Messages From the Small Intestine Carried by Extracellular Vesicles in Prediabetes: a Proteomic Portrait

Author(s) Names

Inês Ferreira[1,2,3#], Rita Machado de Oliveira[1#], Ana Sofia Carvalho[1], Akiko Teshima[1], Hans Christian Beck[4], Rune Matthiesen[1], Bruno Costa-Silva[3,*,#], Maria Paula Macedo[1,5,6,*,#]

Author(s) Address Information

1. *Chronic Diseases Research Centre, CEDOC, NOVA Medical School, (NMS/FCM), Lisbon 1169-056, Portugal*
2. *Bioengineering – Cell Therapies and Regenerative Medicine PhD Program, Instituto Superior Técnico, University of Lisbon, Lisbon 1049-001, Portugal*
3. *Champalimaud Research, Champalimaud Centre for the Unknown, 1400-038 Lisbon, Portugal*
4. *Centre for Clinical Proteomics, Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Denmark*
5. *APDP-ERC Portuguese Diabetes Association Education and Research Centre, Lisbon 1250-189, Portugal*
6. *Departament of Medical Sciences, University of Aveiro, Aveiro 3810-193, Portugal*

Symbol

[#] Equal contribution

[*] Corresponding author(s)

E-mail: bruno.costa-silva@research.fchampalimaud.org (Costa-Silva B),

paula.macedo@nms.unl.pt (Macedo MP)

CEDOC, NOVA Medical School / Faculdade de Ciências Medicas (NMS / FCM),

Universidade Nova de Lisboa,

Campo Mártires da Pátria, 130, 1169-056 Lisboa, Portugal

Phone: (+351) 21 880 3017

Fax: (+351) 21 880 3028

Abstract

Extracellular vesicles (EVs) mediate communication in physiological and pathological conditions. In the pathogenesis of type 2 diabetes, inter-organ communication plays an important role in its progress and metabolic surgery leads to its remission. Moreover, gut dysbiosis is emerging as a diabetogenic factor. However, it remains unclear how gut senses metabolic alterations and whether this is transmitted to other tissues via EVs. Using a diet induced-prediabetic mouse model, we observed that protein packaging in gut derived EVs (GDE), specifically small intestine, is increased in prediabetes. Proteins related to lipid metabolism and to oxidative stress management were more abundant in prediabetic GDE compared to healthy controls. On the other hand, proteins related to glycolytic activity, as well as those responsible for the degradation of polyubiquitinated composites, were depleted in prediabetic GDE. Together, our findings show that protein packaging in GDE is markedly modified during prediabetes pathogenesis. Thus, suggesting that prediabetic alterations in small intestine are translated into modified GDE proteome, which are dispersed into the circulation where they can interact with and influence the metabolic status of other tissues. This study highlights the importance of the small intestine as a tissue that propagates prediabetic metabolic dysfunction throughout the body and the importance of GDE as the messenger.

KEYWORDS: Small Instestines; Exosome; Type 2 diabetes; Lipids; Diet

Introduction

Type 2 diabetes (T2D) is the most common chronic metabolic disease characterized by high blood glucose and it accounts for 95% of diabetes cases in adulthood [1]. The escalating incidence of T2D as a consequence of sedentary lifestyle and over-nutrition, imposes a substantial burden to health care systems worldwide. Among the therapeutic interventions, metabolic surgery which targets the small intestine, is the most effective procedure in diabetes remission [2;3]. Prediabetes is a high-risk state for T2D, and it is defined by glycemic levels higher than normal, but lower than diabetic limits [4]. This stage can be seen as a fork in the road, if left untreated, the likelihood for progression to diabetes is high. Importantly, with timely diagnosis and with patient's compliance to lifestyle intervention, prediabetes can be reversed [5]. Obesity is a potent risk factor for diabetes. Moreover, a diet rich in fat and sugar, with large intake of finely processed grains and starchy carbohydrates is directly associated with this disease [6].

Blood glucose homeostasis requires a constant communication between insulin-secreting and insulin-sensitive organs. Failure in the coordination between these organs can lead to a rise in blood glucose levels and to prediabetes. Recently, extracellular vesicles (EVs) emerged as an important component of organismal communication and have been implicated in the pathophysiology of diabetes [7]. These small vesicles are able to transfer proteins and nucleic acids from a releasing cell to a distant target cell, thus modulating gene and protein expression and leading to functional changes [8-9]. Interestingly, visceral adipose tissue derived EVs trigger systemic inflammation, glucose intolerance and insulin resistance; hallmarks of prediabetes [10]. In obese patients, adipose tissue-EVs were able to modulate insulin responses in hepatocytes and muscle cells [11].

The intestinal system is daily exposed to food, millions of pathogens, and high concentrations of foreign antigens. In the case of the gut EVs, the composition and effects of EVs released by the resident microbiota have been explored [12-13]. For instance, these EVs are released into the gut mucosa and participate in local innate responses to invading bacteria through microbicidal activity [14] and mediate gut permeability through the regulation of tight junctions [15]. While the contribution of microbiota is crucial to maintain intestinal homeostasis, the epithelial cells are a critical component of the intestinal defense system forming a selective permeable barrier, allowing the absorption of nutrients, electrolytes and water, and blocking ingress of toxins and antigens. Epithelial cells in the gut were described to secrete EVs and up today they were described to play a role as antigen-carrying structures [16]. Whether EVs derived from the small intestine play a primordial role as a diabetogenic trigger needs to be elucidated. In fact, the relevance of the gastrointestinal tract in diabetes is elucidated by the fact that metabolic surgery improves glucose homeostasis more effectively than any known pharmaceutical or behavioral approach, causing durable remission in many patients with T2D [17]. Attempts to elucidate the

exact mechanism by which gastro-intestinal surgery reverses T2D have implicated changes in the gut hormones, bile acid metabolism, intestinal nutrient sensing and metabolism, gut microbiota, and other factors [18]. Gut dysbiosis recently emerged as a possible player in the development of T2D [19]. Despite the literature on the impact of microbiota in T2D onset and development, the role of the small intestine EVs, independent of EVs derived from the biota is still elusive. While gut derived EVs (GDE) are considered to play an important role in intestinal diseases [20] it is not known how GDE protein content is modulated by a western diet. Therefore, it is timely to understand the importance of the small intestine as a metabolically active organ in prediabetes and the means by which it communicates with other tissues via EVs. Here we aim at characterizing the differences in the GDE proteome of diet-induced prediabetic mice compared to that of healthy mice fed standard chow.

Results

Description of the prediabetes mouse model

First, we established a mouse model that recapitulates many features of prediabetes [21]. High fat and sugar feeding leads to obesity, hyperinsulinemia and glucose intolerance. Therefore, we used an hypercaloric diet (HD) which composition is 58% fat and 18 % sucrose, in contrast with normal-chow diet (NCD) that is 11% fat with no added sugar. After 12 weeks of diet, HD mice demonstrate significantly increased body weight (Figure 1A, 1B) and an abnormal intrahepatic fat accumulation – a hallmark of liver steatosis (Figure 1C). They also developed glucose intolerance, evaluated by a glucose tolerance test in the previous week of sacrifice and correspondent area under the curve for each group (Figure 1D, 1E) (**Figure 1**).

Characterization of intestine derived extracellular vesicles

Once the prediabetic phenotype was validated in the diet-induced obese animals, we evaluated the presence of EVs in small intestine. We stained cross sections for CD81, a commonly used EV-marker, and the presence of this protein was detected in similar levels in the lumen of the intestine in both conditions (NCD and HD fed mice - Figure 1F). Moreover, western blot was performed to confirm the enrichment of CD63, a commonly used EV marker, in GDE from NCD and HD when compared with the tissue (Figure 1G). Next, GDE were characterized by nanoparticle tracking analysis and total amount of protein was quantified by BCA. The mean size of the particles, in both conditions, was around 100 nm (NCD=152.8±67.5; HD=128.6±50.7) (Figure 1H). While the number of EVs is decreased in HD animals (Figure 1I), protein cargo was higher in GDE isolated from HD-fed mice (HD-GDE) than in NCD-fed (NCD-GDE) mice (Figure 1J). Interestingly, the amount of protein per GDE was higher in prediabetic mice than in controls (Figure 1K) (**Figure 1**).

