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► **To cite this version:**

Sabine Bahn, Marlis Herberth, Dagmar Koethe, Tammy Mk Cheng, Natalia Dorothea Krzyszton, et al.. Impaired glycolytic response in peripheral blood mononuclear cells of first onset antipsychotic-naïve schizophrenia patients. *Molecular Psychiatry*, 2010, 10.1038/mp.2010.71 . hal-00550606

HAL Id: hal-00550606

<https://hal.science/hal-00550606>

Submitted on 29 Dec 2010

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1 **Running title:** Impaired glycolytic function in schizophrenia

2 **Title:** Impaired glycolytic response in peripheral blood mononuclear cells of first onset antipsychotic-
3 naïve schizophrenia patients

4

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19

20 **Keywords:** schizophrenia, proteome, glycolysis, insulin, GLUT1

21 **Abstract**

22 Little is known about the biological mechanisms underpinning the pathology of schizophrenia. We
23 have analysed the proteome of stimulated and unstimulated peripheral blood mononuclear cells
24 (PBMCs) from schizophrenia patients and controls as a potential model of altered cellular signalling
25 using liquid-chromatography mass spectrometry proteomic profiling. PBMCs from patients and
26 controls were stimulated for 72h *in vitro* using staphylococcal enterotoxin B. In total, 18
27 differentially expressed proteins between first onset, antipsychotic-naïve patients and controls in the
28 unstimulated and stimulated condition were identified. Remarkably, 8 of these proteins were
29 associated with the glycolytic pathway and patient-control differences were more prominent in
30 stimulated compared to unstimulated PBMCs. None of these proteins were altered in chronically ill
31 antipsychotic-treated patients. Non-linear multivariate statistical analysis showed that small subsets of
32 these proteins could be used as a signal for distinguishing first onset patients from controls with high
33 precision. Functional analysis of PBMCs did not reveal any difference in the glycolytic rate between
34 patients and controls despite increased levels of lactate and the glucose transporter-1 (GLUT1), and
35 decreased levels of the insulin receptor in patients. In addition, subjects showed increased serum
36 levels of insulin, consistent with the idea that some schizophrenia patients are insulin resistant. These
37 results show that schizophrenia patients respond differently to PBMC activation and this is manifested
38 at disease onset and may be modulated by antipsychotic treatment. The glycolytic protein signature
39 associated with this effect could therefore be of diagnostic and prognostic value. Moreover, these
40 results highlight the importance of using cells for functional discovery and demonstrate that it may not
41 be sufficient to measure protein expression levels in static states.

42

43 **Introduction**

44 Despite extensive research into schizophrenia, questions remain unanswered about the biological
45 mechanisms underlying the disease pathology. Investigations have been hampered due to the complex
46 and multifaceted nature of the illness which is manifested as a diverse spectrum of symptoms ranging
47 from cognitive deficits to behavioural and emotional distortions. Patients suffer from impaired social
48 cognition and communication skills, poor physical health and have a higher risk of committing suicide
49 compared to the general population(1). Although such clinical information is important, it is not
50 sufficient for improving current diagnostic procedures since it does not alleviate the problem of
51 subjectivity. However, it is likely that behavioural abnormalities are reflected at the molecular level
52 and as schizophrenia is a multi-factorial disorder(2) these biological abnormalities could be
53 manifested not only in the brain but also in genetic and proteomic signatures present throughout the
54 body tissues and biofluids including the endocrine organs, cerebral spinal fluid (CSF), blood serum
55 and blood cells.

56 Screening studies conducted at the genetic level have revealed a few credible risk gene candidates for
57 schizophrenia, although most of the findings have not been replicated(3). The onset and progression
58 of the clinical phenotype is not likely to be due to genetic alterations alone as the complexity of the
59 disorder suggests that multiple genes are involved. Moreover, the prevailing hypotheses suggest that
60 the progressive nature of the illness is manifested through the interaction of genes with environmental
61 factors(4).

62 In order to capture both genetic and environmental changes, many researchers have studied the
63 disorder using proteomic platforms. Early studies employing these technologies focused on *post-*
64 *mortem* brain tissues and these identified abnormal expression of proteins associated with several
65 biochemical pathways including metabolism and the immune system(5, 6). However, these effects are
66 likely to be confounded by anti-psychotic drug-treatment, poor diet and unhealthy life style associated
67 with chronic stages of the disease(7). A recent study by Guest and co-workers showed that insulin and

68 insulin-related peptide levels were increased in serum of first onset, antipsychotic-naïve patients
69 indicating that metabolic abnormalities may already be present prior to disease manifestation(8).

70 In the present study, we established proteomic signatures using liquid chromatography mass
71 spectrometry (LC-MS^E, expression mode) profiling for unstimulated and stimulated peripheral blood
72 mononuclear cells (PBMCs) isolated from first onset antipsychotic-naïve (AN) schizophrenia patients.
73 Unstimulated PBMCs from antipsychotic-treated (AT) chronically ill schizophrenia patients were also
74 investigated by LC-MS^E in order to determine which markers may be normalised by treatment and
75 which may be indicators of underlying disease state. PBMCs were chosen as the tissue source in this
76 study due to reports of immune dysfunction in schizophrenia patients (9, 10) and since previous
77 studies have demonstrated that such cells are useful peripheral sources of biomarkers for studies of
78 psychiatric illnesses (11, 12). In addition, numerous studies have shown that the brain and blood cells
79 show a number of parallel responses which suggest that the PBMCs may be a useful surrogate of
80 brain function (13, 14). To investigate peripheral metabolic and immunological alterations associated
81 with the onset of disease, PBMCs from AN patients were also stimulated *in vitro* and then analyzed
82 using LC-MS^E profiling. Stimulation of PBMCs results in activation of various signalling cascades
83 including the triggering of metabolic pathways (15) and could help to identify subtle differences in
84 cell function including changes in the proteome (16). The resulting proteomic fingerprints were
85 characterised by functional analysis *in silico* and validated by mechanism of action studies *in vitro*.

