REVIEW

by Prof. Albena Parvanova Todorova-Georgieva, Ph.D
Department of "Medical Chemistry and Biochemistry", Medical University-Sofia

REGARDING:

PhD thesis for awarding the scientific degree "Doctor (PhD)"
in scientific specialty "Genetics",
Professional field 4.3 . "Biological Sciences",
Higher education area 4 . "Natural Sciences, Mathematics and Informatics"

of Dr. Milena Petrova Stoyanova

on the topic: "Genetic Diagnostic Study among Pediatric Patients with Hereditary Pathology Who Received Genetic Counselling"

Brief biographical data

Dr. Milena Petrova Stoyanova was born in 1978 in the city of Yambol. She graduated with a degree in Medicine in 2002 at the Medical University - Varna, and in 2009 she was elected as an assistant professor in the educational scientific sector of Medical Genetics at the Department of Pediatrics and Medical Genetics, Medical University - Varna (since 2015, an independent Department of Medical Genetics). In 2010, Dr. Milena Stoyanova acquired a specialty in Pediatrics, and in 2018, a specialty in Medical Genetics. Dr. Milena Stoyanova was enrolled as a full-time PhD student by order No. R-109-430/16.07.2018. She has publications in Bulgarian and foreign scientific journals and has participated in 4 scientific projects. By order of the Rector of the Medical University - Varna No. P-109-329/ 01.08.2022. a date for public defense has been scheduled and the scientific jury for reviewing the dissertation work has been appointed.

The PhD thesis submitted to me for review is properly structured, according to the accepted standards for awarding the scientific degree "Doctor". The dissertation covers 149 pages, is illustrated with 34 figures and 17 tables, 246 literary sources are used, of which 25 are in Cyrillic and 221 are in English.

The introduction presents the social significance of hereditary diseases and congenital anomalies, which, although rare, often affect children and are associated with serious adverse effects for both children and their families, health systems and society. Attention is drawn to the importance of genetic testing and genetic counselling in incurable cases, where prenatal diagnosis is the only option to prevent genetic diseases. An accurate genetic diagnosis is also important for preparing the patient for new therapeutic approaches, which are already available, although the number of successfully treatable genetic diseases is still small.

The literature review begins with an overview, general characteristics and classification of hereditary and congenital pathology in childhood. The PhD student presents the genetic and non-genetic causes that can affect a child's growth, development and health. A detailed review of the types of pathologies is given: chromosomal (including copy number variations), genomic and monogenic disorders, mitochondrial diseases, genomic imprinting, uniparental disomy, types of mosaicism, dynamic mutations (including the phenomenon of anticipation), and multifactorial conditions. A substantial part is devoted to the epidemiology of hereditary diseases worldwide and in Bulgaria, citing the most significant Bulgarian works on the subject. The significance of childhood morbidity and mortality and the distribution of genetic pathology among hospitalized children are also analyzed, with foreign and Bulgarian studies.

The next major section is devoted to diagnostic methods for suspected genetic pathology. Genetic counseling and laboratory diagnosis are deployed step by step, including genealogical method and family history data collection, physical examination and laboratory tests. Cytogenetic methods, the gold standard for the diagnosis of chromosomal aberrations, are presented in detail, and the number of detected pathologies with and without Down syndrome is presented separately. The data for Bulgaria are compared with other international studies. Molecular cytogenetic methods, FISH and comparative genomic hybridization, PCR, MLPA and sequencing (including whole genome and exome sequencing) are then presented, and the possibilities and disadvantages of each technology are presented. Percentages of pathologies detected using whole exome sequencing are presented. Available software and computer programs for the assessment of dysmorphic features, as well as databases, are presented in summary.

A historical review of genetic counseling as a highly specialized activity in the diagnosis of genetic pathology has been made. The emergence and organization of genetic counseling in Bulgaria is compared with the genetic counseling developments from abroad. The indications and sources for referral to medical genetic counselling offices are described, as well as the stages of genetic counselling and communication with the family or patient. Emphasis is placed on the fact that genetic counselors are involved in long-term follow-up and support in each specific case.

The "Literature review" section ends with a summary of the literature data, which logically leads to the formulation of a working hypothesis.

In general, the presentation of the literature review demonstrates that the author skillfully handles a large number of sources and diverse information. The PhD student precisely analyzes and interprets the information provided, ultimately presenting it in a systematic manner.

