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Review of PhD thesis

by Anna Ligęzka

„Clinical and basic investigations on aldose reductase inhibitors in Congenital Disorders of Glycosylation"

presented for the Council of Disciplines of Pharmaceutical Sciences Jagiellonian University Collegium Medicum in Cracow

The supervisors of the revised thesis are Dr. Eva Morava-Kozicz and Dr. Wirginia Krzyściak. The study was conducted in the international cooperation Department of Clinical Genomics at Mayo Clinic in Rochester, Minnesota, USA, and the Department of Medical Diagnostics of Pharmaceutical Faculty at the Jagiellonian University Collegium Medicum in Cracow.

The subject of the thesis by Anna Ligęzka is focused on the congenital disorders of glycosylation (CDG). The most common reason for CDG is the decreased activity of phosphomannomutase 2 (PMM2-CDG). This subtype of CDG was described for the first time in the eighties. Congenital disorders of glycosylation are rare and heterogenous diseases, which are genetically determined dysfunctions characterized by metabolic abnormalities in the glycosylation pathways of proteins and lipids. It is already known that the glycosylation process plays an essential role in the proper functionality of the different cell compartments in the human body. Its disturbance leads to serious and varied clinical manifestations. It includes the symptoms of multiorgan failure such as hypotonia, inverted nipples, abnormal fat distribution, and a wide range of neurological symptoms. Differences in the clinical phenotype, including unspecific symptoms, make this group of diseases difficult to diagnose. Furthermore, there is no enzyme assay developed for the diagnosis of CDG types. Transferrin isoform profile testing is sometimes used to identify protein N-glycosylation disorders, although transferrin glycosylation does not always reflect the clinical condition. The N-glycosylation screening is conducted with mass spectrometry of serum transferrin. Moreover, the diagnosis of different variants of PMM2-CDG is supported by gene sequencing, CDG gene panels, and whole-genome- or whole-exome sequencing.

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So far, only retrospective studies of the natural history of the disease have been conducted, while knowledge about the clinical diversity and progression of the disease, as well as therapeutic efficacy, seem to be still limited. There is no approved treatment for PMM2-CDG, as well as there is no reliable biomarker known. Therefore, the topic taken up by Anna Ligęzka in her doctoral thesis is very up-to-date and provides modern research directions aimed at improving the therapeutic effectiveness and quality of life of patients suffering from CDG.

The aims of the studies were:

- the development and validation of new biochemical diagnostic tools/techniques and therapeutic biomarkers for clinical trials;

- the restoration or at least improvement of appropriate glycosylation in CDG with a model drug such as epalrestat in a preliminary single-patient study;

- the assessment of coagulation abnormalities in patients with CDG;

- the definition of the natural history of CDG patients and reported outcomes in the prospective field.

The dissertation is composed of three articles, including two published and revised articles and one preprint. All articles refer to a specific and original theme and focus on several aspects related to it. The total impact factor of two publications within the revised doctoral thesis is 15.478 according to the Journal Citation Reports. The score of the Polish Ministry of Science and Education (MEiN) is 300 and refers to two of the three submitted articles.

The articles of the dissertation include:

1. Ligezka AN, Radenkovic S, Saraswat M, Garapati K, Ranatunga W, Krzysciak W, Yanaihara H, Preston G, Brucker W, McGovern RM, Reid JM, Cassiman D, Muthusamy K, Johnsen C, Mercimek-Andrews S, Larson A, Lam C, Edmondson AC, Ghesquière B, Witters P, Raymond K, Oglesbee D, Pandey A, Perlstein EO, Kozicz T, Morava E. Sorbitol is a severity biomarker for PMM2-CDG with therapeutic implications. **Annals of Neurology 2021**; 90(6): 887-900, doi: 10.1002/ana.26245 (IF_{2021} = 11.274, MNiE/MNiSW = 200);

2. de Graef D, Ligezka A, Rezents J, Mazza GL, Preston G, Schwartz K, Krzysciak W, Lam C, Edmondson A, Johnsen C, Kozicz T, Morava E. Coagulation abnormalities in a prospective cohort of 50 patients with PMM2-Congenital Disorder of Glycosylation. **SSRN 2023**, doi:10.2139/ssrn.4370624;

3. Ligezka AN, Mohamed A, Pascoal C, Dos Reis Ferreira V, Boyer S, Lam C, Edmondson A, Krzysciak W, Golebiowski R, Perez-Ortiz J, Morava E. Patient-reported outcomes and quality of life in PMM2-CDG. **Molecular Genetics and Metabolism 2022**; 136(2): 145-151, doi: 10.1016/j.ymgme.2022.04.002 (IF_{2021} = 4.204, MNiE/MNiSW = 100).