147 **Proteomics Study**

148 EVs protein cargo provides valuable information regarding the cellular environment where they
149 are produced [22]. We proposed that a diet induced dysmetabolic environment modulates the
150 protein composition of GDE. Thus, to identify the impact of the diet-induced prediabetic gut
151 milieu on GDE protein cargo, we isolated GDE (Supplementary Figure1) from mice under HD
152 and respective controls and analysed it by mass-spectrometry (MS) (Supplementary Figure 2A).
153 Potential contribution of gut bacteria EVs was ruled out by mass spectrometry analysis, which
154 showed absence of bacterial proteins (Supplementary Figure 2B), and culture of gut conditioned
155 media in antibiotics-free agar, which did not display bacterial growth (Supplementary Figure 2C).
156

157 **Bioinformatics analysis of the MS identified proteins**

158 After filtering the identified proteins for reverse proteins used for false discovery rate and possible
159 contaminating proteins (e.g. keratin and bovine proteins from cell culture media), we identified a
160 total of 2467 proteins after removing isoforms from the same gene. Intensity based absolute
161 quantitation (iBAQ) was calculated [23]. Excluding proteins with missing quantification values
162 resulted in 2156 proteins (Supplementary file). The raw data was then normalized (Figure 2A).
163 Subsequently, we distinguished between identified and regulated proteins. Regulated proteins are
164 those at least two-fold regulated and with an adj. p-value < 0.05. After applying the
165 aforementioned criteria to our data set, we obtained a list of robustly regulated 558 proteins, which
166 shows that diet-induced prediabetes alters the protein composition of GDEs (Figure 2A). All data,
167 before applying the filtering criteria, is represented in a volcano plot that describes the level of
168 significance and magnitude of changes observed, comparing the HD with the NCD group (Figure
169 2B). The dotted line in the plot represents the threshold of adj.p-value<0.05, and from that line
170 above each individual dot represents a protein within the 558 regulated ones.
171

172 **Gene Ontology**

173 To gain insight into the biological role of the 588 regulated proteins, we performed Gene
174 Ontology and Kyoto Encyclopedia for Genes and Genomes (KEGG) enrichment analysis, which
175 revealed that the vast majority of proteins were related with metabolism. In terms of molecular
176 function, enzyme activity (including isomerases, hydrolases and oxidoreductase) shows up in the
177 top of list (Figure 3A). Most of the enriched pathways for gene ontology biological process
178 emphasized lipid metabolism, highlighting fatty acids (FA) β-oxidation. Curiously, glycolysis-
179 related and proteolysis in cellular protein catabolic processes-related proteins are mostly down
180 regulated (Figure 3B). Considering KEGG pathways enrichment analysis, the affected proteins
181 belong primarily to FA, carbohydrate and amino acid metabolism. Interestingly, alcoholism-
182 related proteins show up with great significance (Figure 3C). In terms of down regulated proteins,
183 based on the KEGG analysis the proteasome and focal adhesions proteins are markedly down
184 regulated. Finally, to assess sub-cellular localization of the GDE protein cargo regulated by diet

185 we used gene ontology cellular component analysis and found that the majority of altered proteins
186 are mitochondrial, followed by the cytosolic ones. Once again, proteasome related proteins were
187 down regulated (Figure 3D).
188
189 **Cluster analysis for GDE regulated proteins**
190 For a more detailed characterization of GDE proteome, we looked into the subset of proteins that
191 were overly altered among groups. For that, we established an adj.p-value<0.0001 as cut-off
192 (Figure 4A). The proteins with that significance are represented in the heatmap (Figure 4B). To
193 gain insight into protein-protein interaction of GDE identified protein cargo, we used the STRING
194 software to link some of the altered proteins and to display the level of relationship between them.
195 The proteins that demonstrate higher level of interaction are the ones related with the proteasome,
196 including both α subunits (PSMA) and β (PSMB). Actin related proteins, which function is to
197 maintain cellular integrity, also display relevant interaction. Moreover, network analysis
198 highlights strong tendency towards metabolic-related proteins (Supplementary Figure 2). The fold
199 change of all altered proteins varied up to 2-fold when comparing HD with NCD GDE. Within
200 metabolic pathways, the altered proteins are mainly involved in nutrient metabolism, such as
201 glucose, FAs and amino acids. In addition, cell integrity, focal adhesion and proteasome-related
202 proteins are all down regulated (Figure 4C and Supplementary Figure 4). Key GDE proteins up
203 and down regulated by HD are also represented in their subcellular location (**Figure 5**).
204 Noteworthy to mention, proteins related with carbohydrate metabolism, which ensures the supply
205 of energy to the cells (PFKL, ALDOA, ALDOB, PGAM2, ENO1, PGM5), with the tricarboxylic
206 acid (TCA) cycle (DLST, IDH1). cholesterol homeostasis (ACAT1, ACAT2) and proteasome
207 (PSMA1, PSMA2, PSMA3, PSMA4, PSMA5, PSMA6, PSMA7, PSMB1, PSMB2, PSMB3,
208 PSMB8, PSMB10) are down regulated by HD. On the other hand, proteins related with lipid
209 homeostasis (ACOT1 ACOT2, ACOT3, ACOT5, ACOT6), FA mitochondrial β-oxidation
210 (HDHA, ACADVL, ACADL, ECHS1, ACAA2), very-long chain FA peroxisomal β-oxidation
211 (ECH1, ACOX1), amino acids metabolism (GSTA1, GSTA2) and antioxidant defence (ALDH2,
212 ALDH1B1, PRDX6, GSTA4, GSTM2) are majorly upregulated. **Table 1** describes all the
213 proteins up and down regulated according to their biological function.
214
215
216 **Discussion**
217 The relationship between diet composition and the incidence of metabolic disorders is a public
218 health concern. One of the first organs and by far the largest one to contact with food and its
219 derivates is the gut, as a consequence it is where the first organismal response should be found
220 [24]. Here, we report that GDE isolated from animals fed an HD, mimicking the western one,
221 have a distinct proteome that strongly reflects the intestinal dysmetabolic milieu. We found

222 numerous pathways associated with macronutrients altered in GDE isolated from HD-fed mice;
223 of great relevance the ones related with lipidic and carbohydrate metabolic processes.
224
225 From the enrichment analysis we observed that both glycolysis-gluconeogenesis and pyruvate
226 pathways are within the most affected ones (Figure 3B). Rate limiting enzymes for glycolysis
227 were found to be less abundant, namely hexokinase (HK) and phosphofructokinase (PFKL), thus
228 suggesting changes in glucose utilization and lactate production. In accordance, in diabetic rats,
229 phosphorylation of glucose is inhibited in tissues such as the heart, skeletal muscle and liver [25;
230 26; 27; 28]. Interestingly, in hearts from diabetic rats, PFK and HK down regulation is reverted
231 by an oral anti-diabetic drug - metformin [29]. We suggest that the observed decreased levels of
232 PFKL in prediabetic GDEs is reflecting a similar down regulation in the small intestine of
233 prediabetic mice and is an early event in the progression of the disease [30]. Down in the
234 glycolysis pathway, pyruvate dehydrogenase A1 (PDHA1), which belongs to the PDH complex,
235 was upregulated in HD GDEs, indicating a possible compensation in the pathway given the
236 importance of acetyl-CoA for the TCA cycle and for many other proteins and lipidic biochemical
237 reactions. PDH is fundamental for metabolic flexibility, the ability of switching from
238 carbohydrates to lipid fuel as energy sources, and the inability of cells to do so is an important
239 hallmark of T2D [31]. Our results suggest that in the early stages of the disease gut cells tackle
240 the insult of HD feeding by changing the source of fuel. The decrease in glycolysis might be
241 explained by the low amount of fibers provided by the diet; since the percentage of fibers in HD
242 is considerably reduced and in part replaced by sucrose. Likewise, fibers have a beneficial effect
243 on NCD mice, by improving intestinal integrity, enhancing energy expenditure and reducing
244 inflammation [32]. In fact, because HD has increased sucrose, we envisage that sucrose is being
245 directed to an alternative pathway, such as de novo lipogenesis, known to also occur in the gut
246 [33].
247
248 In accordance with the high lipidic content present in the HD, we observed that lipid metabolism
249 (biosynthesis of unsaturated FA and FA elongation) is vastly altered. The excess of fat in the diet
250 is known to cause increased fatty acids uptake, which can be either metabolized for the production
251 of energy or, when in excess, will be stored. Several proteins involved in mitochondrial β-
252 oxidation (HADHA, ACADVL, ACADL, ECH1, ECHS1), which generates acetyl-CoA to feed
253 the TCA cycle, and peroxisome β-oxidation (ECH1, ACOX1), major fatty acids degradative
254 pathway, are upregulated in GDE by HD, suggesting that fatty acids are favoured as energy source
255 over glucose (Figure 5). In addition, over-activation of lipid metabolism related pathways is a
256 common feature of obesity and T2D, leading to a state of hyperglycemia [34; 35; 36]. Moreover,
257 a family of acyl-CoA thioesterases (ACOTs) were particularly upregulated; these are
258 intermediaries in the decision whether fatty acids are directed to the TCA cycle or stored, (Figure
259 5, Supplementary Figure 4) [37; 38]. Altogether, this suggests that HD shifts the burden of energy