The "Purpose" section which evaluates the effect of the activity of genetic counseling as an approach to clinical genetic diagnosis in persons with suspected hereditary pathology in childhood for a period of ten years is precisely and clearly formulated, and the path to its achievement can be followed logically in the tasks listed below.

The tasks are adequately formulated to achieve the set purpose.

The "Materials and Methods" chapter presents the patient group and the referral indications in detail. The target group included 3124 patients referred for medical genetic counseling and/or genetic analysis. The different biological materials on which the relevant tests were performed are presented.

Diagnostic methods are described in detail, including clinical methods (documentary, genealogical method and family history taking, physical examination) ; laboratory methods (cytogenetic analysis; DNA analysis) and dysmorphology databases and online-based software programs. The statistical methods selected for data analysis were well described: non-parametric Chi-square test and Fisher's exact test; regression and graphical analysis using Graph Pad Prism 9 statistical data processing software.

In general, the "Materials and Methods" section is well structured, the methods are presented in detail, allowing them to be used as a guide and replicated in other studies. A wide range of

methodologies was used, which is one of the strengths of the work submitted to me for review.

The "Results" section is laid out in a logical sequence, with the results described in detail, systematized in tables and illustrated with a large number of figures. Patients are initially described by year, sex and age, and by referral sources, and the results are illustrated with multiple figures. Subsequently, the patients are presented according to indications and were classified into 6 groups. According to the degree of clinical probability, some of the groups are also divided into subgroups. The results are visualised with appropriate figures to show which group the greatest number of patients fell into.

Subsequently, a selected group of 968 patients who underwent active diagnostic (laboratory and consultative) work-up was characterized. The cytogenetic studies performed are presented in detail, the chromosomal abnormalities detected are described, and they are visualized schematically stratified by age, sex, and indication groups. About 14% of chromosomal pathologies were identified. Aneuploid conditions accounted for 53% of the pathologies detected, structural disorders on autosomes accounted for 27%, and some of these cases are proven to be familial. Fragility of the X chromosome was found in 3.5% of the examined individuals. Numerical and structural disorders of the sex chromosomes were found in 14.5% of the examined patients. The sex distribution in this category shows a three times higher frequency in girls, the difference being statistically significant. Discrepancy between phenotypic and genotypic sex was found in 1% of the subjects.

In the group of single-gene diseases, DNA analysis revealed cases of cystic fibrosis (21 children), Wilson's disease (6 patients) and beta-thalassemia (6 children with thalassemia major and 14 with thalassemia minor).

Among the genetic predispositions, cases of celiac disease, Gilbert's syndrome and thrombophilia have been identified by DNA analysis.

In the group of pharmacogenetic defects, DNA tests for glucose-6-phosphate dehydrogenase deficiency were performed, and polymorphisms in the TPMT, NR3C1, and NALP1 genes were investigated to refine thiopurine and corticosteroid therapies.

The results of diagnostic and consultative work are presented in detail and summarized. Some of the patients with established pathologiy are presented in detail regarding the clinical manifestations. The results of the application of MLPA analysis for diagnostic purposes in

patients with malformative syndromes and neurodevelopmental disorders are presented. Thus, 5 pathological results were established (9.3% of the patients studied) , corresponding to the clinical symptoms. The five cases are described in detail clinically and in terms of genetic findings.

The results of the microarray analysis and MLPA carried out outside the Laboratory of Medical Genetics, Varna are presented in summary form in the thesis. The pathological genetic findings are presented in detail. Interesting data are presented in table 13, where we see the distribution of patients with a DNA analysis and revealed pathology by groups (growth and/or sex development disorder, suspected single-gene non-metabolic disease, probable inherited metabolic disease, uncknown dysmorphic syndrome, developmental delay). A comparison of the percentage of detected pathology in individuals with NGS and another type of DNA analysis (Sanger sequencing, PCR, MLPA of a gene/panel of genes, etc.) showed no statistically significant difference. Table 14 presents an impressive list of proven rare diseases/syndromes using molecular genetic methods for a period of 10 years. The percentage of revealed pathologies is summarized as AR (41.6% of the pathology), AD - 33.7%, X - linked (7.9%) and mitochondrial diseases (2.2%). Imprinting disorders were found in 7.8% of subjects (14.6% of pathology disclosed).

The results of selective metabolic screening and enzyme analysis for inborn errors of metabolism are presented in a table by diagnoses. Lysosomal storage disease was found in half of the diagnosed cases.

