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INTRODUCTION

The most frequently encountered inherent lysosomal storage disorder in man is glycosylceramidosis, better known as Gaucher disease. The disease is marked by deficiency in glucocerebrosidase activity, which catabolizes glycosylceramide to ceramide and glucose. The clinical presentation of Gaucher disease is heterogeneous with respect to age, nature and the progression of symptoms. Manifestations are usually accompanied by abnormalities in serum composition; however, these are not attractive marker candidates since their prevalence varies between patients. Gaucher patients are normally treated using enzyme supplementation therapy to alleviate symptoms. Recently, an enzyme has been discovered that is elevated in serum of symptomatic patients and seems to be a sensitive indicator of Gaucher disease and a potential tool to monitor treatment efficacy.

Results are presented on the label-free, quantitative LC-MS analysis of human serum samples from patients under-going therapeutic treatment for Gaucher disease. An absolute protein concentration has been determined for Chitotriosidase – a disease specific biomarker for symptomatic Gaucher patients. The absolute quantification results were used to develop protein signature profiles, which allow for the analysis of a single condition, thus, removing the need to compare to a control. For the analysis of a single condition, the absolute protein amounts were estimated and expressed as a ratio versus a protein spike providing both an instrument specific absolute concentration response factor and specific condition signatures.

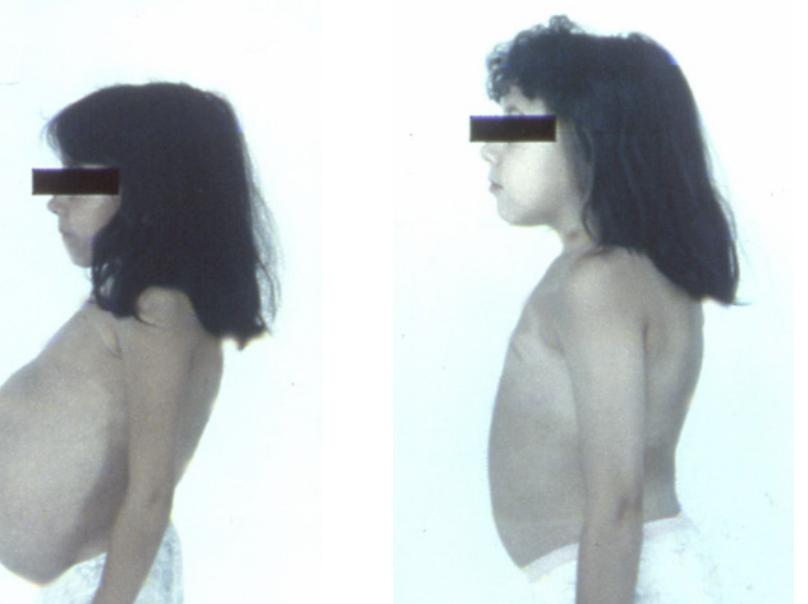


Figure 1. Effect of 1 year of enzyme replacement therapy (spleen functionality restored)

METHODS

Sample preparation

Patient serum samples – control, before treatment and after treatment – were diluted and solubilized prior to depletion with a 10 cm x 4.6 mm multi affinity removal system column (Agilent Technologies, Palo Alto, CA, USA), which removes the six most abundant serum proteins. Serum samples – both depleted and undepleted – were subsequently denatured with RapiGestTM SF surfactant (0.1%) (Waters Corp.), reduced (10 mM DTT), alkylated (10 mM IAA) and enzymatically digested with trypsin, 1:50 (w/w) enzyme:protein ratio.

LC-MS conditions

LC-MS quantification experiments were conducted using a 1.5 hr reversed phase gradient from 5 to 40% acetonitrile (0.1% formic acid) at 250 nL/min on a nanoACQUITY UPLCTM System (Waters Corp.). An Atlantis[®] 3 μm C18 75 μm x 15 cm nanoscale LC column (Waters Corp.) was used, with all samples run in triplicate. Typical on-column sample loads were 0.5 μg protein digest.

The Q-ToF PremierTM mass spectrometer (Waters Corp.) was programmed to step between normal (5 eV) and elevated (25–40 eV) collision energies on the gas cell, using a scan time of 1.5 seconds per function over the m/z range 50–1990. Protein identifications and quantitative information were generated by the use of dedicated algorithms (Waters[®] Protein Expression Informatics), and searching against a Human species-specific database.

