Prof. Matthias Bochtler

International Institute of Molecular and Cell Biology &
Trojdena 4, 02-109 Warsaw, Poland
Institute of Biochemistry and Biophysics
Pawinskiego 5a, 02-106 Warsaw, Poland
Tel: 0048 22 5970732
Fax: 0048 22 5970715
e-mail: mbochtler@iimcb.gov.pl

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Opinion on the PhD thesis by Magdalena Widziołek-Pooranachandran

Magdalena Widziołek-Pooranachandran has presented a PhD thesis on "*In vitro* and *in vivo* modelling of the pathogenicity of *Porphyromonas gingivalis*". The main part of the thesis establishes a zebrafish model for *P. gingivalis* infection.

At first look, zebrafish, especially early zebrafish embryos, appear to be an odd model for *P. gingivalis* infection, since *P. gingivalis* is not a major pathogen of concern for zebrafish under physiological conditions. However, zebrafish has many advantages as an infection model. Early zebrafish embryos (up to 4 or 5 days post fertilization (dpf), depending on temperature) are not legally considered as protected animals, and hence there is much less ethical concern about the use of this animal model. The transparency of the embryos makes the system ideally suited to study the systemic consequences of *P. gingivalis* infection. Zebrafish, unlike the invertebrate *Drosophila* model, possesses both innate and adaptive immunity. Zebrafish even has (continuously replacing) denture, with the first tooth row appearing 48 hours post fertilization (hpf), and the first replacement teeth forming at about 80 hpf. Like mammals, zebrafish has a chambered heart, but in contrast to mammals, there are only two chambers (atrium and ventricle) and not four chambers. Zebrafish husbandry is done at the ideal body temperature of 28 °C, which is lower, but only manageably so, than mammalian body temperature.

Magdalena Widziołek-Pooranachandran's thesis is conventionally structured. It starts with a clearly written abstract of the thesis contents, in Polish and English. This is followed by an extensive introduction, a concise statement of the research hypothesis, and a more detailed outline of aims. After an extensive methods section, which is commendable for the breadth of methods ranging from bacteriology and microbiology to work with transgenic zebrafish, the main results are presented. The results section is a pleasure to read because it is very logically structured and well-illustrated. The thesis ends with a very reasonable discussion, concluding remarks, and an extremely extensive bibliography. The nearly 400 citations in the bibliography are I think the record in any thesis that I have reviewed so far. Here, it is worth emphasizing that the very long reference list is not the result of undisciplined or unfocused writing. The cited references are all very logically connected to the experimental work that has been carried out, and reflect the breadth of the approach and the diversity of methods. In the following, I will briefly comment on key parts of the thesis.

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The introduction provides a very comprehensive discussion of *P. gingivalis* pathogenesis, which shows an excellent understanding of the physiology of the pathogen, and discusses key virulence factors that are subsequently investigated further in the thesis. The introduction also shows that Magdalena Widziołek-Pooranachandran has an excellent understanding of pathogen host interactions. This part of the introduction emphasizes the role of the intracellular lifestyle of *P. gingivalis*, the penetration through endothelial barriers, and the interaction with phagocytic cells. My only slight reservation about the introduction concerns the lack of regard for the physiological relevance of *P. gingivalis* infection in the model animal. In mammalian guts, *Bacteroidetes* tend to dominate together with *Firmicutes* (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5187364/). In contrast, *Bacteroidetes* are very poorly represented in zebrafish, although they are not so underrepresented in other fish (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3176511/). This aspect would have merited some discussion, since it suggests that *D. rerio* may possess strong mechanisms of immune suppression against *Bacteroidetes*, which could target *P. gingivalis* in particular. How do infection doses compare in mammalian models and in zebrafish?

The first part of the results section covers the development of the zebrafish infection model. Magdalena Widziołek-Pooranachandran's steps towards this goal are very logical. She starts out by finding a compromise temperature (30 °C) that is high enough for *P. gingivalis*, and low enough for zebrafish to develop, and she establishes several routes of infection. For very understandable practical reasons, all of these routes are different from the infection route that starts from the denture. Once it has been established that *P. gingivalis* can infect zebrafish with systemic consequences, Magdalena Widziołek-Pooranachandran continues with a demonstration that the virulence of different *P. gingivalis* strains in mammals is largely recapitulated in zebrafish and that live bacteria are required, supporting the relevance of the model. Next, Magdalena Widziołek-Pooranachandran demonstrates that wild type *P. gingivalis* are much less susceptible to the innate immune response than the triple gingipain mutant, because they are much less likely to be taken up by phagocytic cells. She then demonstrates in a cell culture model and in fish that *P. gingivalis* enriches in heart tissue after systemic infection, can invade endothelial cells in a gingipain-activity independent process, and increases endothelial permeability, by gingipain-activity dependent cleavage of surface proteins. Towards the end of the results section, Magdalena Widziołek-Pooranachandran also investigates the effect of T9SS mutants (particularly Δsov) on virulence, and finds reduced persistence and increased phagocytosis, as for the gingipain mutants.