The following are the results of an active diagnostic counseling in a selected group of patients with genetically unspecified disease, achieved using online-based software programs. This type of programs proves to be an indispensable assistant in the diagnosis of children with malformative syndromes and suspected genetic disease. More than 200 patients have been processed this way over a 4-year period, and the definitive diagnosis is among the top 10 offered by the program.

In the "Discussion" chapter, the data obtained are competently analyzed, on the one hand, the author offers her own analysis and interpretation, and on the other hand, comparing them with the data from the world literature. The paper ends with a conclusion and future directions.

Based on the results obtained and their detailed interpretation, the following conclusions are formulated:

1. Regarding the descriptive-epidemiological data and indications, it can be concluded that children's contingent constitutes almost half (46.3 %) of all persons registered in the genetic structure, most were referred by two of the specialised paediatric clinics and the most common clinical indication for referral was "probable chromosomal disease with or without disorders of sex development".
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.

   • Selective screening (by DNA analysis), for known single-gene diseases, hereditary predispositions and pharmacogenetic defects: the largest share and diagnostic detection rate are in patients with suspected cystic fibrosis.

   • Molecular-genetic methods have the following applications and characteristics: in patients with unknown cause for DD/ID with dysmorphism, the application of microarray analysis is leading and should be applied as a method of first choice; in patients with DD/ASD (predominantly male) without dysmorphic phenotype, FRAXA screening is indicated; in suspected single-gene disease with an emphasis on neurological/neuromuscular symptoms, the application of DNA sequencing is relevant; an unknown clinical diagnosis requires the application of whole-exome or clinical exome sequencing.

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 a preference for laboratory (genetically and metabolic) confirmed cause of the disease
4. The genetic diagnostic activity (laboratory and counselling) significantly contributes to 34.2% of the selected group (10.6% of all covered)
5. The directions for improving the approach to conducting a clinical genetic evaluation of a hereditary disease 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 and expanding the panel of laboratory options for suspected clinical diagnoses

At the end of the present work, the author presents the contributions, divided into three thematic groups : (1) scientific contributions of an original nature with an emphasis on those carried out for the first time in Bulgaria (1.1) systematised scientific-practical research on descriptive epidemiological characterisation of the contribution of the overall activity of a genetic structure in service of a paediatric contingent of patients and (1.2) evaluation of the medical genetic activity for establishing a genetic and/or clinical diagnosis of an unknown/unconfirmed clinical disorder specifically in childhood patients (2) confirmatory contributions with emphasis on (2.1) the leading role of laboratory diagnostics in suspected genetic disease as an effective method for detecting chromosomal pathology in a prenatal diagnostic procedure, (2.2) the need to upgrade the application of conventional cytogenetics with modern high- resolute molecular genetic methods for diagnosis and (2.3) the importance of clinical phenotyping and patient selection as a stipulation for diagnostic success; and (3) contributions of an applied nature with emphasis on (3.1) optimization of methods for molecular genetic analysis and the introduction of computer programs with dysmorphic databases into diagnostic practice and (3.2) deriving guidelines for improving the awareness and approach to clinical genetic evaluation of inherited disease during clinical consultation.

The mentioned contributions strongly indicate the importance of the dissertation work.

Dr. Milena Stoyanova has published three articles, related to her thesis, one in English and two in Bulgarian. In all publications, the PhD student is the first author. In addition, the PhD student presented 3 participations in scientific forums.

IN CONCLUSION:

Dr. Milena Petrova Stoyanova is an excellent specialist in the field of pediatric genetics and medical-genetic counseling and presents herself as a scientist with publications in Bulgarian and international journals. The PhD thesis is the result of a thorough and precise scientific research activity, supported by appropriate statistical analyses and an eloquent description of the results and conclusions. The PhD student's critical interpretation of the results is highly impressive and has important scientific significance.

The PhD thesis submitted to me for review fully meets the requirements for awarding the scientific degree "Doctor", defined by the Rules for the Academic Staff Development at

Medical University "Prof. Dr. Paraskev Stoyanov" - Varna and fully meets the European requirements for awarding this scientific degree.

Based on the high quality of the PhD thesis and my excellent direct impressions of the work of Dr. Milena Petrova Stoyanova, I give a positive assessment of the dissertation work and strongly recommend to the members of the Scientific Jury to award her the scientific degree "Doctor".

19.09.2022 Prof. Albena Todorova, DSc