases are presented with facial-cranial anomalies, which shows the critical role of the dysmorphologist. At the same time, according to Hurst, the dysmorphologist bares the difficult task of channelling the anthropomorphic knowledge, and the vocabulary used to achieve an accurate diagnosis (*Hurst, 2018*). According to Gripp et al., data from the analysis of facial dysmorphic marks can complement the summary of the clinical phenotype and provide information beyond the personal experience of the consulting specialist/dysmorphologist (*Gripp et al., 2016*). Better phenotyping in patients with dysmorphism enables better genomic interpretation, especially when performing WES/WGS. In the near future, laboratories might require photographs to accompany the written phenotypic description on requisition forms (*Hurst, 2018*). It is noteworthy that facial recognition software cannot replace the role of the dysmorphologist but rather improves the accuracy of the dysmorphology physical examination and provides an additional diagnostic resource.

6. CONCLUSION AND DIRECTIONS FOR FUTURE WORK

Medical-genetic counselling is essential in the service approach of the multidisciplinary diagnostic process for revealing the genetic aetiology of diseases and predispositions in a patient and his family. This is related both to the appointment and interpretation of genetic tests and to the counselling of relatives for their reproductive behaviour and genetic prevention.

Genetic counselling and testing of children is one of the main activities of the LMGV, with an established tendency towards a significant increase over the years in the number of performed MGCs and/or referred samples/patients for analysis. The Children's Clinics at the University Hospital (at the premises of which the medical-genetic office is based) have a leading place in regards to referrals for genetic services for the children's contingent. We report a low and very low share of referrals from the Child and adolescent psychiatry clinic and general practitioners (0.7%). This is mainly explained by their lack of awareness regarding the meaning and importance of genetic counselling and laboratory tests in clarifying the etiology of genetic diseases. The most frequent *clinical indication* for referral (mainly for cytogenetic analysis) for all registered individuals was the conditionally defined group "probable chromosomal disease with or without sexual development disorders", including the subgroup "growth abnormalities without concomitant symptoms".

The differentiation of a selected group of patients with a predominantly unclear/unconfirmed diagnosis referred for MGC was intended to distinguish it from predominantly laboratory work (in many cases without direct patient/family contact). A leading indication in these patients (almost 50%) was the suspicion of unclear dysmorphic syndrome/MCA with or without MR, associated with substantial diagnostic difficulties.

Of the laboratory genetic/metabolic studies, CA of lymphocyte cultures presented the largest share, which revealed pathology in 13.6% of all examined, with a predominance of autosomal recessive pathology (53.0%). In the group of actively consulted, the detected pathology was 7.5%, primarily autosomal structural disorders. The microarray analysis showed a high detection rate in individuals with an unclear diagnosis and MCA with or without an ID/DD (24.2%). Although the small number of patients studied, with one or more previous tests, this result reinforces the recommendations for its use as the method of choice. MLPA analysis also finds an important application, mainly as a screening method among individuals suspected of a recognisable microdeletion/microduplication

syndrome or with an unexplained developmental delay/MCA; its detection rate of the screening carried out by us was 9.3%. Under discussion is the need to popularise the molecular genetic screening approach – predominantly for boys with an DD/ID, even without the presence of other symptoms for FRAXA syndrome.

The number of examined patients with suspected *monogenic pathology* was small. However, the summarised result of the application of various molecular genetic methods contributed to the discovery/clarification of diagnoses in 53.3% (89 of 167 examined). Different types of molecular genetic methods have been applied, depending on the suspected disorder and the type of the most common mutations, primarily DNA sequencing (Sanger and NGS). The follow-up by years of the conducted molecular genetic analyses showed a statistically significant fivefold increase in the last three years compared to the first three of the analysed period, especially noticeable for sequencing through NGS technologies. Their application provides an opportunity to significantly shorten the diagnostic process for patients with rare/ultra-rare genetic diseases. Our results show a high diagnostic contribution (48.1%) of NGS technologies, mainly in suspected (less obscure) diseases, albeit in a limited number of individuals.

Most patients referred for MGC had an unclear or unspecified cause of MCA/dysmorphism/ID. A vital and frequently used diagnostic resource in the practice of medical geneticists was the software online-based programs and dysmorphology databases, with a particular contribution of the publicly available online application *Face2Gene*. It helped diagnose patients with rare and ultra-rare syndromes. The use of such facial recognition software for better phenotyping in patients with dysmorphism is increasingly being discussed, allowing better genomic interpretation, especially when performing WES/WGS.

To improve the approach in conducting a clinical genetic evaluation of a hereditary disease, it is necessary:

• More targeted information and education of psychiatrists, general practitioners, and paediatricians from pre-hospital care about referral indications, the possibilities and limitations of genetic testing and the benefit of genetic counselling for the index patient and the family in general.

