REVIEW

From Associate Professor Viktoria Stefanova Levterova, PhD, Head Department "Microbiology" and NRL "Molecular Microbiology", NCIPD, Sofia

On a dissertation work presented to the scientific jury formed by order № 583/01.12.2023 of the Director of NCIPD, for the acquisition of educational and scientific degree "DOCTOR" in the scientific specialty "Microbiology" - code 01.06.12. In the field of higher education 4. "Natural sciences, mathematics and informatics". Professional direction 4.3. "Biological Sciences"

Autor: Borislava Ilieva Tsafarova

PhD form: full-time doctoral student

Topic: *"Microbiological, electron-microscopic and molecular-biological methods for studying the pathogenesis of sarcoidosis"*

Scientific supervisor: Prof. DSc, Stefan Panaiotov, NCIPD

The presented for official defense dissertation work on the topic "Microbiological, electron-microscopic and molecular-biological methods for studying the pathogenesis of sarcoidosis" by Borislava Ilieva Tsafarova, gives me the reason to formulate my review as follows:

Relevance of the topic

Sarcoidosis is a multisystem granulomatous disease of unknown etiology. Clinical manifestations are nonspecific and diverse. In over 90% of the cases, a pulmonary form is manifested, affecting the lungs and/or intrathoracic lymph nodes. The course of the disease ranges from self-limiting to debilitating and associated with early mortality. It is presumed that sarcoidosis is triggered by an unknown antigen(s) in people with abnormal immune responses and a genetic predisposition. Attempts to establish the etiological agent of the disease have been ongoing for more than 140 years. Some researchers consider sarcoidosis an autoimmune

disease, others point to a non-infectious agent from the surrounding, domestic, or work environment as a triggering factor. Others speculate the involvement of one or several different microorganisms in the pathogenesis of sarcoidosis. Currently, more and more data point to the infectious etiology of the disease or the development of the disease following dysbiosis of the microbiome. Multiple investigators have demonstrated the presence of microorganisms in sarcoid specimens. Since to date, no single etiological agent of sarcoidosis has been proven, it is possible that various types of microorganisms participate in the pathogenesis of the disease. Most evidence has been accumulated for representatives of the genus Mycobacterium (*Mycobacterium tuberculosis*) and for *Cutibacterium acnes*. Borislava Tsafarova focuses on researching the microbial involvement in the etiology of sarcoidosis. Her main goal is to study the microbiome in patients with sarcoidosis and control subjects using molecular, cultural and microscopic techniques.

Research on the topic

The dissertation is constructed in a traditional form with relevant sections - introduction, review of the literature, aim and objectives, materials and methods, results and discussion, conclusions, contributions and cited literature. The results of each completed task are accompanied by a discussion. The dissertation contains 169 pages, 42 figures and 11 tables. The bibliography includes 213 cited sources. A high percentage of them are from the last few years, which emphasize its relevance.

The review of the literature is written informatively and presents a detailed overview of the state of research on the problem. The scientific trends and hypotheses for clarifying the pathogenesis of sarcoidosis are described.

The materials and methods section presents a detailed description of the applied methods. The methods are diverse and interdisciplinary. They include microbiological cultivation, molecular techniques, scanning and transmission electron microscopy, other microscopic techniques and next-generation sequencing. For the purposes of the dissertation, pairs of materials were examined - blood and tissue biopsy samples from 13 patients with suspected sarcoidosis, and blood, biopsy and bronchoalveolar lavage samples from 31 patients suspected of sarcoidosis. For some of the experiments involving blood culture and electron microscopy, blood from seven healthy volunteers was used. Other examined materials included 20 formalin fixed paraffin embedded (FFPE) biopsy material from patients with pulmonary sarcoidosis, and 19 FFPE tissue materials from patients with pulmonary tuberculosis.

For the set tasks and sub-tasks, important and substantial results have been achieved. The results are illustrated with 42 figures and 11 tables. The description of the results is accurate and clear and is written in good scientific language. Each result is discussed, facilitating reading and comprehension. The visualization of the results is professionally crafted. The photographic material is clear and with good contrast. The description of the figures is detailed and understandable.

The doctoral candidate successfully completes her two complex tasks:

Task 1. Analysis of the blood and tissue microbiome of patients with sarcoidosis through the cultivation of blood from patients with sarcoidosis and control subjects under normal and stressful conditions. Microscopic morphological analysis of microbial species in cultured whole and lysed blood. Extraction of DNA from cultured (blood) and uncultured clinical materials (blood and biopsy) from patients with sarcoidosis, and control subjects, and conducting microbiome analysis through next-generation sequencing.

