Review of the Doctoral Thesis

by Prof. Dr. Nadezhda Petrova Madjirova, DM, DMSc, part-time lecturer at University of Plovdiv “Paisii Hilendarski”

of Tihomir Iliev Vachev, Assistant Professor at the University of Plovdiv - Paisii Hilendarski, Plovdiv, Faculty of Biology, Department of Plant Physiology and Molecular Biology for the award of the degree "Doctor of Science", Field of higher education: 4 "Natural Sciences, Mathematics, and Informatics" Professional field: 4.3 "Biological Sciences" Scientific specialty: "Molecular Biology"

Dissertation thesis: Comparative genomic, transcriptomic and proteomic research in neurodevelopmental disorders

1. Subject of review

The dissertation was discussed and directed to the defense of a meeting of the extended Department Council of the Department of Plant Physiology and Molecular Biology at the Faculty of Biology of the University of Plovdiv - "Paisii Hilendarski".

2. Brief biographical data

Tihomir Vachev was born in 1981, graduated with a Bachelor's degree in Biology (2001-2005) and a Master's degree in Molecular Biology and Biotechnology at the University of Plovdiv Paisii Hilendarski. His career began in 2007 at the same university. He held the position of Biologist Junior Expert at the University of Plovdiv “Paisii Hilendarski”. In 2012 he obtained a Ph.D degree - "Molecular Biology" PU "Paisii Hilendarski", in 2013 – Ph.D student - "Medical Genetics". In 2016 he obtained a Ph.D degree - "Genetics" at the Medical University - Plovdiv. The main subjects and professional skills are in the field of Molecular biology, Genetics, Virology, Molecular genetics, Regulation of gene expression. Other areas of his research interests are epigenetic of some mental disorders (autism and schizophrenia); molecular aspects of mental illness. Here I have a direct observation of the enthusiasm, diligence, and exceptional scientific thinking in the joint research with Dr. Nikolai Popov (my doctoral student). I was impressed by the incredible enthusiasm with which they worked - they finished after a year and a half, if possible, the work will last 3 years. Perhaps even then he had the idea to focus on the genetic manifestations of some mental illnesses - ASD and schizophrenia.

The set of materials presented in electronic format by Tihomir Vachev is in accordance with the Regulations for the development of the academic staff of the University of Plovdiv and includes the following documents:

- Request to the Rector of University of Plovdiv “Paisii Hilendarski” for disclosure of the protection procedure;
- CV in European format;
- Notarized copy of the diploma for higher education (Master's degree);
- Documents from departmental councils, related to reporting of readiness for opening the procedure and preliminary discussion of the dissertation;

- Dissertation work;
- Abstract (in English and Bulgarian);
- List of scientific publications on the topic of the dissertation;
- Copies of scientific publications;
- Declaration of originality and authenticity of the attached documents;
- Reference for covering the minimum national requirements in direction 4.3. Biological sciences. for obtaining the scientific degree "Doctor of Sciences"

3. Relevance of the topic and expediency of the set goals and objectives

Significance of the researched problem: The emphasis of the research idea in the presented dissertation is placed on contemporary molecular genetic issues in neurodevelopmental disorders and in particular ASD and schizophrenia. Hundreds of millions of people around the world currently suffer from mental illness. 10 to 20% of young people have mental health problems and these percentages are higher among disadvantaged categories. Mental disorders and suicides are a source of great suffering for individuals, families and, the community. On the other hand, mental disorders are one of the main causes of disability and a burden on social protection systems, the health sector, and the education sector, the economy, and the labor market.

4. Knowledge of the problem

Over the last nearly seventy years, the etiological paradigms in psychiatry have shown significant development, along with the concept that cognitive and behavioral disorders have an organic "CNS-based" etiology. In 1980, autism was classified as a developmental disorder by accepting the thesis of its biological nature. The term generalized pervasive developmental disorder (PDD) was later introduced in the Diagnostic and Statistical Manual of Mental Disorders - III (DSM), included in the International Classification of Diseases - 10 (ICD).

The diagnosis of ASD is characteristic of all racial, ethnic, and socio-economic groups. The diagnosis is five times more common in boys than in girls. Prior to 1990, most studies reported a total population frequency of 4 - 5 per 10,000 (1/2000 - 1/2500). In the last few years, an increased incidence of autism spectrum disorders has been reported worldwide. According to the Centers for Disease Control (CDC) and the Autism Monitoring Network (ADDM) in the United States, 1 in 88 children is diagnosed with ASD.