Chitotriosidase activity was measured with 4-methylumbelliferyl β-D-N,N',N"-triacetylchitotriose as substrate at pH 5.2

RESULTS & DISCUSSION

Relative Quantification

Figure 2a illustrates low energy BPI chromatograms for 2 of the investigated conditions. Simultaneously, elevated energy chromatograms and spectra were collected. Typical low and elevated energy spectra are shown in Figure 2b. The low energy data are used for the quantification of the peptides and subsequently proteins, whereas the high-energy information is utilized for qualitative, identification purposes.

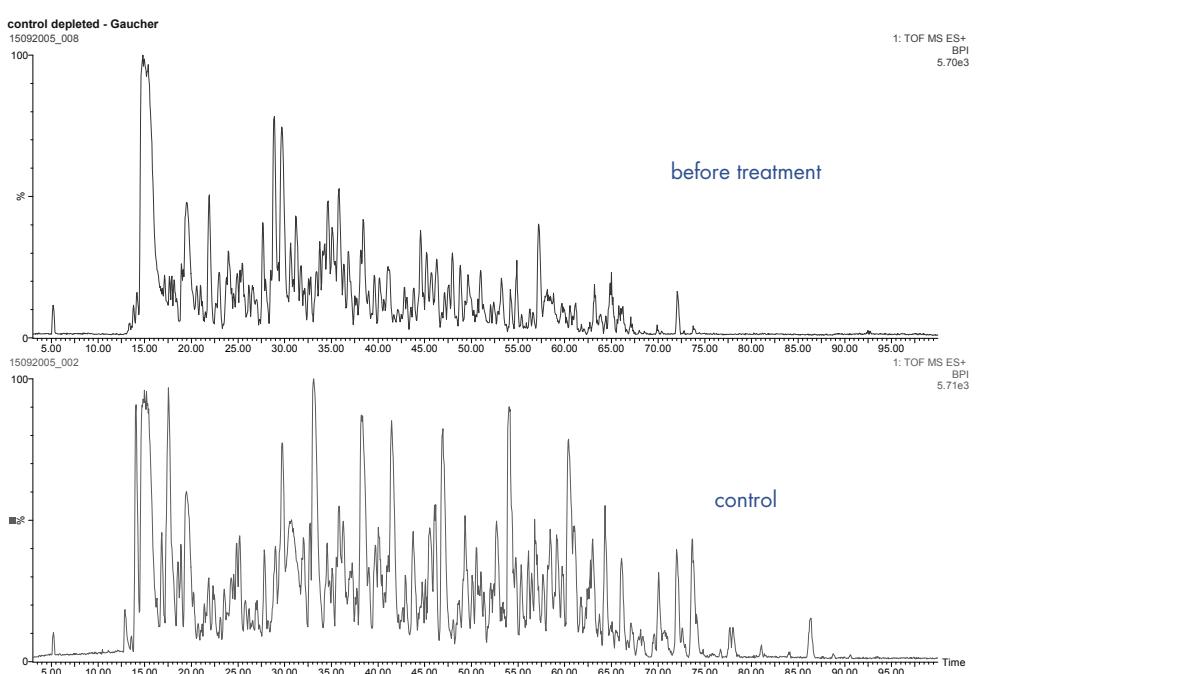


Figure 2a. Low energy BPI chromatograms of depleted serum samples from a before treatment (top) and control sample (bottom) sample