86

87 **Methods**

88 **Study population and demographics**

89 The study was approved by the local ethics committee and conducted from 2007 to 2009 at the
90 University Hospital of Cologne. Subjects comprised 12 first onset antipsychotic-naïve (AN) patients
91 suffering from first-episode paranoid psychosis (DSM-IV: 295.30) and 7 chronically ill antipsychotic-
92 treated (AT) patients (DSM-IV: 295.30), as well as 19 healthy controls (HC) with no family history of
93 schizophrenia or detectable medical, psychiatric or neurological history (**Table 1**). HCs were matched
94 for age, gender, smoking, ethnicity, cannabis use, body mass index (BMI) and education.
95 Psychopathology was assessed on the day of blood withdrawal. In addition, a validation cohort
96 comprising 8 AN, 7 AT schizophrenia patients and 13 HC subjects was recruited. All participants
97 were screened for medical disorders such as diabetes, heart disease, thyroid disease, autoimmune
98 disease, recent infections or current or previous psychiatric illnesses using DSM-IV criteria and gave
99 written informed consent.

100 **PBMC and serum preparation**

101 Blood was collected into 9 mL EDTA S Monovette tubes (Sarstedt; Leicester, UK). PBMCs were
102 isolated by density gradient centrifugation at 750 g for 20 minutes using Ficoll-Paque Plus (GE
103 Healthcare; Amersham, UK) and washed in Dulbecco's phosphate buffered saline (DPBS)
104 (Invitrogen; Paisley, UK). Cells were stored in 90% foetal calf serum (FCS; Sigma; Dorset, UK) and
105 10% dimethyl sulfoxide (DMSO; Sigma) in liquid nitrogen prior to use. For serum, blood was
106 collected into 7.5 mL S-Monovette tubes (Sarstedt). The tubes were placed at room temperature for 2
107 hours for blood coagulation, centrifuged at 4,000 g for 5 minutes and the supernatants stored at -80 °C
108 prior to use.

109 **PBMC stimulation**

110 PBMCs (1×10^7) were thawed in RPMI-1640 medium (Sigma) supplemented with 10% FCS, 1%
111 glutamine, penicillin, streptavidine and 1% DNase (Sigma). For unstimulated PBMCs, cells were

112 washed immediately in DPBS and stored as pellets at -80 °C prior to fractionation. For stimulated
113 PBMCs (AN=8, HC=8), cells were kept in the thawing medium over night at 37 °C under 5% CO₂.
114 The following morning, 7 x 10⁶ cells were stimulated with 1 µg/mL staphylococcal enterotoxin B
115 (SEB; Sigma) and 1 µg/mL CD28 (BD Bioscience; Oxford, UK) in the thawing medium without
116 DNase for 72 h at 37 °C under 5% CO₂. Cell supernatants and pellets were collected after
117 centrifugation at 15 000 g for 4 min. The pellets were washed twice with ice-cold DPBS and stored at
118 -80°C prior to use.

119 **Subcellular fractionation**

120 Subcellular fractions were prepared from unstimulated and stimulated PBMC pellets along with
121 quality control samples (n=8) aliquoted from a single donor. Protein intensity measurements were
122 used to assess variability of the preparation, fractionation and mass spectrometric stages of the
123 procedure. PBMC cytosolic fractions were produced using the ProteoExtract[®] Subcellular Proteome
124 Extraction Kit according to the manufacturer's specifications (Merck; Darmstadt, Germany). The
125 resulting subcellular composition was 71% and 59% soluble proteins in the unstimulated and
126 stimulated samples as determined by Swiss-Prot annotation. Note that only soluble proteins were
127 analysed to keep sample running times under 3 weeks as, after this period, MS detection sensitivity
128 varies between 10-15% introducing noise into the dataset that might hamper data quality (17). Protein
129 concentrations were measured using the BioRad DC[™] Protein Assay (BioRad; Hercules, CA, USA).
130 Proteins were digested using the ProteoExtract[®] All-In-One Trypsin Digestion Kit (Merck) according
131 to the manufacturer's protocol with minor changes. In brief, 4 µL trypsin (Promega, Southampton,
132 UK) was added to samples after addition of blocking agent and samples were incubated for 17 h at
133 37°C with shaking. The reactions were terminated by addition of 1.1 µL 8.8 M HCl (Sigma) and
134 samples stored at -80°C until mass spectrometry analysis.

135 **Liquid chromatography-mass spectrometry (LC-MS^E) and data analysis**

136 The LC-MS^E profiling study was carried out in expression mode (for detailed description refer to
137 (17)) and data acquired as described previously(17). Resulting data were processed with the Waters

138 ProteinLynx Global Server software v2.3 and searched against the human SwissProt v55 protein
139 database (SIB Switzerland) which contained 349480 sequence entries as described previously(17).
140 The total ion current was used for data normalization. The mean intensity coefficient of variation of
141 all proteins detected in the cytosolic fraction of quality control samples was 27%. Processed LC-MS^E
142 data were exported to the software package R (<http://cran.r-project.org>) for filtering and protein
143 intensities were calculated based on methods described previously(17). In brief, criteria for inclusion
144 required the appearance of a peptide in at least two out of three injections per sample and in at least
145 80% of samples in any of the groups. Calculation of protein abundance was based on correlating
146 peptides with a cut-off set to 0.49 (Pearson's correlation)(18). Standard statistical methods were used
147 to investigate data structure and to test for potential experimental artefacts, the need for
148 transformation or exclusion of outlying data. Student's *t*-test was applied to identify differentially
149 expressed proteins ($p < 0.05$). SIMCA-P+ 10.5 (Umetrics; Umea, Sweden) was used for principal
150 component analysis (PCA) to determine the degree of overlap across the groups.

151 ***In silico* pathway analysis**

152 For functional categorization and pathway analysis, statistically significant proteins were analyzed *in*
153 *silico* using the Ingenuity Pathway Knowledgebase (IPKB) software. Assignment of functions and
154 canonical pathways were performed automatically by computational algorithms as described
155 previously(19).

156 **Protein cluster analysis**

157 Protein cluster analysis is a useful computational technique that identifies determinants of a disease
158 which have impact through cooperative function(20). Here, the LC-MS^E protein expression results
159 were subjected to factor analysis (FA) to reduce the multi-dimensional data to the factors which have
160 the highest influence on data structure by considering variance and noise. All combinations of these
161 proteins were tested in simulations to identify those that give the greatest separation between patients
162 and controls. The precision of each combination of analytes in separating patients and controls was
163 tested through a corresponding kernel PCA prediction model. Similar to standard PCA, kernel PCA

164 provides a new projection basis that yields maximal variance in descending order by performing
165 eigenvalue decomposition on the data covariance matrix(21). The prediction boundary of a kernel
166 PCA projection is determined using Fisher's discriminant analysis (FDA). To assess prediction
167 power, each kernel PCA model was built on a randomly selected training set consisting of half patient
168 and half control sample data and then tested on a set consisting of the remaining data (**Fig. S1**).

