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Review of the doctoral dissertation by Mr Grégory Petrazzo, titled „Interplay between senolytic drugs and microbiome preserve cognitive abilities during aging", conducted at the Laboratory of Molecular Bases of Aging and the Laboratory of Cell Biophysics of the Nencki Institute of Experimental Biology, Polish Academy of Sciences, under the supervision of Prof. Jakub Włodarczyk, PhD, D.Sc, and the assistant supervision of Adam Krzystyniak, PhD.

There is an increasing ageing population in Europe. In Poland, by 2050, nearly two-fifths of the population may be over 65. This trend is associated with an increase in the incidence of age-related diseases, including cardiovascular, neurodegenerative, metabolic, and other diseases, increasing morbidity and mortality. At the cellular level, aging is characterized by a number of harmful biological processes. One of the features is chronic, mild inflammation. It is characterized by a sustained increase in the expression of proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). The integrity and selective permeability of the blood-brain barrier may be disrupted. Furthermore, neuroinflammation and dysregulation of the central nervous system occur. The accumulation of senescent cells is a key factor in the aging process. These cells develop in response to genomic instability, DNA damage, oxidative stress, epigenetic changes, and others. In young organisms, they help inhibit cancer development by permanently arresting the cell cycle and preventing the proliferation of damaged cells. However, in older organisms, they accumulate, persist, and continue to secrete proinflammatory cytokines, chemokines, growth factors, and proteolytic enzymes, thereby accelerating aging. This is known as the senescence-associated secretory phenotype (SASP). Currently, therapeutic strategies are being explored to delay aging and its associated pathological changes. One approach is the selective elimination of senescent cells (senolytic therapies) or modulation of their function (senomorphic methods).

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Dasatinib and Quercetin (D+Q) are among the most widely studied senolytic therapies. They can selectively eliminate senescent cells by targeting anti-apoptotic survival pathways. Preclinical studies suggest that this combination may alleviate age-related dysfunctions, improving the functioning of multiple tissues and organ systems.

In his doctoral dissertation, Mr. Grégory Petrazzo, based on behavioral and molecular studies, undertook to investigate the influence of Dasatinib and Quercetin (D+Q) administration on memory parameters in aged Wistar rats, as well as treatment-associated changes in gut microbiota composition and predicted functional profile, microbiota-derived metabolites, intestinal and blood-brain barrier

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integrity, inflammatory status, and neurobiological correlates of cognitive function. The aim of this work is a very interesting and important approach, both from a cognitive and implementation perspective. It is presented clearly, logically, and draws on existing data in this area of research. In light of the extensive experimental and preclinical studies conducted to date, the dissertation demonstrates a solid general theoretical understanding of the mechanisms of action and therapeutic potential of the Dasatinib and Quercetin (D+Q) combination as one of the most widely investigated senolytic interventions.

The work submitted for evaluation consists of 166 pages and has an editorial layout typical of experimental works: Abstract in English and Polish; Abbreviations; Introduction; Materials and Methods; Results; Discussion; Summary and Conclusions; Bibliography; and the PhD candidate's publication. The work ends with a list of figures and tables. All elements of the dissertation are described very well and logically. The Introduction consists of 28 pages, divided into logical themes. It also includes interesting illustrations related to the topic. This approach enhances the educational value of the presented topic. Materials and Methods are described in detail. The experimental model was appropriately chosen. The studies were conducted on age-appropriate Wistar rats. A sufficient number of animals were included in the groups to capture both behavioral and molecular changes. Ethical approval for all experimental procedures was obtained. Moreover, what I personally appreciate about the choice of experimental model is the attention paid to its translatability to a heterogeneous population. The Active Allothetic Place Avoidance Task (AAPAT), the Open Field Test, and other methods, e.g., 16S rRNA microbiome sequencing, GC-MS and UHPLC-HRMS metabolomic profiling, histological and morphometric analyses of intestinal tissues, mucin quantification, qPCR and Western blot-based molecular analyses, epigenetic profiling, as well as DiI-labeled spine imaging for synaptic plasticity assessment, were appropriately selected to address the research questions posed in the dissertation. Moreover, the selection of both behavioral and molecular methods, along with the wide range of goals set, demonstrate the PhD student's research independence and critical approach to the topic. The literature has been correctly applied and is up to date.

The Results are described on 40 pages, divided into thematic subsections.

In the first part of the Results (3.1), the Author demonstrated that aged rats showed significantly impaired spatial memory and learning compared to young rats, using the Active Allothetic Place Avoidance Task. After 8 weeks of treatment with Dasatinib and Quercetin (D+Q), aged rats showed marked cognitive improvement, which included: fewer errors, fewer shocks, and better avoidance learning. These benefits were linked to enhanced cognitive function rather than changes in locomotor activity. Importantly, the improvements persisted for at least 5 weeks after treatment cessation. This suggests long-lasting effects of D+Q on memory and learning. No significant effects were observed in young rats, indicating that the treatment specifically targeted age-related cognitive decline.

