Proteomic biomarkers in Gaucher disease

Łukasz Pawliński ,[1] Ewa Tobór,[1] Maciej Suski,[2] Maria Biela,[3] Anna Polus,[3] Beata Kieć-Wilk[1]

[1]Metabolic Diseases and Diabetology Department, Szpital Uniwersytecki w Krakowie, Kraków, małopolskie, Poland
[2]Department of Pharmacology, Jagiellonian University in Krakow Medical College Faculty of Medicine, Krakow, Poland
[3]Department of Clinical Biochemistry, Jagiellonian University in Krakow Medical College Faculty of Medicine, Krakow, Poland

Correspondence to
Professor Beata Kieć-Wilk; mbkiec@gmail.com

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ABSTRACT

Aims The research work was conducted to find new biomarkers and potential drug targets in Gaucher disease type 1 (GDt1) by analysing the serum proteins.

Methods This study was an observational, cross-sectional analysis of a group of 12 adult participants: six Gaucher disease (GD) patients and six healthy control. Fasting venous blood underwent proteomics analysis and molecular tests. Over 400 proteins were analysed, and in case of significantly different concentrations between the study and control group, we checked corresponding genes to confirm changes in their expression and consistency with protein alteration.

Results We found 31 proteins that significantly differed in concentration between GDt1 patients and a control group. These were mostly proteins involved in the regulation of the inflammatory processes and haemostasis. The levels of proteins such as alpha-1-acid glycoprotein 2, S100-A8/A9, adenyl cyclase-associated protein 1, haptoglobin or translationally controlled tumour protein related to inflammation process were significantly higher in GD patients than in control group, whereas the levels of some proteins such as heavy constant mu and gamma 4 or complement C3/C4 complex involved in humoral response like immunoglobulins were significantly decreased in GD patients. Alteration in two proteins concentration was confirmed in RNA analysis.

Conclusions The work revealed few new targets for further investigation which may be useful in clinical practice for diagnosis, treatment and monitoring GDt1 patients.

INTRODUCTION

Gaucher disease (GD) is caused by an impaired lysosomal glucocerebroside (GBA) degradation. The accumulation of GBA in cells results in their malfunction. The patients' phenotype varies widely despite GD being a single gene mutation disease. Moreover, there are no significant abnormalities in biochemical results (except for changes in blood count and high ferritin values). Other variations, for example in lipidogram, are not characteristic.[1]

In the absence of a clear genotype–phenotype correlation, the identification of new biomarkers and drug targets is still needed. In recent years, many processes in GD aetiopathogenesis such as impaired autophagy and activation of the inflammatory process have been described, suggesting the presence of new, potential biomarkers.[2] However, these observations are based mainly on the results from animal models and research in basic sciences. Until present, there are not many clinical studies proving these theses. Few markers are available for monitoring the patient's response to enzyme replacement therapy (ERT), for example, chitotriosidase activity and lysoGB1 level. Nevertheless, we lack particles that could be useful in diagnosis and monitoring of complications status which could help in making therapeutic decisions. Eventually, we would look for a biomarker of prognostic significance.

Therefore, our work aimed to study proteins profile in GD patients as potential markers of the clinical picture of GD.

MATERIAL AND METHODS

This study was an observational, cross-sectional analysis of a group of 12 adult participants: six GD patients and six healthy control. Patients with GD type 1 (GDt1) were treated in the Metabolic Diseases Clinic in the Jagiellonian University Hospital in Cracow. This study included adult people with GD, confirmed by the positive genetic, enzymatic results and, in two cases, additional histopathological examination. All patients were on enzyme replacement therapy for at least 2 years. The exclusion factors for the analysis were neoplastic diseases, autoimmune diseases, infection and fever. The control group was composed of healthy volunteers, not related to the patients, adjusted with age, sex and body mass index (BMI). The clinical characteristic of groups is presented in table 1.

All the participants underwent a detailed physical examination (ie, age, weight, height, BMI and any disease history). A type of GD was established based on genetic test results. Fasting venous blood and urine was used for a biochemical and molecular biology test. The biochemical tests were carried out in the central Laboratory Department of the University Hospital, according to standard protocol. Proteomics analysis and molecular tests were performed in the OMICRON Laboratory Diagnostics Department and the Biochemistry Department of the Jagiellonian University.