Two articles of the dissertation, Publications 1 and 3, in which Anna Ligęzka is the first author can be found on the list of scientific databases such as PubMed as well as Web of Science, whereas Publication 2, in which Anna Ligęzka is the second author, is available in the open-access preprint repository of Elsevier such as SSRN. For this reason, Publication 3 shows rather initial research results before it appears in scientific journals. According to the information on the website, "This is a preprint article, it offers immediate access but has not been peer-reviewed". All articles are multi-authored, and therefore in relation to the formal evaluation of the work, it would be suggested to specify Anna Ligęzka's percentage share in the attached papers. According to the Authors' contributions, Anna Ligęzka was responsible for data collection and data evaluation. I would kindly ask for at least Anna Ligęzka's declaration and

more details on her tasks within the Frontiers in Congenital Disorders of Glycosylation Consortium (FCDGC), in particular in the studies included in the dissertation. On the other hand, it is worth emphasizing the nature of the doctoral project. I realize that Anna Ligęzka participated in the project of the Frontiers in Congenital Disorders of Glycosylation Consortium, which shares CDG knowledge across a network of 13 centers, including 9 clinical centers. In this case, the difficulty in assessing the percentage share is comprehensible to some extent. This formal bullet point does not affect the scientific soundness of the project in general.

In the introduction, the Author presented the justification for the research and discussed the goals and research methods. The results of the doctoral dissertation were presented in the attached publications. In the end, a discussion of the results and conclusions were included. Last but not least, Anna Ligęzka drew the future directions of the studies based on the doctoral results. In general, 50 patients with CDG due to PMM2 deficiency were enrolled in studies monitoring genetic, laboratory, metabolic, and clinical data.

In Publication 1, Anna Ligęzka performed a proteomic and glycoproteomic analysis of PMM2-deficient fibroblasts treated with epalrestat, which is a known aldose reductase inhibitor. The safety and efficacy of epalrestat were studied in a single-patient trial for 12 months. It should be highlighted that, for the first time, glycoproteomics was used to confirm treatment efficacy in CDG *in vitro*. In this study, it was proved that the enzymatic activity of PMM2 has increased after epalrestat treatment *in vitro* as well as the improvement of overall glycosylation was established. As far as cellular markers of N-glycosylation such as intercellular adhesion molecule-1 (ICAM-1) and lysosome-associated membrane glycoprotein-2 (LAMP-2) were concerned, epalrestat had no effect on their mRNA expression. In particular, the deep multiplexed proteomics and glycoproteomics analysis of fibroblasts from selected patients allowed the Author to identify and quantify over 6,000 proteins with over 60,000 peptides. The details of this analysis were shown in Figures 2 and 3 in Publication 1. Anna Ligęzka's research showed that epalrestat improved the formation of 412 glycopeptides characterized by differential abundance, and 46% of glycopeptides, that had reduced abundance in untreated PMM-deficient fibroblasts, were improved after epalrestat treatment to the control level. Among them, the expression of glycopeptides such as CD63, LAMP-1 and -2, α- and β-integrins or collagen was comparable to control cells after epalrestat application. Based on this, a novel treatment in PMM2-CDG was proposed, which is a great achievement of Anna Ligęzka. It is worth emphasizing that levels of urinary polyols such as sorbitol significantly increased in PMM2-CDG patients and correlated with peripheral neuropathy and disease severity. Therefore, the urine concentration of sorbitol was proposed as a new potential biomarker for assessing the severity of the disease and monitoring therapeutic efficacy in clinical trials.

In Publication 2, Anna Ligęzka analyzed factors related to the coagulation system in PMM2-CDG patients enrolled in the FCDG Consortium study. The observations are important due to the frequent coagulation abnormalities leading to bleeding or thrombotic episodes in patients suffering from PMM2-CDG. In this work, it was concluded that the activity of antithrombin ATIII was below 50% in 62.5% of patients enrolled in the study and no significant changes in the studied coagulation factors were observed over time. The bleeding and/or bruising symptoms were reported in 16% of the cohort, whereas 10% of patients had thrombosis. At the same time, the Author stated that the risk of thrombotic events is minimal if ATIII activity is above 65%. Finally, antithrombin III and factor XI activities were proposed as potential biomarkers to estimate the risk of thrombotic episodes in trials. As far as formal aspects of the preprint should be assessed, I could have found supplementary files neither in the dissertation nor online.