169 **Validation studies**

170 **Immunoblot analysis.** Differential expression of selected proteins identified by LC-MS^E analysis
171 were tested further by immunoblot analysis using soluble PBMC fractions prepared from a separate
172 validation cohort (5 AN schizophrenia, 5 AT schizophrenia and 5 HC subjects; **Table 1**) and primary
173 antibodies against the proteins listed in **Table 3**. All antibodies were purchased from Abcam,
174 Cambridge, UK. Detection and quantification were performed using either the Odyssey Infrared
175 Imaging System (LI-COR; Cambridge, UK). Intensities of immunoreactive protein bands were
176 normalized to those of the β 3-tubulin immunoreactivity in each track.

177 **Insulin/glucose.** Assays were performed in duplicate by the NIHR Cambridge Biomedical Research
178 Centre, Core Biochemistry Assay Laboratory, Addenbrooke's Hospital and the necessary reagents and
179 calibrants provided as described previously(22). In brief, glucose levels were determined
180 spectrophotometrically in 25 μ L serum obtained from 12 AN, 8 AT schizophrenia and 19 HCs (same
181 subjects as for LC-MS^E profiling study) using an adaptation of the hexokinase-glucose-6-phosphate
182 dehydrogenase method on a Dimension RXL Clinical Chemistry System (Dade Behring; Milton
183 Keynes, UK). Insulin was determined in 25 μ L of serum obtained from the same subjects using a two-
184 step time resolved fluorometric assay from Perkin Elmer (Beaconsfield, Bucks, UK).

185 **Lactate measurement.** Lactate concentrations were measured in duplicate in supernatants of
186 stimulated PBMCs (same subjects as for LC-MS^E profiling study) using an assay kit (Biovision;
187 Mountain View, USA). In brief, 50 μ L of cell supernatants were transferred in duplicate to a 96-well
188 flat-bottom plate. Reaction mix buffer (50 μ L; lactate enzyme and substrate) was added to the
189 supernatants and incubated for 30 min at room temperature. The results were quantified at 450 nm
190 using a plate reader (BioRad; Birmingham, UK)

191 **Hexokinase activity.** Hexokinase activity (Sigma) was measured in triplicate in supernatants of lysed
192 PBMCs obtained from the validation cohort (8 AN schizophrenia and 8 HC subjects; **Table 1**). Cell
193 pellets were resuspended in 230 μ L homogenization buffer (150 mM KCl, 5 mM MgCl₂, 5 mM
194 EDTA, 5 mM β -mercaptoethanol), incubated on ice for 30 min and centrifuged at 13,000 g for 5 min.
195 In a spectrophotometer cuvette, 2.28 mL of Tris/MgCl₂ buffer, 0.5 mL 0.67 M glucose, 0.1 mL 16.5
196 mM ATP, 0.1 mL 6.8 mM NAD and 0.01 mL G6PD were mixed and preheated at 30 °C for 6 min,
197 followed by addition of 0.1 mL of the cell supernatants. Hexokinase activity was based on reduction
198 of NAD⁺ in the presence of G6PD and determined spectrophotometrically by recording the increase
199 in absorbance at 340 nm over 10 min.

200 **Cell surface markers:** Stimulated PBMCs (1×10^6) from the validation cohort (8 AN schizophrenia
201 and 8 HC subjects; **Table 1**) were labelled with glucose transporter 1 (GLUT1) antibody conjugated
202 to fluorescein isothiocyanate (FITC; R&D Systems; Abington, UK) and insulin receptor (IR) antibody
203 conjugated to phycoerythrin (PE; BD Bioscience) in DPBS supplemented with 2% FCS (staining
204 buffer). Reactions were incubated for 20 min at 4 °C in the dark and cells washed twice in staining
205 buffer by centrifugation at 1,500 rpm for 3 min. Samples were analysed on the CyAn ADP Flow
206 Cytometer.2 equipped with Summit v.4 software (Dako Cytomation; Copenhagen, Denmark). The
207 percentages of cells expressing GLUT1 or IR were determined using FlowJo software (Tree Star;
208 Ashland, OR, USA).

209 **Statistical analysis:** Statistical analysis for all functional validation assays was performed using two
210 tailed *t*-test in Prism v.5 (GraphPad Software; La Jolla, CA, USA). *P*-values of *P*<0.05 were
211 considered significant.

212

213

214 **Results**

215 **PBMC proteome profiling**

216 PBMCs isolated from AN (n=12) and AT (n=7) schizophrenia subjects and healthy controls (HC=19),
217 consisted of >75% lymphocytes as determined by flow cytometric analysis. No differences in
218 subpopulations of T cells, NK cells, B cells and monocytes were found between schizophrenia
219 patients and HC subjects (data not shown). PBMCs were subjected to subcellular fractionation to
220 enrich soluble proteins. In the unstimulated condition PBMCs from all volunteers were analysed on
221 the LC-MS^E whereas in the stimulated condition only PBMCs from AN patients and HCs were
222 analysed as the primary aim was to identify differentially expressed proteins in the first stages of the
223 disease to eliminate medication effects. LC-MS^E analysis identified 5141 and 7713 peptides
224 (**Supplementary data**) in unstimulated and stimulated PBMCs which translated to 185 and 441 non-
225 redundant proteins, respectively, using the Swiss-Prot database. In total, 18 differentially expressed
226 proteins between AN patients and HCs were identified. Of these, 6 proteins were altered in
227 unstimulated PBMCs, 13 proteins were altered after stimulation with SEB+CD28, and one protein
228 (lactate dehydrogenase B; LDHB) was altered in both conditions (**Table 2**). Note that all proteins,
229 with the exception of CH60, showed increased expression in stimulated PBMCs from AN patients
230 compared to those from HC subjects. None of the proteins that showed significant differences in
231 expression between unstimulated PBMCs from AN and HC subjects were altered in AT patients with
232 the exception of coactosin-like protein (COTL1) which was also significantly decreased in these
233 patients (p=0.03; data not shown). Only those differentially expressed proteins initially identified
234 between AN patients and HCs were considered in AT patients as the identification of differentially
235 expressed proteins between AT patients and HCs would have been confounded by medication effects.
236 Principal component analysis (PCA) was employed to determine whether a separation according to
237 diagnostic group can be achieved based on the differentially expressed proteins. PCA reduces
238 multidimensional datasets by performing spectral decomposition analysis on covariance matrices. We
239 considered the first two dimensions (principal components 1 and 2) that represent the greatest

240 variance/information content of the dataset(23). AN patients showed good separation from HC
241 subjects for unstimulated PBMCs and a greater separation for stimulated PBMCs (**Figure 1**). In
242 contrast, AT patients differed from AN patients in that they clustered more closely with the HC
243 subjects.