• Efforts to extend the application and financial provision of modern genetic research methods in childhood patients with suspected genetic disease – *microarray analysis*, *MLPA* screening for

frequent microdeletion/microduplication syndromes and subtelomeric microaberrations, *screening for FRAXA, NGS.*

• The reported low share of patients with single congenital anomalies referred for MGC necessitates more active search and follow-up, as well as the creation of a *CAs registry for Varna and the region*.

• To support and participate in creating national registries for rare genetic diseases.

• To facilitate the access of patients from remote regions to the highly specialised genetic counselling activity, it is necessary to introduce into the routine practice of the genetic counselling centre telegenetics (genetic counselling at a distance via video conference connection).

• Improving and expanding contacts and collaborations with teams at home and abroad. An important objective will be the active use of the European Reference Networks (ERN), which will offer better opportunities and access for patients with rare diseases to experts to receive an accurate diagnosis and advice on the best treatment and management of their condition.

7. CONCLUSIONS

1. Regarding the **descriptive epidemiological data and indications**, it can be concluded that:

• The children's contingent makes up almost *half* (46.3%) of all individuals registered in the genetic centre. The trend is towards a substantial increase. Predominant is the *youngest age group* (0 – 6 years). One-third (31.0%) are patients with an unclear/unconfirmed clinical disorder necessitating an active diagnostic approach (laboratory and counselling) to establish a clinical and/or genetic diagnosis.

• The vast majority of patients are *referred* by two of the specialised paediatric clinics. The insufficient referrals from other clinics or outpatient specialists in paediatrics and general medicine indicate an unawareness of the importance and appreciation of the object of genetic care – the family, not only the index patient.

• The most common *clinical indication* for referral is "Probable chromosomal disease with or without sexual development abnormalities". In recent years, it has been replaced by "Growth abnormalities without associated symptoms", motivated by current consensus guidelines for initial

testing. A leading indication in patients requiring *diagnostic activity* is single or multiple CAs/with or without ID, which reflects the vast genetic heterogeneity and the difficulties in setting a syndromic genetic diagnosis.

2. The summary and analysis of genetic studies for evaluation of the **diagnostic laboratory** contribution in clarifying the aetiology of hereditary disorders in children showed that:

• *Conventional cytogenetic analysis* is the leading research method (64.9%), with a tendency to decrease on account of the incoming high-resolution genetic analysis methods. The detectability is adequate and statistically significant when a chromosomal disease is suspected but insignificant in patients with an DD/autism spectrum disorder or a growth disorder, which requires specifying the clinical indication with direct referral to other genetic testing methods.

• In *selective screening by DNA analysis* for known monogenic diseases, hereditary predispositions and pharmacogenetic defects, the largest share and diagnostic detection rate are in patients with suspected cystic fibrosis. There is an insufficient number of referrals for suspected beta-thalassemia, which affects the family-oriented character of genetic servicing. The large proportion of individuals detected as high risk for celiac disease and Gilbert's syndrome reinforces the importance of genetic testing for patients' preventive behaviour and family coverage.

• *Molecular-genetic methods* are applied according to the following indications:

- ✓ In unidentified DD/ID with dysmorphism, the application of microarray analysis is leading and should be applied as a method of first choice, followed by MLPA screening for frequent micro-deletion/duplication syndromes and subtelomeric microaberrations;
- ✓ In patient with DD/ASD (predominantly male) without dysmorphic phenotype, FRAXA screening is indicated;
- ✓ In suspected monogenic disease with an emphasis on neurological/neuromuscular symptoms, the application of *DNA sequencing* (primarily *diagnostic panels for exome sequencing*) is relevant;
- ✓ An unclear clinical diagnosis requires the application of *whole-exome* (WES) or *clinical exome* (CES) sequencing.

• *Selective metabolic screening/enzyme analysis* helps diagnose 11.1% of individuals with suspected metabolic disease, which is a resultative selective approach for patients of the youngest age group, with a significantly reduced panel for massive genetic screening of newborns for IEM.

3. **The overall assessment** of the 10-year medical genetic counselling activity in the multidisciplinary diagnostic process of revealing the genetic aetiology of diseases and predispositions in childhood patients amounts to **22.3%**, with the advantage of a *laboratory* (genetically and metabolically) confirmed the cause of the disease -– 17.2% patients over 5.1% with a *clinical* diagnosis (genetic disease/syndrome or single congenital anomaly). Chromosomal diseases account for the largest share of detected pathology (37.1%), followed by monogenic diseases (19.7%) and predispositions (19.3%).