260 provision from carbohydrates toward fat derived from diet, since ACSS2 (a protein that conducts
261 fatty acids to the TCA cycle) is down regulated and PDHA1 (which provides a link between
262 glycolysis, TCA cycle and lipogenesis) is upregulated in order to ensure the energetic need of the
263 cell. Based on recent literature, obesity-induced activation of ACOTs directs fatty acids towards
264 triglycerides synthesis for incorporation into chylomicrons or VLDL particles [39; 40].
265 Noteworthy, some studies consider activation of ACOTs protective against fatty acids over-
266 supply, slowing the flux of fatty acids to downstream metabolic pathways [41; 42; 43]. Whether
267 this is the case in the gut, needs to be further explored. Additionally, intestinal cells in the HD
268 model appear to prioritize production of energy from fatty acids, instead of carbohydrates. This
269 is shown by the upregulation of ATP-citrate lyase (ACLY), an enzyme responsible for the
270 conversion of citrate to acetyl-CoA – the first step in DNL. Overall, the major enzymes found
271 altered within the gut EVs are very much responsible for regulating acetyl-CoA levels in the cells,
272 given that this molecule is a fundamental node in metabolism, in the future it would be of major
273 interest to quantify it in enterocytes.
274
275 Increased fatty acids oxidation promotes mitochondrial dysfunction, as well as fatty acids
276 peroxidation and glucose dysmetabolism, altogether leading to reactive oxygen species (ROS)
277 accumulation to pernicious levels [44]. Oxidative stress is implicated in various diseases
278 including diabetes [45]. We observed several proteins involved in antioxidant defense
279 mechanisms, such as removal of free radicals (PRDX6) and elimination of xenobiotic compounds
280 and products of lipids peroxidation (GSTA4, GSTM2) upregulated in HD-GDE, suggesting an
281 activation of an antioxidant response in the gut cells (Figure 5). Consistent with our model, the
282 presence of numerous isoforms of the aldehyde dehydrogenase (ALDH) family was upregulated
283 in HD-derived GDE. ALDHs are a family of oxidizing enzymes responsible for cellular
284 detoxification by oxidation of biogenic and xenogeneic aldehydes that accumulate through
285 metabolism [46]. In particular, we highlight ALDH1B1 due to its ability to metabolize
286 acetaldehyde and maintain glucose homeostasis [46]. Of interest, ALDH2 has been explored as a
287 pharmacological target, due to its role in cellular damage defense against oxidative stress induced
288 by pathological conditions, for instances high glucose [47].
289
290 Besides glucose and fatty acids, amino acids, in particular lysine, also play important roles in
291 providing energy to gut epithelial cells (Figure 3C) [48]. Lysine, which pathway we see affected
292 in HD-GDE, mediates protein biosynthesis, such as of carnitine, a key factor in fatty acid
293 metabolism [49]. Interestingly, carnitine deficiency due to poor lysine diet induces triglycerides
294 accumulation [50]. Evidence that the HD not only induces obesity, but also a general pathological
295 state arises from the massive alteration of proteasome-related proteins. The proteasome is
296 recognized as an important target in many diseases [51]. Our data showed down regulation of
297 proteasome proteins, which is consistent with decreased proteasome function reported in a broad

array of chronic diseases (Figure 3C; 5) and defective degradation of damaged proteins, a hallmark of neurodegenerative diseases [52]. One hypothesis is that the decreased content of proteasome proteins in prediabetic GDE mirror in the cell of origin. Alternatively, the GDE producing cells can be consuming proteasome to counteract the deleterious effects of oxidative stress, resulting in lower packaging of proteasome proteins in GDEs.

The present study is the first comprehensive analysis of GDE protein content. We found that diet composition strongly modulates GDE proteomic profile. Several lines of evidence support a model in which the metabolic alterations induced by the HD are translated in a similar manner in alterations in GDE protein cargo. These results bring light into the important modification taking place in the small intestine and how it translates into GDE content. We propose that future studies should focus on whether GDE can act in local and distant cellular targets and mediate prediabetes. We also propose that the analysis of GDE in biofluids may represent a strategy to non-invasively detect metabolic modifications in the gut linked with prediabetes and potentially be employed early diagnose and follow-up prediabetes patients.

Material and methods

Animals

Male C57Bl/6J mice were housed in a temperature-controlled room, in a 12-hour light/dark cycle. For induction of prediabetic phenotype, C57BL/6J mice started on a high-fat diet at 6 weeks of age, with free access to food and water, for 12 weeks. Hypercaloric diet (HD) (OpenSource Diet, D12331) composition is 16.5% protein, 25.5% carbohydrate, and 58% fat with 13% addition of sucrose. The control normal chow diet (NCD) (Special Diets Services, RM3) composition is 26.51% proteins, 62.14% carbohydrates and 11.35% fat and no sucrose. At 18 weeks of age, 12 weeks of diet, mice were sacrificed. Mice were anesthetized using 2% isoflurane. Mice were sacrificed under 12 hours of fasting, with a previous feeding period of 2 hours, with the previous 8 hours of fasting. Blood, liver and small intestine were collected from prediabetic mice and respective controls. All animals were treated according with National and European Union Directive for Protection of Vertebrates.

Glucose Tolerance Test

At week 11 of diet GTT was performed. Mice were fasted overnight and weighed in the morning. Mice were then given an intra-peritoneal injection of a glucose solution (20% m/v – Sigma Aldrich) at 2g/kg body weight. Serum blood glucose levels were measured at 15, 30, 60, 90 and 120 min after injection. Blood glucose levels we measured using OneTouch Ultra glucose meter (LifeScan Inc).

335 **Small Intestine Histology**

336 After sacrifice, liver and small intestine were washed in PBS and fixed for histology and a portion
337 of small intestine was snap frozen in liquid nitrogen for later analysis by western blot. The clean
338 gut was placed in 2% Paraformaldehyde with 20% Sucrose, overnight at 4ºC; washed three times
339 in Phosphate Buffered Saline (PBS), 10 min each; transferred to 30% sucrose for 2 hours at 4ºC;
340 left overnight at 4ºC in Optimal Cutting Temperature compound (OCT) with 30% Sucrose (1:1).
341 In the following day, tissues were embedded in 20% Sucrose (1:4) + (OCT) (3:4) in the presence
342 of dry ice and immediately stored -80ºC. Consequently, tissues were sliced into 6 µm thin sections
343 in the cryostat and stored at -80ºC for following applications. Gut samples were used for CD81
344 staining and liver samples for Hematoxylin-Eosin staining.

345

346 **Exosomes Isolation and Nanoparticle Tracking Analysis**

347 After sacrifice the small intestine was removed, washed with PBS and cultured overnight, at 37ºC
348 and 5%CO2, in RPMI medium (Sigma) supplemented with 1% Penicillin/Streptomycin and 10%
349 Fetal Bovine Serum EV-free, to avoid contaminations from serum EVs. In the following day
350 medium was collected. Conditioned medium was submitted to two initial centrifugations (10 min,
351 500 g and 20 min, 3 000 g) to remove any suspended or dead cells in the medium. To remove
352 large EVs, media was centrifuged (20 min, 12 000 g) and the pellet was discarded. The
353 supernatant enriched in small EVs was again centrifuged (2 h 20 min, 100 000 g), and the EVs
354 enriched pellet was collected. For sucrose cushion purification, this pellet was resuspended in 14
355 mL filtered phosphate buffered saline (PBS, Corning 15313581, NY, US) and added the top of 4
356 mL sucrose solution (D2O containing 1.2 g of protease-free sucrose and 96 mg of Tris base
357 adjusted to pH 7.4). A new ultracentrifugation was performed (1 h 10 min, 100 000 g), after which
358 4 mL of the sucrose fraction was collected using a 18 G needle placed at the bottom of the
359 ultracentrifugation tube (away from the pellet). Finally, 16 mL of PBS were added to the collected
360 sucrose/EVs solution and an overnight (16 h, 100 000 g) ultracentrifugation was performed. The
361 pellet containing the isolated EVs was resuspended in filtered PBS. The concentration and size
362 of EVs were analyzed in NanoSight NS300 (NS3000) system equipped with a blue laser (405
363 nm), according to the manufacturer's instructions. EVs were diluted 1:1000 in filtered sterile PBS.
364 Each sample analysis was conducted for 90 seconds and measured 5 times using Nanosight
365 automatic analysis settings. The software calculated the size distribution in nanometers and the
366 concentration in number of particles per mL.

367

368 **Western Blotting**

369 Protein extracts for western blot analysis were obtained using cold lysis buffer (20 mM Tris- HCl
370 pH 7.4, 5 mM EDTA pH 8.0, 1% Triton-X 100, 2 mM Na3VO4, 100 mM NaF, 10 mM Na4P2O7)