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Results 3.2: The Author demonstrated that senolytic treatment with Dasatinib and Quercetin (D+Q) improved hippocampus-dependent learning and memory in aged rats without increasing the overall number of dendritic spines in CA1 neurons. The treatment, instead of promoting large-scale synapse formation, altered the morphology of existing spines, making apical dendritic spines longer and more elongated. The observed changes were associated with enhanced synaptic plasticity. These structural changes suggest that cognitive improvement resulted from functional remodeling of synapses rather than changes in synapse quantity. The effect was specific to apical dendrites, while basal dendrites remained unaffected. Overall, the findings indicate that D+Q may rejuvenate hippocampal function through subtle synaptic remodeling.

Results 3.3: The Author demonstrated that senolytic treatment with Dasatinib and Quercetin (D+Q), in aged rats, partially reversed age-related epigenetic alterations in the hippocampus. After eight weeks of treatment, the repressive histone mark H3K9me3 decreased by about 50%. At the same time, H3K27me3 increased by about 20%. This demonstrates the restoration of a more youthful chromatin state. It supports synaptic plasticity and memory-related gene expression. The molecular changes coincided with improved spatial learning and memory, observed earlier in the study. The findings suggest a possible causal pathway in which the removal of senescent cells leads to chromatin remodeling and subsequent synaptic recovery. However, no significant changes were found in the chromatin-associated proteins Lamin B1 and HMGB1.

Results 3.4: In subsequent experiments, using SA-β-gal staining, the Author attempted to evaluate the burden of senescent cells in the brain after treatment. It is a widely recognized marker of cellular senescence. This method allowed comparison of the quantity and distribution of senescent cells among the experimental groups. The findings showed no significant differences in SA-β-gal activity or chromatin-associated senescence markers between groups within the CA1-CA3 region of the hippocampus. This indicates that the overall level of detectable brain cell senescence was consistent across groups. However, functional improvements were still observed, suggesting these benefits occurred independently of measurable reductions in senescent cell burden.

Results 3.5: The Author demonstrated that treatment with the senolytic treatment D+Q (Dasatinib + Quercetin) altered the gut microbiota composition of aged Wistar rats. The most vital finding was a significant increase in *Lactobacillus acidophilus*. This is a beneficial bacterium associated with gut barrier integrity, immune regulation, and production of health-promoting metabolites. Several other potentially beneficial bacterial groups have also responded to treatment. At the same time, it reduces taxa linked to inflammation and dysbiosis. However, the *Firmicutes*-to-*Bacteroidetes* ratio remained unchanged. These suggest a selective rather than broad reshaping of the microbiota. Functional analysis did not reveal significant differences in metabolic pathways, likely due to high inter-animal variability.

Results 3.6: The Author showed that senolytic treatment with Dasatinib and Quercetin (D+Q) in aged Wistar rats alters the profile of gut-derived metabolites in a significant way. In feces, there was a broad reduction in short-chain fatty acids (SCFAs), suggesting decreased microbial fermentation activity or altered substrate availability. At the same time, levels of several bile acids were reduced, indicating modifications in host-microbiota co-metabolism, and enterohepatic circulation. In serum, the systemic effect was limited, with only formic acid showing a significant

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decrease. It may be a sensitive marker of metabolic changes induced by D+Q. Correlation analyses revealed that specific bacterial taxa are associated with SCFA and bile acid profiles. These may indicate a functional restructuring of the gut microbiome under senolytic treatment.

Results 3.7: The Author showed that Dasatinib and Quercetin (D+Q) induce strong remodeling of the intestinal barrier in aged Wistar rats in a region-specific way. It affects epithelial structure, mucus production, tight junctions, and inflammatory signaling. In the small intestine, D+Q increased mucosal thickness and enhanced protective mucus features, including upregulation of MUC1 while leaving MUC2 largely unchanged. In contrast, the large intestine showed reduced mucosal thickness and segment-dependent decreases in mucin content. These may indicate a different adaptive response in the colon. Tight junction analysis revealed increased scaffold protein ZO-1, alongside decreased claudin-1, suggesting a rebalanced but functionally competent epithelial barrier. Overall, D+Q reduced pro-inflammatory markers (TNF-α, IFN-γ) and increased anti-inflammatory IL-10, indicating a shift toward a more anti-inflammatory and regenerative intestinal environment with strong anatomical compartmentalization.

Results 3.8: The results presented in this section show that aging in rats is accompanied by a pronounced systemic proinflammatory state, in line with the phenomenon known as "inflammaging." Older animals displayed increased circulating levels of several cytokines and chemokines, including IL-6, TNF-α, IL-1α/β, MCP-1, and MIP-1α, while the anti-inflammatory cytokine IL-10 was reduced. This inflammatory pattern distinguished aged rats from young controls, with TNF-α, IL-6, and MCP-1 contributing most strongly to the separation. Administration of Dasatinib combined with Quercetin (D+Q) partially mitigated these age-related changes by decreasing major SASP-associated inflammatory mediators such as IL-6, IL-1α, IFNγ, and IL-17A. In parallel, D+Q treatment enhanced regenerative and anti-inflammatory factors, including EGF, suggesting an overall reduction in systemic inflammatory load.