Schizophrenia has existed since ancient times. There is a huge amount of literature devoted to it and there is almost no unexplored area to clarify the etiology and pathogenesis. A number of clinical, microbiological, chronobiological, immunological, bacteriological, infectious, viral, chemical, biochemical, enzymatic, genetic, epigenetic, and many other studies have been performed. Over the last few decades, the efforts of geneticists and psychiatrists have focused on finding genetic/epigenetic factors to trigger it. Most psychiatrists have observed families with a heavy workload. As of 2011, schizophrenia affects about 0.3-0.7% of people at some point in their lives or 24 million people worldwide. The disease affects 1.4 times more often men and occurs earlier (between 20 - 28 years) than in women (between 26 - 32 years). This determines the significance of the topic.

The existence of standardized methods for characterization and profiling of mRNA molecules (RNA Seq) and miRNA molecules (Small RNA sequencing) as well as fast and universally applicable approaches to quantitative analysis (quantitative RT-PCR) suggest that modern

science has new methodological approaches to study the pathogenesis of diseases that have the potential to detail our knowledge in the field. Over the last decade, a number of pieces of evidence have accumulated to support the hypothesis that disrupted regulation of miRNA regulatory networks may be at the root of neurodevelopmental disorders. Due to the fact that miRNAs can be packaged in exosomes and microvesicles and thus secreted into extracellular fluids, they can circulate in body fluids and be transported to distant tissues and organs. Some of the results presented in this study cover expression studies of serum miRNA molecules in the individual analysis of patients diagnosed with PAS compared to a control group of healthy children.

5. Research methodology

The main objectives of the present study are: The main goal stated in the dissertation described as conducting comparative genomic, transcriptomic, and proteomic studies in neurodevelopmental disorders can be divided into several main goals:

1. To confirm established genomic variants characteristic of ASD in the studied cohort, showing a tendency and supporting the hypothesis of genetic heterogeneity in ASD.

2. To identify specific genomic variants in the analyzed sample of patients diagnosed with ASD that have not been identified to date. Regarding the first goal of the study, the data obtained support the existence of pathogenetic mutations in the studied samples, some of the characteristics of ASD.

3. To identify candidate miRNA molecules in the analyzed sample of patients diagnosed with ASD. Regarding the second goal of the study, the data obtained support the existence of differentially expressed miRNA molecules in the studied samples, some of which are characteristic of ASD.

4. To identify candidate mRNA molecules in the analyzed sample of patients diagnosed with ASD that are characteristic of ASD.

5. To identify candidate mRNA molecules in the analyzed sample of patients diagnosed with schizophrenia.

6. To identify specific protein molecules in the analyzed sample of patients diagnosed with ASD.

In order to meet the goals set in the research, the dissertation sets the following tasks:

1. Conducting a comparative expression analysis of protein-coding geniuses (RNA Sequencing) in ASD.

2. Conducting a comparative expression analysis of small RNA molecules (Small RNA sequencing) in ASD.

3. Conducting a comparative proteome analysis - Isobaric Tag for Relative and Absolute Quantification (ITRAQ) in ASD.

4. Carrying out large-scale exome sequencing (Whole Exome Sequencing) in ASD.

5. Conducting a comparative expression analysis of protein-coding geniuses (Digital Gene Expression) in schizophrenia.

6. Conducting comparative expression (qRT-PCR) analysis of protein-coding geniuses in ASD.

7. Conducting comparative expression (qRT-PCR) analysis of miRNA in ASD.

8. Conducting comparative expression (qRT-PCR) analysis of protein-coding genes in schizophrenia.

9. Conducting ROC (Receiver Operating Characteristic) analysis of differentially expressed protein-encoding genes and miRNA molecules in ASD and schizophrenia.

6. Characteristics and evaluation of the dissertation

Since the development of the dissertation is interdisciplinary, which is evident in the selection of reviewers from different universities and disciplines, in relation to this part of the work I could not give a competent answer because the applied methods are not in my qualification. However, I would like to emphasize that the statement regarding the choice of patients, the applied clinical-psychological and diagnostic tests, as well as the evaluation of patients, are adequate, correctly selected, and used. The high professionalism of the contingent research team should not be overlooked either.