Proteins that were revealed to differ significantly ($p<0.05$) between the study and control group were selected for further investigation. Corresponding genes expression was checked to discover whether the protein profile differences are caused by altered genes activity or post-translational modifications.

Proteomics analysis

The quantitative analysis was performed by the isobaric tag for a relative and absolute quantitation (iTRAQ) method (Sciex, Framingham, Massachusetts). First, the samples were purified using HemoVoid resin (Biotech Support Group, Monmouth Junction, New

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Table 1 Clinical characteristics of study participants with GD (n=6) and control (n=6)

	GD patients	Control	P value
Sex (F/M)	4/2	4/2	1.0
Age (years)	23–73 (median 34)	28–60 (median 37)	0.97
First symptoms (age)	1–55 (median 16)	N/A	N/A
Weight (kg)	39–71 (median 63)	59–89 (median 71)	0.53
Height (cm)	138–175 (median 162)	164–181 (median 169)	0.73
BMI (kg/m^2)	20.5–25.5 (median 24.2)	20.9–29 (median 23.7)	0.84

BMI, body mass index; GD, Gaucher disease.

Jersey) to remove haemoglobin contamination. Cells were lysed in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 1% DTT (1,4 Dithiothreitol) with the mix of protease inhibitors; Sigma, St Louis, Missouri), vortexed, incubated at 25°C for 30 min and then centrifuged at 12 000×g for 15 min. The protein concentrations were determined in the harvested supernatants using a Coomassie Plus assay (Thermo Scientific, Waltham, Massachusetts). One hundred micrograms of the calculated protein content of each sample were precipitated overnight with ice-cold acetone (Sigma) (1:6 v:v). Next, the proteins were solubilised, trypsin-digested and iTRAQ-labelled according to manufacturer's instructions. Samples were randomly assigned to iTRAQ reagents and combined to corresponding 8plex assemblies. For data normalisation, each 8plex assembly contained a common internal reference generated by combining equal amounts of protein from all the samples included in the measurements. Next, labelled peptides were fractionated by strong cation-exchange (SCX) chromatography, after which flow-through fraction and 11 consecutive injections of the elution buffer (5–500 mM ammonium acetate) were collected. Thus, the labelled peptides from each 8plex assembly were distributed across 12 SCX fractions. Each fraction was then injected onto a PepMap100 RP C18 75 µm i.d. × 50 cm column (Thermo Scientific) via a trap column PepMap100 RP C18 100 µm i.d. × 2 cm column (Thermo Scientific) and separated using a 4-hour 5%–45% B phase linear gradient (A phase: 2% Acetonitrile (ACN) and 0.1% formic acid (FA); B phase: 80% ACN and 0.1% FA) operating at a flow rate of 300 nL/min on an UltiMate 3000 HPLC system (Thermo Scientific) and was applied online to a Velos Pro (Thermo Scientific) mass spectrometer through a nano-electrospray ion source. Spectra were collected in full scan mode (400–1500 Da), followed by 10 Higher Energy Collisional Dissociation MS/MS scans of the 10 most intense precursor ions from the survey full scan. These were analysed by the X!Tandem[3] and Comet[4] search engines, statistically validated with PeptideProphet and integrated with iProphet[5] under the Trans-Proteomic Pipeline (TPP)[6] suite of software (Institute for Systems Biology, Seattle, Washington). The Peptide False Discovery Rate (FDR) was estimated by Mayu[7] (TPP), and peptide identification with an FDR below 1% was considered to be correct matches. Imputation of the missing values in peptide abundances was performed in a MaxQuant[8] environment on the log2-transformed normalised iTRAQ, which reports intensities with a criterion of at least 75% of the values present for a peptide in the data set by drawing the values from the normal distribution, with parameters optimised to mimic a typical low abundance measurement. DanteR[9] software was used for protein quantitation and the statistical analysis of iTRAQ-labelled peptides. ANOVA was performed at the peptide level using a linear model with the Benjamini and Hochberg FDR correction used to adjust p values. Protein fold change was reported as a median value of corresponding unique peptides. $P<0.05$ was considered significant. In the analyses on the spectrometer, it was possible to identify 4043 unique peptides that met the criteria for quantitative analysis. These peptides allowed for the identification of 732 proteins in all samples, including the identification of only one unique peptide per protein. As quantitative analysis requires a minimum of two peptides from one protein, the number of proteins for which it was possible to perform the test based on two or more unique peptides was 452.

