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To the Chair of the scientific jury appointed by order No. 583/01.12.2023
of the Director of the National Center for Public Health and Analyses,
Prof. Dr. Iva Hristova, MD, PhD

REVIEW

on the procedure for acquiring the scientific degree of "Doctor" with candidate **Yordan Krasimirov Hodzhev**, a regular doctoral student at the National Center for Infectious and Parasitic Diseases in the field of higher education 4. Natural Sciences, Mathematics, and Informatics, professional direction 4.3 Biological Sciences, in the scientific specialty "Microbiology."

Thesis title: "The Microbiome and Bioinformatic Analysis in Studying the Pathogenesis of Sarcoidosis"

Scientific supervisor: Prof. Stefan Panayotov

Reviewer: Assoc. Prof. Ivan Nikolaev Ivanov, MD, Head of the National Reference Laboratory for Antimicrobial Resistance Control and Monitoring, Dept. of Microbiology, National Center for Infectious and Parasitic Diseases

The submitted application documents are correctly prepared and in compliance with the Law on the Development of Academic Staff in the Republic of Bulgaria and its regulations at the National Center for Public Health and Analyses.

I declare that I have no conflict of interest under Art. 4, Para. 5 of the Law on the Development of Academic Staff in the Republic of Bulgaria.

Career development.

Yordan Hodzhev completed his secondary education in Veliko Tarnovo. Since 2000, he has been a student at the Faculty of Biology at Sofia University, majoring in Molecular Biology, and in 2006 he obtained a Master's degree in Biophysics. After completing his higher education, he worked as a teacher of biology, mathematics, physics, and chemistry for the lower secondary level and as a biostatistician in a clinical trial company. Since October 2020, he has been a regular doctoral student in the laboratory for "Especially Dangerous Bacterial Infections," "Microbiome" sector, Dept. of Microbiology at the National Center for Public Health and Analyses.

Relevance of the work.

Sarcoidosis is a rare inflammatory disease, characterized by the development of granulomas in various organs. It was described more than 130 years ago. The lungs and lymph nodes are predominantly affected. Unfortunately, the pathogenesis of the disease is unknown.

The potential involvement of various microbial species in the pathogenesis of sarcoidosis has been proposed years ago. In sarcoidosis, histological similarities are observed with granulomatous lesions associated with tuberculosis, brucellosis, etc. Epidemiological and microbiological studies suggest that, in some patients, microbes or their products may trigger an immune response leading to the formation of a sarcoid granuloma. New studies show the immunomodulatory role of the lung microbiome in sarcoidosis. The relationship between the blood microbiome and sarcoidosis has not been studied. The role of the blood microbiome in the pathogenesis of many diseases is a relatively new scientific direction. Interdisciplinary research combining microbiological, immunological, clinical, and bioinformatics knowledge is necessary to clarify these multifaceted associations. Advances in bioinformatics and blood microbiome research have opened new vistas for clarification of the sarcoidosis pathogenesis.

Dissertation summary

The structure of the dissertation and bibliography, written on 128 pages, contains 24 figures and 10 tables. A total of 121 literary sources are used, most of which are from the last 10 years. In terms of volume, structure, and formatting, the dissertation fully meets the requirements for obtaining the educational and scientific degree of "doctor." The dissertation is structured as follows: Introduction - 3 pages with a graphical abstract of the dissertation; Literature Review - 31 pages; Goals and Objectives – 1 page; Materials and Methods 17 pages; Results and Discussion - 30 pages, Conclusion - 8 pages, and Contributions - 4 pages.

The literature review concisely describes the state of research on the issue.

Goals and objectives. The candidate aims to investigate the hypothesis that microbial imbalance or related quantitative changes in specific representatives of the blood microbiome may contribute to the development of pulmonary sarcoidosis or even induce it. The goal and the six tasks are precisely formulated, reflecting the main aspects and essence of the dissertation work.

Aim and Objectives. The doctoral candidate aims to explore the hypothesis that microbial imbalance or related quantitative changes in specific representatives of the blood microbiome may contribute to the development of pulmonary sarcoidosis or even induce it. The aim and six tasks are precisely formulated, reflecting the main aspects and essence of the dissertation work.