Results 3.9: The Author showed that senolytic treatment with Dasatinib and Quercetin (D+Q) did not significantly alter mRNA levels of key blood–brain barrier tight junction genes, but it did increase the protein levels of core BBB structural components, particularly in the frontal cortex. The most pronounced structural change was increased expression. A clear structural improvement was observed in the expression of ZO-1 and occludin, while claudin-1 levels increased to a lesser extent. These changes indicate better preservation of blood-brain barrier integrity, although the effect differed across brain regions and was more pronounced in the frontal cortex than in the cerebellum. Regarding neuroinflammation, D+Q treatment did not result in a general reduction in pro-inflammatory cytokines. Instead, it appeared to promote a more anti-inflammatory environment, mainly by significantly elevating IL-10 protein expression, particularly in the frontal cortex. The treatment also influenced gut-brain axis communication. The therapy also affected signaling within the gut-brain axis. This was associated with lower GPR43 expression and a slight increase in GP. The treatment influenced communication along the gut-brain axis, leading to reduced GPR43 expression, while GPR41 expression showed a tendency to rise. This pattern may indicate changes in signaling pathways associated with short-chain fatty acid receptors. R41 expression suggests changes in pathways linked to these receptors. These findings may indicate a selective alteration of signaling pathways associated with short-chain fatty acid receptors. Overall, the findings suggest that D+Q

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improves brain health not by suppressing global inflammation, but by region-specific strengthening of the blood-brain barrier and targeted adjustments in immune and metabolic signaling.

I evaluate the work positively. However, there are several issues that need clarification to increase the clarity of the interpretation of the results and the transparency of the presented analyses:

1. There is a lack of studies on female rats, which would be particularly important given the high incidence of dementia and Alzheimer's disease in women. I understand that the research presented may be continued due to the importance of the topic. However, please discuss any possible differences in the results that may appear in studies on female rats compared to the results obtained on male rats.
2. It should be clarified how the observed increase in spine length and length-to-head-width ratio was translated into an enrichment of „thin + stubby" spines. In classical spine classification, stubby spines are typically characterized by low aspect ratio and short morphology rather than elongated protrusions. Would the observed phenotype be more consistent with thin or filopodium-like spines instead?
3. What direct evidence shows that the change in H3K27me3, rather than changes in H3K9me3 or other chromatin modifications, is functionally linked to improved plasticity-related gene expression and behavior. For example, through locus-specific ChIP-seq (e.g., at LTP-associated genes) or experiments showing that independent manipulation of H3K27me3 is sufficient or necessary to reproduce or block the behavioral effects of D+Q.
4. How might chronic administration of Dasatinib and Quercetin (D+Q) alter intestinal mucosal histoarchitecture in the small and large intestine compared with the cyclic dosing regimen used in the study?

Formal errors and typos:

- Page 9. Abstract: - syntax error, it is „*eliminację starzenie komórkowe...*", it should be: „*eliminację starzenia komórkowego*" in the genitive case
- Page 62. Post-Treatment Cognitive Performance: a typo „avo5idance"

The above comments do not affect my assessment. I rate this work very highly. The author has completed a tremendous amount of work with accuracy and precision, maintaining an exceptionally critical approach to his own results. The work submitted for review demonstrates a very high level of substantive merit, and the results obtained make a significant contribution to understanding the mechanisms underlying the actions of senolytic compounds on the microbiome, cognitive changes, and intestinal and blood-brain barrier integrity. At the same time, the dissertation offers an original solution and fills a knowledge gap regarding the mechanisms of action of tested senolytics in the CNS and throughout the body.

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In conclusion, I declare that the doctoral dissertation meets the requirements specified in Article 187 of the Act of 20 July 2018 – the Law on Higher Education and Science (Journal of Laws of 2024, item 1571, as amended). Therefore, I request that the Scientific Council of the Institute of Experimental Biology admit Grégory Petrazzo, M.A., to the next stages of the proceedings for the award of the doctoral degree.

Considering the high substantive level of the dissertation, the timeliness, significance, and translational value of the research presented, I request that the doctoral dissertation be distinguished.

Podsumowanie w języku polskim:

Powyższe uwagi nie wpływają na moją ocenę. Oceniam tę pracę bardzo wysoko. Autor wykonał ogromną ilość pracy, wykazując się dokładnością i precyzją, zachowując przy tym wyjątkowo krytyczne podejście do własnych wyników. Praca przedłożona do recenzji charakteryzuje się bardzo wysokim poziomem merytorycznym, a uzyskane wyniki wnoszą istotny wkład w zrozumienie mechanizmów leżących u podstaw działania związków senolitycznych na mikrobiom, zmiany poznawcze oraz integralność bariery jelitowej i krew-mózg. Równocześnie rozprawa stanowi oryginalne rozwiązanie i wypełnia lukę w wiedzy na temat mechanizmów działania badanych senolityków, zarówno na OUN jak i obwodowo.

Podsumowując, stwierdzam, że rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U.2024 poz. 1571 z póź. zm.). W związku z powyższym, wnioskuję do Rady Naukowej Instytutu Biologii Doświadczalnej o dopuszczenie mgr. Grégory Petrazzo do dalszych etapów postępowania w sprawie nadania stopnia doktora.

Jednocześnie biorąc pod uwagę wysoki poziom merytoryczny rozprawy, aktualność, znaczenie i translacyjność zaprezentowanych badań, wnoszę o wyróżnienie pracy doktorskiej.

