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Reviewer's Report on the Thesis entitled Advancing Transcriptomics-based and AOP-anchored Predictive Models for Carbon Nanotube Inhalation: the Case Studies on Acute Phase Signaling–driven Inflammation by Mr. Sattibabu Merugu

The doctoral dissertation by Mr. Sattibabu Merugu has been carried out at the Faculty of Chemistry of University of Gdańsk (Poland) under the scientific supervision of prof. dr hab. Tomasz Puzyn and dr Karolina Jagiełło. The dissertation formally consists of seven main chapters, which include: i) Introduction, ii) Research Problems, Hypotheses, and Objectives, iii) Research Methodology, iv) Results and Discussion, v) Conclusions, vi) References, and vii) Supplementary Materials presenting scientific achievements. It also includes a summary in both Polish and English, along with a list of abbreviations. The entire presentation is given on 141 pages. The first chapter plays an introductory role. It presents the structural classification of single-walled (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), with their definitions along with the discussion of their key properties such as chirality, mechanical and electrical characteristics, stability, size and aspect ratio, structural rigidity, surface chemistry, potential for functionalization, as well as the presence of impurities and residual catalysts. Another subchapter focuses specifically on the applications of carbon nanotubes and their market implications. These applications are wide-ranging, spanning fields such as electronics, agriculture, biomedicine, catalysis, and photovoltaic technologies. While I would have appreciated more examples related to nanomedicine and biology, I acknowledge that a balanced selection of applications was necessary. The rapid development of the global carbon nanotube market is, in my view, remarkable. The next section of the introduction addresses the mechanisms of CNT-induced toxicity, which is a crucial topic directly related to the dissertation's objectives. It discusses oxidative stress and the generation of reactive oxygen species, frustrated phagocytosis and chronic inflammation, genotoxicity, and pulmonary fibrosis. Although each of these areas could be further expanded, the discussion provides a well-balanced overview within the scope of a review. Following this, the chapter explores issues related to safety assessment, experimental data, and the selection of descriptors in Quantitative Structure Activity

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Relationship (QSAR) modeling. The final part introduces machine learning methods used in data-driven modeling, with particular focus on Hierarchical Clustering Analysis (HCA), Principal Component Analysis (PCA), Kernel-weighted Locally Polynomial Regression (KwLPR), and tree-based learning techniques. These theoretical tools are applied in the research described in this work. In my opinion, this chapter is written in a comprehensive way, so that even a non-specialist can gain a lot from it. I read it with pleasure. The literature part refers to 87 publications out of 146 presented in the bibliography section. The concept and work plan are presented concisely. The experimental data, derived from external sources including animal and transcriptomic studies were taken from the outer source, and the software utilized in the study is also specified.

The dissertation is built upon the hypothesis that carbon nanotubes with similar physicochemical properties and transcriptomic profiles can induce comparable molecular initiating events within the acute phase response signaling pathway, ultimately resulting in analogous downstream adverse outcomes at the tissue level. The central research question driving this work is how can transcriptomic data and machine learning techniques be integrated to develop predictive models of acute phase-driven inflammation caused by carbon nanotube (CNT) inhalation exposure. There are three main objectives of the work. The first one concerns the impact of multi-walled CNT's properties on lung pathologies and atherosclerosis through acute inflammation, the second one concerns a comparative analysis of single-walled and multi-walled carbon nanotubes (SWCNTs and MWCNTs) to identify both similar and distinct structural features influencing the acute phase response pathway, while the third one concentrates on models and analysis of the impact of metal impurities in CNT's as potential factors influencing transcriptomic disturbances that drive inflammation and fibrosis mechanisms. A global AOP-informed Nano-QSAR model is developed to quantitatively associate specific metal contaminants in CNTs with gene expression alterations, particularly within the acute phase response signaling pathway. Concerning the first task the student developed an AOP-anchored Nano-QSAR model to predict molecular-level inflammatory responses that may lead to lung fibrosis and atherosclerosis following inhalation exposure to multi-walled carbon nanotubes (MWCNTs). Using the Kernel-weighted local polynomial regression (KwLPR) algorithm, the physicochemical properties of 14 MWCNTs were linked to transcriptomic responses of genes involved in the acute phase response (AR) signaling pathway. Importantly, the results indicated that the aspect ratio and specific surface area are the key determinants of CNT induced toxicity. This is a very interesting conclusion, and I find it particularly important to note that nanotubes characterized by lower aspect ratios and higher specific surface areas exhibit reduced toxicity in relation to their ability to disrupt the acute phase response signaling pathway. The other factors that can influence biological response are surface modifications, residual metal impurities, rigidity or formation of a protein corona. The developed model provides mechanistic insights into nanomaterial toxicity of nanomaterials and represents a novel in silico new approach methodology (NAM) to support risk assessment. The second part of the work focuses on a comparative analysis of single-walled and multi-walled carbon nanotubes (SWCNTs and MWCNTs) to identify both common and distinct structural features influencing the acute phase response pathway. This is a very interesting part, but it should be noted that the relatively small number of SWCNTs included in the analysis (only seven systems) may limit the conclusions in some way. Hierarchical Cluster Analysis (HCA), Principal Component Analysis (PCA), and Pearson correlation was applied to 21 CNTs within an Integrated Approaches for testing and assessment (IATA) framework to integrate structural descriptors with biological response data. The analyses revealed six mechanistically coherent clusters driven by factors such as surface area, surface functionalization, and metal oxide impurities. PCA distinguished S- and MWCNTs primarily by metal enrichment (PC1) and surface characteristics (PC2), both of which correspond to molecular initiating events (MIEs) linked to chronic pulmonary outcomes such as fibrosis and atherosclerosis. Importantly, for MWCNTs, structural features such as aspect ratio, specific surface area, surface functionalization and metal impurities were strongly linked to transcriptomic perturbations and fibrogenic responses. In the case of SWCNTs, although surface area and functionalization were robust predictors of toxicity, the relationships appeared more complex and did not follow the linear trends. Metal impurities have been shown to be a particularly important factor in SWCNT toxicity. This suggests that SWCNTs possibly may require distinct mechanistic modeling approaches. However, as pointed above, the limited number of SWCNTs studied, along with insufficient data on their length and diameter, limits the validity of these conclusions. Despite of this I really appreciate this part of the thesis. Finally, the last part of research investigates how metal impurities in CNTs contribute to transcriptomic disturbances driving inflammation and fibrosis after inhalation exposure. A global AOP-informed Nano-QSAR model has been developed to quantitatively link specific metal contaminants to gene expression changes within the acute phase response (AR) pathway. It's construction was, in opinion, rather challenging and required a lot of work. It supports the mechanistic understanding of metal-driven toxicity and enhance predictive modeling for CNT hazard assessment.