The presented dissertation consists of 391 standard typewritten pages distributed as follows: Abbreviations: 11-13 pages; Introduction: 3 pages; Literature review - 100 pages. This section is written in great detail, covering the world's leading theoretical views on schizophrenia, BD, and autism, which have been extensively commented on. The dissertation discusses the genetic, epigenetic, prenatal, and natal factors of schizophrenia and autism, the age of the parents, especially the father, the family burden. Comment on various pathogenetic hypotheses in schizophrenia (GABA, glutamatergic, dopaminergic, jumping analyzes, variations in copy number, genes and loci, histone modifications, the study of protein-encoding genes, activation of maternal immune function, and general characteristics and RNA molecules, etc.). In autism, the author emphasizes social deficits and behavioral theories with various histological and biochemical changes, are signals, mirror neurons, environmental influences, vitamin D and folic acid, chromosomal aberrations, number of copies, overrepresentation of rare variants of mutations and miRNA molecules as biomarkers.

It seems to me that the consideration of bipolar disorder (BD) with a major depressive episode is not necessary or can simply be mentioned very vaguely. It is an indisputable fact that different genes are involved in this disorder, which explains the diverse clinical picture.

Opinion - I have no special critical remarks regarding the terminology and theoretical aspects of schizophrenia and autism. Among the many hypotheses listed, there is no mention of the chronobiological theory of schizophrenia, according to which there is a pronounced desynchronization of biological rhythms. This theory is closely related to genetics - cell division is encoded and in endogenous psychoses, there is an acceleration/lag or desynchronize of the biological clock. It seems to me that the mention of BD and the major depressive episode should be very brief, although different genes are involved in this disorder. I think this part could be shorter. There is no doubt, however, that the dissertation has made

a very complete **and in-depth reference, which is difficult for him to part with.**

Literature sources: the number of used literature is representative (666 authors in Latin only). I think that if the authors were numbered, quoting them would reduce the volume of the pages. The correctness in quoting the authors is observed. Bulgarian authors working abroad in this field are also included. The style is emphatically scientific, sophisticated, clear, and precise, there are no inappropriately used expressions and grammatical inaccuracies. Very rarely I have encountered errors that I do not consider spelling, but rather an error when typing an adjacent letter on the keyboard - e.g. p. 8, 2nd paragraph… modern, instead of the modern, p. 9 (14 line top pacity, instead of the patient)), second-line bottom (meanings, instead of the meaning), p. 11 (4 line top: The design of of), fig. 12 top row (new criteria instead of criteria), fig. 31, p. 218 (circular presentation, instead of circular), fig. 36, p. 226 (Difference in the level of….). These small and insignificant mistakes do not reduce the value of the dissertation.

Materials and methods: covers 65 pages (128-195 pages). The design of the present study and the form of informed consent has been approved by the Commission for Scientific Ethics at MU - Plovdiv. Written informed consent was obtained from 33 patients, in the NPH - Pazardzhik and 25 healthy volunteers. They conducted a Mini-International Neuro-psychiatric Interview and a routine psychiatric and somatic examination. The interview was conducted by a certified psychiatrist, and the diagnosis of paranoid schizophrenia was assessed on the basis of DSM IV-TR (Diagnostic and Statistical Manual of Mental Disorders-IV revised edition) in order to exclude any other mental disorders in controls. All enrolled patients were diagnosed with schizophrenia according to the international classification DSM-IV (American Psychiatric Association. 1994; Diagnostic and Statistical Manual of mental disorders, 4th ed.). The obligatory criterion for inclusion in the sample is that the participants have not received any therapy (even psychotropic) within 1 month before taking blood samples. Individuals with other chronic medical and current acute somatic/neurological diseases, alcohol abuse, or drug addiction are excluded from the samples.

The study design and the informed consent form has been approved by the ethics commission at MU - Plovdiv. A Mini-International Neuropsychiatric Interview, a routine psychiatric examination by a certified psychiatrist, and a complete history based on the criteria (DSM IV-TR; Diagnostic and Statistical Manual of Mental Disorders, IV Revised Edition) are included to exclude any other mental disorders in controls. The patients in the present study were selected within the project "Autism and specific speech development disorder - comparative genomic studies to identify a common pathogenetic basis" № NFSR-B01/21 at the Department of Pediatrics and Medical Genetics, MU - Plovdiv. Project "Analysis of the expression profile of non-protein-coding - microRNA genes in schizophrenia" and respectively project "Study of serum levels of microRNA biomarker molecules in children with autism in order to create a platform for non-invasive molecular diagnosis of autistic patients" № NO -12/2013.