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Szczecin, 13.06.2026

Review of the Doctoral Dissertation
by Grégory Petrazzo, M.Sc.
entitled **"Interplay between senolytic drugs and microbiome preserve cognitive abilities during aging"**

The doctoral dissertation by Mr. Grégory Petrazzo, M.Sc., entitled *Interplay between senolytic drugs and microbiome preserve cognitive abilities during aging*, was prepared at the Laboratory of Molecular Bases of Aging and the Laboratory of Cell Biophysics of the Nencki Institute of Experimental Biology, Polish Academy of Sciences. The dissertation was supervised by Prof. Jakub Włodarczyk, Ph.D., D.Sc., with Dr. Adam Krzystyniak, Ph.D., acting as auxiliary supervisor .

The subject of the dissertation is the assessment of the effects of senolytic treatment with dasatinib and quercetin, referred to as D+Q, on cognitive function, gut microbiota composition, microbial metabolites, intestinal barrier integrity, blood–brain barrier integrity, and selected inflammatory and neurobiological markers in aged Wistar rats.

This review has been prepared with reference to the requirements for doctoral dissertations specified in Article 187 of the Act of 20 July 2018, Law on Higher Education and Science. Therefore, the assessment focuses on three essential aspects: whether the dissertation demonstrates the candidate's general theoretical knowledge in the relevant discipline, whether it shows the ability to conduct scientific work independently, and whether it constitutes an original solution to a scientific problem.

1. Assessment of whether the dissertation demonstrates the candidate's general theoretical knowledge

The dissertation demonstrates broad theoretical knowledge in the fields of aging biology, cellular senescence, senolytic interventions, gut microbiota, the gut–brain axis, microbial metabolites, neuroinflammation, and biological barrier function. The author discusses mechanisms of aging, the role of senescent cells, the senescence-associated secretory phenotype, chronic low-grade inflammation associated with aging, as well as the role of microbiota and microbial metabolites in the regulation of cognitive function.

The dissertation is well situated within the current research field of healthy aging and geroscience. The author attempts to connect central and peripheral mechanisms of cognitive aging, which is a valuable and timely approach. The theoretical background includes both senolytic treatment and gut–brain communication, which corresponds to the interdisciplinary

character of the work. The rationale for using D+Q as a senolytic intervention is presented, and the importance of microbiota-derived metabolites and barrier integrity is also addressed.

However, the introductory section requires some reorganization. In particular, the part concerning the gut–brain axis is at times inconsistent and insufficiently connected with the main hypothesis of the dissertation. The issue is not only the length of this section, but rather the lack of a clear structure and a consistent link with the central topic of the work, namely cellular senescence and senolytic treatment. The author discusses microbiota, metabolites, intestinal barrier function, blood–brain barrier function, inflammation and cognition, but these elements are sometimes presented as a loose collection of information rather than as a logically developed justification for the study.

This section should be revised so that the reader can clearly follow the conceptual sequence: aging and cellular senescence — SASP/inflammaging — intestinal and blood–brain barrier dysfunction — microbiota and microbial metabolites — cognitive decline — potential effects of senolytic treatment.

Despite this reservation, I assess that the dissertation demonstrates the candidate's general theoretical knowledge to a sufficient degree. The knowledge presented is broad, up-to-date and relevant to the scientific problem addressed in the dissertation.

2. Assessment of whether the dissertation demonstrates the ability to conduct scientific work independently

The dissertation shows that the candidate is able to plan and conduct a complex experimental study involving an animal model, pharmacological intervention, behavioural testing, microbiome analysis, metabolomics, histology, gene and protein expression analysis, and assessment of selected neurobiological markers. Such a broad methodological scope demonstrates the candidate's ability to work in an interdisciplinary scientific environment and to integrate results obtained at different biological levels.

A strong aspect of the dissertation is the use of a natural aging model in Wistar rats and the application of the Active Allothetic Place Avoidance Task as a behavioural test of cognitive function. The author also reports important methodological elements, including random assignment of animals to treatment groups, blinding of investigators responsible for behavioural, histological and molecular analyses, and an a priori power calculation. These are important features indicating awareness of methodological standards in preclinical research.

Nevertheless, several methodological issues require clarification. First, the randomization procedure should be described in more detail, preferably with reference to the SYRCLE risk of bias tool. The dissertation states that animals were randomly assigned to groups, but it should be clarified whether randomization also included cage allocation, order of procedures, order of sample collection, order of laboratory analyses and data analysis. It should also be specified

whether allocation concealment was applied and whether all outcome assessments were performed under blinded conditions.

Second, the possible cage effect should be addressed, especially in the microbiome analysis. In microbiome studies, co-housing conditions may significantly influence microbial composition, and this issue is important for the assessment of methodological reliability.

Third, the dissertation indicates the involvement of several collaborators and external laboratories. This is not a criticism in itself, since contemporary interdisciplinary research often requires collaboration. However, in the context of assessing independent scientific work, the candidate should clearly define which parts of the study were performed independently and which were carried out by collaborators or external units. This applies particularly to animal experiments, administration of D+Q, behavioural testing, sample collection, DNA/RNA/protein isolation, histological analyses, 16S bioinformatics, statistical analyses, preparation of figures and interpretation of data.