In Publication 3, Anna Ligęzka assessed patient-reported results in the context of the correlation between clinical assessment of disease severity and quality of life in the cohort of patients with PMM2-CDG. She described the Patient-Reported Outcomes Measurement Information System (PROMIS) and for comparison used the standard CDG severity tool Nijmegen Progression CDG Rating Scale (NPCRS). In this study, she attempted to find correlations between clinical symptoms, including neurological and physical ones, and the general activity of patients as well as their quality of life. In particular, such factors as anxiety, depressive symptoms, fatigue, and satisfaction in social life were taken into consideration. This point of view represents a novel insight into the important aspects of disease burden. Based on this study, PROMIS was proposed as an informative additional tool to measure the CDG disease burden of patients and their caregivers.

When evaluating the doctoral thesis, it is worth paying attention to the fact that Anna Ligęzka's manuscripts, especially 1 and 3, were published in journals from the JCR list, which means they were previously revised in the peer-review procedures of these journals. These publications show the high scientific value of the achievements and are correct in terms of the study design and methodology.

However, there are some questions, which I would like to ask for.

1) Were only T-scores used to compare the cohort to US general population? Did the Authors include the control group of patients as a reference for a group of patients diagnosed with glycosylation disorders?

Could the PhD Candidate refer to the Polish population? What is the percentage of patients with CDG in Poland?

2) In the introduction of the dissertation as well as in Publication 1, Anna Ligęzka mentioned that the research was conducted on fibroblasts from patients suffering from PMM2-CDG. Could the PhD student develop the details concerning fibroblast acquisition, their origin, and identification in the primary cell line?

3) In Publication 1, Western blots and real-time quantitative polymerase chain reaction (RT-qPCR) were used to determine the expression levels of the protein and mRNA of ICAM-1 and LAMP-2 *in vitro*. Are these cellular proteins reliable and specific for evaluation? Which proteins, from the clinical point of view, play the greatest role in the diagnosis of patients with PMM2-CDG?

4) In Publication 1, in the section on Proteomics and glycoproteomics (4.1.3.4.A.) liquid chromatography-tandem mass spectrometry (LC-MS/MS) was described. The solvent B was used in the gradient elution. What kind of solvents were used in LC-MS/MS analysis? What parameters of LC-MS/MS were used in the pharmacokinetic study of epalrestat in plasma (4.1.3.6.A)? Could the PhD Candidate provide more details on the conditions and parameters of gas chromatography/mass spectrometry (GC/MS), which were used for urinary polyol analysis (4.1.3.1.)?

5) In section 5.1 on Future directions, Anna Ligęzka declared clinical studies of epalrestat involving a larger cohort. Did the PhD Candidate consider testing coagulation factors after epalrestat treatment in patients with CDG?

In conclusion, the most important achievements of the revised thesis are:

- description of a biomarker in PMM2-CDG correlating with disease severity;

- proposal of a novel treatment with epalrestat in PMM2-CDG, showing safety and efficacy in a single-patient trial;

- an indication of the diversity of glycopeptides in PMM2-CDG using for the first time glycoproteomics as a tool confirming treatment efficacy in CDG *in vitro*;

- identification of over 6,000 proteins and glycopeptides through proteomics and glycoproteomics *in vitro*;

- the first studies on coagulation abnormalities in PMM2-CDG patients;

- an indication of ATIII and factor XI as potential biomarkers in clinical trials;

- description of the Patient- Reported Outcomes Measurement Information System (PROMIS) used for the first time in order to assess the quality of life in patients suffering from PMM2-CDG;

- proposal of PROMIS as an informative additional tool to measure CDG disease burden.

The PhD Candidate, Anna Ligęzka, is the author of 17 already published articles, which is proof of her scientific engagement as well as maturity.

Anna Ligęzka's doctoral dissertation meets the formal and substantive requirements of the Law on Higher Education and Science published on the 20th of July 2018 (section V, chapters 1 and 2, art. 186, 187 points 1-4). Based on this, I am applying to the Council of the Discipline of Pharmaceutical Sciences of the Jagiellonian University Collegium Medicum to accept this dissertation and allow Anna Ligęzka to the next stages of her doctoral development. Moreover, I am convinced of the high scientific value of the presented dissertation and its contribution to the development of the discipline. Taking into account the entire scientific achievements of Anna Ligęzka, I am applying for a special award for her doctoral thesis.

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