The presented study included 40 children with a generalized developmental disorder, aged 3 to 11 years (mean sample age was 6.86). For the purposes of the study, the diagnostic criteria of DSM IV-TR were used and the patients were diagnosed according to the classification of the American Psychiatric Association. To confirm the diagnosis of ASD in each patient, a detailed psychiatric examination was performed using ADI-R, CARS, and GARS. Following the publication of DSM 5, the diagnosis of ASD was reconfirmed according to the new criteria.

The control group included 30 healthy children aged 3 to 11 years (mean age of the sample was 6.43), after signing an informed consent of the parents.

The author describes in detail the method of blood sampling, the method of secretion of samples, extraction of total RNA from peripheral blood, the isolation of samples from total RNA by applying the PAXgene Blood miRNA Kit. Extraction of total DNA from peripheral blood using QIAmp DNA Blood Mini Kit Spin protocol (Qiagen). The principle of the procedure and the individual steps are described sequentially and completely in a detailed protocol, and the results are presented in Tables 8 (pp. 146-148), 9, and 10. The author uses the method of real-time polymerase chain reaction -PCR (Quantitative Reverse Transcription Real-Time PCR) can be used for quantitative, semi-quantitative, and qualitative analysis. The method allows for reliable detection and measurement of the generated PCR products in each cycle of the polymerase chain reaction process. Each stage of their implementation, as well as the applied methodology and equipment, are precisely selected and described in detail.

The obtained results are presented in illustrative figures. Finally, it concludes with a standard and detailed bioinformatics analysis. The author performs RNA sequencing (Transcriptomic Analysis - Methodology) and performs qualitative assessment and filtering of sequencing data, quantitative analysis of gene expression and study of gene coverage, as well as levels of gene expression. Quantitative proteome analysis - Isobaric Tag for Relative and Absolute Quantification (ITRAQ) in ASD, in fig. 13 (p. 175) states the principle of quantitative proteome analysis (iTRAQ) analysis, emphasizing the advantages of the iTRAQ method.

According to the author, the labor-intensive approach to complete sequencing of all coding regions has the potential to become both a clinically relevant study for genetic diagnosis and the identification of DNA variants with potentially significant pathological effects in diseases of unknown etiology. The aim of this approach is to identify functional changes in the genome that are responsible for the development of a specific pathology. Large-scale exome sequencing is fast becoming the technique of choice when it is necessary to identify new genetic variations that underlie diseases, such as a number of forms of cancer, and a number of psychiatric diseases such as schizophrenia, ASD, and others.

One key feature of identifying specific DNA variants (SNPs, etc.) through the use of next-generation sequencing technologies is that the methods involve variables at a number of levels in the analysis, which are subject to correction by increasingly optimized mathematical programs and algorithms for analysis. Validation of the data from the performed transcriptome analysis is performed using quantitative Real-Time PCR analysis. Five differentially expressed genes were subjected to individual quantitative Real-Time PCR analysis in all samples from patients diagnosed with schizophrenia and a control group of healthy individuals. The change in gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Results: cover 72 pages (196-268 pages)

The generalized analysis of the data obtained from the complete exome DNA sequencing demonstrates the presence of mutations in the ASD group, leading to the formation of non-functional proteins. The main mutations can be divided into several separate categories:

1. Mutations leading to the appearance of termination codons, 2. Mutations leading to the loss of termination codons, 3. Mutations leading to the appearance of serious non-synonymous

substitutions, 4. Mutations related to the shift of the reading frame (frameshift mutation) - deletions or insertions. In the group of children diagnosed with ASD, it was noted that mutations leading to stop codon formation were associated with genes such as MOB3C (MOB kinase activator 3C), associated with the binding of metal ions. Cytochrome P450 Family 4 Subfamily B Member 1 (CYP4B1), a gene encoding a member of the cytochrome P450 superfamily of enzymes. Cytochrome P450 proteins catalyze reactions related to drug metabolism and the synthesis of cholesterol, steroids, and other lipids.