244 **Characterisation of differentially expressed proteins *in silico***

245 **Ingenuity pathway analysis**

246 The Swiss-Prot accession codes of differentially expressed proteins were uploaded into the IPKB
247 database to obtain information on relevant biological functions. The most significant ($p=9.46E-04$)
248 canonical pathway associated with the differentially expressed proteins (GPI, LDHB) in unstimulated
249 PBMCs was glycolysis. The same pathway was also the most significant ($p=1.6E-11$) for stimulated
250 PBMCs which was associated with a higher number of differentially expressed proteins [(LDHB,
251 PGK1, ALDOC, TPIS, GAPDH, PGAM4, Pyruvate kinase isozyme R/L (PKLR) **Figure 2**].
252 ALDOC and TPIS were also significantly associated with the fructose/mannose ($p=5.32E-04$) and
253 inositol ($p=1.05E-03$) metabolism.

254

255 **Protein cluster analysis**

256 To investigate the potential differential effects on glycolysis further, we applied a nonlinear
257 multivariate statistical approach to identify combinations of glycolytic proteins that have high
258 precision for distinguishing patients from controls (**Figure S1**). The top 20 protein combinations
259 producing the best prediction models for separating AN patients from HC subjects were identified.
260 The median precision prediction values were obtained after 1000 simulation tests for all combinations
261 to avoid biased predictions associated with small sample sizes. The clusters generally gave lower
262 prediction values for unstimulated compared to stimulated PBMCs, supporting the case that glycolysis
263 is linked to immune response (**Table 4**). This is also consistent with previous studies which have
264 shown that glycolysis in immune cells is triggered after stimulation *in vitro* (24). The same general
265 result was obtained using sample sets of equal sizes (7 HC, 7 AN) to confirm that there was no bias

266 introduced by different sample sizes (data not shown). Clusters involving enolase 1 (ENO1) and
267 GAPDH gave high precision results for the stimulated samples although ENO1 was initially not found
268 by LC-MS^E to be differentially expressed in patients. This demonstrates the power of the techniques
269 for identifying molecules that exhibit patterned behaviour. To confirm that these clusters were
270 specific for the stimulated state, we chose the top 5 clusters for stimulated PBMCs and used these to
271 predict the diagnostic group of the unstimulated samples. This showed that the prediction results were
272 consistently lower for unstimulated PBMCs (**Table 4**).

273 **Functional validation**

274 **Immunoblot validation of differentially expressed proteins**

275 As the main outcome of the LC-MS^E analysis was the high number of altered glycolysis-related
276 proteins selected biomarker candidates associated with this pathway were also measured by
277 immunoblot analysis using an independent PBMC sample cohort comprised of 5 AN and 5 AT
278 patients as well as 5 HC subjects (**Table 1**). Reproducibility was assessed by comparing the fold
279 changes obtained from the LC-MS^E and immunoblot analyses (**Table 3**). In unstimulated PBMC
280 samples, increased expression levels of cytosolic non-specific dipeptidase 2 (CNDP2) and LDHB
281 were confirmed when comparing AN patients with HC subjects. Expression levels of these proteins
282 did not differ between AT patients and HC subjects, consistent with the LC-MS^E results. In contrast,
283 decreased expression levels of glucose 6-phosphate isomerase (GPI) in AN patients was not
284 confirmed by immunoblot analysis (**Table 3**). In stimulated PBMC samples, increased expression of
285 triosephosphate isomerase (TPIS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and LDHB
286 was confirmed by immunoblot analysis although that of aldolase C (ALDOC) and phosphoglycerate
287 kinase 1 (PGK1) was not. Note that most of the findings did not reach statistical significance ($P < 0.05$)
288 as immunoblot validation was carried out using only 5 samples per group. However, many of the
289 changes showed borderline significance and all, except for ALDOC, showed consistent directional
290 changes with those found in the LC-MS^E profiling study.

291 **Serum analytes**

292 Insulin signaling regulates glycolysis in most tissues(25). As the majority of the differentially
293 expressed proteins that we identified are associated with glycolysis, the circulating serum levels of
294 insulin and glucose were measured in the same subjects from which the PBMCs were derived.
295 Glucose levels were not significantly different between AN and HC subjects ($p=0.3$) and were in the
296 normal range of glycemia ($<7.8\text{mmol/L}$) (**Figure 3A**). This is an important indicator since the patients
297 were not fasted at the time of blood withdrawal. In contrast, insulin levels were increased 1.5-fold in
298 AN patients compared to HC subjects ($p=0.0013$), confirming previous findings(8). Neither glucose
299 nor insulin levels were significantly different in chronically ill AT patients.

300 **PBMC insulin signalling markers**

301 Engagement of the T cell receptor (TCR) and co-ligation with CD28 leads to enhanced glucose
302 transport and glycolysis(26). In lymphocytes, the major glucose transporter and regulator of glucose
303 uptake is GLUT1, an insulin-independent transporter that is up-regulated on the cell surface after T
304 cell stimulation(26). We therefore analysed the expression of GLUT1 and the insulin receptor (IR)
305 using stimulated PBMCs from 8 AN patients and 8 HC subjects (**Table 1**). The percentage of
306 GLUT1-expressing PBMCs was increased 1.3-fold in AN patients when compared to HCs ($p=0.022$)
307 and the percentage of PBMCs expressing the IR was decreased 1.1-fold in the same subjects
308 ($p=0.017$) (**Figure 3B**).