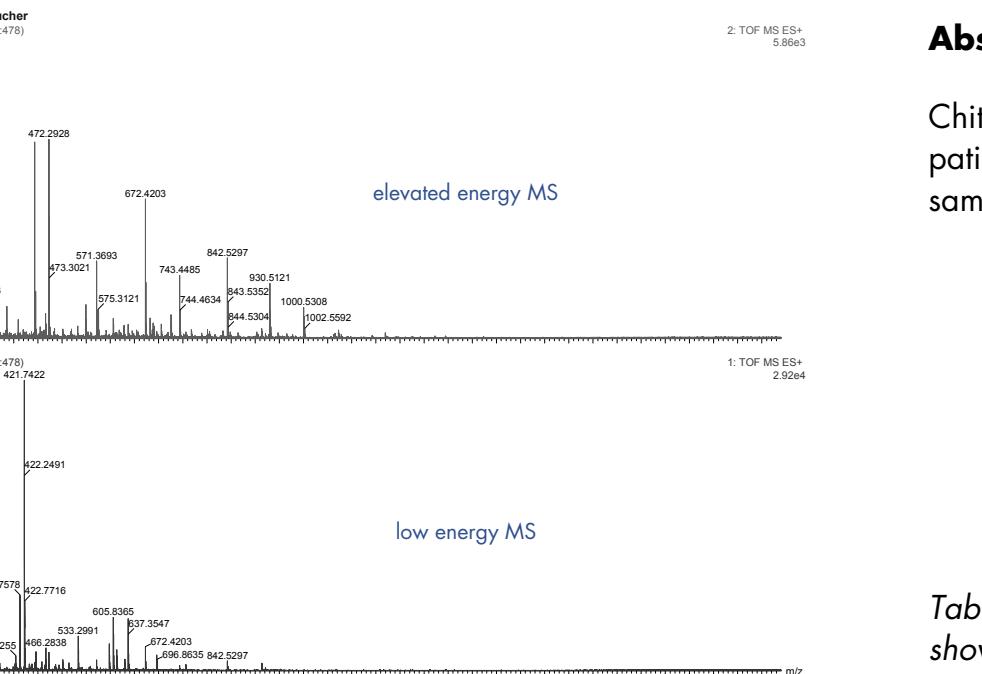


Figure 2b. Low and elevated energy mass spectra from the component eluting at 29.7 min from the depleted, before treatment sample, top pane Figure 2a

The total number of proteins identified – depleted and undepleted sera – was equal to 92 ($n \geq 2$ replicates and ≥ 2 fragments/precursor). Figures 3 and 4 show identification of a regulated protein and the relative quantification results of the depleted, respectively.

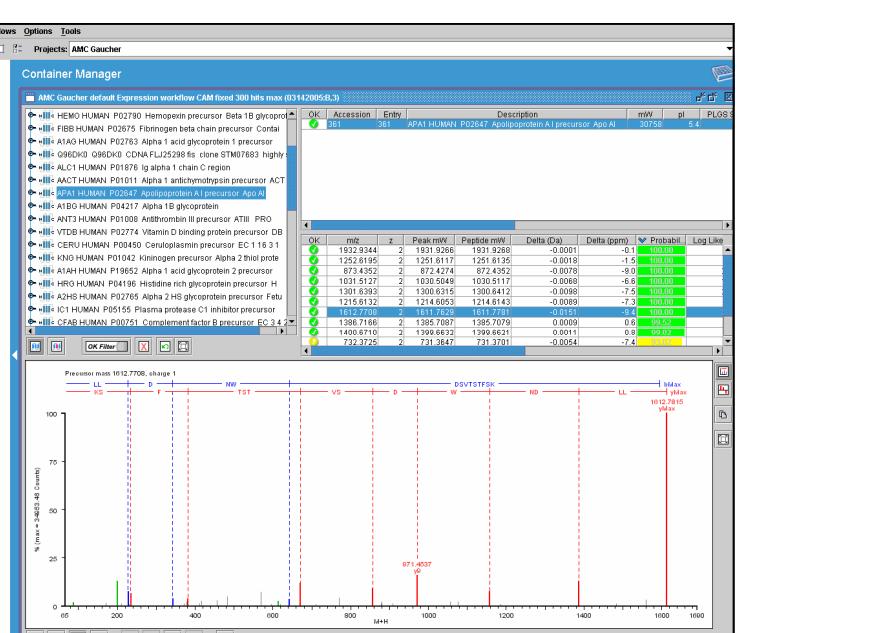


Figure 3. An example of a high-energy identification of a regulated protein in the after treatment serum sample