Materials and Methods. The materials and methods are described in detail and correctly, indicating the high level of the candidate's competence.

Results and Discussion. The conducted research reveals details about the involvement of the blood microbiome in the pathogenesis of sarcoidosis. The blood microbiome analysis provides original data for new diagnostic markers for identifying and monitoring sarcoidosis. An innovative approach to studying and evaluating the involvement of the blood microbiome in the pathogenesis of sarcoidosis is proposed.

Results summary:

(1) The candidate conducts a bioinformatics analysis of the core blood microbiome in healthy individuals, which is compared with the blood microbiome of patients with sarcoidosis. Through culturing blood samples from healthy individuals, resuscitation of latent microbial cells is performed under stress conditions - temperature different from physiological (43°C) and toxic stress with high concentrations of synthetic vitamin K (1g/l, water-soluble menadione sodium bisulfite). In cultured and uncultured blood samples from healthy individuals, analyzed through targeted sequencing of 16S rRNA and ITS markers, 241 bacterial OTUs and 272 fungal OTUs are identified. The dominant bacterial types among uncultured samples were Proteobacteria 92.97%, Firmicutes 2.18%, Actinobacteria 1.74%, and Planctomycetes 1.55%, while among cultured samples Proteobacteria were 47.83%, Firmicutes 25.85%, Actinobacteria 16.42%, Bacteroidetes 3.48%, Cyanobacteria 2.74%, and Fusobacteria 1.53%. Proven fungi of types Basidiomycota, Ascomycota, and unidentified fungi were respectively 65.08%, 17.72%, and 17.2% among uncultured samples, while among cultured samples they were respectively 58.08%, 21.72%, and 20.2%. Other authors confirm the dominant presence of latent Proteobacteria in the blood of healthy individuals (Paisse et al., 2016). Notably, other studies related to cultural stress resuscitation of microbial species in blood are presently not known. The laboratory is the first in the world to prove the existence of a normal fungal microbiome in the blood of healthy individuals. Bacterial diversity in the blood of healthy individuals and patients with sarcoidosis is investigated at the individual and group level and is presented through Sankey and Krona diagrams. The statistical analysis conducted by the candidate proves a significant increase in the richness of bacterial alpha-diversity in patients with sarcoidosis, using the Shannon and Simpson indices compared to healthy controls. The Bray-Curtis index for difference in taxonomic composition is used as an indicator of beta-diversity between patient and healthy groups. The principal coordinates analysis (PCoA) clearly illustrates the distinction between the two groups.

(2) Visualization of the quantitative and qualitative composition of the blood microbiome. Blood samples from 7 patients with sarcoidosis and 22 healthy individuals were studied using a 16S

metagenomic approach. The results are visualized using Sankey and Krona circular diagrams. The Sankey diagram shows the actual diversity of sequences, while the Krona illustrates the relative richness.

(3) The candidate investigates quantitative parameters that reflect changes in the microbiome composition leading to sarcoid pathology. Quantitative indicators of dysbiosis in the blood microbiome are identified. Sankey and Krona diagrams show four dominant types: Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes. Microbiome analysis of sarcoidosis patients identifies several specific genera - Cutibacterium, Corynebacterium, and Veillonella. The genera Dietzia and Acinetobacter are predominant among healthy individuals. Using the Mann-Whitney test, the candidate establishes a significant increase in five bacterial genera in patients with sarcoidosis: Veillonella, Prevotella, Cutibacterium, Corynebacterium, and Streptococcus.

(4) The candidate develops a machine learning algorithm that creates a classification tree of sequencing data. The model calculates and sorts the contribution of each microbial type to differentiate sarcoidosis patients from healthy individuals. The classification model is based on the quantity of OTU sequences for each taxon. Proteobacteria stands out as the most significant in the classification model, followed by Firmicutes and Bacteroidetes. Representatives of the Actinobacteria type do not contribute to the model. The machine learning model for identifying potential key taxa associated with microbiome dysbiosis leading to sarcoidosis has practical applicability for researching microbial dysbiosis in diseases with unclear infectious pathogenesis.