309

310 **PBMC glycolysis markers**

311 The alterations in GLUT1 expression described above, and the expression changes in glycolytic
312 proteins are not sufficient to explain a functional change in glycolysis. Glucose uptake and the role of
313 glucose within a cell are regulated by phosphorylation of glucose and this process is controlled by
314 hexokinase. Therefore, the activity of this enzyme was measured in supernatants of lysed stimulated
315 PBMCs obtained from 8 AN patients and 8 HC subjects (**Table 1**). No difference was observed in
316 hexokinase activity between the two groups ($p=0.9$, data not shown). In addition, we measured lactate
317 levels in cell supernatants of stimulated PBMCs since previous studies have shown that induction of

318 glycolysis results in production and secretion of lactate from lymphocytes(27). **Figure 3C** shows that
319 lactate levels were significantly increased in AN patients compared to HC subjects ($p=0.014$).
320

321 **Discussion**

322 The aim of this study was to identify altered proteomic signatures and molecular pathways to broaden
323 our understanding of the pathophysiology underlying schizophrenia throughout different stages of the
324 disease. PBMCs from first-onset AN and from chronically ill AT patients were profiled using a non-
325 hypothesis driven LC-MS^E screening approach. It was important to include different patient subtypes
326 since the aetiology and pathology are not known and the course of the disease may vary throughout
327 life which is likely to be reflected by changes in molecular markers. Chronically ill AT patients are
328 considered to be an established state of the illness whereas first-onset AN patients represent the first
329 stages of disease without the confounding physiological effects of medication. The use of PBMCs for
330 LC-MS^E profiling allowed downstream functional validation of protein hits and for identification of
331 immunological and metabolic abnormalities.

332 Proteomic profiling of unstimulated PBMCs resulted in the identification of 6 proteins that were
333 differentially expressed between the first onset patients and controls, whereas 13 differentially
334 expressed proteins were identified after stimulation. Eight of these proteins were associated with
335 glycolysis and were altered predominantly only in the case of the stimulated PBMCs. None of these
336 proteins, with the exception of COTL1, showed differential expression in unstimulated PBMCs when
337 comparing the chronically ill AT patients to controls, suggesting that at least some of these proteins
338 may be normalized under long-term disease conditions or by treatment with antipsychotic
339 medications. Changes seen in COTL1 were interesting since this protein has been previously
340 associated with autoimmune disorders (28). Considering the association of this protein with F-actin,
341 changes in COTL1 expression could be indicative of effects on cytoskeletal reorganisation (29). Also,
342 differential protein expressions of ALDOC, GAPDH, PGAM, HNRPK, PGK1 and PPIA in
343 schizophrenia brain tissue have previously been reported further supporting our findings (30, 31).
344 Further longitudinal studies should be carried out to determine whether these could be suitable as
345 biomarkers for distinguishing patients from controls at the earliest stages of the disease or as
346 responsive markers for monitoring antipsychotic treatment status. However, as sample numbers were

347 low, it will be necessary to validate these findings using larger sample cohorts and samples from
348 patients before and after drug treatment. Moreover, it would be of interest to investigate protein
349 signatures in stimulated PBMCs obtained from various schizophrenia subtypes. We demonstrated that
350 cellular/immunological conditions such as stimulation of PBMCs is recommended for these studies as
351 this appeared to increase the separation between the disease and control states. These results highlight
352 the importance of using cells for functional discovery of molecular pathways and demonstrate that it
353 may not be sufficient to measure cellular protein expression levels in unstimulated states. The effect
354 on HSP72 in unstimulated PBMCs was intriguing considering that SNPs in this gene have been linked
355 previously to schizophrenia (32) and expression of the gene has been shown to be altered in
356 schizophrenia post-mortem brain (33). Also, alterations in expression of the protein have been
357 associated previously with mitochondrial dysfunction in PBMCs (34). Here, protein clusters involving
358 ENO1, GAPDH, GPI, PGM2 and TPIS, which are all key enzymes of the glycolysis pathway,
359 resulted in the highest precision values for this separation between disease and control in stimulated
360 cells.

361 Glycolysis provides the energy for immune cells to exert a full immune response(35) by acquiring
362 metabolic substrates such as glucose from the circulation(15). However, immune cells are not capable
363 of regulating the uptake of circulating metabolic substrates autonomously but instead this is controlled
364 by hormones, cytokines or engagement of antigen and co-stimulatory receptors(15). In this study,
365 circulating glucose levels in first onset patients were relatively normal although insulin levels showed
366 a significant elevation. This suggested that at least some of these patients were insulin resistant,
367 consistent with our recent findings of elevated levels of insulin and other insulin-related peptides in a
368 different cohort of patients(8). This means that the bioenergetic demands that maintain normal cellular
369 functions *in vivo*, such as glucose uptake, activation of glycolysis and general regulation of insulin
370 signalling, requires increased secretion of insulin from pancreatic β cells. This has important
371 implications since numerous studies have suggested that too much insulin can have deleterious effects
372 on brain function(36). For example, hippocampal volumes appear to be reduced in diabetic patients
373 and in insulin resistant individuals with high circulating insulin levels(37). Also, hyperinsulinemia has
374 been implicated in the pathogenesis of Alzheimer's disease and associated with phenomena such as

375 aberrant phosphorylation of filamentous proteins, translocation of signalling molecules, increased
376 central nervous system inflammation and β -amyloid plaque deposition(38).

377 The normal mechanism of stimulation by activation of the CD28 co-receptor triggers signaling
378 cascades that overlap with those induced by binding of insulin to its receptor. In this case, stimulation
379 resulted in increased expression of glycolytic proteins in first onset schizophrenia patients, along with
380 increased numbers of GLUT1-expressing and decreased numbers of insulin receptor-expressing
381 PBMCs. Although the increased GLUT1 expression suggests that glucose uptake might also be
382 increased, it does not necessarily suggest an increase in glycolytic flux as this is controlled mainly by
383 phosphorylation of hexokinase one of the main rate limiting enzymes in the glycolytic pathway(27).
384 Consistent with this, we did not find any change in hexokinase activity in the first onset patients.
385 However, it is possible that the observed increase in expression of glycolytic proteins and increased
386 production of lactate may compensate for perturbations of this pathway. A previous report from our
387 group showed decreased expression of glycolytic proteins at the transcriptomic level and increased
388 lactate concentrations in the prefrontal cortex of schizophrenia subjects (39).

389 Glycolysis is a fundamental feature of all cells and is therefore associated with a range of cellular
390 responses which have been associated with schizophrenia. This includes effects on the immune
391 system(35), cytoskeletal abnormalities (40), synaptic plasticity(41) and neurogenesis (42).
392 Abnormalities in glucose metabolism and the link to metabolic syndrome in schizophrenia patients
393 has been known for decades(7) with evidence deriving from genes associated with glycolysis and
394 signs of abnormal glucose metabolism, including changes in glucose transporter expression(12, 43). It
395 has been believed for decades that the link between metabolic syndrome and schizophrenia derives
396 solely from side-effects of antipsychotic medications(44). However, a study by Stone and co-workers
397 supports the view that glycolytic abnormalities are inherent to the disease rather than deriving solely
398 from environmental or pharmacological influences, as shown in this study(43). We also show that
399 insulin levels are elevated prior to disease manifestation and we demonstrate for the first time that
400 patients' cells, most likely deriving from an insulin resistant environment, are compromised at the
401 level of glycolysis when stimulated *in vitro*.

