ADAM MICKIEWICZ UNIVERSITY IN POZNAŃ
Faculty of Biology
Institute of Molecular Biology and Biotechnology

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Referee report concerning the **Habilitation thesis of Irena Đapić, Ph.D.** entitled as: *"**Development of tools based on mass spectrometry for proteomic analysis of biological materials**".* International Centre for Cancer Vaccine Science, University of Gdańsk.

Education and professional achievements

Irena Đapić has been awarded an MSc degree in chemistry in 2008, at the Department of Chemistry, Faculty of Science, University of Zagreb - Croatia, presenting the thesis entitled as „*Adsorption of bovine serum albumin on previously formed polyelectrolyte multilayer*" (supervised by Prof. Davor Kovacevič). She has later been conferred the PhD degree (2014), in the same department for the doctoral thesis work titled: „*Development and validation of the biochemical indicators of the skin barrier function*". (Supervisors: Prof. Ivone Jakasa, PhD and Renata Kobetič, PhD). Dr Đapić has been since employed in various institutions in Croatia, as well as abroad. These include positions of a Scientific and Teaching Assistant (2009-2014) in the Laboratory for Analytical Chemistry, a postdoctoral researcher in the same laboratory (2014-2018), intertwined by the second postdoctoral position at the Biomolecular Systems Analytics group, van't Hoff Institute for Molecular Sciences, University of Amsterdam, in the Netherlands. Since July 2018, Dr Đapić is employed as the Principal investigator (PI, Chemical Biology Group Leader) at the International Centre for Cancer Vaccine Science, University of Gdańsk, Poland.

Scientific achievement

The candidate has presented the scientific achievement in the form of a dissertation related to the development of mass spectrometry (MS) techniques and computational methods dealing with various steps of MS analysis. Such pipelines are useful in the analysis of disease-related phenomena, especially concerning the highly genetically variable and molecularly complex disorders, and in cases when the archived material is scarce, and often collected for other than proteomics purposes. The dissertation is based on five peer-reviewed original articles and one review article in a middle to high rank, mostly specialized journals (years 2017-2022). Their impact factors (IF), Ministry of Science and Higher Education (MNiSW) journal scoring values, and the number of citations were adequately listed by the candidate, and included those from Web of Science and Google scholar, according to the given guidelines. In four articles, the candidate is the first or shared first author, while in one, the corresponding last author. The summarized 5-year IF of publications belonging to the scientific achievement is 32.523, which represents a good level of achievement. These articles were cited 68 times (accessed on 19/05/2023, WoS), demonstrating a relatively decent degree of

international recognition, which is important when topics with mostly technical content are being evaluated.

The mass spectrometry analysis of human tissues is mostly based on archived specimens preserved as formalin-fixed paraffin (FFPE) tissue blocks or less commonly as fresh frozen (FF) samplings. FFPE specimens are commonly stored at room temperature and stable over long periods of time (even over 100 years in pathology archives). FFPE tissues allow also for lower storage costs as compared to FF specimens, however, formalin fixation introduces chemical modifications, i.e. Lysine methylation (+14 Da), methylene (+12 Da) and methylol (+30 Da) modifications and evokes protein cross-linking, which often impedes the further proteomic analysis. Therefore, the development of methods for efficient extraction of proteins, those which deal with limited amounts of cells and tissue material (i.e. LCM-captured tissues and dried blood spots), as well as minimizing protein loss and introducing the proper methods for protein digestion during preparatory phase is of importance in quantitative proteomics, especially in a clinical setup. Taking these challenges into account the candidate has focused on *three objectives*, namely, *i*) dealing with proteome profiling of small tissue amounts, *ii*) developing the methods for rapid protein digestion, and *iii*) introducing the methods for proteome profiling utilizing a limited number of cells. As such, these objectives are coherent in their nature and represent the compact and focused methodology addressing the nature and complexity of the proteomes harvested from small amount of solid tissues or liquid biopsies, which are usually collected in clinical scenarios.

In the first article of the scientific achievement (Dapic I. *et al*. Evaluation of Fast and Sensitive Proteome Profiling of FF and FFPE Kidney Patient Tissues. *Molecules* 2022, 1137), the candidate has utilized the FFPE kidney tissues and assessed their appropriateness for use in clinical proteomics by comparing them to FF specimens. Specific methods for tissue proteomic profiling and three different detergents for proteins extraction were tested. Importantly, the use of proper extraction buffer is of importance in such analyses, as it was suggested, it might be tissue type and its' size dependent. In the second article of the dissertation (Pirog A. *et al*. Comparison of Different Digestion Methods for Proteomic Analysis of Isolated Cells and FFPE Tissue Samples. *Talanta* 2021, 233, 122568) different protein processing protocols including filter-aided sample preparation (FASP), in-solution digestion (ISD) and pressure cycling technology (PCT) with a Barocycler were utilized for two different sets of biological samples, which included isolated immune cell subsets (CD4+ T-cells) and glioblastoma FFPE tissue samples. Furthermore, analysis of the samples was conducted in two independent laboratories, on two LC-MS platforms. Not surprisingly, it was established that classical ISD method is a cheap alternative to PCT and FASP-based methods, which identified, for example, the highest number of integral membrane proteins. Moreover, it was validated that an extensive cross-linking introduced by

