Szczecin, 5th August 2025

The review of the doctoral thesis of Ms. Konkonika Roy, M.Sc. entitled *„The dark side of endotoxin tolerance: increased susceptibility to cancer"* under the supervision of dr hab. Sylwia Wrotek, prof. NCU and the co-supervision of Dr Tomasz Jędrzejewski from the Department of Immunology of the Nicolaus Copernicus University in Toruń. The review has been prepared due to the vocation of the Biology Discipline Council of the Faculty of Biological and Veterinary Sciences Nicolaus Copernicus University in Toruń dated at 12th June 2025, signed by dr hab. Dariusz Smoliński, prof. NCU.

I. Basic characteristics of the doctoral thesis

The doctoral thesis of Ms. Konkonika Roy, M.Sc. is composed out of three original – two papers published, and one paper submitted to prestigious journals in the field of biology. It is important to note, that – as claimed by Ms. Konkonika Roy and other authors – the authorship and contribution of Ms. Roy, M.Sc. was pivotal for the creation of these papers. Below is the list of the papers, along with Ms. Roy's, M.Sc. contribution:

1. ***Roy K.**, Kozłowski H.M., Jędrzejewski T., Sobocińska J., Maciejewski B., Dzialuk A., Wrotek S.: Endotoxin Tolerance Creates Favourable Conditions for Cancer Development. Cancers 15 (20), 5113, 2023* (IF_{2023} 4,5; punkty MEiN 200)

   In this paper, Ms. Konkonika Roy is the first and corresponding author (together with Prof. Sylwia Wrotek). She took lead in designing and conducting key experimental procedures – performed the cell survival assays, investigated migratory capacity and assessed the clonogenic potential of the cancer cells. She

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also conducted ELISA assays, flow cytometric analysis. She also did the data curation, interpretation, analysis and writing the paper, prepared figures and proofreading.

2. ***Roy K.***, *Jędrzejewski T., Sobociński J., Spisz P., Maciejewski B., Hövelmeyer N., Passeri B., Wrotek S.: Divergent impact of endotoxin priming and endotoxin tolerance on macrophage responses to cancer cells. Cellular Immunology 411-412, 104934, 2025* (IF_{2024} 3,7; punkty MEiN 100)

   In this paper, Ms. Konkonika Roy, M.Sc. is also the first and corresponding author (together with Prof. Sylwia Wrotek) and her role is also a primary one, that is she conducted an in vitro study – made the analysis of gene expression and protein levels, performed nitric oxide production assays and ROS analysis. Moreover, she designed and executed co-culture experiments with 4T1 cancer cells and assessed macrophage phenotypes using the flow cytometry. She was also responsible for data curation, interpretation and statistical analysis and participated in writing and reviewing process.

3. ***Roy K.***, *Maciejewski B., Jędrzejewski T., Spisz P., Sobocińska J., Di Pentima M., Passeri B., Wrotek S.: Endotoxin tolerance enhances breast cancer aggressiveness and alters inflammatory marker expression in tumor and spleen of mice. International Immunology 2025* (submitted on the 21st of May 2025) (IF_{2025} 5,0; punkty MEiN 140)

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In this paper, that is submitted to the journal, Ms. Konkonika Roy, M.Sc. was responsible for planning and executing the in vivo experiments, developed the endotoxin tolerance model in mice. She collected tissues for analysis, evaluated gene expression levels and contributed to the writing process. As in the previous papers, she (together with Prof. Sylwia Wrotek) is a corresponding author of this paper.

Moreover, at the end of the PhD thesis, the Author included list of her achievements – 2 papers published (excluding the papers from the thesis), 5 conferences' attendance, additional funding and internships. I highly values the achievements of the candidate showed in this section.