Mutations leading to stop codon loss include genes such as Fasciculation and elongation protein zeta 2 (FEZ2) associated with neuronal binding, Actinin alpha 3 (ACTN3) associated with neuronal connectivity, Neurofibromin 1 (NF1) associated with neurofibromatosis type 1, and autism syndrome. The dissertation identifies 337 mutations in genes leading to a shift in the reading frame between genes such as Caspase 9 (CASP9), from the caspase group, Ubiquitin-like-conjugating enzyme ATG3 (ATG3), ubiquitin-like conjugating enzyme, Fibroblast Factor Receptor4 (FGFR4-fibroblast growth factor 4) and others.

From the performed ITRAQ proteome analysis the dissertation demonstrates the presence of differentially expressed proteins between the analyzed groups.

A total of 351 proteins were identified from the iTRAQ-LC-MS / MS assay. Compared to the control group, a total of 60 differentially expressed proteins were identified, including 24 with increased expression and 36 with decreased expression. Decreased expression proteins include SERPINE2 glia-derived nexin (GDN), intellectin 1, immunoglobulin J, angiogenin, fibulin, apolipoprotein C-II, apolipoprotein C4 (APOC4), apolipoprotein F, complement C4-A protein, periostin, plastin. While proteins with increased expression include: Apolipoprotein C-II, Apolipoprotein C4 (APOC4), Apolipoprotein F, as well as a number of protein components of the complement system: Complement C4-A protein, Complement C1q subcomponent subunit A and others. The author also showed an annotation on clusters of Orthologous Groups of Proteins (COG), gene ontological (GO) analysis of differentially expressed proteins which are beautifully illustrated in several pie charts.

From the analysis of the miRNA expression profile by sequencing small RNA molecules in RAS, the dissertation demonstrates the presence of differentially expressed small RNA molecules (expression profile) in RAS compared to a control group of children in peripheral blood shows the presence of specific changes characteristic of the disorder. The author presents data from a standard bioinformatics analysis for the profiling of small RNA molecules by the reference genome. Finally, the author presents the results of the individual analysis of the expression profile of miRNA molecules from peripheral blood and serum in children diagnosed with ASD. The study thus conducted provides evidence that peripheral blood is a suitable and easily accessible tissue (material) for studying the dynamics in the expression of candidate biomarkers in ASD.

In the results of the performed transcriptome analysis by RNA-Sequencing in ASD, the dissertation demonstrates the presence of differentially expressed small RNA molecules (expression profile) in ASD. Twenty-two RNA transcripts showed differential expression with statistical significance $p < 0.001$, $FDR \leq 0.001$, and $\log2 \geq 1$. In ten genes, the author found an increased level of expression, while another 12 genes showed decreased levels of expression. Additional validation of the data from the performed transcriptome analysis is performed by

the author with the help of quantitative Real-Time PCR analysis. He subjected the 10 differentially expressed genes in all samples from children diagnosed with ASD and a control group of healthy children to individual quantitative Real-Time PCR analysis.

Results of the transcriptomic profiling of protein-encoding genes in peripheral blood in schizophrenia

After a preliminary large-scale expression analysis (DGE) between the analyzed groups, the dissertation identified a significant number of differentially expressed genes in schizophrenia (genes with increased expression were 1012, and respectively 2582 genes with reduced expression). From the preliminary data obtained from the DGE analysis, the dissertation subjected to individual analysis candidate genes showed the greatest changes in the expression profile. To evaluate the characteristics of differentially expressed genes, DICER, FEZ1, DRD4, GRIN2 as potential biomarkers in schizophrenia, the dissertation performs ROC analysis to determine the diagnostic accuracy of the studied protein-coding genes. The diagnostic sensitivity of the DICER, FEZ1, DRD4, and GRIN2D genes was determined at 72.4%, 55.2%, 86.2%, and 48.3% respectively, with the corresponding specificity estimated at 83.3%, 87.5%, 91.7%, and 83.3%.

Discussion:

The discussion covers 64 pages (269-334 pages).