preservation procedure in FFPE tissues had an effect on modification of methylation of Lys residues (adding +14 Da to the mass). The third article of the scientific achievement (Weke K. *et al*. MicroPOTS Analysis of Barrett's Esophageal Cell Line Models Identifies Proteomic Changes after Physiologic and Radiation Stress. *J Prot Res* 2021, 20, 2195-2205) dealt with Microdroplet Processing in One pot for Trace Samples (microPOTS) technology that was used for the analysis of the proteins from small number of the Barrett's esophageal cells cancer cell lines upon stress. MicroPOTS technology allowed identifying over 1500 proteins, including many hydrophobic ones, from only around 200 cells after exposure to chemical and radiation stress. It was pointed out that such technology could be relevant for detecting the putative biomarkers in small amounts of cancer samples. The fourth article of the scientific achievement is the review article published in well-recognized journal of high impact (Dapic I. *et al*. Proteome Analysis of Tissues by Mass Spectrometry. *Mass Spectrometry Reviews* 2019, 38, 1–39). The review aimed to in-depth scrutinize the procedures for successful detection and quantitative assessment of proteins in small tissue biopsies, taking into account the relevant tissue conservation methods and chemical/physical methods of protein extraction using various detergents (e.g. cationic acid-labile surfactant Rapigest and ProteaseMax, and an anionic acid-labile surfactant PPS Silent), and protein extraction methods, i.e. well recognized methods of ultrasonication, pressure based systems or less used picosecond infrared laser method (PIRL). Since publishing in 2019, the review has been well cited (20 times, accessed on 19/05/2023, WoS), which in reviewers' opinion is a true indicator of quality and importance of the selected topic for MS clinical research. The peer-reviewed research article by Dapic I. *et al*. titled "Fast and Simple Protocols for Mass Spectrometry-Based Proteomics of Small Fresh Frozen Uterine Tissue Sections" *Analytical Chemistry* 2017, 10769–10775, represents the fifth article of the scientific achievement. In this article, the candidate has developed the analytical methods for fast and reliable analysis of FF human uterine tissues from laser capture microdissected (LCM) human material cut at various tissue thickness. Several in-solution digestion protocols with different protein extraction buffers (containing urea and various percentage of acetonitrile with/without SDS), and digestion times (five protocols) were tested. Interestingly, the lower number of identified proteins with the buffer containing SDS was attributed to precipitation of proteins or incomplete removal of the detergent. The additional trypsin digestion step (protocols 3 and 5) demonstrated a better overlap between the replicates. This article, published in a well-recognized journal *Analytical Chemistry* has been cited 15 times since 2017 (accessed on 19/05/2023, WoS), which is a relatively good number for a technical paper. The sixth peer-reviewed article has been published in the renowned *Journal of Chromatography A* (Wouters, B.*, Dapic I.* *et al*. A Cyclic-Olefin-Copolymer Microfluidic Immobilized-Enzyme Reactor for Rapid Digestion of Proteins from Dried Blood Spots. *Journal of Chromatography*

A 2017, 36–42). In this research article, Dr Đapić was the first shared author. They main objective of this study was to implement a microfluidic immobilized enzyme reactor (IMER), which could be utilized for rapid protein digestion. The device was implemented for offline digestion of selected marker proteins of different molecular weight, namely cytochrome c (11.7 kDa), myoglobin (17 kDa), S1-casein (23 kDa), ovalbumin (42.8 kDa), bovine serum albumin (66.5 kDa), transferrin (75.2 kDa), and catalase (240 kDa), and dried-blood-spots, significantly shortening digestion times from overnight to few minutes. Importantly, IMER use shortened the sample preparation from 22.5 hours for in-solution digestion to 4 hours. This methodological article has been cited 19 times since 2017 (accessed on 19/05/2023, WoS).

In summary, the presented articles constituting a pivotal part of the achivement represent a solid, concise methodological ground for the further development of methods dealing with the proteomic sample preparation in clinical mass spectrometry. In reviewer's opinion, the candidate has successfully gathered and developed the methodologies and tools, which can be utilized by other researchers in the proteomics field. Thus, **this part of the dissertation clearly fulfils the criteria** set for obtaining the habilitation degree.