402

403 In this study, we have identified biomarkers associated with altered immune response which can be
404 useful for distinguishing schizophrenia subjects from healthy controls. Similar studies of other
405 psychiatric disorders with overlapping symptoms are required to determine whether these effects are
406 specific for schizophrenia. This is important as it is possible that the observed effects represent the
407 proteomic profile of a more generalized neurological or neurodegenerative process. Two of the
408 enzymes reported in this study, ALDOC and TPIS, have been shown to be altered in the brains of
409 subjects with varying disorders such as schizophrenia, Alzheimer's disease, Parkinson's disease and
410 Huntington's disease (45). In addition, enolase-associated pathways have been linked to
411 neurodegenerative disorders (46).

412

413 One note of caution that should be addressed arises from the small sample numbers used in this study.
414 These were limited by the fact that large clinical centers (~1500 patients) recruit only 10-20 AN
415 patients per year who are free of substance abuse or co-morbidities such as heart and thyroid disease
416 or type II diabetes. The small number of samples did also not allow for internal subgroup
417 comparisons. Arbitrary division of the groups would result in a loss of statistical power and are,
418 therefore, unlikely to provide meaningful results. In addition, there is a technical complication
419 associated with the fact that high numbers of samples require running periods over several weeks on
420 the LC-MS^E system. Running times longer than 3 weeks can result in alterations in sensitivity of
421 detection by 10-15%, reducing data quality(17). Further limitations of the study include the small fold
422 changes obtained for differentially expressed proteins. This is most likely due the small sample
423 numbers, the non-homogeneous cell composition of PBMCs and the heterogeneity of the disorder,
424 which most probably consists of a wide spectrum of related conditions with overlapping
425 symptomology. It is also possible that the differences in the proteomic response after stimulation may
426 represent a primary alteration in the immune response in schizophrenia subjects which leads to the
427 observed alterations in glycolysis under the additional burden of immune activation (47). However, it
428 is likely that the current results are valid given the different converging lines of evidence which
429 suggest alterations in the insulin signaling and glycolytic pathways. Furthermore, we employed a

430 nonlinear multivariate statistics method which confirmed that glycolysis was a major pathway
431 affected in the disorder. Moreover, this analysis showed that even biomarkers that have only subtle or
432 non-significant expression changes, show high precision as a cluster for identification of first onset
433 schizophrenia subjects.

434

435 In conclusion, we found increased expression of proteins associated with the glycolysis pathway in
436 first onset, antipsychotic naive schizophrenia patients. We also showed that small clusters of these
437 proteins can be used to discriminate schizophrenia patients from control subjects with high precision.
438 Most importantly, the alterations in glycolysis were seen to occur predominantly after PBMC
439 stimulation as opposed to the situation in unstimulated cells. This highlights the importance of using
440 functional cell investigations for mechanistic studies in schizophrenia. Using such approaches in
441 future studies could help to identify and verify peripheral signatures and molecular pathways
442 associated with schizophrenia and may generate much needed biomarkers for diagnostic and
443 prognostic purposes and potentially facilitate the development of novel therapeutics.

444

Acknowledgements

446

447 The authors wish to acknowledge all volunteer patient and control blood donors. We thank all
448 members of the Bahn laboratory for helpful discussions. We are grateful to Keith Burling from NIHR
449 Cambridge Biomedical Research Centre, Core Biochemistry Assay Laboratory, Addenbrooke's
450 Hospital for performing the insulin/glucose assays. This research was supported by Stanley Medical
451 Research Institute (SMRI). MH is supported by the Cambridge European Trust. No conflict of interest
452 exists.

453

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- 627

628 **Tables**629 **Table 1 - Demographic details of study cohorts**

Demographic parameter	PBMCs (LC-MS ^E)		PBMCs (validation [#])	
	HC	SZ	HC	SZ
Number (n)	n=19	n=19	n=13	n=15
Age (y)*	34.5±7.2	29.7±8.9	31.6±12.0	30.0±10.4
Type (AN/AT)	N/A	12/7	N/A	8/7
Gender (m/f)	12/7	14/5	5/8	6/9
BMI *	23.4±3.0	23.8±2.7	22.6±2.5	24.2±2.1
Smoking (yes/no)	7/12	9/10	10/3	10/5
Cannabis (yes/no)	16/3	13/6	10/3	8/7

630 *(mean±SD)

631 [#]Sample numbers were subject to quantity of PBMCs obtained by a single donor and split across the validation
632 assays (hexokinase assay, cell surface marker analysis, immunoblot analysis).633 Abbreviations: PBMCs, peripheral blood mononuclear cells; HC, healthy control; SZ, schizophrenia patients;
634 AN, first-onset, antipsychotic-naïve patients; AT, antipsychotic-treated, chronically ill patients; SD, standard
635 deviation; m, male; f, female; y, years; BMI, body mass index

636

637 **Table 2 - Differentially expressed cellular proteins between antipsychotic-naïve patients and healthy**
638 **controls**639 PBMC soluble extracts from AN patients and HC subjects were analyzed by LC-MS^E. Accession number (Acc),
640 protein identification, *P*-value and fold change (AN/HC) are indicated for differentially expressed proteins
641 identified in either unstimulated or stimulated PBMCs

Acc	Gene	Description	<i>P</i> -value [#]	Fold change (AN/HC)
Unstimulated PBMCs				
Q96KP4	CNDP2	Cytosolic non-specific dipeptidase	.005	1.2
Q14019	COTL1	Coactosin-like protein	.02	-1.2
P06744	GPI	Glucose-6-phosphate isomerase	.05	-1.2
P54652	HSP72	Heat shock 70 kDa protein	.02	-1.4
Q9Y4F4	K0423	Uncharacterized protein KIAA0423	.003	1.1
P07195*	LDHB	L-lactate dehydrogenase B	.04	1.2
Stimulated PBMCs				
P09972	ALDOC	Fructose bisphosphate aldolase	.01	1.2
P10809	CH60	60 kDa heat shock protein	.04	-1.4
P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	.03	1.2
P61978	HNRPK	Heterogeneous nuclear ribonucleoprotein	.01	1.3
P07195*	LDHB	L lactate dehydrogenase B	.02	1.3
Q7Z406	MYH14	Myosin 14	.04	1.2
Q9Y2K3	MYH15	Myosin 15	.02	1.2
P43490	NAMPT	Nicotinamide phosphoribosyltransferase	.04	1.3
P00558	PGK1	Phosphoglycerate kinase 1	.003	1.4