The scientific research presented in the present dissertation is the complex scientific research first of its kind, which includes comparative genomic transcriptomic and proteomic studies conducted on a clinically selected group of Bulgarian patients diagnosed with ASD and compared with a control group of healthy individuals. The authors sought to link a number of candidate genes to certain diseases. The overall expression profile of miRNA molecules in peripheral blood from patients with ASD is discussed in detail in the study. Using the small RNA molecule sequencing method, a change in the expression of a large number of miRNA molecules is found. The approach used contributes to the understanding of the role of miRNA molecules and related biological processes in the etiopathogenesis of ASD. A new focus in the study of biomarkers in schizophrenia and ASD is the approach based on the study of the molecular profile of biomolecules from peripheral tissues and in particular miRNA molecules in peripheral blood, reflecting the physiological state. The use of miRNA-specific qRT-PCR analysis confirms that dysregulation of miRNA molecules plays a role in the pathogenesis of ASD.

Although blood is not the ideal tissue for ASD research, peripheral blood that is readily available is usually the subject of research in neurodevelopmental disorders - ie. transcriptional sequencing of blood cells is essential for diagnosis in patients diagnosed with ASD. In addition, the integrative approach also makes it possible to identify rare inherited variants with functional consequences that could contribute to the development of the phenotype. Integrative analyzes of genomic and transcriptional data may be important in understanding the mechanism of multifactorial diseases. Asst Prof. Vachev admits that whole exome sequencing can improve the diagnosis of problematic cases.

The proteomic approach used provides encouraging preliminary data that should help stimulate the continued search for etiopathology in ASD and new therapeutic approaches

through peripheral blood testing. It turns out that brain tissue is not particularly suitable in the search for biomarkers for the prevention, diagnosis of ASD in clinical practice. The presented analysis can be considered as a preliminary pilot study, which would require an extension of the sample, as well as additional individual validation of the identified proteins in ASD. The obtained data demonstrate marked heterogeneity, the connection of the immune system, the dysregulation of basic cellular processes, and evidence for the multifactorial model in the pathogenesis of ASD.

7. Contributions and significance of the development of science and practice

With regard to the contributions of scientific and methodological nature in his work, the dissertation successfully applies the new generation sequencing technology "Whole-Exome Sequencing" for analysis of genetic variability in the coding regions of genes in patients diagnosed with ASD. New generation sequencing technologies "RNA-Sequencing" and "Small RNA-Sequencing" have also been successfully applied to identify differentially expressed protein-encoding and miRNA genes in patients diagnosed with ASD.

In order to study possible changes in protein level occurring in patients with ASD, the technology for conducting comparative proteomic analysis "Isobaric Tag for Relative and Absolute Quantification (ITRAQ") was used to analyze the proteomic profile in patients with ASD. While for the purpose of identifying differentially expressed protein-encoding genes in patients diagnosed with schizophrenia, the dissertation successfully applies Digital Gene Expression Tag Profiling (DGE) technology. The created collection of DNA samples from patients diagnosed with ASD and schizophrenia, as well as their respective cohorts from healthy individuals represents a significant contribution of an applied nature, providing an opportunity for larger future genetic studies in the field.

8. Evaluation of the dissertation publications

The list of publications attached to the procedure for obtaining the scientific degree "Doctor of Science" includes 9 publications in Bulgarian and international journals. The quarters of the publications are reported according to the metrics of the scientific journals referred to in the Scopus Scimago Journal Rank (SJR) https://www.scimagojr.com/journalrank.php. Two of the publications are with Q2, four with Q3 and two with Q4. One of the journals (Biodiscovery) in which the dissertation has a publication does not have a quartile but due to its connection with the topic of the dissertation the publication is presented in the list of publications. The candidate has met the minimum required points by groups of indicators for the scientific degree "Doctor of Science" - of the required 350 points on indicators A, B, G, D - the candidate has 432 points.

9. Personal participation of the author

I consider the personal contribution of the dissertation to be the main one, referring to the fact that in all of the mentioned publications related to the dissertation is a leading author.

10. Summary

The summary is designed according to the requirements in structural, content, and technical aspects in Bulgarian and English. The summary with their content accurately reflect and reveal the main points and ideas in the dissertation. The presented summary abstracts in

Bulgarian and English present the most important results of the study. The presented summaries are prepared according to the respective requirements.

11. Critical remarks and recommendations

Critical remarks - it seems to me that the volume of the dissertation is quite large, which I explain with the huge literary reference on the problem, which is difficult for him to part with. Recommendation - the author could consider this huge and original work, the only one of its kind in our country, to be published as a book based on a defended dissertation to be used by more scientists in medicine, psychiatry, genetics and biology.