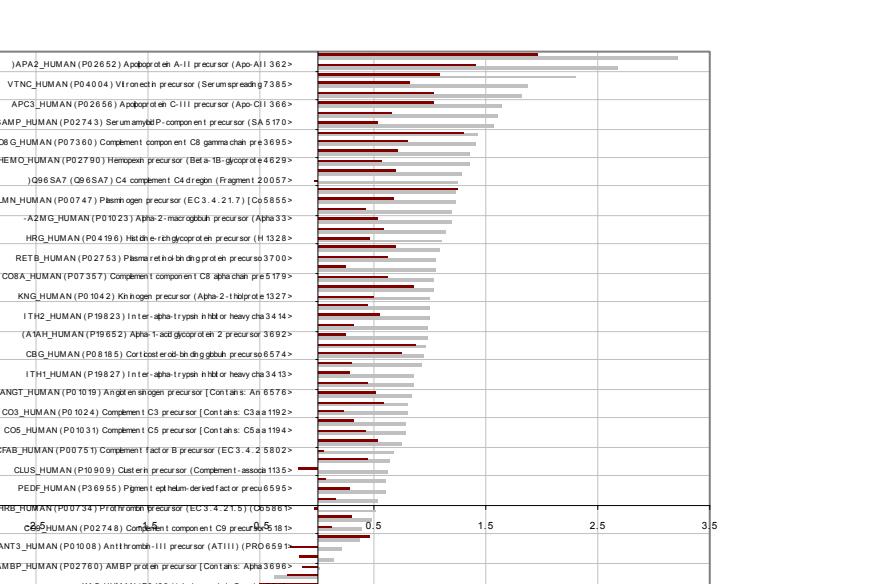


Figure 4. Relative protein concentration (ln ratio) of the before and after treatment (grey & red bars respectively) depleted serum samples versus the control

Absolute Quantification

Chitotriosidase is a known biomarker for symptomatic Gaucher disease patients and was positively identified in all before treatment serum samples, as illustrated below in Table 1.

M+H	stdev mDa	mass precision (ppm)	replication rate		
			before treatment	after treatment	control
1003.5394	0.0016	1.6	3	0	0
1120.6200	0.0020	1.8	3	0	0
1171.5692	0.0037	3.2	3	0	0
1444.7467	0.0024	1.7	3	0	0
2297.1311	0.0042	1.8	3	0	0

Table 1. Replication rate of 5 tryptic fragments from Chitotriosidase, showing the measured mass precision with the retention time/accurate mass cluster

The absolute concentration and enzyme activity of Chitotriosidase was determined by means of a recently published absolute concentration formula:

$$\frac{\sum_{i=1}^n \text{normalized peptide intensity protein } x}{\sum_{i=1}^n \text{normalized peptide intensity internal standard}} \cdot [\text{internal standard}]$$

The absolute concentration of detected Chitotriosidase was found to be equal to 1.6 fmol/μL in the presence of 0.5 μg of depleted serum, corresponding to an enzyme activity of 39,500 nmol/mLh. The activity measured by means of an enzymatic assay was 31,800 nmol/mLh.

Clustering

The ability to determine absolute concentrations allows the data to be clustered to evaluate the data quality (Figure 5a) and identify trends (Figure 5b).

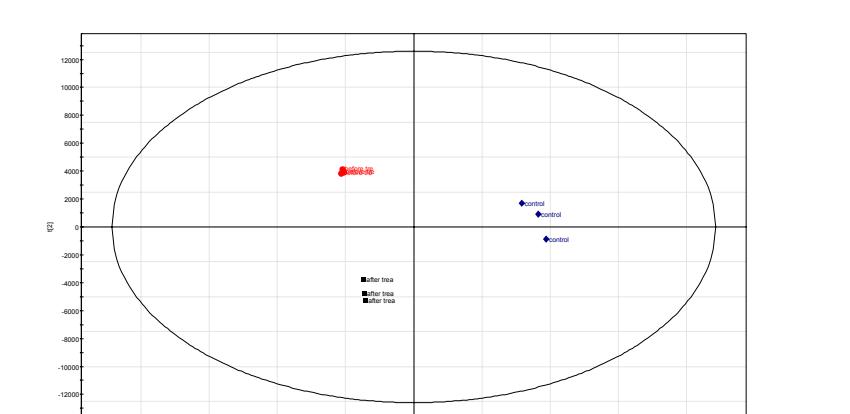


Figure 5a. PCA of depleted serum utilizing the intensity of accurate mass/retention time clusters (peptides) for each injection

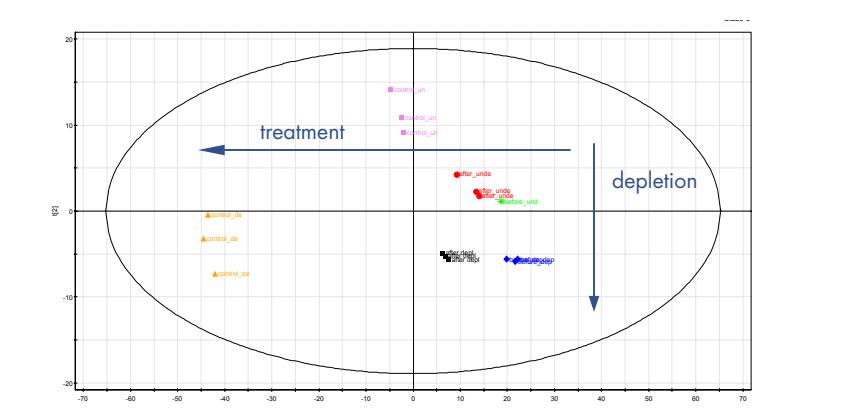


Figure 5b. PLS-DA of both depleted and undepleted samples utilizing the measured absolute protein concentrations determined from serum

