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Annex No. 10 to the MU Directive on Habilitation Procedures and Professor Appointment Procedures

HABILITATION THESIS REVIEWER'S REPORT

Masaryk University	
Applicant	RNDr. Jan Balvan, Ph.D.
Habilitation thesis	*Cell Death and Cellular Secretion: Key Factors in the Cancerogenesis*
Reviewer	Assoc. Prof. Univ. Doc. Irma Schabussova, PhD
Reviewer's home unit, institution	Medical University of Vienna

Scientific Relevance of the Topic and Research Strategy

This habilitation thesis focuses on several closely related areas of cancer research, including autophagy, tumour–microenvironment interactions, cancer cell survival mechanisms, and extracellular vesicle (EV) biology. These topics are highly relevant to current oncology research and are addressed through a coherent and well-defined scientific programme.
Dr. Balvan has developed a distinct research focus centred on three interconnected questions: how different forms of cancer cell death can be identified using advanced imaging approaches; how autophagy contributes to tumour cell survival and therapy resistance in head and neck squamous cell carcinoma (HNSCC) and prostate cancer; and how phosphatidylserine-positive (PS+) EVs reflect intracellular stress and influence communication within the tumour microenvironment.
The choice of HNSCC as the principal disease model is well justified. HNSCC is among the most common malignancies worldwide and is characterised by considerable molecular heterogeneity, making it both biologically complex and clinically challenging, particularly in recurrent and metastatic disease. The overall structure of the research programme is clear and logical. The work progresses from methodological development to mechanistic studies and finally to potential translational applications. It begins with the development of quantitative phase imaging (QPI)-based approaches for detecting cell death, continues with investigations into the role of autophagy in tumour adaptation and resistance, and culminates in the characterisation of PS+ EVs and their potential use as biomarkers in HNSCC. The research aims and hypotheses are clearly formulated, and the individual chapters are well aligned with the publications on which the thesis is based.

Methodology

Dr. Balvan shows genuine and wide-ranging methodological competence. The most technically innovative contribution is the use of quantitative phase imaging (QPI) and multimodal holographic microscopy (MHM) to study cancer cell death in real time without labels. These techniques use the intrinsic optical properties of cells to track changes in mass distribution, motility, membrane morphology, and nuclear appearance, all without fixing,

staining, or phototoxic labelling of the cells. This addresses a well-known limitation of conventional fluorescence-based assays. Pairing these imaging data with deep-learning classifiers trained on simultaneously acquired fluorescence channels as ground truth, and introducing the Cell Dynamic Score (CDS) as a quantitative descriptor of population-level cell dynamics, represents a methodologically mature approach.

For the EV studies, Dr. Balvan uses Tim4-receptor/phosphatidylserine affinity capture (MagCapture™ PS) to isolate PS+ EVs. This is a sensible choice: PS is constitutively exposed on the outer leaflet of EV membranes, and Tim4-based capture achieves higher specificity than the differential ultracentrifugation routinely used for bulk EV isolation. EV characterisation follows the MISEV2018 guidelines of the International Society for Extracellular Vesicles, covering nanoparticle tracking analysis (NTA), cryo-electron microscopy, western blotting for standard markers, and mass-spectrometry proteomics with data deposited in ProteomeXchange. The characterisation is thorough and meets current international standards.

Across the publication record, the candidate also draws on flow cytometry, real-time cell analysis (xCELLigence/RTCA), cytokine multiplex arrays, mitochondrial RNA profiling, and standard biochemical and molecular biology techniques. Statistical approaches are appropriate throughout, including non-parametric tests where distributions warrant them, and multivariate methods such as partial correlation and hierarchical clustering in the more complex datasets. The thesis openly acknowledges the use of AI language tools (DeepL Translate, DeepL Write, ChatGPT-4o) for grammatical and stylistic editing. This use was confined to language polishing and had no bearing on the scientific content, interpretation, or conclusions.

Originality of Results

The thesis makes original contributions in three areas. The first is the QPI and holographic microscopy platform for real-time, label-free discrimination of apoptosis from lytic cell death subtypes including necroptosis, ferroptosis, and oncosis. The CDS is a genuinely new quantitative parameter that had no direct precedent in the literature at the time of publication. Combining it with a deep-learning classifier trained on simultaneous fluorescence ground-truth data is methodologically novel, and the fact that the platform has been validated across cytotoxic drugs, antiviral nucleoside analogues, and nanomaterials adds weight to its originality.