The conclusions, eight in number, are precisely formulated and adequately reflect the achievement of the goals and tasks, emphasizing the most important findings of the dissertation work. The conclusions complement numerous studies proving the presence of microbial species in the tissues, blood, and body fluids of sick and healthy people. The published results describing the fungal microbiome in the blood of healthy individuals are still unique in the scientific literature, except for studies of the tissue microbiome in patients with various oncological diseases, where bacteria and fungi are proven (Nejman et al., 2020). The role of the blood microbiome in human health is an open field for innovative research with the potential to become a new discipline in medical microbiology.

The dissertation contains original fundamental and applied scientific contributions. The work demonstrates that the candidate possesses in-depth theoretical knowledge in the field of bioinformatics and the ability for independent scientific research. Four fundamental contributions are formulated:

1. Experimental evidence demonstrating the relationship between the taxonomic composition of the blood microbiome and the development of pulmonary sarcoidosis has been presented.

2. A hypothesis for the interaction between the blood and lung microbiome in the pathogenesis of sarcoidosis is proposed.

3. For the first time, the presence of a fungal microbiome in the blood of healthy individuals is proven both culturally and metagenomically.

4. The quantitative assessment of dysbiosis in the blood microbiome is an indicator of the development of pathological processes.

There are also four applied contributions:

1. A bioinformatic approach has been developed to determine the composition of the blood microbiome in healthy adults.

2. Methods for visualization and assessment of the quantitative and qualitative composition of the blood microbiome in individual patients are proposed.

3. An effective bioinformatic method for comparative analysis of the taxonomic composition of cultured blood samples under different nutritional media and stress conditions for resuscitation of latent blood microbiomes has been developed.

4. The applied classification model for machine learning allows for the determination of key microbial taxa with the most likely contribution to the pathogenesis of various diseases associated with microbial dysbiosis.

I have no significant critical remarks on the structure, results, and description of the dissertation work. The scientific style could be clearer in some parts of the text. Minor linguistic shifts due to editing are noted. Conclusion 1 is described as a result, not as a fundamental contribution. The limitations of the conducted research as discussed by the candidate are agreed upon.

Publications and the personal contribution

The candidate Yordan Hodzhev has published six articles in impact factor and impact rank journals on the topic of the dissertation. Four are in journals with impact factors such as *Frontiers in Cellular and Infectious Microbiology* – IF 6, Q1, *Microorganisms* – IF 4.1, Q2, *Computational and Structural Biotechnology* – IF 6.1, Q1, and *Biotechnology and Biotechnology Equipment* – IF 1.6, Q3. A cited poster in a journal with an impact factor is included, which according to Appendix 1 of the National Center for Public Health and Analyses (NCPHA) regulation for the application of the Law for the Development of the Academic Staff is equivalent to publications with an impact factor. The journal in which the cited poster is published is *ERJ Open Research* with an impact factor of 4.6. The total impact factor is 22.559, with 14 citations. The results have been reported at 15 international and 7 national scientific congresses. The publications are directly related to the goals and results of the dissertation work. In all works, Yordan Hodzhev is the first or lead author. Yordan Hodzhev has participated and is participating in four scientific projects as an executor. According to the NCPHA and National Agency for Scientific Research and Innovation (NASRI) regulations, 200 credit points are required for the defense of the educational and scientific degree of "doctor." The credit points accumulated during the doctoral studies are 1239, far exceeding the requirements.

It is evident that the candidate Yordan Hodzhev has made a significant contribution to the development of the dissertation work. He has made a special effort to master and apply innovative bioinformatics methods to solve a difficult scientific problem.

Conclusion.

The candidate Yordan Hodzhev has achieved the set goals and tasks. The dissertation work is innovative and has scientific and applied significance. The presented dissertation work, publications, and other documents from the candidate meet all scientometric criteria according to the Law for the Development of the Academic Staff for the defense of a dissertation for the educational and scientific degree "doctor."

I confidently give my positive assessment and recommend to the Scientific Jury to award the educational and scientific degree "doctor" to Yordan Krasimirov Hodzhev in the field of higher education 4. "Natural Sciences, Mathematics, and Informatics", direction 4.3 "Biological Sciences" in the scientific specialty "Microbiology."

Sofia, 15/01/2024

Prepared the review:

/Assoc. Prof. Ivan Ivanov/