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**REVIEW**

**The doctoral dissertation of Mauro Galli entitled**

**"Impact of the expression modulation of Aquaprin 9 on proteomic profile and oxidative stress homeostatasis in HepG2 model of hepatic lipid overload"**

**conducted under the supervision of: dr hab. med. Piotr Zabielski**

Metabolic associated fatty liver disease (MAFLD) is an inflammatory liver disease affecting 25% of the world population and 15-49% of Europe. The scale of problem is best reflected by the fact that MAFLD now affects every third inhabitant of the United States of America or Great Britain, and in the next 15-25 years it may lead to cirrhosis of the liver in as much as a quarter of the currently affected. In some European countries, this disease is already the leading indication for liver transplantation. According to the current guidelines (including the consensus of Polish scientific societies, Hypertension in Practice 2022; 8 (2): 1-26), MALFD should be treated as a component of the metabolic syndrome, with a complex and multifactorial pathogenesis including insulin resistance, lipotoxicity, oxidative stress, genetic factors, endocrine activity of adipose tissue, or disorders of the intestinal microbiota. Due to this complexity, there are no currently approved drugs in this indication, and ongoing clinical trials are exploring various metabolic pathways, e.g. insulin resistance, lipotoxicity, activation of inflammation and fibrosis. However, despite the completion of many studies, there is still no effective treatment approved by regulatory agencies other than lifestyle modification, bariartic surgery, treatment of other components of the metabolic syndrome, and in advanced cases liver transplantation.

The doctoral dissertation presented to me for the review deals with the very current subject of MAFDL / NASH pathogenesis, especially in terms of the mechanisms of lipotoxicity, oxidative stress and the central place of the relatively poorly characterized transport protein AQP9, belonging to the aquaporin family. This work is particularly interesting because it includes two scientific layers, i.e. optimization of the proteomics methodology (non-targeted DIA analyzes) and an experimental one indicating new aspects of the AQP9 protein, i.e. the regulation of oxidative stress in conditions of lipid overload of hepatic cells in ex-vivo and in-vitro conditions. The whole dissertation is crowned with a comprehensive metabolomic analysis in the conditions of steatosis ± increased expression and silencing of APQ9. Importantly, this analysis may contribute to further studies of the microenvironment in liver cells (HEpG2) under lipid overload conditions.

The dissertation is written in English, it is extensive with 144 pages, it has a classic layout and, apart from the list of abbreviations, tables and figures, it includes an introduction, aims and goals, a very precise description of the methodology used, results, discussion, conclusions, abstract and references. The results are additionally enriched with 4 tables, 35 figures and tables in the supplement section.

The introduction is broad and meticulously written. The author precisely describes the mechanisms linking obesity with insulin resistance, the importance of free oxygen radicals in homeostasis and cell pathology, and in more detail the function and clinical significance of aquaporin proteins and their relationship with insulin resistance and diabetes. The second part of the introduction, deserving special mention, describes the techniques of mass spectrometry in an accessible but very accurate manner, especially the comparison of DDA vs DDI data acquisition methods.

Magister Mauro Galli sets 3 main goals of his doctoral dissertation, which are not only scientifically consistent but, above all, innovative. Importantly, not only were all the goals achieved at the later stages of the dissertation, but also the results obtained go beyond them.

The methodology used in the dissertation is innovative and, in part, it also becomes the scientific result of this dissertation. In ex-vivo experiments author used liver specimens obtained from extremely obese patients undergoing bariatric gastrectomy with diabetes (n = 3), insulin resistance (n = 3) and without diabetes (n = 3). The obvious shortcoming of such donor selection

is the lack of material (biopsies or resections) from patients without liver pathology (e.g. elective cholecystectomy), which could create a metabolomic profile under control conditions (no liver disease). Additionally, due to the very small study group, a detailed clinical description would be recommended (not only in terms of concomitant diseases, but also the medications used and the histologic advancement of the liver disease, esp. cirrhosis), so that the homogeneity of the groups of patients could be assessed. I also did not find information whether the participants of the study had signed informed consent. The project received a positive opinion of the Bioethics Committee of the Medical University of Bialystok (R-I-002/609/2018 and APK.002.107.2021).

The limitations in the availability of clinical material and possible individual variability are overcome by author by conducting a further and substantial part of the experiments in in-vivo conditions using commercial HepG2 cells and using a self-validated lipid overload model. At this point, I would like to highly rate the precision, and hence the scientific ethics of Mauro Galli. The scientific methodology is particularly advanced as in addition to the above-mentioned proprietary techniques of mass spectrometry, MSc Galli also uses staining of cells for the presence of lipids, assesses the severity of apoptosis and applies experiments for the presence, location and dynamics of H2O2 formation in the cell.