Fourth, the presentation of results should be improved. The dissertation relies heavily on figures, whereas tables with numerical results are largely missing. Figure legends are not always sufficiently clear as to whether the data are presented as means, medians, SD, SEM, IQR, 95% CI or another measure of variability. Given the broad scope of analyses and relatively small group sizes, summary tables with numerical results would considerably improve the transparency of the work and facilitate assessment of effect sizes and biological relevance.

Despite these reservations, I assess that the dissertation demonstrates the candidate's ability to conduct scientific work. The above comments concern mainly the need for methodological clarification, clearer presentation of data and more precise definition of the candidate's individual contribution. They do not undermine the overall scientific value of the study.

3. Assessment of whether the dissertation constitutes an original solution to a scientific problem

The dissertation addresses an original and timely scientific problem: whether senolytic treatment may influence cognitive function during aging through mechanisms related to the gut–brain axis, microbiota, microbial metabolites, inflammation and biological barrier integrity. This approach is original because it goes beyond the classical view of senolytics as agents eliminating senescent cells and attempts to place their effects within a broader framework of gut–brain communication.

The author reports that D+Q treatment was associated with improved cognitive performance in aged rats, changes in gut microbiota composition, including an increased relative abundance of *Lactobacillus acidophilus*, alterations in microbial metabolite profiles, and changes in markers of intestinal barrier integrity, blood–brain barrier integrity and inflammation. The originality of the dissertation lies primarily in the attempt to integrate several levels of observation, i.e.

behavioural, microbiological, metabolomic, immunological, barrier-related and neurobiological.

In this respect, the dissertation represents an original attempt to solve a scientific problem concerning the relationship between senolysis, microbiota and cognitive function in aging.

However, some conclusions should be formulated more cautiously. The microbiome analysis is based on 16S rRNA sequencing. This method is useful for taxonomic profiling, but it does not allow direct conclusions about the functional potential of the microbiota or the actual activity of metabolic pathways. Therefore, observations concerning microbiota and metabolites should be interpreted mainly as associative rather than causal.

It should also be noted that not all results unequivocally support a direct senolytic mechanism. For example, the lack of a significant change in hippocampal SA-β-gal activity despite improved cognitive function is an important observation. This should be more explicitly addressed in the discussion and conclusions. The author should more clearly distinguish which effects may be directly related to reduced senescence, which may reflect modulation of inflammation, and which may be secondary to changes in microbiota or microbial metabolites.

The discussion also requires revision. In several places, citations are missing, particularly where the author provides mechanistic interpretations concerning D+Q treatment, microbiota, microbial metabolites, intestinal barrier function, blood–brain barrier function, neuroinflammation and cognitive outcomes. Moreover, some sections begin with senolytics and then move to studies on probiotics, prebiotics or faecal microbiota transplantation without a sufficiently clear transition. Such comparisons may be justified, but the author should explain why data from microbiota-targeted interventions are being discussed in a dissertation focused on senolytic treatment.

Some conclusions are also closer to a summary of results than to true scientific conclusions. The final conclusions should more clearly indicate what scientific problem has been addressed, which findings are directly supported by the data, which are mechanistic interpretations, and which remain hypotheses requiring further validation.

Despite these reservations, I assess that the dissertation constitutes an original solution to a scientific problem. Its originality lies in the attempt to demonstrate that senolytic intervention may influence cognitive function in aging in association with microbiota, microbial metabolites, inflammation and biological barriers.

Specific comments

1. The introductory section on the gut–brain axis should be reorganized, as it is at times inconsistent and insufficiently connected with senescence and senolytic treatment.

2. The discussion should include missing citations, especially in sections concerning mechanistic interpretation of D+Q effects, SCFAs, bile acids, intestinal barrier integrity, blood–brain barrier integrity, neuroinflammation and cognitive function.
3. The discussion should be more strongly focused on the central conceptual axis of the dissertation: senescence — SASP/inflammaging — microbiota — biological barriers — cognitive function.
4. Sections concerning probiotics, prebiotics and faecal microbiota transplantation should be better justified in the context of a dissertation focused on senolytics.
5. Tables with key numerical results should be added, especially for behavioural, microbiome, metabolomic, barrier-related and inflammatory outcomes.
6. In the microbiome section, diversity indices should be presented more fully, particularly alpha-diversity measures and difference of significance with providing data on statistics
7. Figure legends should be standardized and should clearly indicate (in caption) whether data are presented as means, medians, SD, SEM, IQR, 95% CI or another measure.
8. The conclusions should be more clearly separated from the results and should have a more interpretative character.
9. The randomization procedure, blinding, allocation concealment and potential cage effect should be clarified.

Questions to the PhD candidate

1. How exactly was animal randomization performed, and did it include cage allocation, order of procedures and order of analyses?
2. Were there any methods implemented to minimise the risk of bias?
3. Was the cage effect considered as a potential confounding factor in the microbiome analysis?
4. Why were full alpha-diversity values, such as Shannon, Simpson and observed ASVs/OTUs, not presented in the results?
5. Were beta-diversity differences statistically confirmed, for example using PERMANOVA?
6. Which effects of D+Q does the author interpret as directly related to reduced senescence, and which as indirect effects mediated by inflammation, microbiota or metabolites?
7. Which parts of the dissertation were performed independently by the PhD candidate, and which were carried out by collaborators or external laboratories?

