

Statement to PhD. thesis entitled “Metabolomics of clinically important *Aspergillus fumigatus* and *Rhizopus microsporus* in the diagnoses of invasive fungal infections” elaborated by Rutuja Hiraji Patil, MSc; Department of Analytical Chemistry, Palacký University, Olomouc.

The research presented in PhD. thesis is a part of a conceptually approached research focused on the characterization of *Aspergillus fumigatus* and *Rhizopus microsporus* siderophores and mycotoxins with respect to use in the diagnosis of invasive fungal infections, especially to distinguish between colonization and invasion. The work also deals with inter-kingdom interactions of *A. fumigatus* with bacterial pathogens (*Pseudomonas aeruginosa*), host cells (neutrophils) and polymycovirus AfuPmV-1.

The dissertation is logically outlined and synoptically organized. It opens with a theoretical part that clearly summarises the studied issues in 20 pages. Here the author presents current knowledge on *A. fumigatus* infections, its secondary metabolites and interactions. It also deals with mucormycoses and *R. microsporus* secondary metabolites.

Subsequently, the author clearly and comprehensibly defines the goals of the work.

The experimental part includes the methodology used and the chapter Results and discussion and it is completed with a final Conclusion.

The results achieved and their discussion are divided into four parts, each of which summarises and comments on results already published in related papers. In the first part, the author presents a comprehensive paper focused on infectious metallomics and the use of microbial metallophores as promising biomarkers of invasive infectious diseases.

In the next part, she studied the presence *R. microsporus* siderophores, rhizoferrin and its analogues, *in vitro* and in patient's urine by new analytical method, Liquid Chromatography-Mass Spectrometry.

The third part is aimed to study *A. fumigatus* growth stage-specific siderophore and mycotoxin and its production during interaction with neutrophils, *P. aeruginosa* and polymycovirus AfuPmV-1.

The last part of the chapter Results and Discussion deals with the application of the newly developed methods and achieved knowledge in clinical practice, specifically for the diagnosis of invasive aspergillosis.

The thesis further includes a summary in Czech and English language, list of abbreviations, *curriculum vitae* including list of scholarly works and the bibliography of 170 publications, predominantly recent ones.

Scientific contribution of the dissertation and its significance for the practice

Over the last few decades, invasive fungal infections such as invasive aspergillosis and mucormycosis have taken on greater importance, mainly due to increased morbidity and mortality in immunocompromised and critically ill patients. Thus, these infections become a more and more serious medical problem of today. For the successful therapy of these serious infections, their rapid and reliable diagnosis is essential to enable the prompt initiation of targeted antifungal therapy. Currently used diagnostic methods have significant limitations, so reliable diagnosis of these infections is complicated. All currently used diagnostic tests for the detection of biomarkers (such as galactomannan, 1,3- β -D-glucan, specific antibodies, DNA detection) are of limited use for the diagnosis of proven invasive aspergillosis. Therefore, it is

necessary to look for other markers that can be used for the diagnosis of these infections. For the mentioned reasons I consider the topic of the thesis highly topical and useful.

A significant contribution of the author of this thesis is the use of new advanced LC-MS methods for the analysis of the metabolome of *A. fumigatus* and *R. microsporus* respectively, focusing mainly on siderophores and mycotoxins. The author monitored the dynamics of siderophores, eventually mycotoxins, production depending on the growth phase and morphotype of *A. fumigatus* as well as the study of the dynamics of their production during aspergillus interactions with *P. aeruginosa*, host neutrophils and polymycovirus. She also verified her observations in real human and animal clinical samples. The author has comprehensively assessed the possibilities of using these substances as new suitable biomarkers of invasive mycoses with sufficient specificity and sensitivity.

Having perused the presented thesis I can say that all the goals were achieved. I appreciated the thesis introduces a lot of new knowledge and the results have been published in high-quality journals, where these publications have undergone a rigorous peer review. The total list of the student's publications includes 4 peer-reviewed original papers in journals with IF, (one of which is first-authored), all journals belong to the second quartile of the relevant discipline. She is also the first author of a review article in an IF journal, Q1. Furthermore, she has authored nine presentations at international conferences. The quality of her papers is reflected in the response among the professional community, currently 16 citations without self-citations, where I would like to point out that these are papers published very recently, in the years 2020-2023.