The results are presented very meticulously, also with descriptions of those without statistical significance and enriched with clear figures and photos. The first part of the results concerns the optimization of the DIA method and includes the settings of the instrument output parameters, optimization of the liquid chromatography gradient, collision energy and of the STW settings. This part of the dissertation will be fascinating reading for people who independently perform mass spectrometry. In the following parts, M.Sc. Gallo conducts research with an optimized methodology on liver samples taken from patients, and after selecting protein candidates for further studies carries them out in an in-vitro model. Among others, the author shows increased expression of aquaporin 9 (AQP9), FAB4, LPIN2 and HMOX2 in patients with glucose intolerance as compared to the to the nondiabetic group, which forms a coherent environment, suggesting the influence of AQP9 on the regulation of fatty liver and, hypothetically, on the synthesis of diacylglycerols and activation of oxidative stress. The obtained clinical results are innovative as there are only single reports of AQP9 expression in patients with fatty liver disease. Rodriquez A et al. (Int J Obes 2014) found decreased AQP9 expression in the liver (PCR, WB, histochemistry)

in people with glucose intolerance compared to normoglycemic subjects in a similar population of 66 bariatric patients. Furthermore they have shown a decreased AQP9 expression in the liver in patients with NALFD versus those without steatosis, which correlated negatively with the degree of inflammation and steatosis, indicating a possible compensatory mechanism. I would like to ask M.Sc. Gallo to refer his results in more detail in comparison to the above-cited work. Subsequently, the student conducts a series of elegant and consistent experiments in the HepG2 cell lipid overload model. Mgr Gallo shows, inter alia, lipotoxicity of FFA and glycerol, the effect of APQ9 silencing on the inhibition of the of HepG2 steatosis, activation of apoptosis with an overexpression of AQP9 and, in my opinion, the most interesting, the kinetics of activation of oxidative stress (H2O2) in lipid overload during the 20-hour incubation of HepG2 cells. The presented results are innovative, consistent with the few studies already published (including Wang Ch. Et al. Int J Mol Med. 2013; 32: 1159) and have great potential for their translation into clinical medicine. The crowning achievement of the PhD student's work is a comprehensive proteomic analysis of HepG2 cells under control conditions, lipid overload, silencing and AQP9 overexpression, which may be the starting point for further publications and analyzes and is an absolutely unique achievement. Another very interesting setup would be to conduct and compare such an analysis on human clinical material vs HepG2 cells. The obtained results are innovative on a global scale, and have not only cognitive significance, but also therapeutic potential, as APQ9 could be a possible therapeutic target for the treatment of hepatic steatosis.

The results are critically confronted with the state of the art in the Discussion. The author gives the context of his observations in the light of published works. Importantly, a large part of the discussion brings the reader closer to the nuances of the metabolomics methods and the modifications used by the author, which greatly improves the possibility of a critical evaluation of the results obtained. On the other hand, and which requires a clear emphasis in author's future publications, APQ9 in the light of the latest reports may be important factor in carcinogenesis (Transl Cancer Res. 2021 Apr; 10 (4): 1826), as well as has numerous immunomodulatory functions (Front Oncol. 2021 Nov 5; 11) : 770565), which are difficult to assess in a cellular model, and may limit the applicability of this protein as a potential molecular target.

In the summary of his doctoral dissertation, Magister Mauro Galli formulated correct conclusions, which are justified by the analyzes carried out. The conclusions are not only of a scientific value nature, but also potentially practical, and may be the starting point for further

research. When assessing the dissertation presented to me, I would like to emphasize the scientific independence and vast methodology of the doctoral student, the enormous contribution of work and the ability to logically use scientific tools and experiments in order to comprehensively prove their hypotheses. This dissertation not only meets the requirements for doctorates, but could also be a habilitation thesis.

**In conclusion, the doctoral dissertation by M.Sc. Mauro Galli entitled "Impact of the expression modulation of Aquaprin 9 on proteomic profile and oxidative stress homeostatasis in HepG2 model of hepatic lipid overload" I rate very highly and consider it valuable scientifically and important from a scientific and clinical point of view. In my opinion it meets the requirements set out in the appropriate acts (Ustawa z dnia 20 lipca 2018r. Prawo o szkolnictwie wyższym i nauce, Dz. U. z 2021r., poz. 478). Therefore, I am applying to the High Senate of the Medical University of Bialystok for the admission of M.Sc. Mauro Galli to the next stages of his doctoral dissertation.**

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