Final conclusion

After reviewing the doctoral dissertation by Mr. Grégory Petrazzo, M.Sc., I conclude that:

1. the dissertation demonstrates the candidate's general theoretical knowledge in the fields of aging biology, cellular senescence, senolytics, gut microbiota and the gut–brain axis;
2. the dissertation demonstrates the candidate's ability to conduct scientific work, including the planning and implementation of a complex experimental study using behavioural, biological and molecular methods;
3. the dissertation constitutes an original solution to a scientific problem by assessing the relationship between senolytic treatment, microbiota, microbial metabolites, biological barriers, inflammation and cognitive function in an aging model.

The critical comments presented above mainly concern the organization of the narrative, methodological clarification, presentation of numerical results, completion of missing citations and more cautious mechanistic interpretation. They do not undermine the overall scientific value of the dissertation.

Therefore, I assess the doctoral dissertation positively and conclude that it meets the requirements for doctoral dissertations specified in Article 187 of the Act of 20 July 2018, Law on Higher Education and Science. I recommend that Mr. Grégory Petrazzo, M.Sc., be admitted to the next stages of the doctoral degree procedure.

Joanna Żyderle

17.06.2026

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Review of the doctoral thesis

PhD candidate: Grégory Petrazzo, M.Sc.
Title: Interplay between senolytic drugs and microbiome preserve cognitive abilities during aging
PhD Thesis Supervisor: Prof. Jakub Włodarczyk, Ph.D., D.Sc.
Auxiliary supervisor: Dr. Adam Krzystyniak, Ph.D.
Institution: Nencki Institute of Experimental Biology, Laboratory of Molecular Bases of Aging and Laboratory of Cell Biophysics

The doctoral thesis submitted by Grégory Petrazzo, M.Sc., investigates the effects of senolytic treatment with a combination of dasatinib and quercetin on key mechanisms involved in the pathogenesis of age-related cognitive decline. The hypothesis assumes that this intervention may influence cognitive aging not only through direct effects on inflammation and tissue homeostasis, but also through modulation of the gut microbiota, microbiota-derived metabolites, and the integrity of the intestinal and blood-brain barriers. The topic of this experimental work is clinically relevant, particularly in light of global population aging and the projected increase in the prevalence of age-related conditions.

The thesis has been prepared in the form of an extensive monograph comprising 166 pages. It is well organized and follows a typical layout for PhD theses in the field of neuroscience, including five main sections: I. Introduction, II. Materials and Methods, III. Results, IV. Discussion, and V. Summary and Conclusions. The title page is followed by a list of funding organizations, acknowledgements, a table of contents, abstract in English and Polish, and a list of abbreviations. The thesis concludes with a bibliography comprising 263 relevant references, a list of the PhD candidate's publications, and lists of figures (33) and tables (7).

In the **Introduction**, the Author defines aging as a biological process, introduces *cellular senescence* as one of its key hallmarks, and outlines diseases in which aging acts as a contributing factor. This section also discusses epidemiological projections and the economic burden associated with global population aging. It is commendable that, in addition to global data, the Author also provides information on demographic trends in Poland. The Introduction concisely describes key mechanisms involved in the pathogenesis of age-related conditions, including cellular senescence, chronic inflammation, and barrier dysfunction. Importantly, this subsection introduces key concepts and terminology that have shaped the field of aging research over the past two decades and have gained particular prominence in recent years, including inflammaging, senescence-associated secretory phenotype (SASP), and senotherapeutics. It concludes by presenting the main aim of the thesis, namely "to determine whether senolytic treatment with D+Q can preserve or restore cognitive function during aging through mechanisms involving the gut–brain axis", followed by five specific objectives. Overall, this section provides a concise scientific overview of the research field and appropriately introduces the topics analysed later in the doctoral thesis.

Two issues drew the reviewer's attention in this section:

- Subsection 1.3 reviews available senolytic interventions, focusing specifically on dasatinib (D) and quercetin (Q). A valuable aspect of this fragment of the thesis is that it emphasizes not only the beneficial effects of D+Q intervention, but also its limitations, including pharmacokinetic constraints, context-dependent effects, possible interactions with the host immune system, and potential off-target effects on non-senescent cells. However, the above-mentioned issues are primarily mechanistic concerns. It is somewhat disappointing that the Author did not discuss clinically reported general adverse effects following D+Q intervention, including fatigue, nausea, decreased appetite, headache, malaise, diarrhea, sleep disturbances, and anxiety (PMID: 36857968). These effects are generally classified as non-serious adverse events, but they are nevertheless relevant when assessing the translational potential and tolerability of D+Q-based senolytic interventions. The risk of more serious adverse events is mainly associated with dasatinib, a tyrosine kinase inhibitor originally developed and approved for the treatment of chronic myeloid leukemia, particularly when administered in repeated or long-term regimens (PMID: 19536317).
- In subsection 1.5, the Author writes: "The rationale of this thesis is therefore that senescent cell clearance may influence cognitive aging not only through direct effects on inflammation and tissue homeostasis, but also through modulation of the gut microbiota, microbiota-derived metabolites, and the integrity of the intestinal and blood-brain barriers." I do not agree with presenting this statement as rationale. A rationale is a justification or line of reasoning behind a study, in other words it explains *why the thesis is worth undertaking* or *why a given line of inquiry is warranted*. The quoted statement reads rather as a **hypothesis**, understood as an *assumption* or *premise to be tested*. This hypothesis is then examined in the doctoral thesis.