The overall scientific soundness of the papers included in the doctoral dissertation of Ms. Konkonika Roy. M.Sc. is very high. All papers are published (or submitted) in highly recognized scientific journals from the discipline of biology, listed on JCR list. In my opinion the construction of the doctoral dissertation is proper. The subject of the doctoral dissertation is crucial and up-to-date. Endotoxin tolerance (ET) is a mechanism that develops in organism due to repeated or prolong endotoxin exposure, for example during infections caused by Gram-negative bacteria. Due to the phenomena of a weakend febrile response being a hallmark of ET, a hypothesis exists, that ET may be responsible for the reduced fever-related immune response observed in cancer patients. In the light of the above, the aim of the study was to investigate, if the ET promotes cancer progression. Specific objectives were:

- examining the impact of ET on macrophages and cancer cells, and this was achieved in paper 1;
- studying the effect of the tumor microenvironment on endotoxin-tolerant macrophages, done in paper 2;
- examining the effect of ET on tumor development in mice, performer and submitted in paper 3.

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I conclude, that the aims of the PhD thesis have been correctly stated and properly implemented. The PhD candidate demonstrated a clear understanding of the research objectives and employed appropriate methodologies to achieve them. The outcomes of the work are coherent with the initial goals and contribute meaningfully to the field of study.

The study was performed with the use of two systems: in vitro and in vivo with the use of several methods, showing the high performance and abilities of Ms. Konkonika Roy, M.Sc.

Practical aspect of the PhD thesis is unquestionable – the role of ET in the progression of cancer is a clinical issue that might be of use in further research and therapeutic development. Understanding this mechanism could contribute to identifying new prognostic markers or treatment targets. The findings have potential translational value and may inform future clinical strategies in oncology.

The overall impression of the doctoral dissertation is highly positive. The aforementioned arguments clearly demonstrate the solid methodological foundation and in-depth subject knowledge of Ms. Konkonika Roy, MSc. Her work reflects a strong commitment to scientific rigor and contributes meaningfully to the field.

My remarks for this formal part of the thesis are:

- The dissertation does not include statements from other authors. In my opinion, their presence would be an additional and strong argument supporting the leading role of the doctoral student.

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II. Substantive evaluation of the work with a discussion of the individual works comprising the doctoral dissertation

Paper 1: ***Roy K.****, Kozłowski H.M., Jędrzejewski T., Sobocińska J., Maciejewski B., Dzialuk A., Wrotek S.: Endotoxin Tolerance Creates Favourable Conditions for Cancer Development. Cancers 15 (20), 5113, 2023*

This paper includes the results from in vitro model of ET macrophages. The aim of the paper was to investigate the impact of ET on macrophage behaviour and its role in cancer development. The results showed the suppression of TNFα and IL-6 in both monocultures – Mo_{ET} and coculture of these macrophages with cancer cells; increase in survival capacity of cancer cells cultured with Mo_{ET} and increase expression of CD80 in Mo_{ET} comparing to other cells. The thourough investigation of macrophage-cancer cells crosstalk with different methodology led to the conclusion, that ET may promote cancer progression. Moreover, the macrophages' susceptiblity to endotoxin changes over time – showing the ability of reprogramming for macrophages in the context of endotoxin-tolerance. As the Author stated, this is the first report on such an issue in literature. Looking at the data from this paper, it is natural that the Authors decided to go into the vivo model, this is a very bright way of experimenting. On the basis of the results, I would like the candidate to answer what are the potential clinical implications of macrophage reprogramming in the context of endotoxin tolerance for cancer therapy?

Article 2: ***Roy K.****, Jędrzejewski T., Sobociński J., Spisz P., Maciejewski B., Hövelmeyer N., Passeri B., Wrotek S.: Divergent impact of endotoxin priming and endotoxin tolerance on macrophage responses to cancer cells. Cellular Immunology 411-412, 104934, 2025*

This paper, done in a very fruitfull and well-establish cooperation (yet, the statements of the authors are more than needed). This stage of the PhD thesis aimed to determine, if ET triggers

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a shift in macrophage polarization and metabolism in vitro, by evaluating the response of ET macrophages upon contact with cancer cells. The results showed the following characteristics of Mo_{ET} – suppression of proinflammatory mediators such as NO, iNOS, COX-2, elevation of ROS levels, increased capacity of survival, CD163 expression and notable metabolic flexibility. The results, based on surface marker analysis, demonstrated a shift toward the M2 phenotype in breast cancer-associated macrophages – an immunosuppressive profile. These findings indicate that endotoxin tolerance (ET) not only alters macrophage function but may also provide a survival advantage, potentially reshaping the tumor microenvironment in a distinct and significant manner.