Clustering Driven Gene Ontology Searches

Figure 6 illustrates K-means clustering of accurate mass/retention time pairs, grouping peptides that lead to the same protein identification, and possibly proteins involved in the same biological process. The top left pane shows a cluster that was low in intensity before treatment and increased in intensity after treatment and in the control sample, illustrating the effect of treatment.

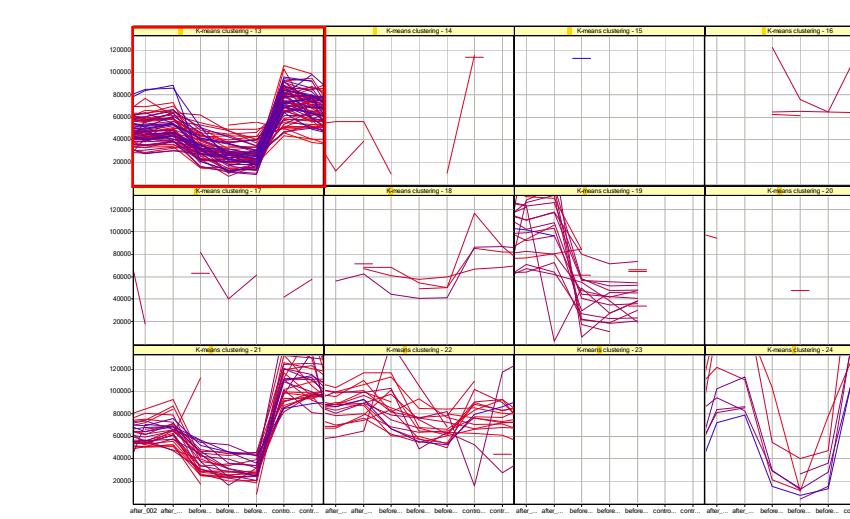


Figure 6. K-means clustering of accurate mass/retention time pairs. Order: triplicate injections of the after treatment, before treatment and control depleted serum

The accurate mass/retention time pairs identified in the top left corner of Figure 6 were selected for a gene ontology search, with the result shown in Figure 7. The majority of the identified proteins had an associated GO annotation, with Figure 8 displaying the molecular function distribution.

Term	Retrieved Genes	Annotated Genes	p-value
Gene Ontology			
cellular_component	25 (0)	23976 (0)	1
biological_process	25 (0)	18846 (0)	1
molecular_function	22 (0)	21031 (0)	1
binding	18 (1)	16087 (844)	0.0001
catalytic_activity	5 (0)	1090 (0)	0.511
enzyme_regulator_activity	11 (0)	982 (7)	1.45e-10
enzyme_inhibitor_activity	11 (0)	359 (23)	2e-015
protease_inhibitor_activity	11 (0)	252 (2)	1.49e-17
endopeptidase_inhibitor_activity	11 (1)	224 (1)	1.40e-07
exopeptidase_inhibitor_acti...	2 (2)	36 (2)	0.00943
serine-type_endopeptidase_inhi...	8 (0)	132 (128)	1.4e-013
chymotrypsin_inhibitor_activity	1 (1)	1 (1)	0.00875
elastin_inhibitor_activity	1 (1)	1 (1)	0.00875
cathepsin_inhibitor_activity	1 (0)	1 (0)	0.00020
wide-spectrum_protease_inhibi...	1 (1)	2 (2)	0.00175
signal_transducer_activity	5 (0)	4187 (399)	0.299
receptor_bonding	3 (3)	95 (86)	7.63e-005
receptor_activity	1 (0)	2932 (2048)	0.935
transmembrane_receptor_activity	1 (0)	1988 (119)	0.837
scavenger_receptor_activity	1 (1)	53 (53)	0.054
transporter_activity	5 (1)	2142 (565)	0.0345
No (or no valid) Annotation	2	0	0

Figure 7. K-means clustering driven gene ontology search based upon the highlighted/selected group of clusters in Figure 6