4. **The genetic diagnostic activity** (laboratory and counselling) significantly *contribut*es to 34.2% of the selected group (10.6% of all covered) to build the genetic and/or clinical diagnosis in cases of an unclear/unconfirmed clinical disorder. The largest share of detectability accounts for patients suspected of *monogenic (non-metabolic) disease*, and the smallest – is for individuals with delayed NPD. Laboratory-made diagnoses prevail. The role of the MGC specialist is related not only to the guidance and interpretation of laboratory results but also to diagnosing rare dysmorphic syndromes using software online-based programs and dysmorphology database applications.

5. The guidelines for improving the approach for conducting a clinical genetic evaluation of a hereditary disease in the overall multidisciplinary care of the patient are related to the identification of a recognised need for:

• Increasing the activity, trust and referrals towards the genetic units, primarily from the pre-hospital network of specialists in paediatrics, psychiatry and general medicine, by improving their awareness of indications for referral, the importance of etiological clarification of the diagnosis in the index patient and involving the relatives in reproductive risk counselling discussions.

• Expanding the panel of laboratory options for presumptive clinical diagnoses through the application of modern high-resolution genetic methods as indicated by the genetic counsellor and the panel for metabolic disorders in the IEM screening program for newborns. Their application improves laboratory diagnostic options, despite the financial and, in some cases, analytical limitations of a routinely applied approach.

• The critical role of the genetic counsellor is also related to shortening the diagnostic search for patients with rare and ultra-rare dysmorphic syndromes. This enables an adequate MGC in the future reproductive plans of the family (and close relatives) and discussion on preventive medical approach options.

8. THESIS CONTRIBUTIONS

Original contributions

• For the first time in our country, systematised scientific-practical research on descriptive epidemiological characterisation and evaluation of the contribution of the overall activity of a genetic structure in service of a paediatric contingent of patients for an extended period of time is carried out and presented.

• The present work is the only published study in our country for evaluation of the medical genetic activity (laboratory and counselling) for establishing a genetic and/or clinical diagnosis of an unclear/unconfirmed clinical disorder in patients, specifically in childhood patients. The study results are the groundwork for comparative population studies and deliberate efforts in the field of medical care for patients with suspected genetic disorders in various epidemiological studies at a national and international level.

• The present work is one of the few studies in our country based on the diagnostic success rate of genetic laboratory methods and counselling tools to compare and derive the most effective indications for guidance when searching for the etiological genetic diagnosis.

Confirmatory contributions

• The leading role of laboratory diagnostics for etiological clarification in suspected genetic disease/disorder as an effective method for detecting chromosomal pathology in the prenatal diagnostic procedure has been confirmed.

• The need to upgrade the use of conventional cytogenetics with modern high-resolution molecular-genetic methods for diagnostics has been confirmed; the financial and logistical support of the university structures has been acknowledged.

• The importance of clinical phenotyping and selection of patients in the specialised paediatric clinics, genetic counselling offices and expert centres for rare diseases has been confirmed as a stipulation for diagnostic success.

Contributions of applied nature

• Methods for molecular genetic analysis (MLPA, molecular genetic screening for FRAXA, Real-time-PCR) were optimised. Dysmorphology database programs and applications were mastered at the Laboratory of Medical Genetics Varna to serve patients in need of genetic assistance from a broad region of the country.

• Guidelines for improving the awareness and the approach in conducting a clinical genetic evaluation of a hereditary disease, for clarification through laboratory tests as per indications (new generation sequencing, microchip analysis, etc.) and computer dysmorphology programs when serving patients in the medical genetic counselling centre.

9. PUBLICATIONS AND CONFERENCE PARTICIPATION ON THE SUBJECT OF THE THESIS

FULL-TEXT Scientific Publications

1. Stoyanova M., M. Tsvetkova, M. Levkova, V. Miteva, D. Yahya, V. Yotova, M. Georgieva, L. Angelova. Cytogenetic research in children – the experience of the Laboratory of Medical Genetics - Varna for a period of 10 years. Paediatrics, 2021, volume 63, issue 1, pages 31-34.

2. Stoyanova M, Hachmeriyan M, Levkova M, Bichev S, Georgieva M, Mladenov V, Angelova L. Molecular screening for fragile X syndrome in children with unexplained intellectual disability and/or autistic behaviour. Folia Medica. 2022 Feb 28;64(1):27-32.

3. Stoyanova M., L. Angelova, M. Levkova, M. Georgieva, V. Yotova. Practical aspects and effectiveness of genetic counselling in children with hereditary pathology. General medicine. XXIV, issue 1, 2022, 30-36.

Scientific announcements – conference reports, ABSTRACTS:

1. Stoyanova M., L. Angelova, M. Hachmeriyan, M. Levkova, V. Miteva. Single-gene pathology in paediatric patients referred for genetic counselling for a period of five years; European Human

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