The second part of the results section looks at the effects of *P. gingipain* mutants on bacterial morphology by SEM and TEM, and on infection of cultured cells. Specifically, Magdalena Widziołek-Pooranachandran worked with two types of endothelial cells, HuDMEC (Human

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Dermal Microvascular Endothelial Cells), HMEC-1 (a Human Microvascular Endothelial Cell line), with macrophages (RAW 264.7) and with oral keratinocytes (TIGK). The main findings are: Gingipains contribute to endothelial permeability by cleaving surface proteins. Some *P. gingivalis* mutants are more capable to form biofilms than the wild-type strain (despite lower virulence). As expected, the *P. gingivalis* mutants are cleared more effectively than the wild-type. The mechanisms underlying this difference are investigated by a dual dye assay, which measures both acidity (by a pH sensitive rhodamine dye) and cellular viability (by quantifying intracellular esterase activity, resulting in the de-quenching of the dye CFSE).

Altogether, the results section of the thesis is impressive by breadth of methods that are used. The work includes biochemical assays, microbiology, zebrafish biology, various advanced optical imaging techniques, scanning and transmission electron microscopy, cell culture work (endothelial assays), and flow cytometry. I would also like to highlight the careful statistical analysis of experimental data. Quantifications are consistently done with proper error analysis and the real spread of raw data is illustrated (rather than hidden, as it often is). Comparisons are always backed up with an assessment of their significance, both for single variables and Kaplan-Meier survival curves.

I have no major criticism of the results part, but a number of questions to clarify my own thinking.

- There is a curious mismatch between the near complete loss of virulence in the gingipain R-ab/K triple mutant on the one hand (Fig. 12), and the results of pharmacological inhibition of gingipains on the other hand (Fig. 13). The interpretation is that the inactive gingipains contribute to virulence. Another interpretation could be that after inhibitor treatment, new gingipains are expressed, which are not affected by the inhibitor any more. Can Magdalena Widziołek-Pooranachandran comment on the chemical mechanism of inhibition (are the inhibitors covalent, what is the nature of the electrophile, are the inhibitors expected to be stable and persistent?). Also, was it attempted to restore virulence in the triple null mutant using the inactive versions of the enzymes to definitely prove that the inactive gingipains contribute to it?

- On p. 81, Magdalena Widziołek-Pooranachandran states that at 32 hpf (infection at 30 hpf + 2h of incubation), the embryo relies primarily on innate immunity. It is intuitively plausible that this is correct, but I have had a hard time finding a formal justification in the literature. At what developmental stage/at what time can V(D)J recombination first occur? And when does adaptive immunity start to become functionally relevant?

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- On p. 85, Magdalena Widziołek-Pooranachandran describes experiments with pu.1 morpholino, which depletes the myeloid lineage. The myeloid lineage does not only include the phagocytic cells, but also red blood cells. This suggests to me that oxygenation of tissues could be altered, and that the obligatorily anaerobic *P. gingivalis* may profit from poorer oxygenation of tissues in the knockdown, perhaps as much or more as from reduced phagocytosis.

- On p. 63, Magdalena Widziołek-Pooranachandran describes the use of the Griess test to determine the NO concentrations. My understanding is that the Griess test detects nitrite (NO_2^-), by formation of a diazonium salt, which then reacts with the reporter amine naphthylethylenediamine to create the dye. It is not clear to me why nitrite (NO_2^-) can be taken as a measure of nitric oxide (NO) production. In this context, note that HNO_2 is NOT the direct hydration product of NO (formally H_2NO_2, a non-existent chemical entity). Can Magdalena Widziołek-Pooranachandran explain the nitrogen oxide chemistry and clarify the link?

The discussion section summarizes the experimental results, and puts them in perspective. The discussion is reasonable, although not as carefully done as the introduction in terms of organization, and it is the only part in which I have found editorial errors (e.g. p. 123, "provies bacteria", "Aantibiotic therapies", "increasing efforts has been invested", etc). However, these small glitches do not tarnish the overall impression that the thesis has a high editorial standard.

On the whole, my impression of Magdalena Widziołek-Pooranachandran's PhD thesis is extremely positive. The work is innovative and novel, particularly the part in zebrafish. Given that zebrafish has external fertilization, is transparent, genetically tractable, and not under animal rights protection for the first five (at 30 °C, four) days of development, it is clearly attractive as a *P. gingivalis* infection model and the work is likely to be well cited. I was also impressed by the breath of methods and clear presentation.

I have found at least three publications by Magdalena Widziołek(-Pooranachandran) in Google Scholar that relate directly to the content of the thesis (and a few others under the same name, perhaps resulting from prior work). For one of these publications, entitled "Zebrafish as a new model to study effects of periodontal pathogens on cardiovascular diseases", Magdalena Widziołek-Pooranachandran is the first author. This work has appeared in a solid journal (Scientific Reports). Given the prospects of the zebrafish model for *P. gingivalis* research, and the standing of the Potempa group in this field, I am a bit surprised that it has not been possible to publish this work more prestigiously. Despite the lack of a

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high profile publication, I strongly feel that the breadth and quality of the work justifies awarding Magdalena Widziołek-Pooranachandran not only a PhD title, but also a distinction.

With best regards

Matthias Bochtler