Evaluation of other scientific achievements

Dr Irena Đapič has published in total 21articles, in which in recent two she was the last, corresponding author (Zhou M. *et al*., *Anal. Chem*. 2020, 92 (10), 7087–7095; IF= 6.986 and Weke K., *et al*. *Anal. Chim. Acta* 2022, 1204, 339695). The research towards her MSc degree resulted in two publications, while during the course of doctoral degree she has published five articles. Following PhD degree in 2014, Dr Đapič has published 14 articles, of which six is included as part of the scientific achievement (four first or shared first author publications and two in which she was the last, corresponding author). Research of Dr Đapič has been cited in total **298 times** (282 times excluding self-citations; access on 19/05/2023; WoS), while her Hirsch index (**HI**) from the same database **equals 10** (not 11 as stated in the **Annex 5**). Recent Google scholar parameters, also provided by the candidate, list in total **471 citations** and **HI of 12** (access on 19/05/2023). Dr Đapić has also contributed to one book chapter and one monograph (citation incomplete), published in 2021. **In summary, these parameters are somewhat moderate concerning the time of obtaining the PhD thesis (2014), and taking into account the maternity leave periods.**

Concerning the management of international and national research projects and participation in such projects as a project executor, Dr Đapič has listed only two projects, which include an equipment grant (funding for Orbitrap Exploris 480 Mass Spectrometer, Polish Foundation for Science; 2019) and the study of an immobilized enzyme reactor, in collaboration with Micronit; 2017. Judging from the published articles and gathered experience by the candidate this part seems to be

less developed, and **requires stronger attention in the future**, **to successfully conduct the research and gather adequate funding as the PI**.

Dr Đapič, has listed nine achievements (parameter International and National awards for scientific or artistic achievement), of which majority relates to her successful PhD thesis work, with the last one awarded in 2015. Concerning the oral presentations at the national and international level, only eight were enumerated, with the last one given in 2020 at the local Computational Mass Spectrometry and Therapeutics conference, Gdansk, 13 January 2020. Concerning the participation in European programs and other international and national programs, Dr Đapič has mentioned four programs, which occurred during her active doctoral and postdoctoral period in the Netherlands. No other programs after year 2015 are given. Regarding the participation in international and national scientific conferences, 15 of such events were stated since 2007 (the last one at 1the 1th International Conference of Contemporary Oncology; 13-15 March 2019, Poznan, Poland; please note that the year is not given! [Please also refer to https://iccvs.ug.edu.pl/blog/11th-international-conference-of-contemporary-oncology-in-poznan/]). Concerning the participation in the organizational committees of international and national scientific conferences, Dr Đapič has mentioned five of such events with the biggest one in 2017 (OurCon V Mass spectrometry imaging conference held on 25-28 September 2017 in Doorn, the Netherlands), as a key person responsible for organizing the scientific program.

Dr Đapić is a member of Advisory board of *Rapid Communications in Mass spectrometry* (since 2018; verified at Editorial board RCMS, accessed at 19/05/2023), and a member of Croatian proteome organization (is this another name for *Croatian Proteomics Society* (CROPROT), a part of EuPA?) as well as Association for natural science research (**further information is not provided**).

Judging all above, this demonstrates rather a **relative inexperience of the candidate at the international forum** as a speaker, organizer and a senior researcher.

Teaching achievements and in the field of the popularization of science or art

The Dr Đapić has a long teaching career, started as a third year student at the Department of Chemistry, Faculty of Science at the Uni. of Zagreb where she participated as a teacher at the course „Laboratory exercises in physical chemistry“. She later taught the first year students of Food technology and biotechnology and Nutrition (2009-2015), and conducted laboratory exercises and seminar classes (2018). At the Uni. of Amsterdam (postdoctoral period, 2015-2017) she participated as teacher at several courses at the BSc and MSc levels: She has supervised several BSc and MSc students (two towards MSc thesis, five towards BSc degree, 3 towards Literature thesis, three taking part in the Project Scheikunde). Since the beginning of the employment at the ICCVS, Gdańsk, Dr

Đapić has been/is involved in supervision of three PhD students and four postdoctoral researchers. Regretfully, three of these supervisions were interrupted due to either students' personal reasons or maternity leave. Dr Đapić has also served as ad-hoc reviewer in multiple proteomics, mass spectrometry journals (5 listed) and took part in organizing workshops towards popularization of science (2011-2013), in Zagreb, Croatia. Taken together, **the candidate possess the good level of teaching experience demonstrated by multiple educational activities at different levels and steps of her career, in several countries**, well qualifying her to be a supervisor of various theses and to further conduct educational activities at the higher academic level.

Taking into account the overall achievements of Dr Irena Đapić, I believe that she fulfils the formal criteria for obtaining the degree of habilitated doctor, and further the requirements of the ACT Law on Higher Education and Science; Journal U. 2018, item 1668 of July 20, 2018. Having stated this, I also wish to pinpoint some deficiencies, which were described in detail in the report given above.

Hereby, I submit to the High Scientific Council of the Biological Sciences discipline at the University of Gdańsk, an admission of Dr. Irena Đapić to further stages of the habilitation procedure.

Sincerely yours,
Maciej M. Łałowski

Signed by / Podpisano przez:
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