371 in the presence of protease inhibitors (completeTM, Mini, EDTA-free Protein inhibitor cocktail
372 tablets, Roche, Sigma). Gut tissue and GDE were homogenized in the lysis buffer and sonicated
373 three time for 30 seconds each at 10 µm amplitude, incubated on ice between each sonication
374 step. Lysates were centrifuged at 18.000 g for 10 min at 4°C. Soluble fraction was collected and
375 protein concentration was determined using the PierceTM BCA Protein Assay kit (Thermo
376 Fisher). For protein expression analysis, equal amounts of total protein (20 µg) were mixed with
377 sample buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 8% β-mercaptoethanol,
378 bromophenol), before being denaturated at 95ºC for 10 min. Samples were then resolved by an
379 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were
380 subsequently electrotransferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P
381 Membrane, PVDF, Millipore) for 20 min at 25 mA and 2,5 mV in a Trans-Blot Turbo TM
382 Transfer System (BioRad). Membranes were blocked for 1 hour at RT with 3% BSA (Thermo
383 Fisher) in tris-buffered saline with 0,1% Tween (TBS-T) (blocking solution). Membranes were
384 incubated overnight at 4ºC, with CD63 antibody (Santa Cruz Biotechnology) prepared in 3% BSA
385 in TBS-T, diluted 1:1000. Membranes were washed three times with TBS-T and incubated for 1
386 hour at RT with the secondary antibody anti-rabbit antibody diluted 1:5000 (Santa Cruz
387 Biotechnology). Blots were developed with ECL (ECL Prime, GE Haelthcare) according to
388 manufacturer's instructions. Chemidoc Touch (Biorad) was used to detect chemilumunescence.
389 Band intensities were quantified usinf ImageLab software and normalized by β-actin as a loading
390 control.
391
392 **Nano-LC-MSMS analysis**
393 Peptide samples were analysed by nano-LC-MSMS (Dionex RSLCnano 3000) coupled to a Q-
394 Exactive Orbitrap mass spectrometer (Thermo Scientific). Briefly, 5 µL of sample was loaded
395 onto a custom made fused capillary pre-column (2 cm length, 360 µm OD, 75 µm ID) with a flow
396 of 5 µL per min for 7 min. Trapped peptides were separated on a custom made fused capillary
397 column (20 cm length, 360 µm outer diameter, 75 µm inner diameter) packed with ReproSil Pur
398 C18 3-µm resin (Dr. Maish, Ammerbuch- Entringen, Germany) with a flow of 300 nL per minute
399 using a linear gradient from 92 % A (0.1% formic acid) to 28 % B (0,1% formic acid in 100
400 acetonitrile) over 93 min followed by a linear gradient from 28 % B to 35 % B over 20 min at a
401 flowrate of 300 nl per minute. Mass spectra were acquired in positive ion mode applying
402 automatic data-dependent switch between one Orbitrap survey MS scan in the mass range of 400
403 to 1200 m/z followed by HCD fragmentation and Orbitrap detection of the 15 most intense ions
404 observed in the MS scan. AGC target value in the Orbitrap for MS and MSMS scans were
405 1,000,000 ions at a resolution of 70,000 at m/z 200 for MS scans, and 25,000 ions at m/z 200 for
406 MSMS scans. Peptide fragmentation in the HCD cell was performed at normalized collision
407 energy of 31 eV. Ion selection threshold was set to 5,000 ions for MSMS analysis, and maximum

injection time was 100 ms for MS scans and 200 ms for MSMS scans. Selected sequenced ions were dynamically excluded for 60 seconds.

MSMS analysis

Mass accuracy was set to 5 ppm on the peptide level and 10 Da on the fragment ions. A maximum of four missed cleavages was used. Carbamidomethyl was set as a fixed modification. M oxidation, N-terminal protein Acetyl, Q Deamidation and N Deamidation were set as variable modifications. The MSMS data was searched against all reviewed mouse proteins from UniProt with concatenation of all the sequences in reverse maintaining only lysine and arginine in place. The data was searched and quantified with both MaxQuant [53] and VEMS [54].

Statistical Analysis

Data are presented as means ± SEM. GTT curves, bar plots, volcano plots, heatmap and linear regression analyses were done using GraphPad Prism 8 (GraphPad Software). Differences significance was calculated through unpaired student's t tests with Welch's correction; one-way ANOVA followed by Tukey-Kramer multiple comparison tests. Differences were accepted as statistically significant at $p<0.05$.

Author's contributions

IF and RMO carried out the animal model of disease, western blotting and drafted the manuscript. IF did the isolation of the extracellular vesicles and characterization by NTA; gut histology and performed bioinformatics analysis, together with MS, for graphical representation. ASC prepared GDE for MS analysis and RM was responsible for the mass spectrometry analysis and bioinformatics. Mass spectrometry was performed by HCB. MPM and BCS conceived the study, participated in its design and coordination and edited the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared no competing interests, except for MPM who serves on advisory board for Gilead Sciences.

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592 # Figure legends

593 **Figure 1 High fat diet induces prediabetes in mice and increases protein cargo in gut**
594 **derived EV**

595 **A**. Diet plan schematic representation. **B**. Body weight (n=20 per group). **C**. Representative Hematoxylin-
596 Eosin images of liver histological sections of NCD and HD mice. White areas indicated by black arrows
597 are lipid droplets, revealing steatosis in the liver of mice subjected to HD. **D.** Plasma glucose profile (md/dl)
598 along a glucose tolerance test (GTT) at different time points after glucose bolus (0, 15, 30, 60, 90, 120
599 minutes) at week 17 of age. **E**. Mean area under the curve of the GTT. Results are expressed as mean ±
600 SEM; n=20 per group. Unpaired t test with Welch's correction for B and D; ****p<0.0001. **F**. Gut cross
601 sections, EVs are labelled in green (CD81) and nuclei in blue (DAPI). NCD gut tissue in the left and HD
602 gut tissue in the right. CD81 quantification in the gut. **G**. Immunoblotting of gut tissue and gut derived EV
603 (GDE) isolated from NCD and HD mice. Protein extracts prepared from gut (10 μg) and from GDE (15
604 μg) were analyzed by western blotting using antibodies against EV marker CD63 and β-actin. **H**. Size and
605 concentration distribution of GDE determined by nanoparticle tracking analysis (NTA). A representative
606 NTA graph of GDE of mice fed with NCD (NCD-EV) (left) and HD (HD-EV) (right). **I**. Analysis of the
607 number of particles per mL of sample. **J.** Protein quantification in GDE in mg per mL of sample, obtained
608 by BCA. **K.** Protein content per GDE, represented in mg of protein per particle. Results are expressed as
609 mean ± SEM; n=17 for NCD-EV and n=25 for HD-EV. Unpaired t test with Welch's correction (F, I, J,
610 K); ***p<0.0005, **p<0.005.
611

612 **Figure 2 Study flow of proteomic approach for characterization of gut derived EV**
613 **protein cargos**

614 **A**. Strategy used for selection of proteins to be analyzed with confidence. **B**. Volcano plot representing the
615 fold change and adj.p-value in the gut-derived EV (GDE) isolated from prediabetic mice and control
616 groups. x-axis represents fold change, and y-axis adj.p-value. Dotted line sets the threshold of adj.p-
617 value<0.05. In grey are the non-regulated proteins, in black the 558 regulated proteins.
618

619 **Figure 3 Characterization of prediabetic gut derived EV by GO and KEGG**
620 **enrichment analysis directly against mouse annotation, according to GO terms**

621 **A**. Molecular function analysis of the GDE identified proteins. GO enrichment analysis according to
622 Molecular Function. **B**. Analysis of the main biological regulated by the proteins identified in the GDE.
623 GO enrichment analysis according to Biological Process. **C**. KEGG enrichment analysis for KEGG
624 pathway. **D**. GO enrichment analysis according to Cellular Compartment. On the left panels, changes are
625 displayed as the number of proteins with increased (red) or decreased (blue) levels (horizontal axis). On
626 the right panels, the horizontal axis indicates the significance -log10 (p-value) of the functional association,
627 which is dependent on the number of submitted proteins in the class, the number of proteins annotated in
628 the class, the total number of submitted proteins and the total number of mouse proteins.

629 **Figure 4 Cluster analysis of the top50 altered proteins of gut derived EV in**
630 **prediabetic mice**
631 **A**. Volcano plot representing the fold change and adj.p-value of gut-derived EV (GDE) proteins isolated
632 from prediabetic mice and control groups. x-axis represents fold change, and y-axis adj.p-value. Dotted line
633 sets the threshold of adj.p-value<0.0001, highlighting the most altered proteins in GDE from HD comparing
634 with GDE from NCD. In blue are the most down regulated proteins and in red the most upregulated. **B**.
635 Heatmap representing mean-centered abundance of the most altered proteins decorated in the previous
636 volcano plot. **C**. Violin plot demonstrating the fold changes of the most altered proteins and correspondent
637 cellular functions. Cellular metabolic process (GO) represented in green.
638
639 **Figure 5 Overview of prediabetes impact on gut derived EV proteome**
640 The identified proteins with higher differential expression are represented in the image according to their
641 function and estimated subcellular localization. Red color for proteins upregulated and blue color for
642 proteins down regulated in small intestine derived EV of mice fed with HD compared with controls, mice
643 fed with NCD. ROS, reactive oxygen species; TCA, tricarboxylic acid cycle. Image done in BioRender.