Comments and suggestions

As for formal issues, I do not understand why the author chose this form of the thesis, instead of an annotated set of publications accompanied by an introduction and a review of current knowledge. I suppose it would have been less laborious for the author and clearer for the opponent and other readers. In addition, in the Results and Discussion section, a large number of figures, tables and texts are taken from the author's publications and comments are added. Moreover, in the Materials and Methods chapter, the author refers to her own publications anyway for a number of important information, such as the origin of the human or equine clinical samples etc.

The author is contradicting herself with the statement "Serum GM has substantial false positivity rates caused by early antigen clearance from the blood due to circulating neutrophils" and this statement is not even supported by the chosen reference.

Since this is a full-length dissertation, it would be preferable to include all relevant facts. Referring to already published articles is suboptimal in this case. For example:

- "...identified by phylogenetic analysis of the internal transcribed spacer regions 1 and 2." - some information about the methodology is missing; alternatively, a reference should be provided.

- I would have appreciated more information about "anticoagulated blood" on page 35 - especially about the origin of the blood and its processing, type of anticoagulant, etc.

- For the strain of *P. aeruginosa* on page 35, indicate the origin [see citation 160] or indicate that it is originally *Pseudomonas aeruginosa* ATCC 15692 from American Type Culture Collection.

For reference 170: „J.P. Donnelly, S.C. Chen, C.A. Kauffman, W.J. Steinbach, J.W. Baddley, P.E. Verweij, C.J. Clancy, J.R. Wingard, S.R. Lockhart, A.H. Groll, T.C. Sorrell, M. Bassetti,

H. Akan, B.D. Alexander, D. Andes, E. Azoulay, R. Bialek, R.W. Bradsher, Jr, S. Bretagne, T. Calandra, A.M. Caliendo, E. Castagnola, M. Cruciani, M. Cuenca-Estrella, C.F. Decker, S.R. Desai, B. Fisher, T. Harrison, C.P. Heussel, H.E. Jensen, C.C. Kibbler, D.P. Kontoyiannis, B.-J. Kullberg, K. Lagrou, F. Lamothe, T. Lehrnbecher, J. Loeffler, O. Lortholary, J. Maertens, O. Marchetti, K.A. Marr, H. Masur, J.F. Meis, C.O. Morrissey, M. Nucci, L. Ostrosky-Zeichner, L. Pagano, T.F. Patterson, J.R. Perfect, Z. Racil, E. Roilides, M. Ruhnke, C.S. Prokop, S. Shoham, M.A. Slavin, D.A. Stevens, G.R. Thompson, III, J.A. Vazquez, C. Viscoli, T.J. Walsh, A. Warris, L.J. Wheat, P.L. White, T.E. Zaoutis P.G. Pappas, Revision and update of the consensus definitions of invasive fungal disease from the European organization for research and treatment of cancer and the mycoses study group education and research consortium. *Clin Infect Dis*, (2019) “, it would be useful to add the electronic source of the citation, or provide a reference from 2020, when the article was published, see below. In addition, if there are too many authors for a publication, it is enough to list the first three authors: J.P. Donnelly, S.C. Chen, C.A. Kauffman et al. Revision and Update of the Consensus Definitions of Invasive Fungal Disease from the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium. *Clin Infect Dis*. 12;71(6):1367-1376.

All abbreviations should be consistently explained and listed in the list of abbreviations:

- Some items that are missing in the list of abbreviations, e.g.: VF in the methodology (later stated to be virus free)/ virus-infected (VI); the list of abbreviations contains FTICR, while in the text the author uses the abbreviation FT-ICR etc.

The title of chapter 4.1 in the text is abbreviated (LC-MS-based characterization of *R. microsporus* siderophores) is distinct from the title given in the table of contents (Liquid chromatography-mass spectrometry (LC-MS) based characterization of *R. microsporus* siderophores).

I would like to emphasize that the above-mentioned comments and minor errors and typos have only a minimal effect on the clarity and readability of this high-quality text.

The opponent questions:

- What is the perspective of the suggested methods to be used in practice? What is the author's estimation of the horizon for the implementation of these methods into routine diagnostics?
- If the developed methods were to be used in practice, what are the equipment requirements for conventional laboratories? What are the estimated costs of these analyses (instrumentations, consumables)?
- Can the author analyse the possible shortcomings of the proposed methods compared to methods already in use?