P62937	PPIA	Cyclophilin A	<.001	1.4
P60174	TPIS	Triosephosphate isomerase	.008	1.5
P30613	PKLR	Pyruvate kinase isozyme R/L	.05	1.2
Q8N0Y7	PGAM4	Phosphoglycerate mutase 4	.05	1.2

642 #Student's *t*-test

643 *Protein was differentially expressed in unstimulated and stimulated fractions

644

645

646 **Table 3 – Immunoblot validation of differentially expressed proteins**

A) unstimulated PBMCs

Antibody	Normalized Intensity [†]			Fold change (AN/HC)		Rep.	P-value [#] (AN/HC)	Immunoblot (AN/AT/HC)
	AN	AT	HC	IB	LC-MS ^E			
	CNDP2	0.46±0.06	0.44±0.09					
GPI	4.70±0.63	4.50±0.50	4.91±1.20	1.0	-1.2	x	0.737	
LDHB	1.67±0.38	1.38±0.34	1.20±0.39	1.4	1.2	✓	0.087	

B) stimulated PBMCs

Antibody	Normalized Intensity [†]		Fold change (AN/HC)		Rep.	P-value [#] (AN/HC)	Immunoblot (HC/AN)
	AN	HC	IB	LC-MS ^E			
	ALDOC	1.22±0.33					
GAPDH	3.29±0.80	2.89±0.78	1.2	1.2	✓	0.268	
LDHB	0.39±0.13	0.25±0.07	1.6	1.3	✓	0.031	
PGK1	4.25±2.97	4.13±2.89	1.0	1.4	x	0.943	
TPIS	0.23±0.02	0.17±0.06	1.2	1.5	✓	0.129	

647 Abbreviations: ALDOC, Aldolase C; CNDP2, cytosolic non-specific dipeptidase 2; GAPDH, Glyceraldehyde 3
 648 phosphate dehydrogenase; GPI, Glucose-6-phosphate isomerase; LDHB, lactate dehydrogenase B; PGK1,
 649 Phosphoglycerate kinase 1; TPIS, Triosephosphate isomerase; IB, Immunoblot; Rep, reproducibility of LC-MS^E
 650 data with (✓) = reproducible and (x) = not reproducible by IB analysis based on non-conflicting fold changes.

651 [†]Shown are the normalized mean intensities averaged over 5 AN, 5 AT patients and 5 HCs ± standard deviation.

652 Antibodies were normalized against tubulin.

653 [#]P-values were calculated using two tailed *t*-test

654

655 **Table 4 – Median precision prediction values of the top 20 protein combinations**

Stimulated PBMCs		Unstimulated PBMCs	
Protein clusters	Median precision (%)	Protein clusters	Median precision (%)
<i>GAPDH+GPI</i>	80 (40-50)*	ENO1+TPIS	60-70
<i>ENO1+GAPDH</i>	80 (50)*	ENO1+PGK1+TPIS	50-60
<i>ENO1+GAPDH+TPIS</i>	80 (50)*	ENO1+GPI	50-60
<i>ENO1+GAPDH+PKM2+TPIS</i>	80 (40)*	ENO1+PKM2+TPIS	50
<i>ENO1+GAPDH+GPI+ PGM2+TPIS</i>	80 (40-50)*	ENO1+PKM2	50
GAPDH+LDHA	70-80	ENO1+LDHA+TPIS	50
GAPDH+GPI+PKM2	70-80	ENO1+LDHA+PGK1+PKM2	50
GAPDH+TPIS	70-80	ENO1+FBP1+GPI	50
GAPDH+LDHA+PKM2+TPIS	70-80	ENO1+GAPDH	50

ENO1+GAPDH+PGM2+TPIS	70-80	ENO1+FBP1+GPI+PGK1	50
ENO1+GAPDH+PGM2+PKM2+TPIS	70-80	ENO1+GPI+PGK1	50
ENO1+GAPDH+LDHA	70-80	ENO1+ PGK1	50
GAPDH+GPI+LDHA	70-80	ENO1+LDHA+PGM2	50
GAPDH+PGM2+TPIS	60-70	GPI+TPIS	40-50
GAPDH+LDHA+PKM2	60-70	GAPDH+GPI+TPIS	40-50
ENO1+GAPDH+LDHA+PKM2+TPIS	70-80	GPI+LDHA+PGM2	40-50
GAPDH+PGM2+TPIS	60-70	ENO1+FBP1+GPI+PKM2	40-50
GAPDH+LDHA+PKM2	60-70	FBP1+GPI	40-50
GPI+PKM2	60-70	ENO1+PKM2+ PGM2	40-50
GAPDH+PGM2+PKM2	60	ENO1+PKM2+PGM2+TPIS	40-50

656 Abbreviations: ENO1, Enolase 1; PKM2, Pyruvate kinase type M2; PGM2, Phosphoglucomutase 2; FBP,
 657 Fructose 1,6-bisphosphate; N/A, not applicable
 658 *Values were obtained by applying the corresponding protein cluster to the unstimulated PBMCs.

659 **Figure legends**

660 **Figure 1 - PCA of differentially expressed cellular proteins in unstimulated and stimulated PBMCs**

661 PCA of the differentially expressed proteins identified in unstimulated (**A+C**, n=5) and stimulated PBMCs (**B**,
 662 n=14) between AN patients and HC subjects. (**A**) The first two principal components account for 49.6% of the
 663 total variance. (**B**) The first two principal components account for 69.5% of the total variance. (**C**) PCA showing
 664 the degree of separation between AN and AT patients and HC subjects in unstimulated PBMCs.

665

666 **Figure 2 - Overview of LC-MS^E-derived cellular proteins involved in glycolysis**

667 Shown is the glycolysis pathway and the proteins detected by LC-MS^E in unstimulated (US) and stimulated (ST)
 668 PBMCs. Proteins were significantly increased (red), decreased (green) or unchanged (blue) in AN patients
 669 compared to HC subjects. N/A = catalytic enzymes that were not detected.