644 # Tables

645 **Table 1 Biological function of the main up and down regulated proteins in HD-GDE**
646 **compared with NCD-GDE by proteomics**

647

648 **Supplementary material**

649 **Supplementary Figure 1 Study flow of proteomic approach for characterization of gut**
650 **derived EV protein cargos**
651 **A**. Schematic representation of the detailed protocol used for isolation of GDE. Image done in
652 BioRender.
653 **B.** Schematic representation of the experimental process of analysis of gut derived EV (GDE).
654 Image done in BioRender. **C**. Bar plot indicating the number of proteins uniquely identified from
655 different species when searched against all proteins from all species in UniProt. **D**. Picture of LB-
656 agar (without antibiotics) petri dish after 24 after hours of inoculation with media where guts were
657 deposit overnight at 37ºC prior to EVs isolation.
658
659 **Supplementary Figure 2 Protein-protein interaction network**
660 The network was built using the STRING online software with a medium confidence level (0.4).
661 Each circle represents one protein. The line thickness indicates the strength of the evidence, with

662 thicker connections indicating higher confidence in the protein-protein interaction. Green circles
663 represent the proteins belonging to cellular metabolic process according to GO enrichment
664 analysis for biological processes.
665
666 **Supplementary Figure 3 Individual protein alteration levels according to its biological**
667 **function**
668 Data plots demonstrating the log fold changes of each protein in the image in accordance to its
669 related pathway.
670
671

Figure 1

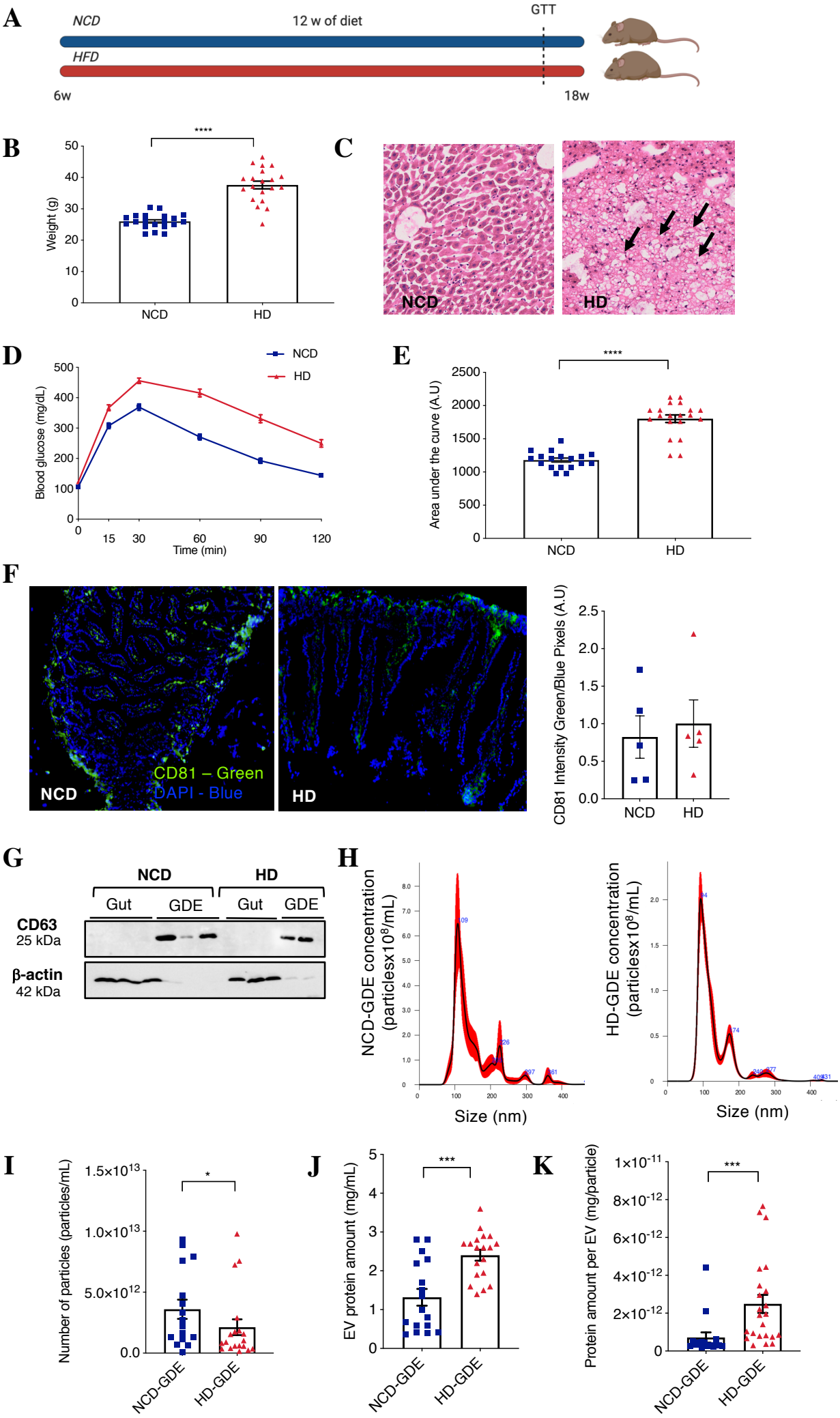
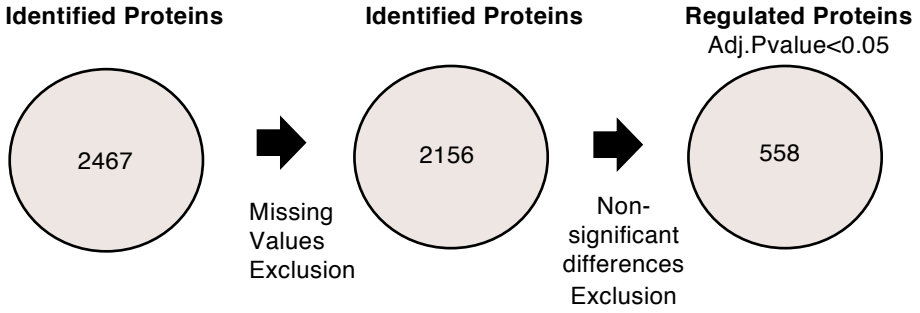

A
NCD
12 w of diet
GTT
HFD
6w
18w
B
****
Weight (g)
NCD
HD
C
NCD
HD
D
NCD
HD
Blood glucose (mg/dL)
Time (min)
E
****
Area under the curve (A.U)
NCD
HD
F
CD81 – Green
DAPI - Blue
NCD
HD
CD81 Intensity Green/Blue Pixels (A.U)
NCD
HD
G
NCD
HD
Gut
GDE
Gut
GDE
CD63
25 kDa
β-actin
42 kDa
H
NCD-GDE concentration (particlesx10^8/mL)
Size (nm)
HD-GDE concentration (particlesx10^8/mL)
Size (nm)
I
*
Number of particles (particles/mL)
NCD-GDE
HD-GDE
J
***
EV protein amount (mg/mL)
NCD-GDE
HD-GDE
K
***
Protein amount per EV (mg/particle)
NCD-GDE
HD-GDE

Figure 2

A


Identified Proteins
2467
Missing Values Exclusion
Identified Proteins
2156
Non-significant differences Exclusion
Regulated Proteins
Adj.Pvalue<0.05
558