Quantitative gene expression analysis

A substantial number of proteins were analysed, and those that significantly differed in concentration between the study and control group were selected for further analysis. Corresponding genes were checked to confirm changes in their expression and consistency with protein alteration.

RNA isolation

Purification of total RNA from human whole blood was performed using the PAXgene Blood RNA Kit, following producer protocol. The RNA quality was analysed using the Tapestation 2200 instrument (Agilent, Santa Clara, California) and quantified by spectrophotometry on the NanoDrop (Thermo Fisher Scientific, Wilmington, Delaware).

Microarray

After RNA quality testing, amplification of RNA was performed using the Illumina Amplification kit (Ambion, Austin, Texas). The Quick Amp labelling kit was used for total RNA labelling according to the manufacturer's protocol. Hybridisation of biotin-labelled cRNA to an Illumina chip was performed according to the manufacturer protocol. Arrays were scanned on a HiScan scanner (Illumina, San Diego, California).

Microarray statistical analysis

A microarray data analysis was done using Gene Spring V.13 (Agilent). To identify differentially expressed genes, we applied a quartile normalisation with background correction to identify the median of all samples for the Illumina chip. Separation of different groups for analysis was carried out by dividing participants into two categories: GDt1 patient and control group, using principal component analysis (PCA). In the three-dimensional view of the PCA plot, the three axes are the three principal components (first three by default). Based on the PCA plot analysis, some of the outlier results were excluded from further microarray analysis to achieve the most homogeneous groups. From the performed microarray hybridisation samples with GD 5 (from 6) and control 4 (from 6) samples were used for further analysis. The one-way ANOVA with the post-hoc Tukey test revealed significantly regulated genes in GD versus control group ($p<0.05$).

RESULTS

Among 452 analysed proteins, 31 significantly differed in concentration between GDt1 patient and control groups. These proteins are presented in table 2. The functional analysis showed that there were mostly proteins involved in the regulation of the inflammatory processes and haemostasis. The levels of proteins such as alpha-1-acid glycoprotein 2, S100-A8/A9, adenyl cyclase-associated protein 1 (CAP1), haptoglobin or translationally controlled tumour protein related to inflammation process were significantly higher in GD patients than in control group,

Table 2 Significant differences in protein concentration in patient and control groups

UniProt accession	UniProt protein name	Fold change
P04220	Immunoglobulin heavy constant mu	–1.263
P01861	Immunoglobulin heavy constant gamma 4	–1.245
P27169	Serum paraoxonase/arylesterase 1	–1.237
P13716	Delta-aminolevulinic acid dehydratase	–1.235
P00450	Ceruloplasmin	–1.207
P00747	Plasminogen	–1.175
P05546	Heparin cofactor 2	–1.173
P02751	Fibronectin	–1.166
P16157	Ankyrin-1	–1.165
P0C0L4	Complement C4-A	–1.159
P01042	Kininogen-1	–1.159
P00734	Prothrombin	–1.143
P01024	Complement C3	–1.100
P00751	Complement factor B	1.116
Q9Y490	Talin-1	1.127
P04114	Apolipoprotein B-100	1.142
Q86U×7	Fermitin family homolog 3	1.164
P12814	Alpha-actinin-1	1.202
P10643	Complement component C7	1.205
P02768	Serum albumin	1.205
P02649	Apolipoprotein E	1.233
Q01518	Adenylyl cyclase-associated protein 1	1.253
P06702	Protein S100-A9	1.289
P19652	Alpha-1-acid glycoprotein 2	1.31
P05109	Protein S100-A8	1.315
P00738	Haptoglobin	1.332
P07237	Protein disulfide-isomerase	1.341
P07737	Profilin-1	1.367
P18669	Phosphoglycerate mutase 1	1.38
P13693	Translationally controlled tumour protein	1.433
P29622	Kallistatin (SERPINA4)	1.468

whereas the levels of some proteins such as heavy constant mu and gamma 4 or complement C3/C4 complex involved in humoral response like immunoglobulins were significantly decreased in GD patients. The summary of the functional characteristics of selected proteins is shown in table 3.