The issues addressed and solved by the doctoral student are both interesting and important, not only in terms of advancing fundamental understanding of the key factors influencing CNT toxicity (critical physicochemical determinants as aspect ratio, surface area, functionalization, specific structure, rigidity or specific metal impurities), but also from the perspective of development of methodology. The models developed in this work, in my opinion, successfully identified key molecular drivers and early key events, demonstrating strong predictive performance. Using machine learning provided mechanistic insights beyond descriptive toxicology with focus on molecular pathways. One of the key tools employed by Mr. Merugu was Principal Component Analysis (PCA). In this context, I am curious about the potential applicability of a related approach, namely Factor Analysis (FA). QSAR models often rely on a large set of molecular descriptors, many of which may be intercorrelated. Factor analysis helps reduce this complexity by transforming the original correlated variables into a smaller number of uncorrelated factors. This simplification can enhance the robustness and predictive performance of QSAR models. Additionally, factor analysis can uncover hidden structures within the data by grouping related variables and identifying factors that reflect underlying chemical or physical properties influencing biological activity. While this approach is conceptually similar to Principal Component Analysis (PCA) used in this work, it can also serve as a complementary method. This question is raised purely out of curiosity. The doctoral thesis demonstrates a high level of proficiency, both in terms of editing and graphics. The dissertation is written in a concise way, successfully balancing the literature review and novel theoretical work. Minor imperfections are rare. Examples include typographical errors such as the incorrect decade of the discovery of SNTs (page 14), Mitsui-7 (page 75), or notations like "Fe2O3" instead of "Fe_2O_3" (pages 91, 92, 94, 104, 109 and 113). Additionally, the subchapters of section 1.2 are not listed in the Table of Contents. I also noted that the parameters n and m, which describe carbon nanotubes (CNTs), are not clearly defined—an illustrative figure or a scheme would suit well. While the bibliography has been compiled with care, there are a few issues with reference numbering. For instance, reference 88 should be 90, and reference 92 should be 94 (see page 56); similarly, references 136 and 137 on page 106 should correspond to references 138 and 139, respectively. Overall, the analyses, including the literature review, although detailed, are described in concise, scientific language. I highly appreciate the way the results are presented - all figures and graphs were prepared with great care. It is worth noting that a significant part related with the thesis has been published in a leading journal: *Small* [**2025**, *21*, 2501185], where the PhD student is the first author. It is worth mentioning that he coauthors two other papers: i) *Current Drug Targets* [**2019**, *20*, 1550-1562] and ii) *IOSR Journal of Pharmacy and Biological Sciences* [**2016**, *11*, 45-50]]. He also presented his work in the form of oral and poster presentations at international conferences/symposia. This demonstrates his dedication to science. To sum up: I am fully convinced that Mr. Sattibabu Merugu's doctoral dissertation contains important elements of scientific novelty. The PhD student defined the research problem well, planned it well and performed quite excellent research.

The doctoral thesis by Mr. Sattibabu Merugu meets all formal requirements of the current Act on Academic Degrees and Titles of Republic of Poland. Therefore, it is with great pleasure that I recommend to the Scientific Council of the Discipline of Chemistry at the University of Gdańsk that he be admitted to the next stages of the doctoral process.

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