B

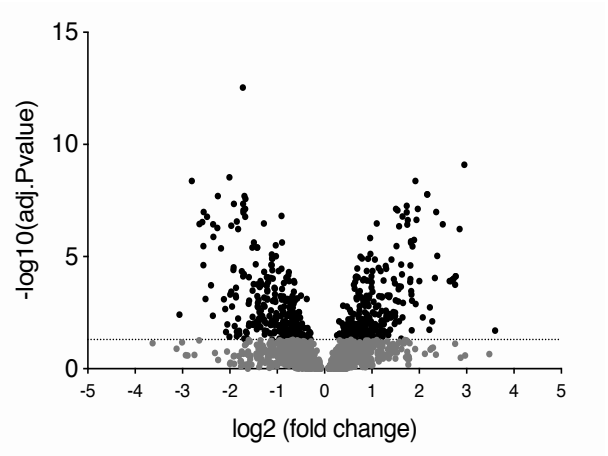
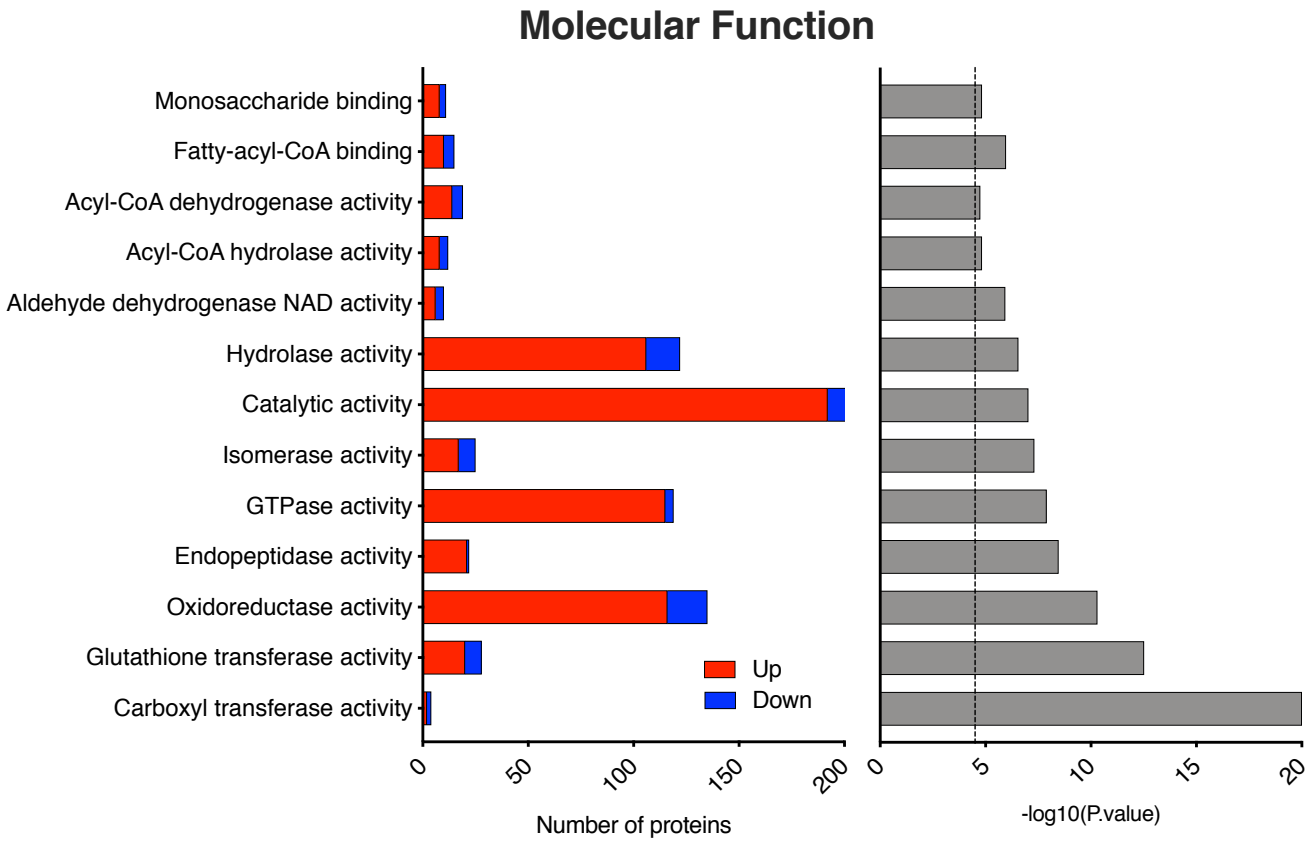
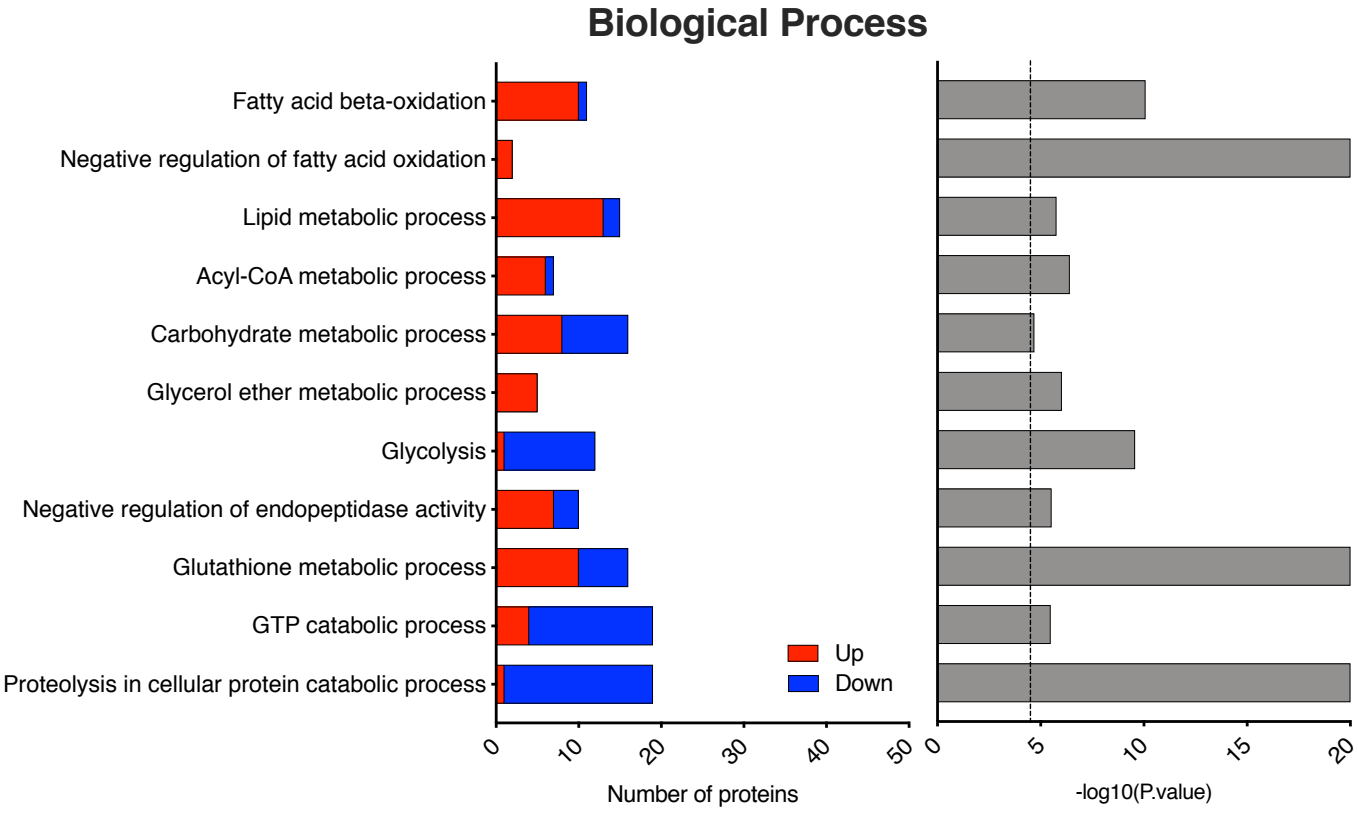

-log10(adj.Pvalue)
15
10
5
0
-5
-4
-3
-2
-1
0
1
2
3
4
5
log2 (fold change)

Figure 3

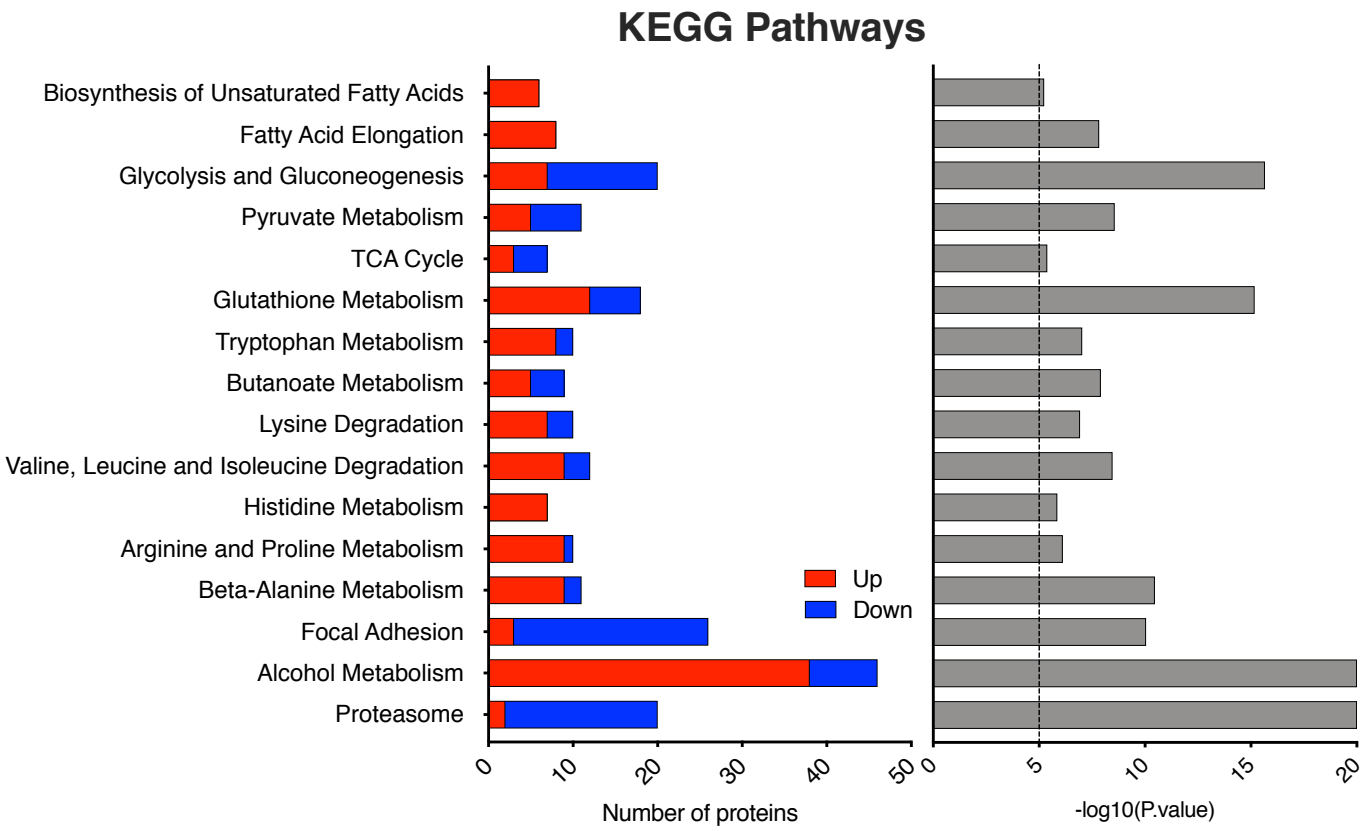
A


Molecular Function
Monosaccharide binding
Fatty-acyl-CoA binding
Acyl-CoA dehydrogenase activity
Acyl-CoA hydrolase activity
Aldehyde dehydrogenase NAD activity
Hydrolase activity
Catalytic activity
Isomerase activity
GTPase activity
Endopeptidase activity
Oxidoreductase activity
Glutathione transferase activity
Carboxyl transferase activity
Up
Down
0
50
100
150
200
Number of proteins
0
5
10
15
20
-log10(P.value)