The second contribution is the review (Raudenska et al. 2021. Molecular Cancer, 20(1):1–27), which offers an original and substantive conceptual synthesis. By mapping the molecular connections between autophagy, lysosomal degradation, endosomal sorting, and EV secretion, it lays out a new framework for understanding why autophagy inhibition in cancer therapy can produce unexpected off-target effects on EV biogenesis and tumour-immune communication. This is a synthetic rather than empirical contribution, but its publication in a leading journal with a high readership gives it real field-level impact.

The third contribution is the experimental finding that the protein cargo of PS+ EVs is selectively and differently reshaped by pharmacologically distinct autophagy modulators. This is an original observation with direct relevance to understanding how cancer cells alter their secretory output under therapeutic pressure, and the candidate is among the first to study PS+-defined EV subpopulations specifically in the context of autophagy modulator treatment in HNSCC.

Applicability for Field Development and Further Research

The methodological work has clear reach beyond the candidate's own group. The QPI-based platform for detecting and classifying cell death is directly applicable to high-throughput drug screening and cytotoxicity testing, as already shown for antiviral nucleosides and nanomaterials. The cell segmentation comparison paper in BMC Bioinformatics (129 WOS citations) and the QPI dynamics paper in Scientific Reports (58 WOS citations) have both been taken up by the wider cell biology and bioimaging communities, which confirms their practical value.

The conceptual framework set out in the Molecular Cancer review (20(1):1-27, 2021) has direct implications for the design and interpretation of clinical trials using autophagy inhibitors, particularly regarding their underappreciated effects on EV biogenesis and immune signalling in the tumour microenvironment. The finding that autophagy modulators alter PS+ EV cargo and can influence fibroblast phenotype through EV-mediated signalling opens up interesting questions about tumour-stroma communication that are worth pursuing further.

In the Conclusion, the candidate outlines several well-grounded directions for future work: proteomic analysis of EVs from HNSCC patient serum after radiotherapy to assess treatment response; development of a salivary EV-based diagnostic device for HNSCC in collaboration with ESSENCE LINE, s.r.o. (TACR project); and prospective biomarker validation studies with Motol University Hospital. These are realistic, clinically motivated goals that follow naturally from the work presented.

Practical Applicability of Results

Several results of the thesis have immediate or near-term practical use. The QPI-based cell death methodology is well suited to preclinical drug development as a non-invasive, real-time platform for assessing how compounds affect living cancer cells. Its use in antiviral compound screening (Eyer et al. 2021, Antimicrobial Agents and Chemotherapy, 65, e01522-20) and nanomaterial safety evaluation (Fojtu et al. 2019, Chemistry - A European Journal, 25, 349-360) shows that it works across different application areas beyond oncology. The QPI and deep-learning combination can be set up in any research or pharmaceutical laboratory that has holographic microscopy equipment.

On the EV side, the PS+ EV isolation and characterisation workflow is reproducible and technically accessible enough to be adopted by other laboratories. The translational pilot study showing differences in mitochondrial RNA content of serum-derived PS+ EVs between HNSCC patients and healthy controls (Bugajova et al. 2024, Scientific Reports 14, 25815) provides an early indication that this approach could, with validation in larger cohorts, develop into a minimally invasive diagnostic and monitoring tool for a cancer type where better biomarkers are urgently needed. The ongoing industry collaboration for a salivary EV-based diagnostic device reflects a genuine commitment to taking these findings from bench to clinic.

Formal Presentation and Language Quality

The English throughout the thesis text and commentary is good. The writing is grammatically correct, terminology is used accurately and consistently, and the scientific argument is expressed clearly. The use of AI language tools (DeepL Write, ChatGPT-4o) for grammatical

and stylistic editing is declared openly and does not raise any concerns about the integrity of the scientific content, which is demonstrably the candidate's own. Some minor typographical inconsistencies are present but do not affect readability. The Czech-language textbook (Bunecna smrt: jeji vyznam ve fyziologii a patologicke fyziologii; Grada Publishing, 2021) shows the candidate's ability to communicate complex biomedical material to a broader educational audience.