Task 2. Investigating the potential involvement of *Cutibacterium acnes, Mycobacterium tuberculosis*, and other mycobacteria and microorganisms in the pathogenesis of sarcoidosis through the study of clinical materials (biopsy, blood, and BAL) from patients with sarcoidosis, using PCR. Additional immunohistochemical examination with monoclonal PAB antibody for the detection of *Cutibacterium acnes* in lymph nodes affected by sarcoidosis.

The obtained results describe the morphology and mechanisms of proliferation in blood microbiota. Borislava Tsafarova has published a study describing the mechanisms of division, using light and electron microscopy. She discovers that blood microbiota form electron-dense and electron-transparent microbial bodies. Visually, she observes and discovers a new mechanism of reproduction of blood microbiota, where daughter cells do not leave the mother cell but grow inside, and new daughter cells proliferate within them. This mechanism of division is called "cell within a cell" and is compared to matryoshka dolls where several smaller dolls can fit inside a larger one.

The doctoral candidate investigates the role of *Cutibacterium acnes, M. tuberculosis*, other mycobacteria, and *Toxoplasma gondii* in the etiology of sarcoidosis using PCR with species- and genus-specific primers. The results show that these microbial species are related to the disease. Based on the metagenomic data from the sequencing of samples (blood, BAL, and biopsy) from patients with sarcoidosis, the relationship of *Cutibacterium acnes*, *Toxoplasma gondii*, *M. tuberculosis*, and other mycobacteria as potential pathogens involved in granuloma formation was studied. Results (unpublished) of *Toxoplasma gondii* in the

pathogenesis of sarcoidosis were obtained. To date, there is no published evidence of the participation of this parasite in the pathogenesis of sarcoidosis in the literature.

In summary, the "Results and Discussion" section is written competently. It is very well illustrated and documented.

The conclusions are correctly formulated and summarize the main points of the work. Borislava Tsafarova formulates five scientific-theoretical contributions of an original nature and four scientific-applied contributions with which I fully agree. The conclusions can be summarized as follows:

1. FFPE tissue samples are not a suitable model for molecular-biological study of the pathogenesis of sarcoidosis. The residual DNA is severely damaged due to treatment with toxic reagents and aging.

2. Blood microbiota differs in morphology, but generally, there are two types: electron-dense bodies and electron-transparent bodies.

3. Electron-dense bodies proliferate by budding or forming chains or grow and release progenitor cells with sizes of 180 – 200 nm.

4. A new mechanism of reproduction called "cell within a cell" has been observed.

5. The role of mycobacteria, *Cutibacterium acnes*, and *Toxoplasma gondii* in the pathogenesis of sarcoidosis requires further studies.

6. A characteristic microbiome profile of the blood and a characteristic microbiome profile of the lung tissue, which differ from each other, are observed.

7. Microbiome analysis shows that other microbial species could also participate in the pathogenesis of sarcoidosis.

Publications and personal contribution of the doctoral candidate

The results have been published in 7 scientific articles on the topic of the dissertation. Three are in journals with an impact factor such as Frontiers in Cellular and Infectious Microbiology, Microorganisms, and Computational and Structural Biotechnology. A cited poster in a journal with an impact factor is applied, which according to Appendix 1 of the NCIPD Regulations for the implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria is considered a publication with an impact factor. The journal in which the cited poster is published is ERJ Open Research and has an impact factor of 4.6. The publications have a total impact factor of 16.359 (or 20,959). The publications have 13 citations to date. The doctoral candidate has presented her results at 16 international and 7 national scientific forums. The number of accumulated credit points is 747. According to the

regulations of NCIPD and the National Agency for Quality in Higher Education, the doctoral candidate exceeds the necessary 200 credit points for the defense of the educational and scientific degree "Doctor". It is evident that the doctoral candidate has made a substantial personal contribution to the research and has worked diligently and in-depth on her dissertation.

I have no critical remarks on the structure, results, and description of the doctoral work.

In conclusion, the dissertation of Borislava Ilieva Tsafarova is a thorough and professional study of the blood and tissue microbiome in sarcoidosis patients and healthy individuals. The dissertation is innovative and has scientific and practical significance. Therefore, I believe that the reviewed dissertation fully meets the requirements of the Law on the Development of Academic Staff in the Republic of Bulgaria, the Regulations for its implementation, and the Regulations of the NCIPD for awarding the educational and scientific degree "Doctor".

I give my **positive assessment** and support the awarding of the educational and scientific degree "Doctor" in the scientific specialty "Microbiology" to Borislava Ilieva Tsafarova.

12.01.2024

Reviewer:

Assoc. Prof. Victoria Levterova, PhD