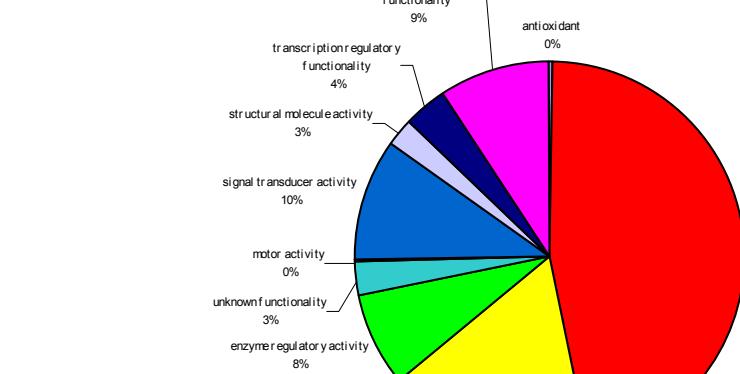


Figure 8. Molecular function distribution as determined from the GO search of all identified proteins in depleted sera across conditions

Protein Signatures

Absolute protein amounts were estimated and expressed as the $^{2}\log$ ratio vs. a protein spike, providing both an instrument specific absolute concentration response factor and condition signatures, which are shown in Figure 9. These signatures do not require comparative analysis and therefore easily extended for larger scale studies.

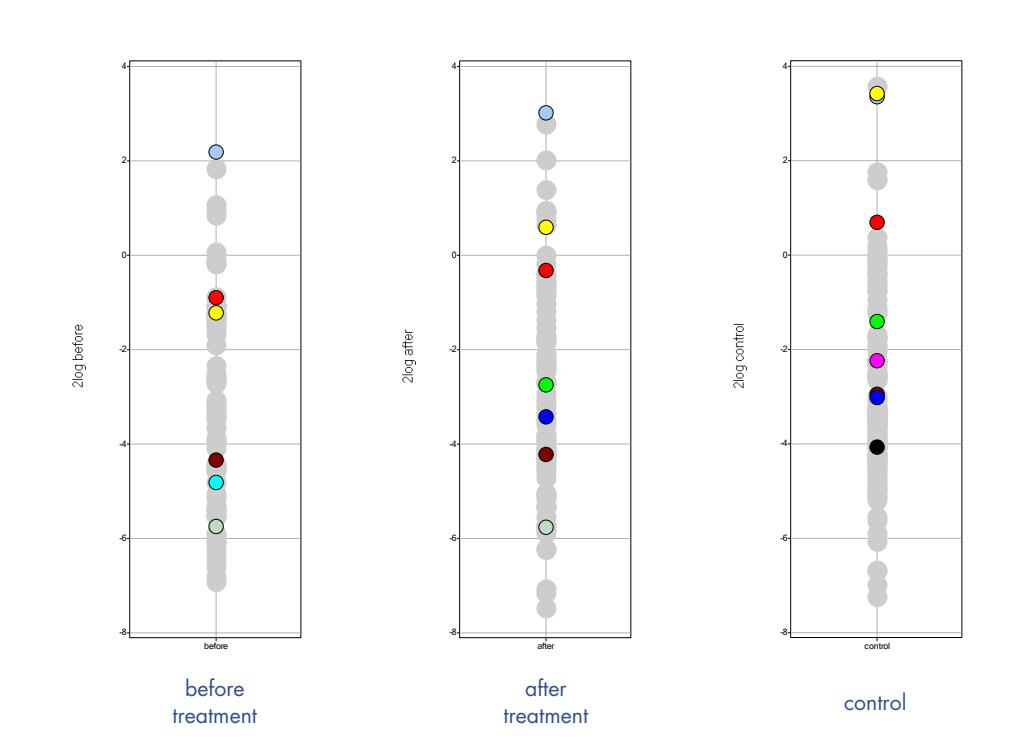


Figure 9. Protein Signatures determined from before treatment, after treatment and control samples depleted serum samples, respectively

CONCLUSION

- A specific Gaucher disease biomarker – chitotriosidase – has been positively identified, quantified and its enzyme activity determined
- Principle component analysis can be applied to both the low and elevated energy data sets to rapidly access data quality and identify trends
- Clustering driven gene ontology searches provide a convenient means for classification of the peptides and proteins that exhibit a similar change profile from a complex data set
- Protein signatures – based on absolute protein concentrations – allow for non-comparative analysis, which could potentially be extended to large-scale clinical research studies

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