B


Biological Process
Fatty acid beta-oxidation
Negative regulation of fatty acid oxidation
Lipid metabolic process
Acyl-CoA metabolic process
Carbohydrate metabolic process
Glycerol ether metabolic process
Glycolysis
Negative regulation of endopeptidase activity
Glutathione metabolic process
GTP catabolic process
Proteolysis in cellular protein catabolic process
Up
Down
0
10
20
30
40
50
Number of proteins
0
5
10
15
20
-log10(P.value)

C


KEGG Pathways
Biosynthesis of Unsaturated Fatty Acids
Fatty Acid Elongation
Glycolysis and Gluconeogenesis
Pyruvate Metabolism
TCA Cycle
Glutathione Metabolism
Tryptophan Metabolism
Butanoate Metabolism
Lysine Degradation
Valine, Leucine and Isoleucine Degradation
Histidine Metabolism
Arginine and Proline Metabolism
Beta-Alanine Metabolism
Focal Adhesion
Alcohol Metabolism
Proteasome
Up
Down
0
10
20
30
40
50
Number of proteins
0
5
10
15
20
-log10(P.value)

D

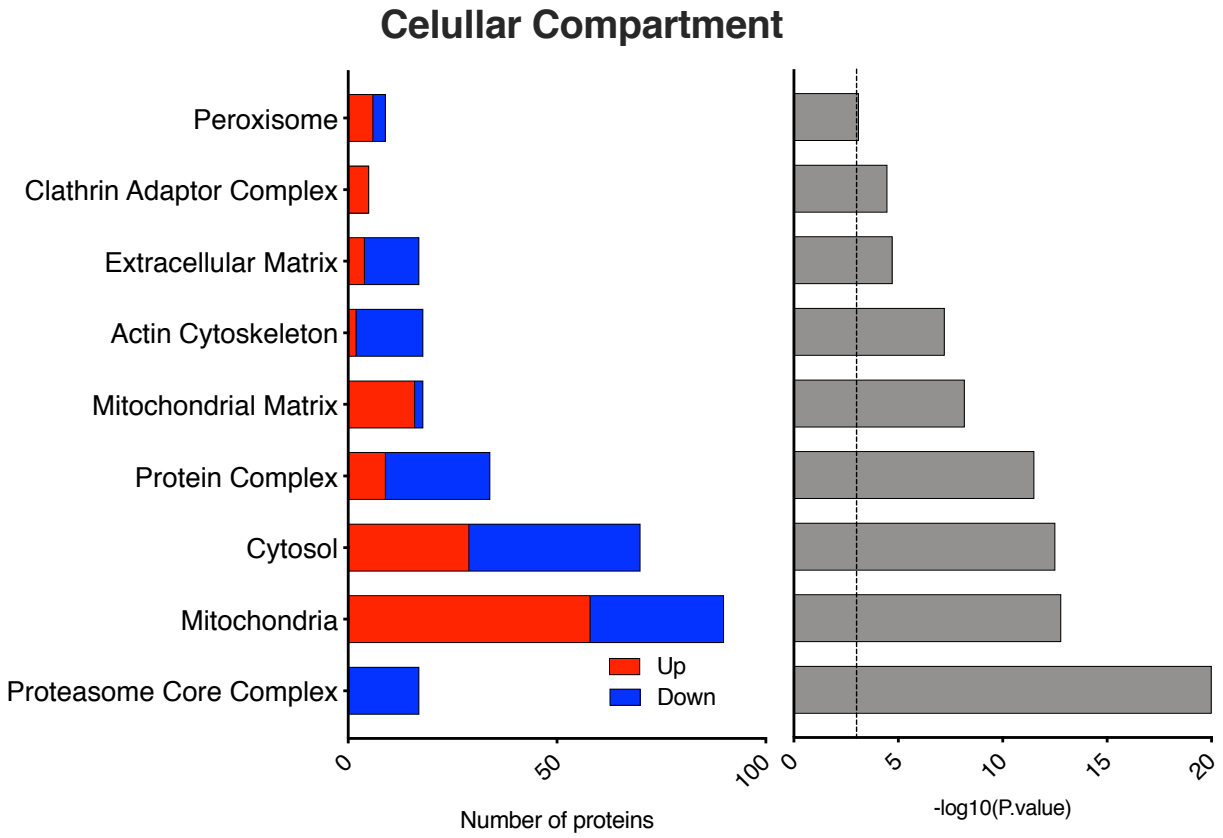
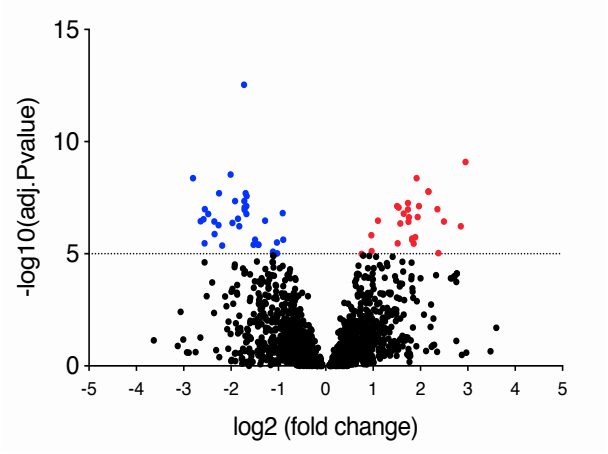
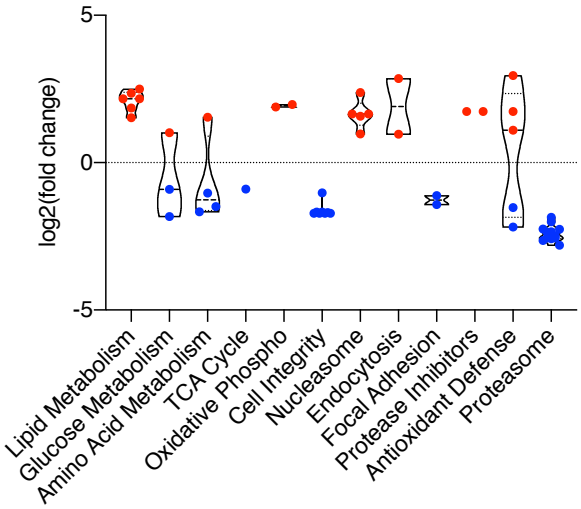

Celullar Compartment
Peroxisome
Clathrin Adaptor Complex
Extracellular Matrix
Actin Cytoskeleton
Mitochondrial Matrix
Protein Complex
Cytosol
Mitochondria
Proteasome Core Complex
Up
Down
0
50
100
Number of proteins
0
5
10
15
20
-log10(P.value)

Figure 4

A


-log10(adj.Pvalue)
log2 (fold change)

C


log2(fold change)
Lipid Metabolism
Glucose Metabolism
Amino Acid Metabolism
TCA Cycle
Oxidative Phospho
Cell Integrity
Nucleasome
Endocytosis
Focal Adhesion
Protease Inhibitors
Antioxidant Defense
Proteasome

