To the Chairman of the Scientific Jury,
appointed by order 582/01.12.23
of the Director of the NCIPD

REVIEW

by assoc. prof. Dr. Tanya Georgieva Dimova, Institute of Reproductive Biology and Immunology "acad. K. Bratanov", Bulgarian Academy of Sciences, Sofia

of a dissertation for awarding the educational and scientific degree "DOCTOR", scientific specialty "Microbiology", Biological Sciences, Natural Sciences, Mathematics and Informatics

To full-time doctoral student **Borislava Ilieva Tsafarova**, Department of Microbiology, NATIONAL CENTER FOR INFECTIOUS AND PARASITIC DISEASES, Sofia

Topic of the dissertation: **"Microbiological, electron microscopic and molecular biological methods to study the pathogenesis of sarcoidosis"**, supervisor: Prof. Dr. Stefan Panayotov, PhD

The review was prepared in accordance with the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria and the Rules for its Application.

Relevance and importance of the topic

Sarcoidosis is a rare inflammatory disease characterized by the development of granulomas in various organs, especially in the lungs and lymph nodes. The clinic of the disease largely depends on the affected organ and can vary from mild symptoms to life-threatening manifestations. Despite significant progress over the past two decades in the diagnosis, clinical evaluation, and treatment of sarcoidosis, unfortunately, the exact etiology of this disease remains unknown. An infectious etiology is suggested, with the involvement of mainly *M. tuberculosis* and *C. acnes*. Current evidence suggests genetic predisposition as possible etiologic factors, as well as an autoimmune response, as symptoms and pain are relieved in response to corticosteroids or immunosuppressive drugs. Several recent epidemiological and microbiological studies raise the hypothesis that, at least in some patients, the microbiota or their products may trigger an immune response leading to sarcoid granuloma formation. The lack of clarity around the etiology of the disease strongly argues for recent PhD thesis. The author also paid due attention to the Bulgarian contribution to the study of this disease by Dr. Emil Kalfin, who introduced a modified method for culturing blood microbiota, which is also used in this dissertation.

Characterization and evaluation of the dissertation work

The dissertation explored the possible infectious etiology of sarcoidosis and one based on microbiome imbalance. The work is written on 169 pages, there are 42 figures and 11 tables and 213 references. The work is well-structured with the following sections – **Introduction** (1 p.), **Literature review** (36 p.), **Aim and objectives** (1 p.), **Materials and methods** (36 p.), **Results and discussion** (65 p.), **Conclusions** (1 p.), **References** (21 p.). The literature review is informative and concise, structured in separate paragraphs. The author introduces the various symptoms of the disease, as well as its unclear etiology and pathogenesis. Therefore, the complication of the diagnosis of sarcoidosis motivates the present study. Recent data on the role of the microbiome in the pathogenesis of this disease is also indicated. The reader slowly is prepared for the new and interesting data obtained during the development of the dissertation work.

The **goal of the thesis** is clearly stated. There are 2 Tasks with 2 subtasks for the realization of the goal. I have a remark about the description of Task 1b:

1b) Isolation of DNA from cultured (blood) and uncultured clinical materials (blood and biopsy) from sarcoid patients and controls and performing microbiome analysis by next-generation sequencing.

In my opinion, the Task could be formulated as follows: Microbiome analysis by next-generation sequencing of sarcoid patients and controls..... Sequencing anyway requires DNA isolation.

In the **Materials and Methods** section, the good selection of research materials such as blood, tissue biopsy and lung lavage from patients suspected of sarcoidosis and blood from healthy volunteers, as well as archival paraffin sections from patients with sarcoidosis and tuberculosis, is impressive. The study included a total of 44 patients admitted to the "St. Sofia" to confirm or reject the diagnosis of pulmonary sarcoidosis, which are summarized by gender, age, diagnosis and accompanying diseases in a table. Patient groups (proven for sarcoidosis), patients with cancer and suspected for sarcoidosis, patients with tuberculosis, as well as a control group - healthy volunteers, were defined. The materials were obtained after completing an informed consent and complying with the established regulations. Relevant and varied methods were used, which were comprehensively described and could easily be replicated by other researchers. Methods such as DNA isolation from blood, tissue and archival paraffin blocks, PCR, agarose electrophoresis, microbiological cultivation of microbiota, specific Heinz corpuscle staining techniques and silver impregnation of sarcoid granulomas, immunohistochemistry, light microscopy, transmission electron microscopy and sequencing 16S and 18S metagenomic targeted and random, bioinformatics analyses. It makes a nice impression that the information about the types of research samples and the methods used is presented through infographics - an attractive way of visualizing text for easier perception by the reader.

The **"Results"** section also includes a **Discussion** of the results, and I think it would be more correct if the section was called "**Results and Discussion**". Presenting the results in groups

with subsequent discussion on them is not unusual and is a matter of choice for the PhD student and her supervisor. This is the easiest and fastest way to compare your own results with those of other authors. The discussion is the most difficult and at the same time the most interesting part of any dissertation work and is indicative of the professional preparation of the doctoral student. In the discussion immediately after each group of results, the student correctly reflected the published data of others in relation to her own results. The results and the accompanying discussion are grouped according to the tasks. The results of a microbiological, electron microscopic and genetic study of the microbiota of healthy volunteers versus those of patients with sarcoidosis – in native and cultured blood samples under stress conditions are shown. The results are illustrated with informative and sufficient set of light and electron microscopic images. The blood microbiota in uncultured samples has been shown to be undetectable by light microscopy, and thus TEM is an extremely important methodology for studying the blood microbiota. An interesting result was the testing of a specific medium for resuscitation and cultivation of the blood microbiota, proved that the majority of the blood microbiota can be cultured and studied. Although the main idea is to study the blood microbiota in patients with sarcoidosis, the relatively high microbial abundance in the blood of healthy individuals is of interest as well. It was determined that the blood microbiota in all tested blood samples from healthy donors reached high microbial biodiversity, a total of 55 bacterial and 39 fungal genera were proven by sequencing. Cultured blood samples show significant numbers of microbial taxonomic units that are not detected in uncultured ones, giving the point to use cultured-uncultured sample pairs when testing microbial biodiversity. Like L-forms, the blood microbiota can reproduce in various ways, which are described in detail in plenty of images. The student describes a new matryoshka-like propagation phenomenon called "cell-in-cell". The existence of blood microbiota in clinically healthy individuals is a very interesting fact, and this is not necessarily associated with an infection or a disease state. Such findings have been the subject of debate in the scientific community recently. These microbial structures are accepted as normal resident microorganisms in the blood - commensals. The author emphasizes that, at the moment, no studies have been found in the scientific literature studying the microbial abundance of the blood and tissue microbiome in sarcoidosis in parallel. In the present pilot study, formation of a “characteristic microbial profile” of lung tissue and a “characteristic microbial profile of blood” was observed, but no clustering was detected between blood and biopsy samples from the same sarcoid patient. The word “characteristic” could easily be replaced by “specific”. Such data deserve further investigation. In order to clarify the possible involvement of *C. acnes* and *M. tuberculosis* in the etiology of sarcoidosis, archival biopsy samples fixed in formalin and embedded in paraffin were examined. For the isolation of DNA from embedded tissue in paraffin two methods were compared to choose more suitable for the subsequent use in PCR detection of *C. acnes* and *M. tuberculosis*. However, the absence of *M. tuberculosis* DNA in all paraffin blocks of tissue from tuberculosis patients is questionable. That is why the author discusses in detail the shortcomings of this type of research for diagnosis and molecular biological studies of sarcoidosis. Acknowledging the limits of one's own research and outlining new research perspectives makes a good impression.

Seven conclusions are formulated, which I think would be better grouped into four or five. Conclusions 2 and 3 could, for example, be combined. Conclusions must be very well stated with accuracy in reflection of the results obtained.

I have the following **remarks and questions** to PhD student Borislava Tsafarova:

1. Do conclusions 2, 3, and 4 apply to the microbiota of sarcoid patients, healthy donors, or to altogether?

I personally lack data comparing the diversity of the blood microbiome in sarcoid patients versus the normal blood microbiome of healthy individuals. And there is such data in the dissertation.

2. Regarding conclusion 6 - doesn't it make sense that the blood microbiome profile would differ from any tissue microbiome?

3. Please explain conclusion 7: The microbiome analysis showed that other microbial species could also be involved in the pathogenesis of sarcoidosis - others, besides which, should be clear in the statement.

4. Based on all knowledge and skills, accepted or rejected hypotheses during the preparation of her dissertation, what is the opinion of the doctorand Borislava Tsafarova about the infectious etiology of sarcoidosis? Does she believe in the existence of *Kalfnela sarcoides*?

The above-mentioned small remarks do not in the least detract from the importance and relevance of the dissertation work, especially since the results have been widely disseminated and discussed in the international scientific community through their publication in highly impacted scientific journals. The results of the dissertation have been published in 7 papers, this is a rare phenomenon recently in Bulgaria, and I congratulate the PhD student, her supervisor and the entire group working on this problem. Two of the articles were published in journals from Q1 (IF - between 4 and 6), one from Q2, three from Q4. One article was published in a non-quartile journal. In four of the articles, the doctoral student is the first author, two are overviews with a single author, the doctoral student. These results have received community appreciation through 13 citations to date.

The Abstract correctly reflects the main results of the dissertation and is adequately formatted on 67 pages.

Doctoral student Borislava Tsafarova has fulfilled the requirements for the collected credits under the credit system for evaluating the doctoral student at the NCIPD. She has accumulated 667 points, which many times exceeds the requirements of ZRSRB and its Regulations.

CONCLUSION: In conclusion, I state that the PhD thesis of doctoral student Borislava Tsafarova was prepared and presented with the necessary scientific competence on the subject. This scientific work meets the requirements for the awarding of the educational and scientific degree "Doctor", laid down in the Law on the Development of the Academic Staff in the Republic

of Bulgaria, the Rules for its Application and the Rules of t NCIPD. I confidently give a positive assessment and recommend to the respected members of the Scientific Jury to make a decision to award the educational and scientific degree "Doctor" to Borislava Tsafarova in the scientific specialty "Microbiology", "Biological Sciences", "Natural Sciences, Mathematics and Informatics".

January 15th 2024

Sincerely yours

assoc. prof. Tanya Dimova