Based on the results of significant changes in protein levels in the analysed groups, most promising, corresponding genes were checked to confirm changes in their expression and consistency with protein alteration. Among checked proteins' genes expression, we revealed that they were in-line with proinflammatory proteins concentration in two cases, namely protein S100-A8 (2.11-fold change) and adenyl CAP1 (1.46-fold change), which confirmed the significant upregulation of these genes in Gaucher group in comparison with control group. We did not find any publications about discussed gene expressions in Gaucher disease in available papers up to the publish date of this article.

Table 3 Functional analysis of proteins significantly changed between analysed groups

Proinflammatory proteins	Anti-inflammatory proteins	Haemostasis
S100A8/S100A9	Kallistatin (SERPINA4)	Plasminogen
Adenyl cyclase-associated protein 1	Haptoglobin	Fibronectin
Alpha-1-acid glycoprotein 2	Apolipoprotein E	Prothrombin
	Kininogen	

DISCUSSION

Quantitative proteomic analysis provides a broad spectrum of data that can help to find biomarkers or potential drug targets.[10] That can further provide an instrument for early diagnosis of disease, disease classification and response to drug target therapy. Identification of specific profile proteins for GD might provide a novel insight into GD pathogenesis and guide individualised treatments.

We analysed serum protein concentrations in GD patients and compared them with the healthy control group. Then we checked for gene expression for some of those proteins, and we confirmed overexpression of two genes coding CAP1 and S100-A8 proteins. Therefore we described the potential effects of the most prominent, altered proteins on patients' phenotype and their possible role as a biomarker in GD.

In our study, according to our knowledge, for the first time, we revealed differences in protein levels in GD patients in comparison with healthy subjects, additionally supported by the analysis of expression of the corresponding genes confirming the differences found.

We revealed an increased concentration level of *proteins S100-A8 and A9* in GDt1 patients. Those proteins play an essential role in the stimulation of inflammatory reaction and immune response which makes up for proinflammatory activity.[11] It was confirmed that in GD, the inflammation process on a cellular level is activated by the mutant *GBA* and its pathogenic substrates on endoplasmic reticulum.[12] High concentrations of S100A8/A9 are also found in cancer development and tumour spread,[13] since in GD population is more susceptible for not only haematological neoplasma as myeloma multiplex or non-Hodgkin's lymphoma but also others, that is hepatocellular carcinoma.[14]

Additionally, S100A8 and S100A9 were found to regulate myeloid differentiation in leukaemia. S100A9 induces acute myeloid leukaemia (AML) cell differentiation, whereas S100A8 maintains the AML immature phenotype.[15] However, till present, no reliable data connecting GD and secondary AML were shown, although such a case was reported.[16]

It is well known that GD patient is significantly more prone to multiple myeloma (MM) development than the general population.[17] Patients with GD also have an increased risk of monoclonal gammapathy (MGUS), which translates into 25–50 times higher risk of MM.[18] In the general population, about 1% of MGUS will pass in MM (no such data are available for GD). S100A9 was described as a chemoattractant for MM cells and inducer for myeloid-derived suppressor cells, which express and secrete inflammatory and pro-myeloma cytokines, including tumor necrosis factor α (TNFα), interleukin (IL)-6 and IL-10.[19] Available data suggest that extracellular S100A9 promotes MM development and that inhibition of S100A9 may have a therapeutic benefit.[20] Therefore, taking into consideration a high prevalence of MGUS in GD and an increased risk of MM, a new biomarker to assess the risk of MM development would be valuable.

In addition, there are reports about GD patients, mostly with D409H mutation, who present prominent aortic calcification.[21 22] The pathogenesis of this complication is still unknown although it was found that S100 protein family can accelerate the aortic valve sclerosis and might be a missing link between those two.[23]

In conclusion, we discovered a high concentration of S100-A8/A9 proteins in GD patients which might be associated with intensified inflammation conditions and increased risk of MM, AML and aortic calcification in them. We pointed out S100-A8/A9 as a potential biomarker and treating target.