B

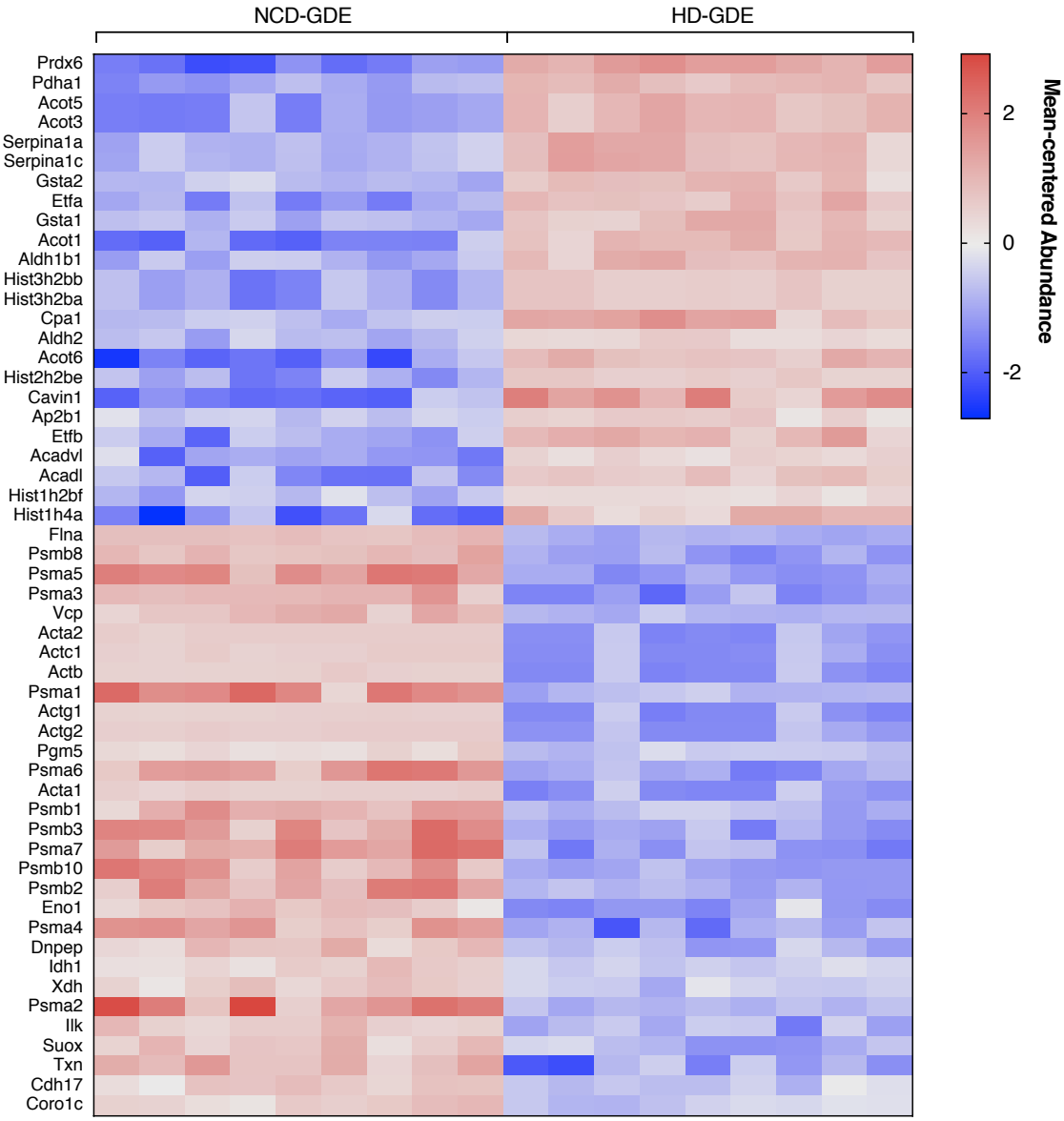

NCD-GDE
HD-GDE
Prdx6
Pdha1
Acot5
Acot3
Serpina1a
Serpina1c
Gsta2
Etfa
Gsta1
Acot1
Aldh1b1
Hist3h2bb
Hist3h2ba
Cpa1
Aldh2
Acot6
Hist2h2be
Cavin1
Ap2b1
Etfb
Acadvl
Acadl
Hist1h2bf
Hist1h4a
Flna
Psmb8
Psma5
Psma3
Vcp
Acta2
Actc1
Actb
Psma1
Actg1
Actg2
Pgm5
Psma6
Acta1
Psmb1
Psmb3
Psma7
Psmb10
Psmb2
Eno1
Psma4
Dnpep
Idh1
Xdh
Psma2
Ilk
Suox
Txn
Cdh17
Coro1c
Mean-centered Abundance

Figure 5

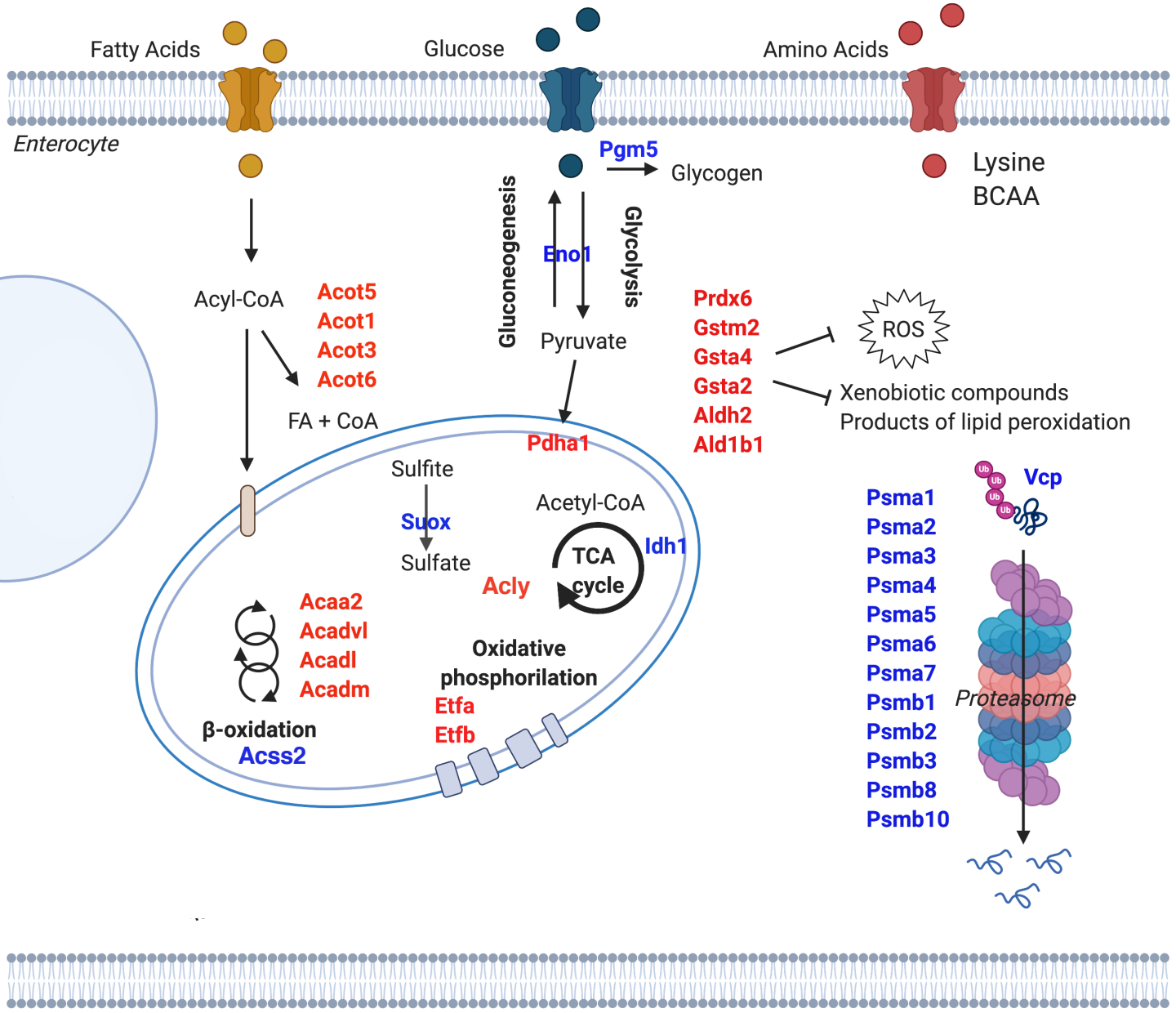

Fatty Acids
Glucose
Amino Acids
Enterocyte
Pgm5
Glycogen
Lysine
BCAA
Gluconeogenesis
Eno1
Glycolysis
Acyl-CoA
Acot5
Acot1
Acot3
Acot6
Pyruvate
Prdx6
Gstm2
Gsta4
Gsta2
Aldh2
Ald1b1
ROS
Xenobiotic compounds
Products of lipid peroxidation
FA + CoA
Pdha1
Sulfite
Acetyl-CoA
Suox
Sulfate
TCA
cycle
Idh1
Acly
Acaa2
Acadvl
Acadl
Acadm
Oxidative
phosphorilation
Etfa
Etfb
β-oxidation
Acss2
Vcp
Psma1
Psma2
Psma3
Psma4
Psma5
Psma6
Psma7
Psmb1
Psmb2
Psmb3
Psmb8
Psmb10
Proteasome

Table 1

Biological Function	Up	Down
Lipid Metabolism		
Mitochondrial ß-oxidation	ACADVL; ACADL	
Lipid Homeostasis	ACOT1; ACOT2; ACOT3; ACOT5; ACOT6	
Glucose Metabolism		
Glycolysis		ENO1; PGM5
Conversion of pyruvate to acetyl-CoA	PDHA1	
Amino Acid Metabolism		
C-terminal amino acid release	CPA1	
Protein degradation		VCP
Peptide metabolism		DNPEP
Purine Metabolism		XDH
TCA Cycle		IDH1
Oxidative phosphorylation	ETFA; ETFB	
Cell Integrity		ACTA1; ACTA2; ACTB; ACTC1; ACTG1; ACTG2; CORO1C; FLNA
Nucleosome	HIST1H4A; HIST3H2BB; HIST3H2BA; HIST2H2BE; HIST1H2BF	
Endocytosis		
Caveolar endocytosis	CAVIN1	
Clathrin-dependent endocytosis	AP2B1	
Focal adhesion		ILK; CDH17
Protease inhibitor	SERPINA1A; SERPINA1C	
Antioxidant Defense	PRDX6; GSTA4; GSTA1; GSTA2; ALDH2; ALDH1B1	TXN; SUOX
Proteasome		PSMA1; PSMA2; PSMA3; PSMA4; PSMA5; PSMA6; PSMA7; PSMB1; PSMB2; PSMB3; PSMB8; PSMB10