The second section of the PhD thesis is **Materials and Methods**. The experiments underlying Mr. Petrazzo's doctoral thesis were designed in accordance with high scientific standards. This is supported by the use of animal randomization for treatment allocation, power calculation, and blinding to group assignment during behavioural testing, as well as during histological and molecular analyses. The rich methodological approach deserves particular attention, as it enables verification of the research hypothesis through comprehensive multi-level analyses encompassing intestinal microorganisms and their metabolites, as well as cellular, mRNA, and protein-level assessments in selected parts of the gastrointestinal tract, brain, and serum.

My comments regarding this part of the thesis relate primarily to missing information or a lack of clarity in its presentation:

- The first subsection describes the animal model used in the study. Although it provides a clear rationale for choosing the specific model, it lacks some important details, such as total number of rats used in the study, the animal breeder or supplier, and the place and conditions where the animals were kept during and between behavioural assays.
- A potential source of confusion for the reader is the lack of consistency regarding the age of the animals, as reported in different parts of the thesis. In the first sentence of subsection 2.1, the Author refers to the selection of "adult male Wistar rats" [3-6-months-old rats are generally considered adult, 6-12-months-old rats – mature adult or sometimes early middle aged]. On page 42 there is a reference to an article of Krzystyniak et al. (PMID: 35042834), of which the PhD candidate is a co-author, where two age groups are mentioned: 3-months-old young rats and 18- to 22-month-old aged rats. The following sentence describes 22 months of age as "corresponding to an advanced stage of physiological aging (in humans) (…)". However, at the beginning of Discussion are mentioned "very-old Wistar rats" and in subsection 4.6, the Author discusses the strengths and limitations of using "24-month-old outbred male Wistar rats". It is therefore not clear whether the animals used in the thesis are the same as those described in the Krzystyniak publication, or whether the thesis presents results of a new study. Moreover, there is no explicit statement indicating whether the rats were 22-months-old at the beginning of the experiment (before D+Q intervention).
- Subsection 2.1 does not specify the number of experimental groups included in the study. Most graphs presenting the results in section III include only two groups: VEH and D+Q. However, the baseline cognitive performance of aged rats assessed in the AAPAT was compared with that of young animals (exact age not defined; Fig. 12). In addition, the cytokine and growth factor panel also included 6-month-old rats described as "young" (Fig. 30). As a reviewer, I do not question the inclusion of additional comparisons with other age groups in the thesis. On the contrary, I consider these data valuable. However, the Materials and Methods section should provide complete information on the number of experimental groups and the age and number of the animals included in each group. The clarity of this section could be improved by presenting the study design in schematic form.

- The ethics statement is insufficiently detailed. The Author does not indicate which institutional and national guidelines governed animal handling, nor does he provide the approval number or the name of the ethics committee that issued the permit.
- In the last paragraph of subsection 2.1, the Author justifies the exclusion of females from the study by referring to the potential influence of sex hormones. However, this argument is less convincing in the context of 22-month-old rodents, in which reproductive senescence and age-related alterations in sex hormone profiles would be expected.
- In subsection 2.7, which describes the histological and morphological analyses, I did not find information on the number of sections analysed per animal or per group. This information should be provided because the number of analysed sections directly affects the representativeness of the sampling, the reliability of quantitative histological measurements, and the interpretation of group comparisons. It is also important for assessing reproducibility and potential sampling bias.
- In subsection 2.8, which describes, among other procedures, the qPCR protocol, the Author should include the sequences of primers used in the study and indicate the tools used for their design. If the primer sequences were taken from previously published studies, the relevant references should be provided.

The key findings presented in the **Results** section indicate that the time-limited D+Q intervention improved spatial learning and memory in aged male Wistar rats, with the effect persisting for at least five weeks. Cognitive improvement was accompanied by changes in neuroplasticity, remodelling of the gut microbiota and its metabolites, as well as alterations in inflammatory and barrier-related markers.

Several points in this part of the thesis require clarification:

- The statement “Despite these impairments, aged rats showed day-to-day improvement across the five days of training,” referring to the results presented in Fig. 11, is not fully supported by the data. There are most likely no statistically significant differences in the comparisons: day 2 vs. day 3, day 3 vs. day 4, or day 4 vs. day 5 for any of the analysed parameters in the aged rat group. The only comparison that might indicate a statistically significant difference is day 1 vs. day 5. However, this comparison is not shown in Fig. 11, and no corresponding statistical data are provided in the text. Therefore, the statement, as currently formulated, is not sufficiently supported by the presented data.
- In the legend to Fig. 13, in the sentence describing the calculation of the exploration ratio, the meaning of the word “mischiefs” is unclear. This appears to be a typographical or translation error, and the intended word may be “groups”.
- The subsection 3.2 is entitled “Hippocampal Chromatin Remodelling”. However, this title is misleading, as the subsection does not contain any data on chromatin remodelling. Instead, it focuses on D+Q-induced neuroplastic changes in the hippocampal CA1 sector, specifically dendritic spine number and morphology. Therefore, the title should be revised to accurately reflect the content of the subsection.