12. Personal impressions

Finally, I would like to emphasize the fact that Asst Prof. Tihomir Vachev has two dissertations for the scientific degree "Doctor", one of which is related to the epigenetics of schizophrenia. The dissertation for the award of the scientific degree "Doctor of Sciences" is developed independently and does not repeat the topic and a significant part of the content of the work presented for the acquisition of the educational and scientific degree "PhD". Asst Prof. Vachev's voice has also participated in previous joint studies with Dr. Nikolay Popov related to the genetics of autistic children. The contingent used by patients and control group of persons and the applied methods in the dissertation of Dr. Nikolay Popov do not overlap with the methods and publications presented in this dissertation.

13. Recommendations for future use of dissertation contributions and results

Research in the field of miRNA biology is still in its infancy. The role of miRNA in psychiatric disorders, the inclusion of meta-analyzes of miRNAs covering genetic variation, expression, and biological function, will provide additional valuable information on the potential role of miRNA molecules in ASD and some psychiatric disorders in terms of their diagnosis, etiology, and pathogenesis. Biomarkers based on miRNA molecules can be very useful in distinguishing different subtypes of psychiatric disorders. Current understandings of transcriptional profiling based on the RNA-Seq approach, as well as next-generation sequencing technology, Whole-Exome Sequencing, reveal their usefulness in identifying genetic variants in ASD. MiRNAs undoubtedly prove promising in the diagnosis of CNS disorders as well as in the evaluation of related therapy. The role of miRNA in psychiatric disorders is intense due to ever-improving research approaches.

I would add that not hundreds but thousands of genes are involved in schizophrenic construction. The genetic mechanisms of schizophrenia have been a mystery for decades, and new discoveries are continuing to fill the mystery. It is almost impossible for students to declare the mechanism of genes - what you unlock and why they are so persistent since the complete lines remain a generation. As a result, it is found that one of the reasons for the construction of the psyche is the loss or duplication of many genes. In cell division, chromosomes sometimes exchange identical participants - plus or minus several genes. What is interesting about these specific delegations and duplications is that many of them are not actually owners. They are caused due to new mutations, ie. their parents do not have them and mutations are caused in a child. It is quite possible that some of the changes will be processed. It is no coincidence that psychiatrists have observed schizophrenic users with only one access throughout their lives. So it is quite strange that these huge deletions and

duplications are essentially theoretically data on therapy. Numerous studies show that the same mutations cause not only schizophrenia but also other diseases such as autism and various property disorders. On the positive side, the dissertation opens the door for future research in this direction in order to clarify the pathogenesis of children with ASD. In this regard, future research in the field could focus on a number of genes that are regulated by miRNA molecules studied in previous studies that are involved in neurological diseases and mental disorders.

CONCLUSION:

The dissertation contains scientific, scientific-applied, and applied results, which represent an original contribution to science and meet all the requirements of the Law for development of the academic staff in the Republic of Bulgaria (ZRASRB).The Regulations for application of ZRASRB and the respective Regulations of Plovdiv University "Paisii Hilendarski". The candidate Asst Prof. Tihomir Vachev, after a detailed literature review, builds on what has been achieved so far. From the development of the material, it is clear that he has in-depth theoretical and practical knowledge in the field and is an example of modern interdisciplinary research. The engagement of specialists from different fields shows his skill and ability for good organizational skills and teamwork. These qualities undoubtedly speak of depth and independence in the development of the dissertation. The research conducted with the help of modern methods and the most modern technologies has led to results with a significant contribution to medicine, genetics, and psychiatry, some of which have been published in prestigious international journals. The goals and tasks he has set are clearly formulated and achieved. I am particularly pleased with the fact that the author is not satisfied with what he has achieved and provides guidelines for future research that he could lead.

In view of the above-mentioned, I confidently give my positive assessment of the research presented by the dissertation reviewed above, abstract, achieved results and contributions, and I propose to the esteemed scientific jury to award the scientific degree "Doctor of Science" to Tihomir Iliev Vachev, Asst. Prof. at the University of Plovdiv - Paisii Hilendarski, Plovdiv, Faculty of Biology, Department of Plant Physiology and Molecular Biology in the field of higher education: 4 "Natural Sciences, Mathematics, and Informatics" Professional field: 4.3. "Biological Sciences" Scientific specialty: "Molecular Biology".

23. 12. 2020. Prepared the review:……...
Plovdiv Prof. Dr. N. Madjirova