670

671 **Figure 3 –Metabolic serum and cell markers in schizophrenia compared to healthy controls**

672 Boxplots showing the mean \pm standard deviation of (**A**) circulating glucose and insulin levels in serum of 12
 673 AN, 7 AT patients and 19 HC subjects, (**B**) the percentages of PBMCs expressing GLUT1 and the insulin
 674 receptor after 72 h stimulation with SEB+CD28 in 8 AN patients and 8 HC subjects and (**C**) lactate levels in
 675 PBMC supernatants after 72 h stimulation with SEB+CD28 of 8 AN patients and 8 HC subjects. * $P < 0.05$,

676 ** $P < 0.01$

Figure 1

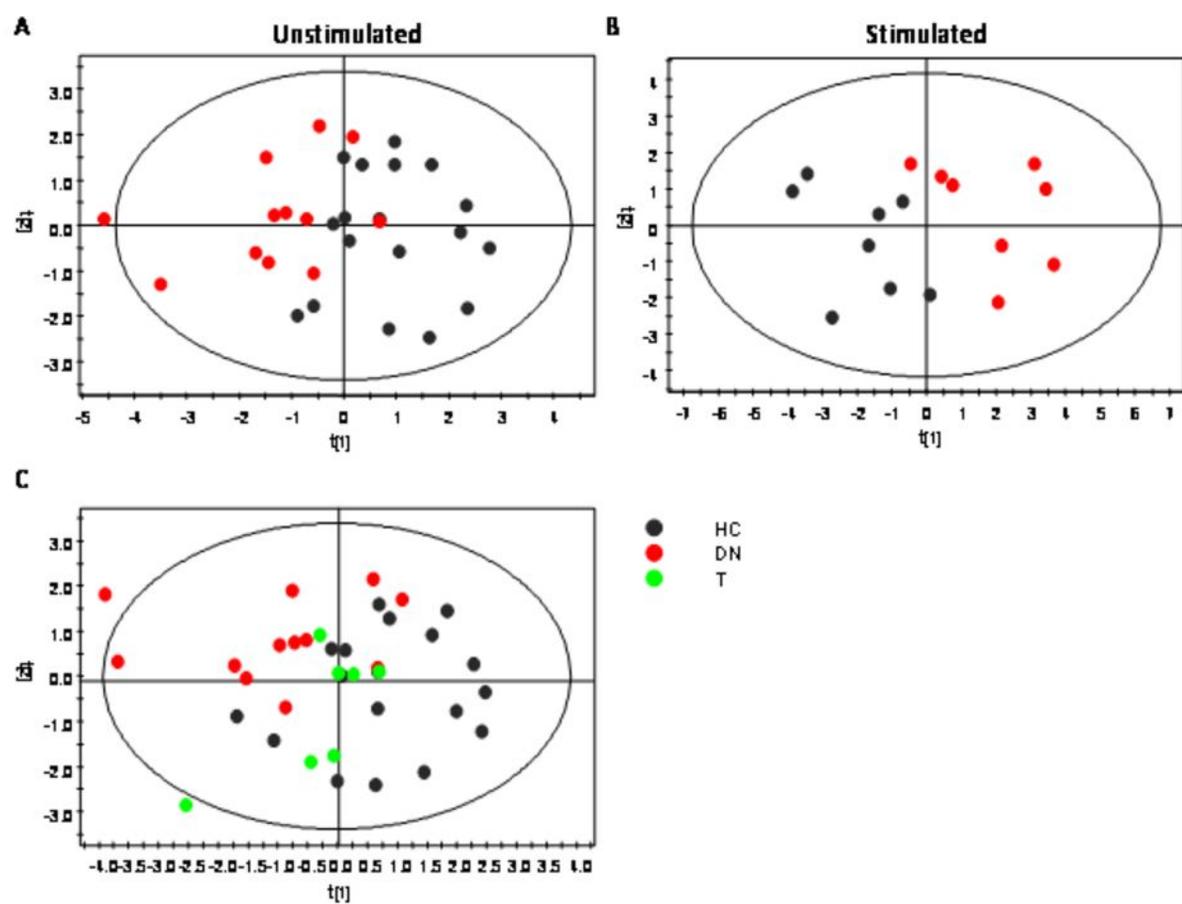


Figure 2

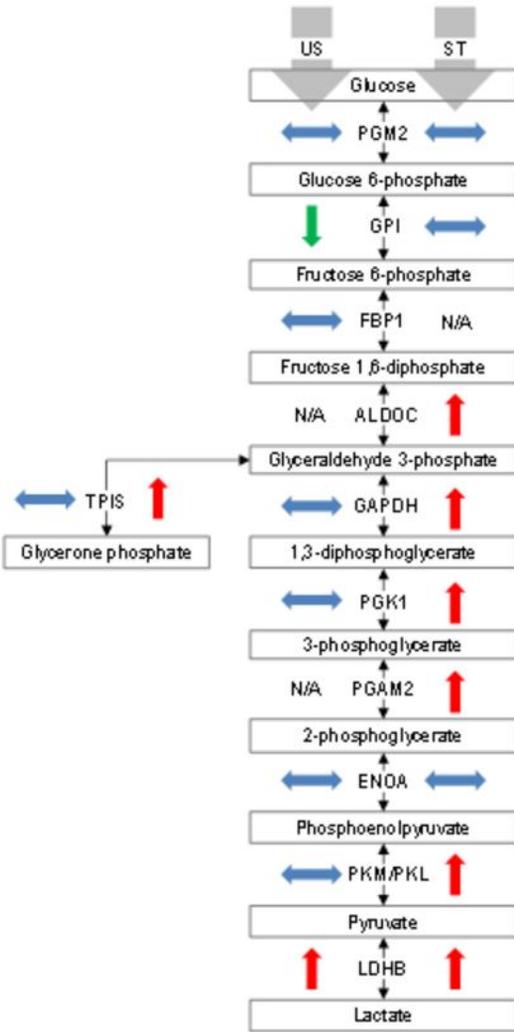
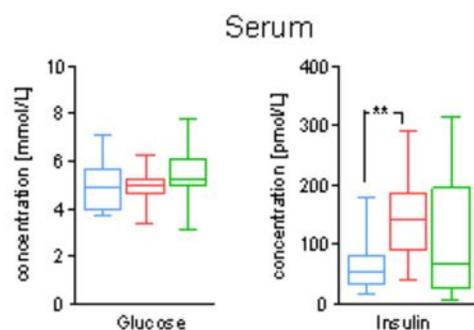
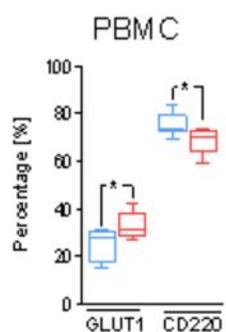


Figure 3

A



B



C