- Metabolomic analyses revealed that the D+Q intervention induced changes in the faecal concentrations of selected short-chain fatty acids, such as acetic and pentanoic acids, as well as selected bile acids, such as taurocholic acid, in D+Q-treated rats. These results are accurately illustrated in Fig. 22. However, despite the Author's statement that "only formic acid exhibited a statistically significant reduction in the D+Q group compared to controls (Figure 22 C)" [measured in serum], the relevant graph in Fig. 22 shows no difference between the two groups. This statement should therefore be revised or further substantiated.
- In the subsection 3.7, the Author presents the results of morphometric analysis, showing a D+Q-induced increase in mucosal thickness in the small intestine and a decrease in mucosal thickness in the large intestine of aged rats. However, there is a discrepancy between the description in the text and the data presented in Fig. 25. With regard to the large intestine, the Author writes: "Although the observed differences did not always reach statistical significance, the trend was consistent and most pronounced in the distal colon, where the mucosa thinned from an average of 25.8 μm in vehicle-treated animals to 9.5 μm in the treated group (p = 0.0845)." Thus, the text indicates only a trend toward reduced mucosal thickness in the distal colon. However, in the corresponding graph comparing VEH and D+Q groups in the distal colon (Fig. 25 C), one asterisk is shown, indicating that the reduction in mucosal thickness reached statistical significance. This inconsistency should be corrected or clarified by the Author.
- The intestinal tight-junction marker analysis showed no significant changes in Occludin (*ocln*) and ZO-1 (*tjp1*), but a significant decrease in Claudin-1 mRNA expression (*cldn1*) in the small and large intestine. Based on these findings, the Author concludes that D+Q "re-balances" the intestinal tight junction without uniformly reinforcing it. This interpretation should be formulated more cautiously, as the data are based on selected mRNA/protein markers and do not directly demonstrate functional changes in intestinal barrier integrity.
    - The text states: "The rise [of *il10* in the small intestine] was driven chiefly by the ileum, with an additional upward trend in the duodenum (p = 0.09)." However, in the corresponding graph in Fig. 29, the D+Q-induced increase in *il10* gene expression is marked as statistically significant not only in the ileum, but also in the duodenum, as indicated by one asterisk.
    - In addition, the description of IL-10 (*il10*) changes in the large intestine is incomplete. The Author writes: "IL-10: no clear net change was observed when the large intestine was pooled. When split by segment, proximal colon *il10* mRNA showed an upward trend (p = 0.06), but this was not paralleled by a significant protein-level increase." However, panel A in Fig. 29 shows a decrease in IL-10 in the large intestine. This result is not interpreted in the text of Results section.

Importantly, the direction of the inconsistencies does not uniformly favour the expected or more readily interpretable outcomes. I therefore assume that they represent inadvertent shortcomings in the description of the results, or errors introduced during the editing of the thesis, rather than an intentional attempt to manipulate the data.

- Subsection 3.8 describes D+Q-induced changes in the cytokine and chemokine profile in the serum of 6-months-old and 25-months-old rats. The description is accurate, and Fig. 30 clearly illustrates the results. The Author states that serum samples were analysed using a 32-plex Luminex assay. However, Fig. 30 presents only 24 markers. I did not find an explanation as to why the remaining eight markers are not shown. Were their levels below the detection limit, or were they excluded for another reason? This should be clarified by the Author.

The **Discussion** is extensive, substantively well justified, and supported by seven summary tables, six of which present relevant findings from the field. Most of the results obtained in the reviewed thesis are appropriately interpreted and discussed by the Author in relation to the current state of knowledge. Particular attention should be given to subsections 4.6, entitled "Critical Assessment of the Study," and 4.7, entitled "Future Directions." where the Author reflects critically on the study, indicating both its strengths and limitations, outlines the directions of future investigations, and discusses the translational and clinical potential of the study findings.

The substantive comments presented above do not diminish my positive assessment of Mr. Grégory Petrazzo's doctoral thesis, which is of high scientific quality. The PhD candidate accurately presents the research topic and study concept, therefore I positively assess his general theoretical knowledge in neuroscience. The thesis is based on a properly designed study, applies appropriately selected advanced methodology, and for the most part, clearly presents correctly interpreted results and accurately drawn conclusions. Thus, it constitutes an original solution to the stated scientific problem and confirms the PhD candidate's ability to conduct independent scientific work.

I hereby conform that the doctoral thesis entitled **"Interplay between senolytic drugs and microbiome preserve cognitive abilities during aging"** by **Grégory Petrazzo, M.Sc.**, meets the requirements for a doctoral thesis. I therefore recommend proceeding to the next stages of the doctoral procedure, as specified in the Polish text below:

Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U. 2024 poz. 1571 z póź. zm.). W związku z powyższym, wnioskuję do Rady Naukowej Instytutu Biologii Doświadczalnej o dopuszczenie mgr. Gregory Petrazzo do dalszych etapów postępowania w sprawie nadania stopnia doktora.

G. Lietzau

Grażyna Lietzau, Ph.D., D.Sc.