I Wonder, if the PhD candidate can comment on how the potential survival advantage conferred by ET might influence the design of immunomodulatory treatments in cancer?

Article 3: ***Roy K.****, Maciejewski B., Jędrzejewski T., Spisz P., Sobocińska J., Di Pentima M., Passeri B., Wrotek S.: Endotoxin tolerance enhances breast cancer aggressiveness and alters inflammatory marker expression in tumor and spleen of mice. International Immunology 2025*

This paper was designed to further extend on the ET role in the whole organism. The model implemented mice treated with four doses of LPS. This experiment (performed on many different levels and several methods) led to the observation of the absence of febrile response and decreased motor activity in the endotoxin tolerant breast cancer bearing (ETBC) mice in response to a pyrogenic dose of LPS. Moreover, ETBC mice showed early tumor initiation and faster tumor growth in comparison with breast cancer bearing (BC) mice. Also, faster deterioration of health condition and lower leukocytes counts and smaller spleen size in ETBC mice compared to the BC group was noticed. What is also of a great importance, there were changes in the expression of immune-related genes in spleen and tumor tissues between the two studied groups registered. I am very found of the experiment designed for this study and the results, and I would like the PhD candidate to answer if the suppressed febrile response and

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immune dysregulation in ETBC mice could serve as potential early indicators of tumor-promoting immunosuppression in clinical settings in her opinion.

Based on all the work included in her doctoral dissertation, Ms. Konkonika Roy, M.Sc., drew a number of relevant and accurate conclusions:

1. ET impacts the systemic and local immune response;
2. ET reprograms macrophage functions toward a pro-tumorigenic phenotype, altering both metabolic and inflammatory signaling pathways;
3. ET enhances cancer cel aggressiveness;
4. ET accelerates tumor progression in vivo.

I would like to emphasize that I am greatly impressed by the multi-faceted nature and broad perspective of the results that have been noted and described by Ms. Konkonika Roy, M.Sc. In my opinion, the conclusions drawn by the author are appropriate, well-formulated, and consistent with the objectives of the doctoral dissertation. The PhD candidate has undoubtedly identified the right research gap, planned the research well (using a very wide range of in vitro and in vivo studies) and successfully solved the research task. I consider this work to be very ambitious and fully meeting the requirements. Also, the results presented in the papers representing the PhD thesis are showing great biological importance and possible practical character. The results constitute an important step forward understanding the role of ET in the tumor progression. They shed new light on the complex interactions between immune cells and the tumor microenvironment, particularly under conditions of endotoxin tolerance. Such insights could pave the way for identifying novel therapeutic targets or strategies aimed at modulating immune responses in cancer.

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III. Final remarks and conclusion

I hereby confirm that the PhD thesis of Ms. Konkonika Roy, M.Sc. entitled *„The dark side of endotoxin tolerance: increased susceptibility to cancer"* under the supervision of dr hab. Sylwia Wrotek, prof. NCU and the co-supervision of Dr Tomasz Jędrzejewski from the Department of Immunology of the Nicolaus Copernicus University in Toruń is a valuable and a high quality original study of a research problem. The assumptions and objectives of the dissertation are ambitious, accurately formulated, and fully realized. The conclusions drawn are both original and of great substantive value – they not only effectively answer the research questions posed, but also constitute a significant scientific achievement with high potential for practical application. Therefore I confirm, that this PhD thesis **meets all the conditions** specified in Article 187(1) and (2) of the Act of July 20, 2018, on Higher Education and Science (Journal of Laws of 2024, item 1571). I therefore request that Ms. Konkonika Roy, M.Sc. **be admitted** to the further stages of the doctoral procedure.

At the same time, given the exceptionally high standard of the doctoral dissertation I have reviewed, and in particular:

- the clear definition and relevance of the chosen research topic, which addresses a timely and important issue;
- the use of a well-selected and comprehensive set of research methods;
- the ability to formulate meaningful and precise conclusions, as well as to thoughtfully interpret and discuss the findings in relation to existing literature, including confidently addressing new questions arising from the data;
- the demonstrated skill in preparing scientific articles published in respected peer-reviewed journals;
- and the clear practical and societal relevance of the research,

I hereby recommend that the dissertation be recognized with **an appropriate distinction**.

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