Conclusion:

The student Rutuja Hiraji Patil, MSc, demonstrated high level of creative abilities and her thesis unequivocally meets all requirements applied to PhD thesis according § 47 VŠ zákona 111/98 Sb. Therefore, I recommend the thesis for the defence.

Brno, 7th August 2023


Prof. Filip Ruzicka, MD, PhD

Prof. RNDr. Zbyněk Zdráhal, Dr.
Central European Institute of Technology
National Centre for Biomolecular Research
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Brno, July 28, 2023

Review of doctoral thesis

MSc. Rutuja Hiraji Patil

Metabolomics of clinically important *Aspergillus fumigatus* and *Rhizopus microsporus* in the diagnoses of invasive fungal infections

Rutuja Hiraji Patil dedicated her doctoral study to metabolomic characterization of *Aspergillus fumigatus* and *Rhizopus microsporus* which cause wide spectrum of infections with the aim to exploit potential of selected metabolites for rapid, sufficiently specific and sensitive diagnostics of these life-threatening infections in human and veterinary medicine. Thus, the Rutuja's work contributes to the topic with high importance to human society – treatment of serious fungi infections.

She focused namely on siderophores and selected mycotoxins. Her PhD study extends our knowledge about fungi behaviour and metabolite production under different *in vitro* and *in vivo* settings, and provides a basis for further research in the field of infection metallomics or even for its possible practical medical applications in future.

The thesis is written and organized as extended digest of her published work. The theoretical introduction is clear and concise, provided with rich list of references (170). Based on methodical nature of the thesis I missed more detailed description of experimental approaches applied for characterization of siderophores. The reference list suffers from frequent incomplete citations, even in case of her own publications which are referred in experimental and result part (see comments below). The results are presented with adequate discussion but I would appreciate attachment of (or direct links in the text to) author's publications which forms the basis of experimental and result parts of the thesis.

She adequately published results of her work in recognized scientific journals with impact factor. In summary, she is the first author of one research and one review publication and the co-author of another three publications related to her PhD study. She also presented her results in national and international conferences (9 oral and poster contributions in total).

I have following comments or questions:

Page 31 – „...ref [130].“ is not complete. The material should be listed or the publication easily accessible.

Page 31 – „A quadrupole filter to 200 –700 and 500–1500 Daltons...“ – Unit Daltons is not correct.

Page 32 – in Data processing section – Is really ref. [40] dedicated to CycloBranch software?

Page 37 – „...ref [136].“ is not complete. The material should be listed or the publication easily accessible.

Page 39 – „The box plots were built using MS Excel 2016.“. No box plots are in the thesis, only in the original publication.

Page 40 – „Acquity HSS C18/1.8-mm“; „Acquity HSS T3/1.8-mm“ – I believe it should be 1.8 μm .

Page 41 – „...ref [140].“ is not complete. The material should be listed or the publication easily accessible.

Page 41 – „whole lungs were lyophilized...“. Were really whole horse lungs lyophilized?

Page 45 – ref. [145] is the same as ref. [135]

Please explain the reason why you performed separately ethyl acetate extraction and then pooled the extracts with a supernatant after protein precipitation (page 31, section „Metabolite extraction“).

How you can explain absence of hydroxyferricrocin (Hfc) during *A. fumigatus* conidial germination in the interval 7-9 hrs (Fig. 5.8 B) and in the residual fungal mass of *A. fumigatus* in the interval of 14-24 hrs (Fig. 5.9 A)?

What might caused a difference in supernatant concentration of metabolites in a single *A. fumigatus* cultivation in two different experiments (if they are comparable), eg. triacetylfusarinine C (TafC) after 18 hrs incubation – cca 10 $\mu\text{g/ml}$ in Fig. 5.10 B and over 200 $\mu\text{g/ml}$ in Fig. 5.9 B?

You mentioned the proposed method is suitable for rapid diagnostics of IPA. Could you please summarize an average time of the whole procedure and compare it with the other diagnostics techniques?

Could you please assess perspectives of practical utilization of MS of siderophores in medicine, in general. How far we are?

In summary, Rutuja Hiraji Patil proved her capabilities of independent scientific work and I recommend her for receiving of PhD degree.



Zbyněk Zdráhal