The significantly overexpressed gene of *adenyl CAP1* was found, resulting in a high concentration of CAP1 protein in our GDt1 patient. CAP1 is involved in signalling pathways and cell migration.[24] It plays a crucial, regulatory role in activated monocytes by interaction with a human cytokine—resistin and it mediates the inflammatory activity of human monocytes.[25] Although CAP1 is considered an intracellular protein, it was shown that the resistin facilitates CAP1 transport to the cell membrane.[25] Our analysis revealed an increased degree of resistin receptor, although resistin itself was in the normal range. Increased levels of receptors, even with the unchanged level of their agonists, may suggest functional activation of this pathway. The latter mechanism of increased CAP1 level might be extensive cell apoptosis due to GBA overload. These are the preliminary observation and hypothesis, and there is still a need for further study on the molecular level.

Additionally, a positive correlation of CAP1 with hepatocellular (HCC) tumour development and metastasis was identified, as overexpression of CAP1 in HCC specimen in comparison with healthy, adjacent tissue was described.[26] It was stressed that CAP1 could be an independent prognostic factor for patients' survival.[26] A series of 16 cases of HCC in GD patients were described,[27] with a dominating picture of fibrosis and iron overload, although no specific protein or gene expressions were presented. The role of CAP1 in GD is currently unknown.

The upregulated level of *Kallistatin* (kallikrein-binding protein(KBP), Serpin A4) protein was also confirmed. KBP belongs to a group of serine proteinases that can produce kinins promoting local vasodilation.[28] Human tissue kallikrein as a substrate for kallistatin is currently used in some countries in the treatment for diseases with disturbances of blood flow such as acute ischaemic stroke.[29] Therefore, the higher concentration of kallistatin presented in a patient with GD may provide a protective effect on ischaemic diseases, including cardio-protection and nephro-protection.[30] A decrease in mortality rate caused by heart disease in GD was already observed.[31]

Few other proteins such as alpha-1-acid glycoprotein 2, haptoglobin, complement C7 known for their proinflammatory effect[32] were elevated in GDt1. Simultaneously those proteins, including kininogen, paraoxonase and ceruloplasmin, that act as anti-inflammatory agents were reduced. That alteration gives a consistent picture of the uplifted inflammation process in GD.

Patients with GD are characterised by a decreased level of low-density lipoproteins (LDL) and high-density lipoproteins (HDL), with positive dynamic after ERT introduction.[33] The elevated level of *apolipoprotein E* in our study may be the result of treatment with ERT all of the studied patients and confirms its efficacy.

Few other proteins involved in the coagulation process were also found to be significantly changed. In our study, *complements C3 and C4*, which play a central role in the activation of platelets and the complement cascade system[34] were decreased more than half-fold. Published data revealed thrombocytopathy in GD patients in the form of reduced adhesion of platelets.[35] These, together with concomitant thrombocytopenia, result in an increased risk of bleeding. Molecular studies connected an increased activation of complement C5 with glucosylceramide accumulation, tissue inflammatory response and proinflammatory cytokine production in GD.[36]

Moreover, a reduced level of plasminogen, observed in our study, also favours the coagulation process and platelets abnormal function.[37] Regarding abnormalities in the coagulation process, we observed decrease in plasminogen concentration and prothrombin levels. Those changes were not confirmed in genes expression in our study. Hence we speculated that it might be a result of post-translation modifications. However, an influence of all described changes on the whole coagulation process is vogue, due to a complex nature of GD with concomitant thrombocytopenia, abnormal platelet function or liver involvement, and it needs further studies on a molecular level.

CONCLUSION

For the first time, this study provides an in-depth view of protein levels in GDt1 patients with an analysis of direct alteration in selected genes. We concluded that the presented data may be a good starting point for further research on new biomarkers that might help to explain some of the symptoms in Gaucher disease, monitor its progression and give a possible target for further treatment development.

Take home messages

- Gaucher's disease is one of the most common rare diseases.
- Work is ongoing to find new biomarkers and therapeutic targets.
- Our work indicates that proteins will be helpful in treating patients with Gaucher's disease.

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Contributors ŁP, MS, ET, AP and MB performed the research. MS and AP analysed data. BK-W designed the research study and interpreted the data. ŁP and BK-W wrote the paper. All authors contributed to critical revision of the manuscript and approved its publication. BK-W and ŁP are the guarantors of this work.

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ORCID iD

Łukasz Pawliński http://orcid.org/0000-0002-2743-1922

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