Scientific Independence and Publication Record

The candidate's scientific independence is clearly demonstrated by the contribution summaries provided in the Commentary, which detail their involvement in experimental work, supervision, manuscript preparation, and overall research direction across the twelve publications included in the habilitation. In the earlier papers published between 2014 and 2015, the candidate contributed substantially to the experimental work (60–90%) and played a major role in shaping the research direction (40–90%). Over time, these contributions evolved towards increasing scientific leadership. In publications from 2021 to 2024, the candidate serves as corresponding author and is credited with 70–90% of supervision and 90% of the research direction, indicating a clear transition from active contributor to independent research leader. Furthermore, the successful mentorship of junior researchers who achieved first authorship on publications arising from the candidate's projects provides tangible evidence of effective research group leadership and scientific independence.

Critical Remarks

Before concluding, I would like to raise a few points that I think deserve attention. They should be read as suggestions for further development rather than as objections.
– **In vitro scope.** Experimental work is conducted almost entirely in a small set of established cancer cell lines. In vivo validation or experiments using patient-derived primary cultures would considerably strengthen the translational relevance of the mechanistic findings.

– **Functional EV characterisation.** The proteomic changes in PS+ EVs under autophagy modulation and metabolic stress are well described, but the downstream functional consequences of these compositionally altered vesicles on relevant recipient cell types in the tumour microenvironment, such as macrophages, dendritic cells, and T cells, remain largely inferred rather than directly tested. Functional assays in co-culture systems with immune cells represent the most important gap in the current EV research.

– **International profile.** The thesis does not document invited international lectures, bilateral collaborations, teaching responsibilities, supervision of students, or peer-review and editorial service. For a reviewer who does not know the candidate personally, this makes it difficult to form a complete picture of his international standing and academic engagement. That said, based on the candidate's visible and active participation in international conferences, scientific meetings, and professional networks such as the Czech Society for Extracellular Vesicles (CzeSEV), it is evident that Dr. Balvan is a well-recognised and highly regarded scientist within his field. He is also known as an engaged mentor and collaborator. Providing a more systematic overview of these activities in the thesis would have made it easier for reviewers

unfamiliar with the candidate to appreciate the full extent of his international visibility, scientific reputation, and academic contributions.

Reviewer's questions for the habilitation thesis defence:

Question 1. In your cytokine work, you prepare conditioned medium that has been cleared of large EVs and free cytokines, which means the cytokines you detect are associated with small EVs. You cannot tell from your data, however, whether they are sitting on the outside of the vesicle membrane or packaged inside. That distinction probably matters quite a bit biologically, since a surface-bound cytokine and an encapsulated one are likely to interact with recipient cells in very different ways. How do you interpret your cytokine findings in light of that ambiguity, and do you have a sense of how one would go about resolving it experimentally?

Question 2. We know that what ends up inside an EV is not random. Cells are clearly making selective decisions about what to package. From your experience with PS-positive EVs across different stress conditions and autophagy modulators, what do you think are the most important mechanisms driving that selectivity? And where do you see the biggest gaps in our current understanding?

Question 3. Your work focuses on PS-positive EVs as a defined subpopulation, rather than working with bulk EV preparations. That is a deliberate methodological choice, and in your case a well-justified one. How do you think about this more broadly for the field? Should EV research in general be moving away from bulk preparations towards subpopulation-specific approaches, and if so, what are the practical obstacles to doing that routinely?

Recommendation

Having read the thesis, the Commentary, and the full publication record carefully, I have no hesitation in recommending RNDr. Jan Balvan, Ph.D. for habilitation in Pathological Physiology at the Faculty of Medicine, Masaryk University, Brno. The work presented is scientifically strong, methodologically rigorous, and genuinely original. It addresses questions that matter clinically, and it does so with a coherence and ambition that speak to a rsearcher who has built something real over the past decade rather than assembled a collection of papers. Dr. Balvan has demonstrated that he can lead an independent research programme, attract competitive funding, mentor junior scientists, and publish at a high level in international journals. He is, in my view, well ready for the title of Docent, and I recommend it with confidence.

Conclusion

The habilitation thesis entitled "*Cell Death and Cellular Secretion: Key Factors in the Cancerogenesis*" by RNDr. Jan Balvan, Ph.D., **fulfils** requirements expected of a habilitation thesis in the field of Pathological Physiology.

Vienna, 9 June 2026 Signature: