

UNIVERSIDADE DE LISBOA
INSTITUTO SUPERIOR TÉCNICO

**Extracellular vesicles: agents of gut
communication in prediabetes scenario**

Inês Raquel Antunes Ferreira

Supervisor: Doctor Maria Paula Borges de Lemos Macedo

Co-supervisors: Doctor Bruno Costa da Silva

Doctor Cláudia Alexandra Martins Lobato da Silva

**Thesis approved in public session to obtain the PhD Degree in
Bioengineering**

Jury final classification: Pass with Distinction

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O presidente do júri
Homologo



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Doctor Joaquim Manuel Sampaio Cabral, Instituto Superior Técnico, Universidade de Lisboa

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Doctor Fábio Monteiro Fernandes, Instituto Superior Técnico, Universidade de Lisboa

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produce anti-microbial peptides; and stem cells that reside normally in the crypts are responsible for the constant renewal of the epithelial layer. The three structural layers of intestine in charge of protection and stability are: the epithelial layer that is covered by a mucus layer and sits over the lamina propria. The superior zone is filled with bacteria and other microorganisms. The lamina propria is the interface for blood and lymph exchanges and is the microenvironment of a huge population of immune cells. On the right part of the image the dark brown cells represent damaged enterocytes, in those conditions, the permeability of the gut is compromised and the lamina propria is invaded by foreign bodies, activating and increasing the immune response.

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- Figure 30. Kupffer cells favorably take up gut-derived EV after six weeks of injections.** Mice were fed with HFD for 6 weeks and during the same period were injected with 20 μ g of fluorescent-labelled gut-derived EV (GDE) from NCD-fed mice (NCD-GDE) and 20 μ g of fluorescent-labelled gut-derived EV from HFD-fed mice (HFD-GDE). **A.** Flow cytometry plots with gating strategy for the analysis of PBS-injected group, NCD-GDE and HFD-GDE. **B.** Analysis of 71

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Appendix Document Pages

Proteomic raw data with all the proteins identified in gut-derived EV. In red is highlighted the up-regulated proteins; in blue the down-regulated proteins and in purple the missing values; when two colors are present is because one protein was identified as up or down-regulated but it had an acquisition of zero, therefore it was excluded. Description by columns: Pro - protein headers; Sample names - 18 columns with quantitative values named with sample names (9 columns of NCD sample replicas, 9 columns of HFD sample replicas); mean_C - mean expression for controls (NCD-GDE); mean_T - mean expression for treated (HFD-GDE); GN - gene name; logFC - log2fold change; AveExp - average expression; t - t value; PValue - Pvalue; adj.PVal - adjusted P value using BH; B - B-value. 109

List of Abbreviations

α -KC	Alpha-ketoglutarate
Acaa2	Acetyl-CoA acyltransferase 2
Acadl	Acyl-CoA dehydrogenase long chain
Acadvl	Acyl-CoA dehydrogenase very long chain
Acat1	Acetyl-CoA acetyltransferase 1
Acat2	Acetyl-CoA acetyltransferase 2
ACC	Acetyl-CoA carboxylase
Acetyl-CoA	Acetyl coenzyme A
ACK	Ammonium chloride-potassium buffer
ACLY	ATP-citrate lyase
Acot 3	Acyl-CoA thioesterase 3
Acot 5	Acyl-CoA thioesterase 5
Acot1	Acyl-CoA thioesterase 1
Acot2	Acyl-CoA thioesterase 2
Acot6	Acyl-CoA thioesterase 6
Acox1	Acyl-CoA oxidase 1
Acss2	Acyl-CoA synthetase short chain family member 2
Acta1	Actin alpha 1, skeletal muscle
Acta2	Actin alpha 2, smooth muscle, aorta
Actb	Actin beta
Actc1	Actin alpha, cardiac muscle 1
Actg1	Actin gamma 1
Actg2	Actin gamma 2, smooth muscle, enteric
Acyl-CoA	Acyl-coenzyme A
ALD	Alcoholic liver disease
Aldh1b1	Aldehyde dehydrogenase 1 family member B1
Aldh2	Aldehyde dehydrogenase 2 family (mitochondrial)
Aldh5a1	Aldehyde dehydrogenase 5 family member A1
Aldh9a1	Aldehyde dehydrogenase 9 family member A1
Aldoa	Aldolase, fructose-bisphosphate A
Aldob	Aldolase, fructose-bisphosphate B
AMPK	Adenosine monophosphate-activated kinase
ANOVA	Analyses of variance
Ap2b1	Adaptor related protein complex 2 beta 1 subunit
ApoB	Apolipoprotein B

Arg2	Arginase 2
ATP	Adenosine triphosphate
Atp5i	ATP synthase inhibitory factor subunit 1
AUC	Area under the curve
BCA	Bicinchoninic acid assay
BCAA	Branched-chain amino acids
Bdh2	3-hydroxybutyrate dehydrogenase 2
C57Bl/6J	C57 black 6
Cavin1	Caveolae associated protein 1
CCL2	Monocyte chemoattractant protein 2
CCL5	Monocyte chemoattractant protein 5
CD11b	Cluster of differentiation 11b
CD36	Cluster of differentiation 36
CD45	Cluster of differentiation 45
CD63	Cluster of differentiation 63
CD81	Cluster of differentiation 81
CD9	Cluster of differentiation 9
Cdh17	Cadherin 17
ChREBP	Carbohydrate response element binding protein
CoA	Coenzyme A
Coro1c	Coronin 1C
COVID-19	Coronavirus Disease 2019
Cpa1	Carboxypeptidase A1
DAPI	2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride
DG	Diacylglycerol
DGAT1	Diacylglycerol acyltransferase 1
DGAT2	Diacylglycerol acyltransferase 2
Dlst	Dihydrolipoamide S-succinyltransferase.
DNA	Deoxyribonucleic acid
DNL	<i>De novo</i> lipogenesis
Dnpep	Aspartyl aminopeptidase
DPP-4	Dipeptidyl peptidase-4
Ech1	Enoyl-CoA hydratase 1
Echs1	Enoyl-CoA hydratase, short chain 1
Eno1	Enolase 1
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
Etfa	Electron transfer flavoprotein alpha subunit

Etfb	Electron transfer flavoprotein beta subunit
EV	Extracellular vesicle
FA	Fatty acid
FAS	FA synthase
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FFA	Free Fatty Acids
Flna	Filamin A
FSC	Forward side scatter
Fth1	Ferritin heavy chain 1
G3P	Glycerol-3-phosphate
G6P	Glucose-6-phosphate
GCK	Glucokinase
GDE	Gut-derived extracellular vesicle
GIP	Glucose-dependent insulintropic peptide
GK	Glucokinase
GLP-1	Glucagon-like peptide 1
GLUT-2	Glucose-transporter 2
GO	Gene Ontology
Gsta1	Glutathione S-transferase alpha 1
Gsta2	Glutathione S-transferase alpha 2
Gsta4	Glutathione S-transferase alpha 4
Gstm2	Glutathione S-transferase mu 2
GTT	Glucose tolerance test
GWA	Genome-wide association
Hadha	Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alfa
HbA1c	Glycosylated Hemoglobin
HCl	Hydrochloric acid
HDL	High-density lipoproteins
HFD	High fat diet
HFD-GDE	Gut-derived extracellular vesicle from high fat diet-fed mice
HHEX/IDE	Hematopoietically expressed homeobox gene
Hist1h2bf	Histone cluster 1 H2B family member f
Hist1h4a	Histone cluster 1 H4 family member a
Hist2h2be	Histone cluster 2 H2B family member 3
Hist3h2ba	Histone cluster 3 H2B family member a
Hist3h2bb	Histone cluster 3 H2B family member b
HPLC	High performance liquid chromatography

HSP	Heat shock proteins
IDF	International Diabetes Federation
Idh1	Isocitrate dehydrogenase (NADP(+)) 1, cytosolic
IDL	Intermediate-density lipoprotein
IFG	Impaired Fasting Glucose
IFN- γ	Interferon gama
IGF2BP2	Insulin Like Growth Factor 2 mRNA Binding Protein 2 gene
IGF-R	Insulin-like growth factor 1
IGT	Impaired Glucose Tolerance
IL-1 β	Interleukin-1 beta
IL-13	Interleukin-13
IL-4	Interleukin-4
Ilk	Integrin linked kinase
ILV	Intraluminal vesicle
IR	Insulin resistance
ITT	Insulin tolerance test
KC	Kupffer cell
KEGG	Kyoto Encyclopedia for Genes and Genomes
Krt5	Keratin 5
Krt6a	Keratin 6a
Krt75	Keratin 75
LB	Luria-Bertani
LC-MS-MS	Liquid chromatography with tandem mass spectrometry
LD	Lipid droplets
LDL	Low-density lipoproteins
Lgals3bp	Galectin 3 binding protein
LPS	lipopolysaccharide
LXR	Liver X receptor
Mac-1	Macrophage 1
MG	Monoacylglycerol
MHC	Major histocompatibility complex
Mif	Macrophage migration inhibitory factor
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTBE	Methyl-tert-butyl ether
MTTP	Microsomal triglyceride transfer protein

MVB	Multivesicular bodies
Myl6	Myosin light chain 6
Myl6b	Myosin light chain 6B
Na ₃ VO ₄	Sodium orthovanadate
Na ₄ P ₂ O ₇	Sodium pyrophosphate decahydrate
NaF	Sodium fluoride
NAFLD	Non-alcoholic fatty liver disease
NCD	Normal chow diet
NCD-GDE	Gut-derived extracellular vesicle from normal chow diet-fed mice
Ndufv1	NADH:ubiquinone oxidoreductase core subunit V1
nIR	Near infra-red
NO	Nitric oxide
NPC	Non-parenchymal cell
NTA	Nanoparticle tracking analysis
OCT	Optimal Cutting Temperature compound
PBS	Phosphate buffered saline
PBS-T	Phosphate Buffered Saline with Tween-20
Pdha1	Pyruvate dehydrogenase E1 alpha 1 subunit
PFA	Paraformaldehyde
Pfkl	Phosphofructokinase, liver type
Pgam2	Phosphoglycerate mutase 2
Pgm5	Phosphoglucomutase 5
PI3K	Phosphoinositide-3 kinase
PKA	Protein kinase A
PKB	Protein kinase B
Pnlip	Pancreatic lipase
PPAR α	Proliferator-activated receptor alpha
Prdx6	Peroxiredoxin 6
Psbm1	Proteasome subunit beta 1
Psma1	Proteasome subunit alpha 1
Psma2	Proteasome subunit alpha 2
Psma3	Proteasome subunit alpha 3
Psma4	Proteasome subunit alpha 4
Psma5	Proteasome subunit alpha 5
Psma6	Proteasome subunit alpha 6
Psma7	Proteasome subunit alpha 7
Psmb10	Proteasome subunit beta 10
Psmb2	Proteasome subunit beta 2

Psmb3	Proteasome subunit beta 3
Psmb8	Proteasome subunit beta 8
PVDF	Polyvinylidene fluoride
PYY	Peptide YY
RHM	Recruited hepatic macrophages
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SCFA	Short-chain fatty acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
Serpina1a	Serpin family A member 1, alpha-1-antitrypsin1-1
Serpina1c	Serpin family A member 1, alpha-1-antitrypsin1-3
SGLT2	Sodium-glucose transporter 2
SGLT3	Sodium-glucose transporter 3
Slc25a5	Solute carrier family 25 member 5
SLC30A8	Solute Carrier Family 30 Member 8 gene
SREBP1c	Sterol regulatory element-binding protein 1c
SSC	Side scatter
Succlg2	Succinate-CoA ligase GDP-forming beta subunit
Suox	Sulfite oxidase
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBS-T	Tris-buffered saline with 0,1% Tween
TCA	Tricarboxylic acid cycle
TG	Triglyceride
TLR4	Toll-like receptor 4
TNF- α	Tumor necrosis factor alpha
Txn	Thioredoxin
VAT	Visceral-adipose tissue
Vcp	Valosin containing protein
VLDL	Very-low density lipoproteins
WHO	World Health Organization
WT	Wild-type
Xdh	Xanthine dehydrogenase

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Abstract

Western-style diet is a leading cause of metabolic diseases, specifically type 2 diabetes (T2D). T2D is a systemic disease that cannot be seen from the perspective of one only single organ affected. While the liver is a fundamental player in global glucose and lipid metabolism; experimental evidences show that rearrangements of gastro-intestinal anatomy can directly affect glucose homeostasis, and not only through weight loss. In particular, metabolic surgery, initially designed to promote weight loss, can significantly improve glucose homeostasis more effectively than any known pharmaceutical or behavioural methodology, causing total remission of T2D. Therefore, understanding the importance of the intestine as a metabolically active organ is crucial for the development of novel therapies to improve metabolic health. A wide variety of circulating factors, including hormones, cytokines and chemokines work together to orchestrate the systemic response of metabolic organs to diet composition. In the last few years, a new organismal communication mode has emerged. This novel type of systemic communication is mediated by extracellular vesicles (EVs) produced via the multivesicular endosomal pathway and released by every cell. EVs are of great interest because they have the capacity to carry genetic and protein cargoes inside its membranes, along the organism. The high level of technological advance we have nowadays, both in equipment and methods of analysis, enables the detailed characterization of every protein in the cell and in the EVs. The gut plays a poorly explored role in metabolism, partially uncovered by the ameliorations of T2D after metabolic surgery and prior to weight loss. Moreover, EVs participate in the pathophysiology of diabetes. Based on that, we hypothesize that gut derived EVs (GDE) carry a diabetogenic message from the gut to the liver, in response to high fat and sucrose diet, mimicking the western diet.

For this study we used two groups of C57Bl/6J mice fed with normal chow diet (NCD) or high fat diet (HFD). We observed that GDE from HFD-fed mice (HFD-GDE) carry more protein inside compared with GDE from NCD-fed mice (NCD-GDE). Interestingly, by proteomics we revealed that GDE protein cargo are highly affected by diet. Carbohydrates, amino acids and lipids metabolism-related proteins are noticeable affected in HFD-GDE, being the latter ones particularly up-regulated, in opposition to the ones involved in glycolysis. Additionally, proteins involved in fatty acid metabolism, as well as oxidative stress are up-regulated, both are critical pathways underlying prediabetes development. That might also explain why proteins related with the oxidative milieu appear up-regulated in the HFD-GDE. On the other hand, proteasome-related proteins, that are responsible for the degradation of polyubiquitinated composites are extremely down-regulated in HFD-GDE, revealing a typical pathological profile; this will eventually lead to the accumulation of misfolded, damaged and unnecessary proteins causing a disruption in normal cellular function and can even cause cell death. Importantly, decrease proteasome function has been reported in a broad array of chronic diseases.

In a second stage, chronic exposure of healthy mice to HFD-GDE revealed that GDE have affinity to the liver and are preferentially up-taken by the resident macrophages of this tissue, the Kupffer cells (KC), that are described to trigger an inflammatory response under metabolic stress. Moreover, we observed that HFD-GDE mice livers manifested increased levels of triglycerides. Collectively, these evidences further strength that the altered protein content in HFD-GDE are translated into hepatic biological alternation, revealed by the upregulation of lipid metabolism related proteins in HFD-GDE and the concomitant augment in triglycerides hepatic content.

In summary, we demonstrated that GDE are vehicles of communication in the entero-hepatic axis, and disclosed lipids as their relevant cargoes. Thus, corroborating our hypothesis that GDE content is altered by different dietetic scenarios and their cargoes reflect the environment of the producer cell,

which in turn signals to the gut. The proficiency of HFD to alter the proteome of GDE and, these ones to increase the hepatic lipid content indicates that GDE carry lipids from the gut to the liver. Our findings suggest that GDE translate diet alteration into a prediabetic hepatic dyslipidaemia signature.

Key-words: prediabetes, extracellular vesicles, gut, liver, lipids

Resumo

As dietas hipercalóricas, características da sociedade ocidental, estão diretamente associadas ao desenvolvimento de doenças metabólicas como é o caso da diabetes tipo 2 (DT2). Esta é considerada uma epidemia mundial, atingindo números alarmantes e despesas colossais. No sentido de prevenir a DT2 é crucial a deteção e intervenção precoce, nomeadamente na prediabetes, identificada como um estado inicial da doença, no qual ainda é possível reverter o quadro clínico através de alterações do estilo de vida. Apesar do papel fundamental do fígado no metabolismo de glucose e lípidos, um dos órgãos que se tem mostrado particularmente relevante no controlo da doença é o intestino. A cirurgia metabólica, inicialmente desenvolvida para o tratamento da obesidade mórbida, mostrou ter elevadas taxas de êxito na remissão da DT2. De salientar, que esta remissão ocorre antes da acentuada perda de peso, indicando que o intestino por si desempenha uma função primordial no controlo da DT2; contudo o mecanismo molecular subjacente a este controlo ainda não está clarificado. Desta forma o intestino ganha um papel de relevo como órgão metabolicamente ativo e potencial alvo no diagnóstico precoce e intervenção terapêutica na DT2. A homeostasia metabólica é atingida através de uma intrincada comunicação entre os diversos órgãos. Variados mediadores circulatórios, como é o caso de hormonas e citocinas participam na resposta sistémica dos diferentes órgãos metabólicos às alterações externas, nomeadamente à dieta. Nos últimos anos, foi descrito uma nova forma de comunicação no organismo denominadas de vesículas extracelulares (VE). As VEs são produzidas a partir de corpos multivesiculares formados pela via endossomal e posteriormente libertados para o meio extracelular. As VEs transportam, dentro da sua dupla membrana, material genético e proteico, assim como lípidos, de uma célula para outra vizinha, ou distante. Sabendo que os enterócitos libertam VE, neste trabalho avaliamos a hipótese de que o “milieu” intestinal, no contexto da dieta hipercalórica no modelo de prediabetes, é modificado originando alterações do conteúdo proteico das VEs derivadas do intestino (VE-intestinais) que, por sua vez, têm um efeito diabetogénico ao comunicar com o fígado, nomeadamente com as células de kupffer, responsáveis por ativar o processo inflamatório característico da T2D.

Neste contexto, murganhos C57/Bl6J foram expostos a dieta normal e dieta hipercalórica durante 12 semanas, em dois grupos. As VE foram isoladas diretamente do intestino limpo, garantindo que não há contaminação de microbiota, a partir de um processo de separação por gradiente de tamanho, e adicionalmente, de densidade. Estas VE-intestinais foram analisadas pelo seu tamanho e quantidade a partir de um equipamento de rastreio de nanopartículas. Uma caracterização detalhada ao conteúdo proteico das VE-intestinais, foi efetuado por espectrometria de massa e consequente análise proteómica com ferramentas bioinformáticas.

É de evidenciar que o perfil proteico das VE-intestinais de animais submetidos a uma dieta hipercalórica é amplamente alterado, concluindo que há uma clara influência da dieta no conteúdo proteico das VE-intestinais. As VE-intestinais de animais submetidos a uma dieta hipercalórica, para além do número de proteínas ser maior, também o seu perfil proteico está significativamente alterado, quando comparadas com VE-intestinais de animais saudáveis.

Observámos que proteínas específicas envolvidas em vias metabólicas de hidratos de carbono, aminoácidos e lípidos estão significativamente afetadas. Nomeadamente uma diminuição de proteínas associadas à via de degradação de glucose.

No entanto, as vias utilizadas por ácidos gordos encontram-se aumentadas, tanto no que toca ao catabolismo de ácidos gordos para produção de energia como de armazenamento na forma de triglicéridos.

Analogamente às vias associadas ao metabolismo proteico, observamos que vias associadas a aminoácidos essenciais aparecem bastante alteradas. As vias de síntese de aminoácidos de cadeia curta, que são produzidos a partir da atividade microbiana também se encontram aumentadas. É interessante observar que, embora as VE sejam exclusivamente de origem celular intestinal, refletem efeitos da relação simbiótica que a flora intestinal tem com o intestino, evidenciada aqui em consequência do regime nutricional.

Nas VE-intestinais também se observa um aumento, tanto em número como em expressão, de proteínas com função protetora contra o stress oxidativo. Stress oxidativo este muito característico de ambientes hipercalóricos onde as vias lipídicas e mitocondriais estão sobre ativadas. O que está em conformidade com o cenário fisiopatológico em estudo.

Neste seguimento, vemos também, e de uma forma muito clara, que as proteínas do proteossoma, se encontram expressivamente diminuídas nas VE-intestinais de animais submetidos a dieta hipercalórica. Este resultado sugere a função celular estar comprometida, consequente da diminuição da principal via de degradação proteica.

Estabelecido que as VE-intestinais se encontram alteradas pela dieta fizemos, seguidamente, o estudo do seu efeito no organismo. Para avaliação do tropismo das VE-intestinais e do seu potencial efeito fisiopatológico na disseminação da prediabetes, injetámos VE-intestinais, marcadas com fluorescência, em animais wild-type.

Após injeção retro-orbital de VE-intestinais previamente marcadas em animais wild-type, observámos que estas vão preferencialmente para o fígado, em específico para as células de Kupffer, que são os macrófagos residentes deste tecido, com grande capacidade fagocítica, o que facilita a captação preferencial. O papel das células de Kupffer no metabolismo hepático tem sido recentemente evidenciado como principal ativador de inflamação.

Com o objetivo de analisar o papel das VE-intestinais no fenótipo prediabético, injetámos três vezes por semana, durante seis semanas, VE-intestinais de animais submetidos a dieta hipercalórica e VE-intestinais de animais submetidos a dieta normal, e analisámos alguns parâmetros metabólicos. Deste modo, e sabendo que as VE-intestinais vão maioritariamente para o fígado, observámos que, após as seis semanas, os animais não manifestam alterações estatisticamente significativas na tolerância à glucose e na resistência à insulina. Por outro lado, os fígados destes animais apresentam níveis de triglicéridos alterados, com um perfil gradual, ou seja, aqueles que apresentam valores mais elevados de triglicéridos são os animais injetados com VE-intestinais de animais submetidos a dieta hipercalórica.

Estes resultados evidenciam uma relação muito interessante com a análise proteómica feita previamente. Sobressai que VE-intestinais de animais submetidos a dieta hipercalórica contêm um aumento de proteínas envolvidas no metabolismo lipídico. Em concordância, também identificámos uma família de enzimas, as ACOT, que está extremamente aumentada, e participam no direcionamento de ácidos gordos para a síntese de triglicéridos.

Em suma, relativamente à nossa hipótese, observamos que as células intestinais são produtoras de VEs, e que as VE do intestino sofrem alterações em contexto de prediabetes. O intestino como órgão primordial na digestão e absorção dos nutrientes, obriga às células intestinais ajustarem o seu mecanismo face à dieta. As VE-intestinais vão conter mensagens que refletem estas alterações transportando-as para o fígado onde o seu conteúdo vai ser depositado. Visto haver grande acumulação de triglicéridos no fígado dos animais injetados, uma hipótese é que as VE-intestinais estejam a transportar lípidos, provenientes da dieta, do intestino para o fígado. Contudo, precisamos de mais dados para poder tirar essas conclusões.

Finalmente, com este trabalho destacamos o impacto da dieta nas VE-intestinais e no papel que estas pequenas vesículas podem ter como portadores de conteúdos funcionais, dos quais destacamos os lípidos, tanto em contexto de doença como de saúde.

Palavras-chave: Prediabetes, vesículas extracelulares, intestino, fígado, lípidos

I

Chapter

Introduction

1. Prevalence of diabetes and economic impact

Diabetes is a chronic metabolic disease, of multiple etiology, characterized by hyperglycemia as a result of insulin deficiency and/or decreased insulin action. The prevalence of diabetes worldwide is exponentially increasing because of a combined interplay of factors such as socioeconomic, demographic, environmental and genetic. Despite all the diabetes awareness campaigns run by multiple international organizations aiming at informing the public of the causes, symptoms, complications and treatments associated with the condition, western societies are still not totally convinced that lifestyle has a huge impact on health. This is clearly demonstrated by the positive correlation between diabetes incidence and obesity, frequently associated with sedentarism and an unhealthy diet. A poor diet, rich in fat and sugar, with large intake of finely processed grains and starchy carbohydrates have been shown to be directly associated with this disease (Hamdy et al. 2018).

The International Diabetes Federation (IDF) in 2000 estimated 150 M adult people worldwide had diabetes. Shockingly, this number triplicated in 2019 and today there are almost 500 M people with diabetes and the projections for the future clearly indicate that the global impact of this disease will continue to increase substantially (Fig. 1), 11% of global deaths are related with diabetes (IDF 2019). In Portugal, in 2015, The Portuguese National Observatory of Diabetes assessed that 13% of the adult population had diabetes and, approximately, half of those were not diagnosed (Observatório Nacional da Diabetes 2016).

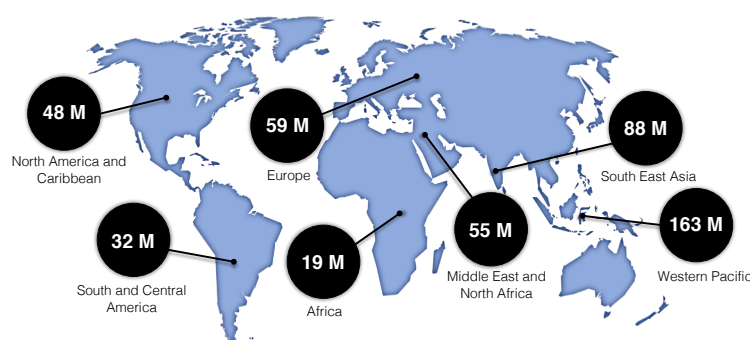


Figure 1. Prevalence of diabetes worldwide in 2019 in adults (aged between 20-79 years). Representation of the number of people living with diabetes around the world, by IDF region, accounting that ~90% of the cases are of type 2 diabetes (T2D) (adapted from IDF, 2019).

An important matter of remark is the health costs due to diabetes. The expenditure has been rising considerably over the years. IDF estimated, in 2019, that total diabetes-related health spending reached 680 billion euros. The economic impact of diabetes is expected to grow year by year (IDF 2019). In Portugal, the last studies in 2015, diabetes predicted costs were near 1100 million euros, that represents 12% of the expenses in health (Observatório Nacional da Diabetes 2016). Clearly by preventing the growth of the disease, by performing high quality fundamental research, we will positively impact not only on global well-being but also on decreasing the public and out-pocket-spending on health; and importantly this may grant access to quality health care systems to underprivileged groups.

2. Diabetes mellitus, the disease

Diabetes mellitus, or diabetes, is a condition characterized by high levels of glucose in peripheral blood due to an impairment in the production of the hormone insulin or in the inability to use it effectively.

Insulin is a hormone produced in the pancreatic β -cells that allows glucose from the bloodstream to enter the body's cells where it is converted into energy. The deficiency or the incapacity of cells to respond to insulin gives rise to high levels of blood glucose – hyperglycemia – which is a clinical indicator of diabetes.

There are two main forms of diabetes, type 1 (T1D) and type 2 (T2D), with the second one accounting for the vast majority (~90-95%) of total prevalence.

Type 1 diabetes (T1D) is an autoimmune disease in which the immune system attacks insulin-producing β -cells of the pancreas. Therefore, these cells produce very few or no insulin at all. The causes are not fully understood but one liable explanation is the combination of genetic susceptibility and an environmental trigger, such as viral infection. There are also evidences of toxins or some dietary products to be implicated (Barnett 2018). This condition can appear at any age, although in children and young people occurs more frequently.

2.1. Type 2 Diabetes

Type 2 Diabetes (T2D) is characterized by hyperglycemia and insulin resistance (IR). One leads to the other in a relation cause-consequence but it is still not clear which one happens first. This state is characterized by an initial intensification of insulin production known as hyperinsulinemia. At this point the disease enters in a stage called intermediate glycemia or prediabetes, that will be discussed in more detail later on. Consequently, in order to keep up with the demand, pancreatic β -cells may start to fail resulting in inadequate production of insulin. At the same time, another factor contributing for hyperinsulinemia is the decreasing rates of insulin clearance over time. Insulin clearance determines the availability of insulin in the systemic circulation. Insulin is produced in the pancreas but 50-80% is degraded, in great extent by the liver, working as a buffer of accessible insulin (Henry 1998; Najjar and Perdomo 2019) (Fig. 2). Frank diabetes occurs when the glucose levels surpass either in fast or postprandially states of homeostasis.

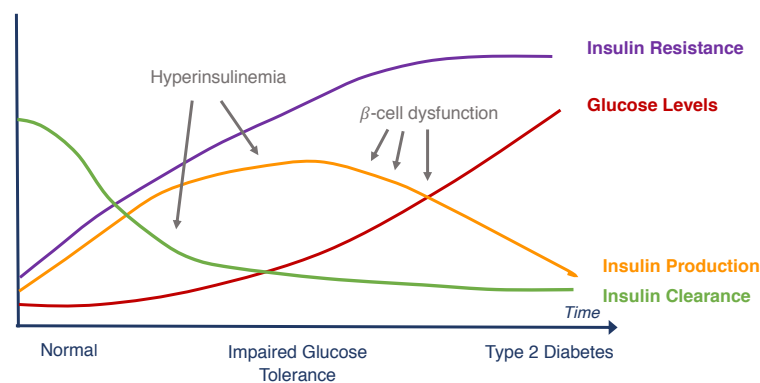


Figure 2. Time-course evolution curves from healthy stage to T2D stage. Curve in purple shows the progress of insulin resistance over time; curve in yellow is about insulin production development along time; curve in green is insulin clearance decreasing; the inter-play of this three determines the curve in red that represents glucose levels growth. The x-axis denotes the advancement with time from normoglycemia to intermediate hyperglycemia and hyperglycemia in T2D (adapted from Henry, 1998).

Frequently, the manifestation of T2D is not noticeable and the condition may be symptomless. Also, the exact time of the onset of T2D is usually impossible to precisely determine. As an effect, there is a long pre-diagnostic period and is estimated that 50-80% of people with T2D in the population may

be undiagnosed. That is why, by the time of the diagnosis, typically there are already more than one associated comorbidity. More importantly, in the prediabetic state some of these comorbidities are already installed (Tabák et al. 2012). The causes of T2D are not entirely understood but it is known that it is triggered by a combination of genetic predisposition and environmental factors.

T2D has a heritability range of 20%-80% and that evidence comes from a variety of population, family and twin-bases studies. Certain mutations in genes are associated with glucose levels control, insulin production and regulation and how it is sensed in the body. The advent of genome-wide association (GWA) studies led to identification of some genes, namely *IGF2BP2*, *HHEX/IDE* and *SLC30A8*, with a role in T2D development (Hansen 2002; Saxena et al. 2007; Zeggini et al. 2007).

Environmental factors known to impact the development of T2D include stress, sedentary lifestyle, obesity and diet, that is already naturally related with obesity. Adoption of western diet patterns, characterized by a high consumption of meats as well as refined foods, sugary beverages and fast-food has shown to be related with increased numbers of T2D in developing countries (Qi et al. 2009).

2.2. Diabetes associated comorbidities

There are several comorbidities associated with T2D explaining its tremendous social and economic impact (Fig.3).

The most common cause of both morbidity and mortality associated are cardiovascular diseases. The risk of coronary vascular disease is extremely increased by insulin resistance, as inflammation, endothelial dysfunction, and glucose has many toxic effects on microvasculature (Gerstein 2015; Paneni et al. 2013). Other diabetes complication is diabetic eye disease, specifically diabetic retinopathy, that is one of the leading causes of blindness worldwide (Bunce and Wormald 2006; World Health Organization 2015). Also, chronic kidney disease is often associated to diabetic patients with hypertension (Coresh et al. 2003; Steinke 2009). Noticeably, one major cause of lower limb amputation is the diabetic foot, that is a consequence of peripheral neuropathy affecting the distal nerves of the limbs, predominantly those of the feet (Moxey et al. 2011; Davies et al. 2006). High blood glucose is also associated to increased blood pressure and high triglycerides (TG) and cholesterol, named dyslipidemia. The combination of these factors is generally called metabolic syndrome, leading to other pathologies like non-alcoholic fatty liver disease (NAFLD), which initial phase is accumulation of fat in the liver known as liver steatosis (Perry et al. 2014) and compromised gut permeability associated with general inflammatory over-state (Gurung et al. 2020). NAFLD includes a spectrum of liver diseases initiated by liver lipid accumulation and progression up to cirrhosis and, in more severe cases, hepato-carcinoma. NAFLD seems to be a risk factor for diabetes and vice-versa: in Europe, the latest numbers estimated that 70% of T2D patients also have NAFLD. Indication of NAFLD increases the occurrence of T2D and rushes the development of its complications. Intrahepatic fat accumulation activates liver inflammation that additionally aggravates dyslipidemia and hypertension. On the other hand, T2D and systemic IR cause increase of free fatty acid (FFA) influx from peripheral organs to the liver, leading to NAFLD progression (Xia, Bian, and Gao 2019).

The gut is directly affected by T2D, specially driven by diet, since the microbiota composition changes. By interacting with dietary constituents, microbiota modulates inflammation and affects gut barrier, causing metabolic endotoxemia, allowing the passage of lipopolysaccharides (LPS) derived from intestinal bacteria to the bloodstream (Gurung et al. 2020). Alterations in intestinal barrier integrity will have consequences on glucose and lipid metabolism, insulin sensitivity and overall energy homeostasis (Chelakkot et al. 2018).

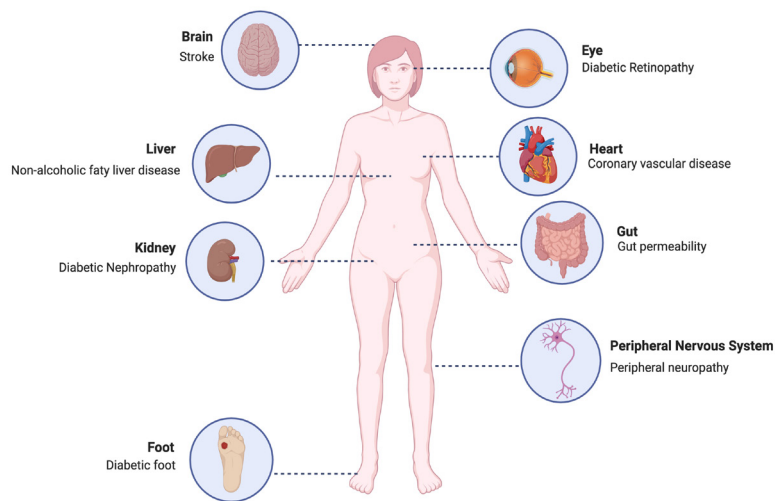


Figure 3. Comorbidities associated with diabetes. Diabetes is manifested by high blood glucose that has a deleterious impact upon the whole organism. The most common complications associated are stroke, in the brain; diabetic retinopathy, in the eye; coronary vascular disease linked to high blood pressure, in the heart; non-alcoholic fatty liver disease, in the liver; diabetic nephropathy, in the kidney; permeability impairment in the gut; peripheral neuropathy, that affects the peripheral nervous system; and finally diabetic foot.

Other critical diseases like cancer are also at greater threat in T2D patients. Mortality rates are increased in these individuals, making T2D a cause of concern in relation to global cancer impact (Tsilidis et al. 2015; Pearson-Stuttard et al. 2018).

Generally, diabetes patients are at major risk of several complications specially because the fluctuations in blood glucose levels compromises the immune system. It is also unclear whether T2D is a predisposing factor to emerging diseases, such as COVID-19 (Fang, Karakiulakis, and Roth 2020).

It is not new that diabetes itself is an epidemic, it has been for years the spotlight of medical sciences, pharmaceuticals, public health, and many others areas of research, however it is not solved and we believe the sooner it is detected, the better it can be fixed or even, avoided.

3. **Prediabetes: the opportunity for intervention**

The diagnosis of diabetes is based on blood glucose concentration. The amount of glucose in circulation results from the balance of its usage and storage that is, fundamentally, the glucose metabolism. Nevertheless, there is a period termed prediabetes or intermediate hyperglycemia that is an early stage of T2D, characterized by blood glucose levels above the normal range, but still below the recommended diabetes diagnostic (Fig. 4). Nevertheless, the levels of systemic glucose are directly related with insulin activity, since it is the responsible for facilitating cellular glucose uptake (Fargion et al. 2005).

The identification of prediabetes is not straight forward, as glucose concentrations increase not only in the fasting state but also can be increased postprandially, which is not usually evaluated in the clinical setting. Figure 5 represents the threshold values used to categorize from prediabetes to diabetes. Prediabetes can be characterized by impaired glucose tolerance (IGT), or impaired fasting glucose (IFG) or both, at a smaller scale. The table is in accordance with the parameters followed by World Health Organization (WHO). American Diabetes Association (AAA) also includes glycated hemoglobin (HbA1c) as a parameter, if people present 5.7% to 6.4%, of IFG.

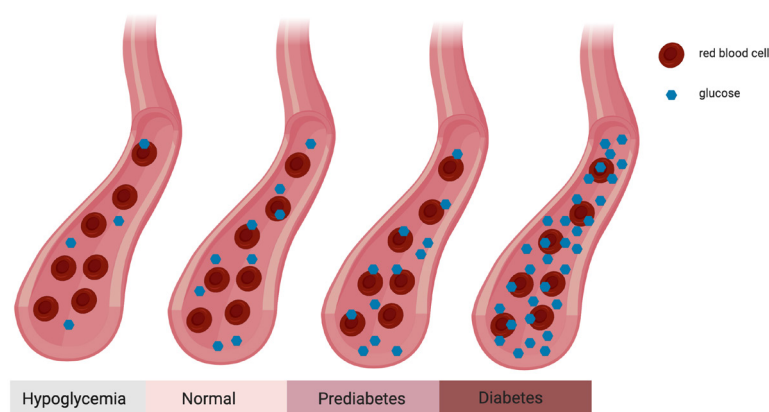


Figure 4. Schematic representation of glycemic profiles in peripheral blood circulation. From left to right it is illustrated the four different scenarios of blood glycemia; hypoglycemia, levels of glucose (in blue) under the necessary; normoglycemia, with balanced ration between red blood cells (in red) and glucose; prediabetic stage, with glucose levels above the expected already evidencing hyperglycemia; and diabetic stage with glucose levels excessively increased (adapted from Observatório Nacional da Diabetes 2016).

Recognition of IGT and IFG is extremely relevant, as they not only represent high risk to develop T2D, but also opens the opportunity for intervention and prophylaxis. Progression from IGT and IFG to T2D depend on the extent of hyperglycemia but it is known that other factors matter, like age, weight, sex and ethnicity (Tabák et al. 2012). The incidence of T2D five years after diagnosis for IGT and IFG are of 26% and 50%, respectively (IDF 2019). There is a big lack of information on the prevalence of prediabetes worldwide, but if we use Portugal as reference, the last studies in 2016, point out for ~30% of the population to have prediabetes (Observatório Nacional da Diabetes 2016). Extrapolating for the globe, this represents more than one third of the people, which, on top of that, are not aware of their condition which may be silently aggravating over time.

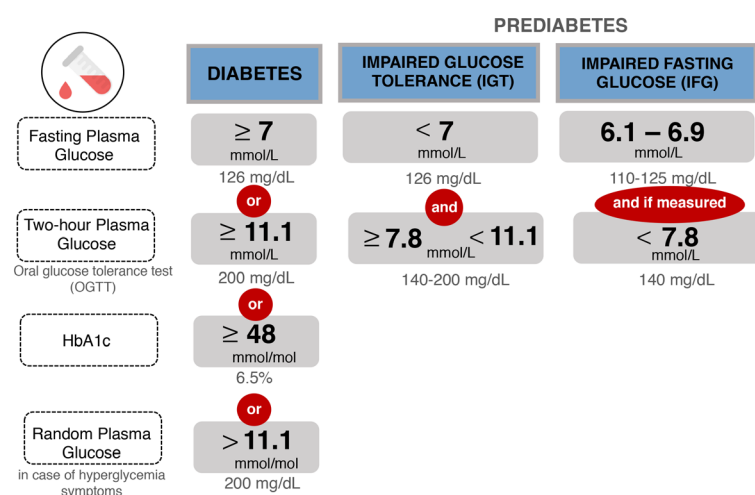


Figure 5. Parameters of diagnostic criteria for diabetes and prediabetes. Diabetes and Prediabetes can be due to impaired glucose tolerance (IGT) or impaired fasting glucose (IFG), or both. It can be diagnosed by one of the four blood glucose measure methods: fasting plasma glucose; two-hour plasma glucose; glycosylated hemoglobin (HbA1c) random plasma glucose. In the grey boxes are the threshold values for each parameter. Fasting is defined as no caloric intake for at least 8 hours and the 2-hour postprandial glucose test should be performed using a glucose load containing the equivalent of 75g anhydrous glucose dissolved in water (adapted from IDF, 2019).

3.1. In health: glucose homeostasis in fasting and fed state

Before understanding the pathological state of diabetes, preceded by prediabetes, when glucose homeostasis is impaired, it is necessary to clarify the physiological mechanism. In order to always have energy available for our basic functional needs, and because humans feed intermittently, our organism built a dynamic network of permanent supply of metabolic fuels – carbohydrates, lipids and proteins – to the tissues. Carbohydrates are broken down into monosaccharides, such as glucose and fructose. Lipids are commonly addressed as fat and are turned into fatty acids (FA). Proteins are degraded into amino acids. Noteworthy, glucose is the main source of energy used by our organs, the brain in particular uses it exclusively. Glucose results from the digestion of carbohydrates, is absorbed in the gut, and it is consequently stored in the form of glycogen in other tissues. Basically, our body has a storage of energy that can be mobilized efficiently to the tissues when in need.

In the fasting state, after an overnight fast, the liver is the responsible for providing glucose, either by breaking down its own stores of glycogen via glycogenolysis or by synthesizing glucose from gluconeogenic precursors via gluconeogenesis. The regulation of hepatic glucose production is largely done by the action of insulin. During prolonged fasting periods, the scenario is different – ketone bodies are used as major fuel. They are formed in the liver from the oxidation of FFA. During fasting, the basal rate of tissue glucose uptake is correspondent to the rate of glucose output by the liver (DeFronzo and Ferrannini 2015).

In response to an increase in blood glucose and amino acids levels after a meal, in the fed state, that fine balance between hepatic glucose production and tissue glucose utilization is disrupted. Maintenance of normal glucose homeostasis is dependent on insulin secretion that is stimulated in response to tissue glucose uptake that suppresses hepatic glucose production. In the presence of insulin, the liver inhibits glucose production and shift its functions to glucose storage (glycogenesis) and usage as energy (glycolysis). The liver, together with the pancreas, regulates the levels of plasma insulin. While these two organs are key regulators of organismal insulin levels the gut also plays a fundamental role in insulin homeostasis. In that context, the gut secretes hormones, addressed as incretins, that will positively increment insulin. The two incretins responsible for that is glucagon-like peptide 1 (GLP-1) and glucose-dependent insulintropic peptide (GIP) that potentiate insulin secretion and hepatic glucose uptake. In the muscle, insulin will promote glucose and FA uptake (Kelley 2005). In the adipose tissue, insulin will inhibit lipolysis and promote glucose uptake to be converted into lipids (adipogenesis), also it will stimulate the uptake of TG and protein synthesis (DeFronzo and Ferrannini 2015). Glucose will also be transported to the β -cells in the pancreas, intensifying insulin production, meaning that insulin has a positive feedback on its own metabolism.

Counteracting insulin action, there is glucagon, another hormone produced in the pancreas, in the α -cells. Glucagon has the opposite effect of insulin, showing up with a central role during the fasting state where it is responsible for keeping the concentration of glucose and FAs in the bloodstream balanced and high enough for our body's need.

Figure 6 summarizes, in a simplified manner the complex regulation of glucose homeostasis by these two hormones: during the fed state – insulin – and when in the fasting state – glucagon (Bedinger and Adams 2015).

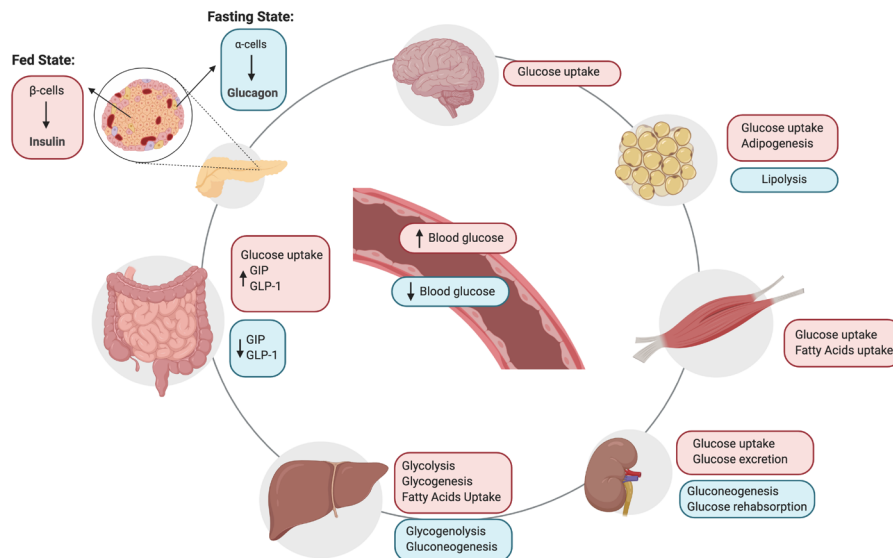


Figure 6. Summary of glucose homeostasis in post-prandial and fasting state. After a meal, glucose reaches the blood mainly through the gut where it is absorbed and incretins are released (GIP and GLP-1). They act in pancreas stimulating the secretion of insulin triggered as soon as glucose reaches the blood. Insulin promotes the uptake of glucose by the peripheral organs, especially muscle and adipose tissue. The kidney, the last line of selectivity, plays an important role regulating glucose levels, when in fasting instead of excretion it reabsorbs glucose to the blood. The liver, in fasting conditions is the main source of glucose, through glycogenolysis and gluconeogenesis, allowing the brain to always have glucose available; in the post-prandial, inhibits glucose production and stores it. At this point the adipocytes start to produce energy by lipolysis. In fasting state, the hormone in charge is the glucagon, that has the opposite effect of insulin. GIP: Glucose-dependent insulinotropic peptide; GLP-1: Glucagon-like peptide 1

3.2. In disease: insulin resistance effect

Now, talking about the disease state, one of the features of T2D is IR, but it is established that IR starts very early in time, even before the disease, and can be an indicator of prediabetes. While it is still a matter of discussion what happens first, IR, hyperglycemia and β -cell dysfunction, are undoubtedly in the core of prediabetes (Kahn 2003). IR by definition is the inability of the body to respond appropriately to circulating insulin, therefore euglycemia is lost.

Again, the usage of glucose depends on the balance between insulin and glucagon production by the pancreas and degradation, done majorly by the liver. This asks for a close inter-organ crosstalk to keep up with the organisms' requirements (Petersen and Shulman 2018) (Fig. 7).

IR in the pancreas leads to a dysregulation of hormone's secretion both by β and α -cells. The latter ones become less responsive to insulin inhibitory effects (Ferrannini, Gastaldelli, and Izzo 2011) and the β -cells, for being under extreme demand of insulin secretion, start to fail leading to the other major feature of prediabetes – β -cells dysfunction (DeFronzo 2009).

After a meal, proteins, lipids and glucose are absorbed by the gut. The gut is responsible for the secretion of a class of hormones, called incretins that play a primary role in the regulation of the amount of insulin that is secreted by the pancreas. The two incretins that share common actions in the pancreas are GLP-1 and GIP (Kim and Egan 2008). Upon a pathologic scenario, there are abnormalities in this incretin axis. In prediabetes it is observed deficiency of GLP-1 and resistance to the stimulatory effect of GIP on insulin secretion (DeFronzo 2009) contributing for the general state of hyperglycemia.

In the post-prandial state, FFA uptake by the liver is increased and consequently FFA hepatic oxidation happens. This oxidation stimulates the activity of key gluconeogenic enzymes and provides

energy (Ferrannini et al. 2004). IFG, one of the phenotypes of prediabetes, characterized by high levels of glucose in the fasting state, is characterized by accelerated rate of hepatic glucose production that is directly related with hepatic IR (Campbell et al. 2020).

Moreover the kidney contributes for this imbalance by increasing glucose absorption back to the blood flow instead of excrete it, therefore further contributing for the aggravation of the hyperglycemia as the glucose transporters SGLT2 in the proximal tubules are overexpressed (Ferrannini, Gastaldelli, and Izzo 2011; DeFronzo 2009).

Adipose tissue IR leads to the continuously FFA stratification, giving rise to an excessive FFA release into the bloodstream. As consequence, FFA start to be preferentially uptaken, instead of glucose, from insulin target tissues. In fact, studies have demonstrated that lipid oxidation rates increase in negative correlation with glucose oxidation, causing a phenomenon named metabolic inflexibility (Kelley 2005).

Skeletal muscle IR causes impaired glucose uptake after a meal and contributes to post-prandial hyperglycemia (DeFronzo 2009). IGT, which is described by high glucose levels in the postprandial state, even though its etiology is complex, appears to be characterized by high muscular IR (Campbell et al. 2020).

In conclusion, our organism functions as a whole system very well organized and regulated. When it comes to its metabolism there are main players with very specific roles. Figure 7 (Alfa et al. 2015) summarizes the participants, and the need of a close communication among each other by using a common route, the blood flow. Dysregulation of any organ will consequently affect the others and a general defective state is created, like the prediabetic state. This leads to a succession of negative feedbacks, accumulating dysfunctional responses and along the time it culminates in an endpoint of disease.

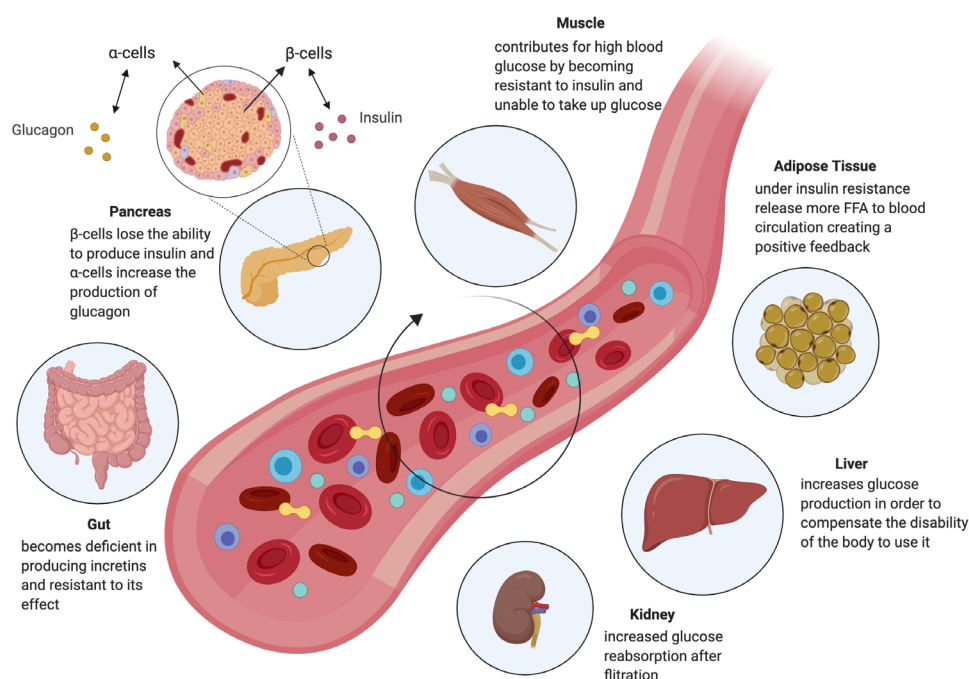


Figure 7. Metabolic organs cross-talk among each other and blood circulation. Representation of the main tissues with metabolic function and the effect of insulin resistance in each one of them. Muscle is the tissue with higher demand of glucose, especially during exercise, adipose tissue is the major store of energy, liver has the ability to produce glucose in fasting, kidney filtrates the glucose from blood, gut is the first tissue absorbing glucose after a meal, and pancreas is where insulin and glucagon are constructed (adapted from www.cisbio.com/diabetes-and-metabolic-disorders) (li, n.d.).

3.3. Clinical approaches

The best way to prevent prediabetes, and avoid T2D, is focus on lifestyle, especially diet and exercise. When it is no longer possible, or hard to keep it for a long-term, the approach needs to be more complex by combining changes in routine with pharmacological interventions. Drug therapy needs to be precise to each case individually and consists, generally, in an initial phase of hypoglycemic agents and, later on, intensification strategies to maintain glycemic control. Invariably the sooner the intervention, the better the outcomes (Marín-Peñalver et al. 2016). This is why early diagnosis of prediabetes is so important – it is the only way of effectively counteract T2D. Several therapeutic approaches have been done for T2D, from oral, to injectable agents, to surgical procedures. The most common ones are the insulin sensitizers, such metformin and thiazolidinediones, that have some evidences of effectiveness. Metformin for example inhibits gluconeogenesis in the liver and improves both hepatic and peripheral IR; thiazolidinediones acts on muscle, adipose tissue and liver to increase glucose utilization, decrease its productions, improving IR (Xia, Bian, and Gao 2019). Injection of insulin analogues with short and long-actions is used in a later stage as treatment for T2D. However, the only procedure able to completely revert T2D is metabolic surgery (Rubino 2008).

3.3.1. Metabolic surgery: the only efficient practice

Given the global social-economic impact of T2D, the pharmaceutical industry has been working for many years on tackling it from all possible ways. Curiously, the only procedure that demonstrated to be capable of T2D remission is metabolic surgery (Pareek et al. 2018; Kashyap et al. 2010; Buchwald et al. 2009; Singh, Singh, and Kota 2015). Surgical operations with intestinal diversion and mainly duodenal-jejunal exclusion, have reliably shown positive effects on glucose homeostasis (Aguilar-Olivos et al. 2016). Essentially, metabolic surgery consists on ways of manipulating gastrointestinal tract (Koliaki et al. 2017).

Bariatric surgery, initially called like that, started to be done to treat obesity (generally characterized by body mass index ≥ 30 kg/m²) (Pareek et al. 2018) but it showed to be effective in inducing remission of T2D before any significant weight reduction (Mingrone and Castagneto-Gissey 2014). Inclusively, the name metabolic surgery comes from the metabolic implications that the surgery revealed to have (Pareek et al. 2018; Batterham and Cummings 2016).

After metabolic surgery, diabetes starts to improve within days to weeks, while weight loss occurs more slowly and later in time. This suggests that the mechanisms of metabolic benefit extends beyond of weight loss by itself (Pareek et al. 2018; Singh, Singh, and Kota 2015). Figure 8 represents a very simplified model of the potential effect of metabolic surgery on glucose metabolism (Pareek et al. 2018). Despite the pathophysiology being not well understood, clearly the gut is a major player in glucose homeostasis.

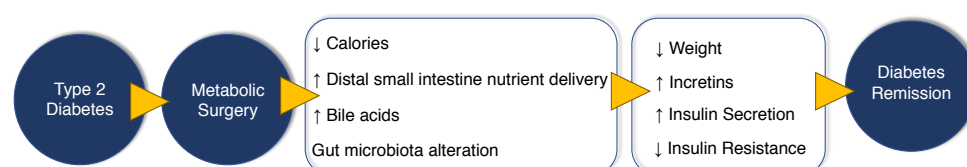


Figure 8. Potential effects of metabolic surgery in glucose metabolism. Several physiological alterations observed after metabolic surgery contribute to its metabolic benefits. Caloric restriction; increased nutrient delivery to the distal part of the small intestine; increased bile acid concentrations; and changes in the gut microbiome contribute to weight loss and favorable hormonal changes that will stop insulin resistance and consequently revert diabetes, eliminating comorbidities associated. Arrow down: decrease; arrow up: increase (adapted from Pareek et al. 2018).

4. The Gut

Many therapies for T2D target several organs, namely the pancreas, adipose tissue, muscle, kidney and liver; curiously, the most effective strategy involves the gut. Gut, or intestine, consist of two very different parts, small intestine and large intestine. We will focus on small intestine, which is responsible for nutrient absorption and metabolism.

4.1. Small intestine anatomic structure and cellular organization

In order to digest and absorb the nutrients the gut has to keep the integrity of its anatomical structure. The small intestine is around 6 meters long and has 2.5 cm of diameter and is composed by three distinct zones. The first 25 cm is the duodenum, curving downwards after leaving the stomach and until the middle of the abdomen; the jejunum accounts for the 2 meters followed and finally the ileum is about 2.5-3 meters long (Fig. 9).

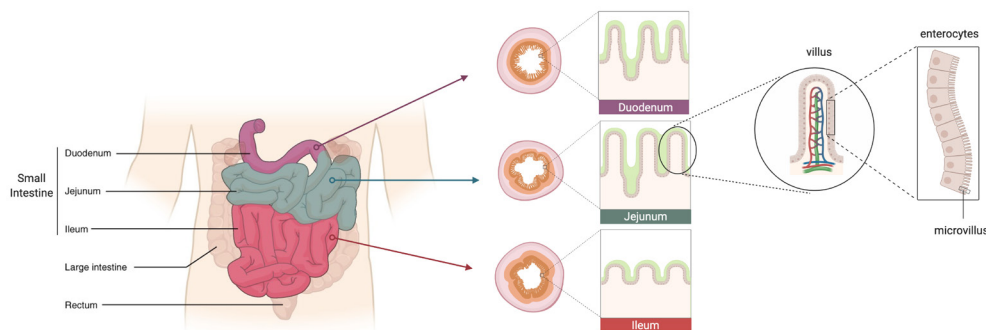


Figure 9. Anatomic representation of the small intestine. The intestine is divided in two parts with some differences in anatomy and behavior – the small intestine and the large intestine. The small intestine comprises three areas: duodenum, jejunum and ileum. The intestine is covered by smooth muscle in the outside. The inside is covered by a mucous (green layer) around each villus. The villi are constituted by enterocytes that also have small villi themselves, the microvilli (adapted from <https://courses.lumenlearning.com/suny-ap2/chapter/the-small-and-large-intestines/>).

The intestine is formed by a single layer of tall columnar cells that are epithelial cells of different types: i) enterocytes, the primary and more predominant; ii) paneth cells that secrete antimicrobial substances; iii) goblet cells which produce mucins, responsible for the mucus layer above; iv) enteroendocrine cells, that secrete hormones; v) tuft cells that have immune function, ready for immunity responses; and vi) stem cells, that are proliferative cells and live in the invaginations what allows the renewal of the epithelial layer by a cycle of migration to the top, maturing along the way (Yang and Liao 2019). They are all connected by transmembrane proteins, known as intercellular junctions, that regulate the transport among them and are composed by gap junctions, tight junctions, adherens junctions and desmosomes (Khan and Asif 2015; Odenwald and Turner 2017). These cells are structurally arranged as finger-like projections called villi separated by invaginations called crypts. The surface of each epithelial cell on the villi has also projections called microvilli. The brush border is composed by all the microvilli together which accounts for a huge surface of contact between the cells and the extracellular environment, allowing nutrient digestion and absorption. Also, these microvilli keep in its plasma membrane digestive enzymes for that process. The stem cells are located in the crypts, intercalated with the endothelial cells, allowing for a continuous replacement of the cells in the villi in a process designated epithelium turnover (Yang and Liao 2019). Above the epithelial layer is a mucus layer that consists of a viscous secretion of goblet cells. Importantly, these two layers together are the first line of defense against the outside aggressions. Under the epithelial layer is the lamina propria with dense network of capillaries

surrounding a channel that is a branch of the lymphatic system. The lamina propria is densely filled with immune cells, making the intestine one of the largest immunological organs in the body, hosting more than 70% of all the immune cells (Kagnoff 1993). Overall, the epithelial cells layer with the mucus layer on top and the lamina propria underneath, provide an ideal microenvironment for chemical digestion, selective permeability and battle against endotoxins and pathogens (Fig. 10). The intestinal mucosa promotes nutrient and water transport and serve as a protective barrier at the same time. The intestine is protected in the outside by layers of smooth muscle around its circumference. The direct interaction with the liver is provided by the venous blood vessels that merge and form the hepatic portal vein (Frayn, 2010).

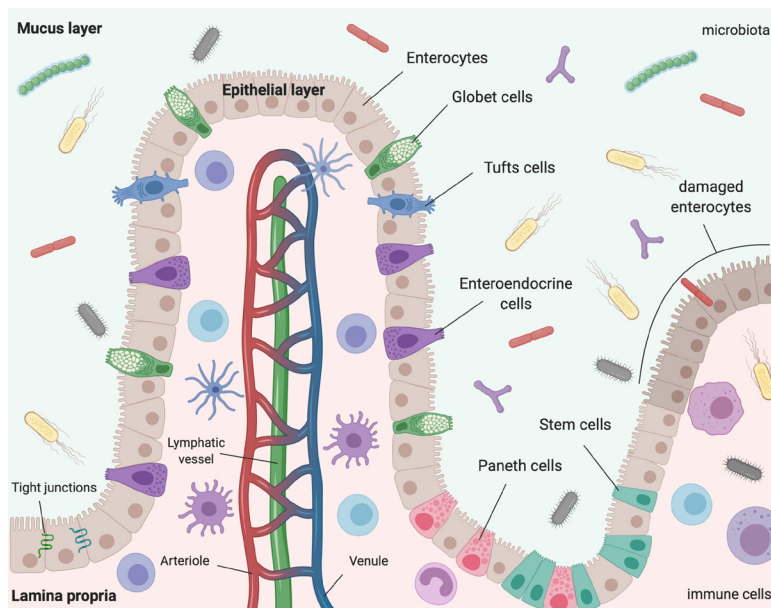


Figure 10. Intestinal villi and surrounding environment. The villi are finger-shaped projections formed by one layer of multiple cell types. These cells vary in shape and function, and are connected by tight junctions. The most abundant cells in the villi are the enterocytes (brown), which primary function is to absorb nutrients; the goblet cells (green) produce mucins that create a mucus layer on top, offering protection and a good habitat for microbiota; tuft cells (blue) have immunity function; the enteroendocrine cells (purple) secrete hormones; paneth cells (pink) produce anti-microbial peptides; and stem cells that reside normally in the crypts are responsible for the constant renewal of the epithelial layer. The three structural layers of intestine in charge of protection and stability are: the epithelial layer that is covered by a mucus layer and sits over the lamina propria. The superior zone is filled with bacteria and other microorganisms. The lamina propria is the interface for blood and lymph exchanges and is the microenvironment of a huge population of immune cells. On the right part of the image the dark brown cells represent damaged enterocytes, in those conditions, the permeability of the gut is compromised and the lamina propria is invaded by foreign bodies, activating and increasing the immune response.

4.2. Gut: the major host of microbiota in our body

Besides its anatomical structure there is other vital element in gut health and function – its microbiota. The immense microbial community that lives in the gastrointestinal tract is a particular feature of this tissue. It is extremely diverse, consisting of a mixture of bacteria, yeasts, protozoa and virus. It is generally agreed that the biggest part of these microorganisms are beneficial to the host, although some of them can be harmful or opportunistically harmful (Aron-Wisniewsky et al. 2020). Naturally, microbiota and the host have a symbiotic relationship extremely diverse, it degrades indigestible components of our diet, harvest energy and nutrients, shape host immune system and maintains the integrity of gut mucosal

barrier as well as xenobiotic metabolism (Narita 2020).

Microbiota population is determined by some individual factors. In adulthood, some factors can alter the microbiome, like changes in diet (David et al. 2014), the use of several types of medication, such as antibiotics (Falony et al. 2016) and metformin (Wu et al. 2017) and some other interventions like metabolic surgery (Zhang et al. 2009; Ramírez-Pérez et al. 2017).

Low diversity in the gut microbiome is associated with obesity and higher prevalence of IR, NAFLD and low-grade inflammation. This pro-inflammatory properties increase mucin-degrading bacteria that leads to gut integrity impairment potentiating inflammation through endotoxemia that is related with IR and T2D (Le Chatelier et al. 2013).

It is not yet well clear but amazingly gut microbiota and the host immune system have co-evolved so closely that they influence each other (Lee and Mazmanian 2010). One evidence of that is observed in germ-free mice experiments, where absence of microbiota leads to defects in the development and function of the immune system (Macpherson and Harris 2004). Also, the dynamic characteristic of gut microbe reveals host susceptibility to infection, inflammatory diseases, and autoimmunity (Narita 2020).

Gut microbiota itself produces small molecules according to diet. From the various substrates (amino acids, lipids, carbohydrates, iron) presented to the intestinal lumen, gut microbes produce specific metabolites. Carbohydrates that are not digestible are fermented by the microbial community to produce short-chain FA (SCFA), of which acetate, propionate, lactate, and butyrate are the most important (Stephens, Arhire, and Covasa 2018; Roy et al. 2006). These metabolites are important factors, influencing host physiology. In the case of bile acids, those are first synthesized from cholesterol in the liver and later metabolized by gut microbiota into secondary bile acids, that are responsible for diverse metabolic pathways in the host, regulating as well gut microbiota composition (Ramírez-Pérez et al. 2017).

Also, the gut-brain axis has been widely explored as a fundamental pathway that connects gut microbiota and its metabolites with central regulation of metabolism, namely appetite and nutrient sensing (Narita 2020). Several evidences have demonstrated the importance of microbiota, such as each individual has his own microbiota signature and that can tell a lot about overall health state.

4.3. Gut: a metabolic organ

The gut performs numerous functions, such as mixing and impulsion of luminal content, absorption and secretion of ions, water, and nutrients, pathogens defense, and waste products elimination (Horváth et al. 2015). Inclusively, gut plays a major role in the regulation of post-prandial glucose state. Digestion and absorption of nutrients consist on a complex set of processes along the digestive tract, where intestine has a central role (Holst et al. 2016).

4.3.1. Gastric emptying

Before absorption into the bloodstream, nutrients are retained for some time in the stomach. The rate of gastric emptying is directly related with the composition and macronutrients of the meal. More recently was found that the rate of gastric emptying is a major factor of overall post-prandial glucose excursion in healthy and T2D individuals, where it shows to be delayed (Phillips et al. 2015). In the stomach, the emptying of nutrients is adjusted in order to have a relatively constant entry rate of the meal into the gut. Generally, the dominant mechanisms that regulate gastric emptying result from the interaction of nutrients with the intestine. Small intestinal feedback is mediated by the digestion

products and is regulated by the area of the tissue that is exposed to nutrients. Gastric emptying is also influenced by GLP-1 and Peptide YY (PYY), that inhibits gastric motility and suppress pancreatic hormone secretion (Little et al. 2006).

Naturally, these mechanisms are all connected and any intervention on those, will affect the rate of nutrient absorption from the gut and, consequently, alter glucose and other nutrients concentrations in the blood.

4.3.2. Incretins

Intestine is responsible for releasing a group of hormones that regulates the release of insulin, causing downregulation of hepatic glucose production and enabling the delivery of glucose in insulin-sensitive tissues. These hormones are called incretins, GLP-1 and GIP. Those are secreted in response to glucose ingestion, amplifying its effect on pancreatic β -cells. The insulin secretory response to incretins is known as the incretin effect; and it accounts for 50% of total insulin secreted after glucose ingestion (Kim and Egan 2008). This response is quite fast, incretins plasma levels start to increase as soon as nutrient intakes. Simultaneously to insulin secretion increasing, GLP-1 and GIP modulate the release of other pancreatic islet hormones, namely glucagon; while GIP enhances glucagon release, GLP-1 suppresses it (Holst et al. 2016). The main nutrients that stimulate incretins secretion are glucose and other carbohydrates; proteins have a weaker effect. Both GIP and GLP-1 are substrates of dipeptidyl peptidase-4 (DPP-4) that degrade and inactivates them (Nauck and Meier 2018).

4.3.3. Intestinal gluconeogenesis

Together with liver and kidney, the gut is one of the few organs where gluconeogenesis can happen (Penhoat et al. 2014). Besides, it was recently described that intestinal gluconeogenesis (IGN) is extremely important maintaining glucose homeostasis in the post-prandial state, with great anti-diabetes and anti-obesity activity (Vily-Petit et al. 2020). Due to its connection to the liver, glucose derived from gut is detected in the portal vein by a glucose receptor in the neural system (sodium glucose co-transporter 3, SGLT3) transmitting that signal to the hypothalamus. IGN is able through the gut-brain-liver axis to minors body weight gain, reduces hepatic glucose production and improves insulin sensitivity (Mithieux 2009). Additionally, IGN has an impact on regulating appetite where gut hormones also interfere, such as GLP-1 and PYY, with an inhibitory action of that feeling. The existence of intestinal sensory nerves sent to the hypothalamus (Grill and Hayes 2012) is another feature of gut explaining the excitatory impulses generated by food presentation and eating experience on that tissue (Alfa et al. 2015). Also, by controlling appetite and energy intake, gut hormones influence the size of adipose stores, which are a major determinant of peripheral insulin sensitivity (Schroeder and Bäckhed 2016).

Some of the effects of metabolic surgery are precisely eliminating or impairing some of these above mentioned mechanisms, namely, gastric emptying, incretin activity and IGN (Holst et al. 2016).

4.3.4. Lipid absorption: chylomicrons

The digestion of lipids begins in the oral cavity, continuing in the stomach where dietary fat and fat-soluble vitamins are emulsified. In the duodenum, bile and pancreatic juice provide pancreatic lipase, bile salts, and colipase that together contribute for the digestion and absorption of lipids (Iqbal and Hussain 2009).

The degradation of fats results in FA and monoacylglycerols that are absorbed in the intestine and synthesized into TG by enterocytes. Most TG biosynthesis within the enterocyte happens alongside with monoacylglycerol (MG) pathway, in which MG and fatty acyl-CoA join to form diacylglycerol (DG). DG acylation leads to the formation of TG, by diacylglycerol acyltransferases (DGAT1 and DGAT2), that are particularly relevant for TG synthesis. During absorption of dietary lipids, FA and MG are released in the lumen and enter in the enterocyte from the apical side. Those originate TGs that are synthesized in the endoplasmic reticulum (ER). TGs are transported from ER in a specialized vesicle, the prechylomicron transport vesicle that is sent to the trans-Golgi network for additional processing. The mature chylomicron fully packaged into another vesicle is directed to the basolateral membrane and is exocytosed, entering the lymphatic vessels located within intestinal villi (Cifarelli and Abumrad 2018).

Intestine produces two types of lipoproteins: chylomicrons and very low-density lipoproteins (VLDL). Chylomicrons transport all the dietary lipids, the VLDL transport the endogenous lipids. Lipoproteins allows lipids to be in suspension and interact with enzymes and receptors on surface of cells. They are composed by an internal hydrophobic core and a peripheral layer composed by amphipathic molecules, such as proteins, phospholipids, and non-esterified cholesterol (Sirwi and Mahmood Hussain 2018).

The absorption of lipids from the intestinal lumen into the enterocytes and their consequent discharge to lymph is a complex process. CD36 has been identified as particularly important mediating FA transport for uptake, and regulating the packaging of lipids into lipoproteins (Drover et al. 2005). There are two mandatory requisites in the assembly of lipoproteins: Apolipoprotein B (ApoB) and microsomal triglyceride transfer protein (MTTP). ApoB is the essential surface structural protein that enables the construction of lipoproteins within the ER. MTTP fosters lipoprotein biogenesis by shuttling neutral lipid from within the bilayer of the ER to an acceptor ApoB molecule (Abumrad and Davidson 2012).

TG is the dominant fat derived from diet synthesized in the gut but other lipids may be metabolized there, namely phospholipids, sterols, such as cholesterol, and others such as fat-soluble vitamins.

Cholesterol of exogenous source, meaning dietary source is absorbed in the intestine and only in its non-esterified form, incorporated into bile acid can be absorbed by enterocytes (Cohen and Fisher 2013).

4.4. Large intestine

This work focuses on small intestine, but the large intestine is essential to optimize small intestine work. Large intestine is about 1.5 meters long and extends from the end of the ileum to the rectum. It contains water, bacteria, residual food particles, shedded epithelial cells, and mucus. Its main functions are absorption of water and there is a considerable bacterial activity for the products that escaped small intestine digestion. Usually the energy we excrete in feces represents only 5% of the energy we ingest and much of its weight consists of bacteria from this part of the gut and materials that are unable to be digested.

While the intestine is the main gate of food we ingest, it has a very close relationship with the liver. In fact, the intestinal tract is supplied along his tunnel with innumerable blood channels that send the blood to the portal vein towards the liver.

5. The Liver

The liver is the largest organ in the body and has a leader metabolic role regulating whole-body homeostasis and energy management. It has a central privileged position in the blood circulatory

system being supplied with two major vessels from below: the hepatic artery and the hepatic portal vein, or simply portal vein. The blood in the portal vein comes directly from the system of blood vessels around the intestine. Thus, the nutrients derived from the diet are directed to the liver, before reaching the general circulation. The pancreatic veins are another important group that join the portal vein in the entrance to the liver and carry blood from the pancreas transporting insulin and glucagon. In the way-out, the blood goes through the hepatic veins which enter the inferior cava vein and points towards the heart (Frayn, 2010).

5.1. The structural heterogeneity of the liver

What allows the liver to be a multi-tasking organ is its heterogeneity in cell composition. Several types of cells can be found in the liver. The most abundant ones are the parenchymal cell: hepatocytes, accounting for approximately 60% of liver cells. The non-parenchymal cells comprise the 40% hepatic cells left, and correspond to the sinusoidal endothelial cells (~20%), the Kupffer Cells (KC) (~15%) and stellate cells (~5%). In a much smaller percentage other types of cells reside in the liver, like fibroblasts, smooth muscle cells, lymphocytes and progenitor cells. Sinusoidal endothelial cells are the interface between blood and hepatocytes, acting as a buffer. KC, that will be explored in more detail later on, are derived from circulating monocytes and have the ability to proliferate locally, to phagocyte and produce cytokines to mediate the inflammatory response. Hepatic stellate cells have the particularity of storing 50-80% of all vitamin A in the body as retinyl ester into lipid droplets (LDs), that in pathological circumstance, such as liver fibrosis, the vitamin A is lost to synthesize components of the extracellular matrix such as collagen, proteoglycan, glycosaminoglycan and adhesive glycoproteins, altering considerably the morphology of these cells (Senoo 2017; Malarkey et al. 2005; Seo and Jeong 2016).

Hepatocytes contain all the necessary machinery for liver metabolic functions. With age the numbers of hepatocytes tend to decrease. Hepatocytes have a very particular organization, which appears in a cross-section as hexagonal units and at each corner of the hexagon are formed by three vessels: small arms of the portal vein, hepatic artery and bile duct. The function of periportal hepatocytes modulates the microenvironment sensed by pericentral hepatocytes and to that is called liver zonation. The hepatocytes on the periphery of each lobule (periportal hepatocytes) are exposed to blood arriving from the portal vein and hepatic artery, meaning these hepatocytes in particular are well oxygenated and well supplied with nutrients, and that is mainly where the oxidative metabolism happens. Thus, gluconeogenesis, the synthesis of glucose, takes place mostly in the aforementioned region, whereas glycolysis occurs in the central part of the lobule (pericentral hepatocytes) (Ben-Moshe and Itzkovitz 2019; MacParland et al. 2018) (Fig. 11).

5.2. Metabolism of the liver

As all the blood leaving the intestine goes into the hepatic portal vein, the liver has the essential role of metabolizing and sorting dietary-derived carbohydrates, lipids and proteins (Sadri et al. 2006). Simultaneously, the liver is responsible for maintaining glucose homeostasis, managing energy storage and supply (Macedo et al. 2014). Moreover, liver also converts lipids into FAs that are stored as TG or synthesized as cholesterol or phospholipids (Maxson & Mitchell 2016). Some of those are packed into lipoproteins and made available for the other tissues. Also, liver metabolizes amino acids or turn them into FAs so it can be used as source of energy. There is a close interaction among the different macronutrient metabolic pathways in order to guarantee well balanced glucose levels and steady supply of energy for cellular demands.

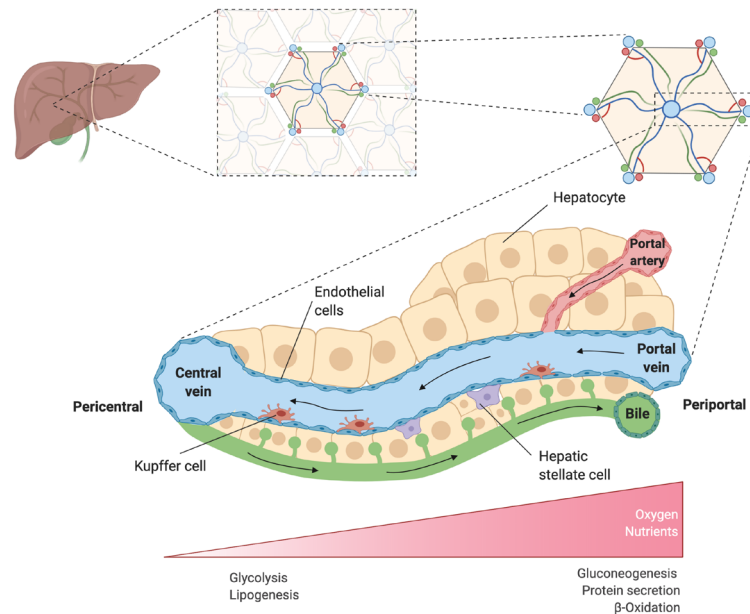


Figure 11. Schematic representation of a cross-section of the liver. The liver structure is made of hexagonal lobules. In each corner of the lobule are located a hepatic portal artery (red), a portal vein (blue) and a bile duct (green). Blood enters the lobules from the corners and flows inwards towards the central vein through blood vessels that are sustained by liver endothelial cells (cells in blue) and might intercalate with Kupffer cells (KC) (cells in red). The main hepatocytes (cells in yellow) are arranged, confer the structure of the lobule and have some hepatic stellate cells as neighbors (cells in purple). Bile canal transport bile acids secreted by the hepatocytes in the opposite direction to blood flow into the bile duct to be sent to intestine. The blood arriving to liver comes well supplied of oxygen and nutrients, along the way to the central vein, the concentration decreases gradually so that will determine the functions of hepatocytes according to their localization. Process like gluconeogenesis, protein secretion and β -oxidation will occur in the periportal area and glycolysis and lipogenesis will be localized to the pericentral area. The specialization of the liver different zones in distinct metabolic reactions is called liver zonation (adapted from Ben-Moshe and Itzkovitz 2019; MacParland et al. 2018).

5.2.1. Carbohydrate metabolism in the liver

In the fed condition, glucose is absorbed from the intestine into the portal vein sending it immediately to the liver and exposing the hepatocytes to high concentrations of glucose. The uptake of glucose by this organ is insulin independent and it is mediated by the glucose-transporter 2 (GLUT-2). Inside the hepatocyte, glucose is phosphorylated by glucokinase enzyme (GCK) to form glucose-6-phosphate (G6P) which is the first step of both glycogenesis (glycogen synthesis) and glycolysis (glucose degradation), therefore being the major regulatory step in glucose uptake. Both, the insulin arriving from the pancreas, and the glucose coming from the gut, promotes glucose storage as glycogen, by stimulating glycogenesis and inhibiting glycogenolysis (glycogen breakdown). In the fed state, G6P can also be metabolized to pyruvate via glycolysis. What happens in glycolysis is that pyruvate can be either oxidized in the tricarboxylic acid cycle (TCA) or converted into lactate to be released. Glycolysis pathway is activated under fed state, but when in fasting state gluconeogenesis is favored. Gluconeogenesis convert non-carbohydrate molecules into glucose. They catalyze opposite functions and the first is stimulated by insulin, while the other by glucagon.

Under fasting conditions, when there is no income of glucose from the ingestion of food, glucose concentration in the blood falls, and liver needs to release it to maintain tight glycemic values

preventing hypoglycemia. First, glycogen is broken down, controlled, simultaneously, by the activation of glycogen phosphorylase and inhibition of glycogen synthase. This is mainly regulated by glucagon. Gluconeogenesis can also use amino acid substrates to synthesize glucose, depending on the supply of nutrients and its rate (Lisa C. Hudgins et al. 2000).

Liver works as a sensor of glucose through the activity of insulin. The insulin secreted by the pancreas, in response to higher levels of glucose in the blood, travels directly to the liver through the portal vein. This means that liver is exposed to much higher insulin levels than the systemic circulation. In this first passage, the liver immediately removes 50 to 70% of secreted insulin from circulation through receptor-mediated endocytosis, a process known as insulin clearance that have been evidenced to be impaired in T2D (Bedinger and Adams 2015). This way, the liver determines the insulin available for the peripheral organs.

Abundance of carbohydrates, such as glucose and fructose, makes the liver convert glucose into FA, in a process called *de novo* lipogenesis (DNL) (Lisa Cooper Hudgins et al. 1996). DNL starts with the conversion by the ATP-citrate lyase (ACLY) of citrate to acetyl-CoA, that is later carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC). FA synthase (FAS) intermediates the conversion of malonyl-CoA and NADH into palmitate that can generate different FA, predominantly TG. These TG can later provide energy via β -oxidation. DNL is regulated by two transcriptional factors, sterol regulatory element binding protein 1c (SREBP1c) and carbohydrate response element binding protein (ChREBP), that are activated by increasing insulin signaling and increased glucose concentrations, respectively, both stimulated by feeding (Sanders and Griffin 2016). The activation of SREBP1 happens via two main pathways downstream of insulin receptor, one causing the phosphorylation of the SREBP1c itself and the other activating the liver X receptor (LXR), both comprising the phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB) (Horton, Goldstein, and Brown 2002). ChREBP is activated by the delivery of glucose into hepatocytes. ChREBP, by its turn, is regulated by dephosphorylation of Ser196 and other protein kinase A (PKA) or adenosine monophosphate-activated kinase (AMPK) phosphorylation sites (Silva et al. 2019; Ortega-Prieto and Postic 2019). SREBP1c and ChREBP act synergistically mediating the response to dietary carbohydrates.

5.2.2. Fat metabolism in the liver

In a healthy scenario, the liver metabolizes great quantities of FA, but stores much lesser amounts.

FA in the liver derives from exogenous or endogenous sources. The exogenous or dietary source, corresponds to the chylomicron TG produced in the intestine that are taken up mainly by muscle and adipose tissue. The remaining are delivered back to the liver where chylomicrons are received by receptor mediated endocytosis, releasing the FA during lysosomal processing (Cohen and Fisher 2013). The endogenous source can be DNL, that happens when carbohydrates are abundant, and the liver converts glucose into FA or direct uptake from plasma, when in fasting.

In fasting periods, when insulin concentrations are low, white adipose tissue enters in lipolysis, increasing the plasma FA pool sending it to the liver (Cohen and Fisher 2013). In the liver, specifically in the hepatocytes, FA are esterified to glycerol-3-phosphate (G3P) and to cholesterol in order to generate TG or cholesteryl esters, respectively. These lipids can be either accumulated in cytoplasmic LDs, or released to the bloodstream in VLDL.

The liver is able to synthesize TG, incorporating it to lipoproteins different of chylomicrons, named VLDL, which are responsible for carrying endogenous lipids in blood. In normal conditions, the liver

stores few amounts of TG but sends considerable FA in the form of VLDL to muscle and adipose tissue (Kawano and Cohen 2013). Also, in the liver, FA may originate other complex lipids, like phospholipids. In fasting periods, FA can be used as local energy suppliers or substrate for ketone bodies (Gray, Tompkins, and Taylor 2014).

Considering cholesterol, a lipid also prevalent from both dietary or endogenous source, is an essential constituent of cell membranes and a precursor of steroidal hormones and bile acids. Cholesterol can either be stored in LD or packaged into lipoproteins, that can vary in density. Based on density there are five categories of lipoproteins, ordered from lower to higher density: chylomicrons, VLDLs, intermediate-density lipoproteins (IDLs), low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs). TGs are packed into chylomicrons, VLDL and IDL, while cholesterol are present in all (Cohen and Fisher 2013).

However, the primary pathways for cholesterol catabolism is bile-acid synthesis. They are produced in the liver from cholesterol and secreted by hepatocytes into the bile ducts and subsequently stored in the gall bladder. Upon ingestion of food, bile flows into the duodenum, where it participates in the digestion of lipid-soluble nutrients. Bile acids are afterwards absorbed by passive diffusion and active transport from the terminal ileum to the liver via portal vein (Thomas et al. 2008).

LDs are dynamic cellular structures that transitorily stock lipids. After white adipose tissue, the liver is the second greatest organ with capacity to store TG in LDs. An overnight fasting is enough to originate these LDs accommodating FA derived from adipose tissue lipolysis (Jr. 2012).

There are multiple ways for FA oxidation derived from hydrolysis of hepatic TG stores, circulating lipids or DNL. The primary route in hepatocytes is mitochondrial β -oxidation, when it comes to short-, medium-, and long-chain FA. Secondly, β -oxidation of very long and branched-chain FA starts in the peroxisomes. Additionally, α -oxidation and ω -oxidation within the endoplasmic reticulum, mediated by cytochrome P450, are alternative pathways for FA oxidation (Lavoie and Gauthier 2006). The expression of genes involved in mitochondrial and extramitochondrial FA oxidation is regulated by peroxisome proliferator-activated receptor α (PPAR α) activity (Ortega-Prieto and Postic 2019).

5.2.3. Amino acids metabolism in the liver

Before being taken up by the liver, dietary proteins are broken down into their component amino acid in the gut. Contrasting with glucose and FA, amino acids cannot be stored in the liver, thus they are metabolized through deamination to provide energy or to be used for synthesis of nonessential amino acids, FA or glucose, by gluconeogenesis. The liver plays a catabolic role in terms of excess of amino acids coming from the diet. The resulting toxic metabolite, ammonia, can be transformed to less toxic urea, and be excreted in urine as a waste product of digestion, by the kidney. On the fed state the supply is done by the nutrients intake, and in the fasting state depends on the net rate body protein breakdown. It is proved that glucose together with amino acids are required to trigger insulin sensitization in the gut, in the fed state, independently of insulin secretion (Afonso et al. 2016).

The word “central” used often to characterize the role the liver plays in metabolism is not euphemistic. Liver is localized in the center of our body and is the first passage of many components that enter in our system, giving to the liver the responsibility of maintaining physiological homeostasis. Therefore, despite the ability to metabolize all the nutrients coming from gut, it also complies with detoxification processes, forming the first line of defense against pathogens and xenobiotics, that were able to cross the intestinal barrier. The population of KC in the liver are the ones in charge of this protection.

5.3. The role of Kupffer cells on metabolism

Macrophages are the effector cells of the innate immunity with major role in inflammation and host defense. Some of the particular features of cells from the monocyte-macrophage lineage are the diversity and the plasticity. In reaction to environmental signals macrophages present great extent in phenotype and function. They have two ways of activation: M1, the classical one, and M2, the alternative one. M1 activation occurs in response to toll-like receptors ligands LPS and interferon- γ (IFN- γ), resulting in the secretion of pro-inflammatory cytokines, reactive oxygen species (ROS) and nitric oxide (NO). The M2 activation happens after stimulation with interleukin-4/interleukin-13 (IL-4/IL-13), promoting tissue renovation and other immune regulatory functions by producing polyamines (Luo et al. 2017).

KC are the hepatic resident macrophages of the liver, comprising the largest tissue with specific population of macrophages in the body (80-90% of tissue macrophages). They can be activated by several endogenous and exogenous stimuli, regulating the phenotype and function of neighboring parenchymal and non-parenchymal cells. The localization of KC in the hepatic sinusoid allows them to efficiently phagocytize antigens coming from the portal vein or arterial circulation. Absence or decreased functional activity of KC may lead to pathogens invasion and systemic inflammation. In the other hand, over-activation of KC, in NAFLD cases, results in uncontrolled inflammatory stated of the liver (Dixon et al. 2016). Changes in the functional activity of KC are associated to the wide variety of disease states in the liver.

NAFLD and alcoholic liver disease (ALD) progression is strongly associated with KC activity. This progression is marked by the manifestation of fatty liver, hepatocyte necrosis and apoptosis, inflammation, nodules regeneration, fibrosis and cirrhosis. Hepatic fibrosis results from dysregulated inflammatory processes in consequence of hepatocellular damage (Dixon et al. 2016; Luo et al. 2017).

In addition to immune function, KC are very important to iron and lipid metabolism. They can uptake lipids and digest them in the lysosome, generating cholesterol and FA. Also, there are evidences that high fat diet (HFD) induces hepatic steatosis and significantly increase hepatic expression of pro-inflammatory cytokines and disturbances of phagocytic functions (Ferrere et al. 2016; Luo et al. 2017). Another fact is that diet composition alters microbiota and that alteration has effects on the inflammatory pattern of the liver double-stating the close communication between gut and liver (Ferrere et al. 2016). KC also appear to have a role on insulin resistance in cases of obesity since its associated with chronic inflammation and altered lipid metabolism (Clementi et al. 2009). There are data inclusively showing that depletion of KC protects against diet-induced steatosis and IR demonstrating an active role they have in mediation factors from diet (Huang et al. 2010).

The direct communication between gut and liver is quite relevant on their individual functions. Thus, there is an inherent need to look at them, especially in the pathologic scenario, in the perspective of interaction and communication. Actually, communication, has been the topic between the lines. T2D is fundamentally a systemic disease, extremely complex, resulting from the dysfunction of many tissues, or a dysfunction that is communicated and translocated from tissue to tissue.

6. Tissue communication

We cannot talk about tissue communication without first mention cell communication. Along evolution cells developed strategies of communication among them through signaling pathways. Small unicellular creatures evolved to big and complex multicellular organisms, and with that, new strategies came along. To successful survive in changing environments, organism had to develop

sensing mechanisms to external conditions and adapt to maintain homeostasis, so the tissues of multicellular organisms had to communicate with each other via many signals. Intercellular signaling can be autocrine, paracrine or endocrine. Autocrine signaling means that a cell secretes a signaling molecule that will bind to receptors and perform its action in the same cell. Paracrine signaling means that molecules will bind to receptors in the nearby cells. Endocrine signaling is when a cell releases a signaling molecule, classically known as hormone, into the bloodstream and binds to receptors in distant cells, exerting their action there. These molecules are soluble factors, with bioactive function, and include hormones, growth factors and cytokines, the latest ones are commonly used by immune cells. We will not look into that, but in the matter of tissue communication, nervous system has a spotlight. Inclusively, metabolic homeostasis results from the interaction between the central nervous system and the peripheral organs, requiring a coordinated release of multiple hormones and signaling molecules. The nervous system combines a vast network of innervation where neurotransmitters and neuropeptides transfer the information in the form of electrochemical messages (Castillo-Armengol, Fajas, and Lopez-Mejia 2019).

Inter-organ communication has always been a topic of great curiosity from the perspective of several diseases, but metabolism, in particular, is a very suitable field to approach that. From peptide hormones to lipids, several molecules coordinate the responses of the tissues to the fluctuating nutritional states. Besides cell-to-cell contact and transfer of secreted molecules, a third mechanism of intercellular communication has recently captured our attention: the extracellular vesicles (EVs).

EVs were discovered long ago, by the middle of the last century. In the late 1980's the term exosome was used for the first time to coin these vesicles but, by then, they were seen as a waste removal mechanism, or a vehicle to excrete unwanted and unnecessary cellular components (Johnstone et al. 1987). Only in 2001 it was demonstrated these EVs have functional activity and after that, many studies about their function, especially in the cancer field, have been published (Maia et al. 2018; Raposo et al. 1996; Janowska-Wieczorek et al. 2001).

Essentially, EVs are double phospholipid bilayer membrane vesicles, whose origin can be from endosomes or plasma membrane, that contain cytosolic and cell-surface proteins, DNA, RNA, lipids and metabolites (Raposo and Stoorvogel 2013; Are 2016). Although the classification of EVs is quite polemic, to some extent a consensus was found by characterize them according to size and cellular origin – endosomal or plasma membrane (Théry et al. 2018). The larger EVs (100-1000 nm in diameter) that are formed and released directly from the plasma membrane of both living or dying cells are generally known as microvesicles or ectosomes. In this class of EV can be also included the apoptotic bodies whose size can go until 5000 nm. The EVs displaying a smaller size (40-160 nm) with multivesicular endosomal origin, that are generated inside multivesicular bodies (MVBs), and are secreted upon its fusion with the plasma membrane are called exosomes (Théry et al. 2018; Are 2016; Maia et al. 2018). EVs are a recent field and the detailed nomenclature is a matter of active discussion. The lack of well-established markers and the heterogeneity of isolation protocols generates a technical issue of debate.

Figure 12 characterizes the different types of EVs and also compare its size and density with other non-EV structures that circulate in the body (Mathieu et al. 2019).

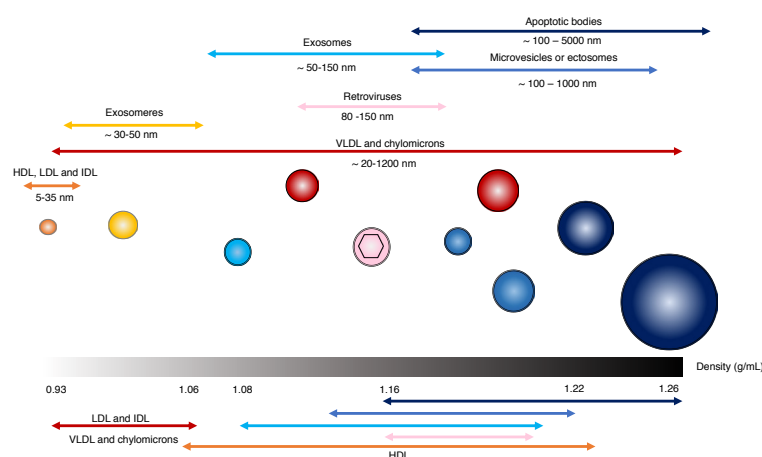


Figure 12. EV subtypes in perspective with other non-EV structures, that can be co-isolated, in terms of size and density. The three groups of EVs according size. Unlike ectosomes and exosomes, the apoptotic bodies are bigger and undergo apoptosis soon after secretion. EVs are being compared with other non-EV structures, present in the organism, that can have similar sizes. Density is a characteristic that should be considered to correctly separate the population of interest. HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein (adapted from Mathieu et al. 2019).

6.1. Extracellular vesicles: biogenesis

EVs are membrane-derived vesicles, being microvesicles and exosomes the most prevalent. Exosomes are formed through the invagination of the plasma membrane that engulfs cell-surface proteins and soluble proteins of the extracellular environment. This results in the formation of early-sorting endosomes, that in some cases can fuse with pre-existing ones. At this point, trans-Golgi network, ER and mitochondrion may contribute to the content that is being encapsulated through close interactions (Van Niel, D'Angelo, and Raposo 2018; Mathieu et al. 2019). The early-sorting endosomes mature to late-sorting endosomes and finally to MVBs, that can be also called multivesicular endosomes. Fundamentally this MVBs are inward invaginations of the endosomal membrane, that were already originated by invagination of the plasma membrane, meaning a double invagination happens. This process originates MVBs with intraluminal vesicles (ILV) inside. This MVBs have normally two potential directions: they can fuse with lysosomes or autophagosomes for degradation or, in the other hand, fuse with plasma membrane to release the ILVs as exosomes (Van Niel, D'Angelo, and Raposo 2018) (Fig. 13). The sequential invaginations, the interactions with other cellular compartments, and the formation of exosomes contributes for the EVs great heterogeneity. They can carry a vast range of cargoes offering a unique package to transport information by providing a safe and protective environment to travel (Yáñez-Mó et al. 2015).

One feature that contributes to EV biogenesis is its membrane curvature. The residents of the membrane are, some way, flexible and move along the membrane, so some molecules will accumulate in certain regions that are energetically favorable, determining its shape and influencing its physiological role, non-specific mechanism manner (Kralj-Iglic 2012; Yáñez-Mó et al. 2015).

It is important to have in mind that EVs are produced both in pathological and healthy conditions, and its biogenesis, degradation or secretion are part of the natural mechanism of a cell.

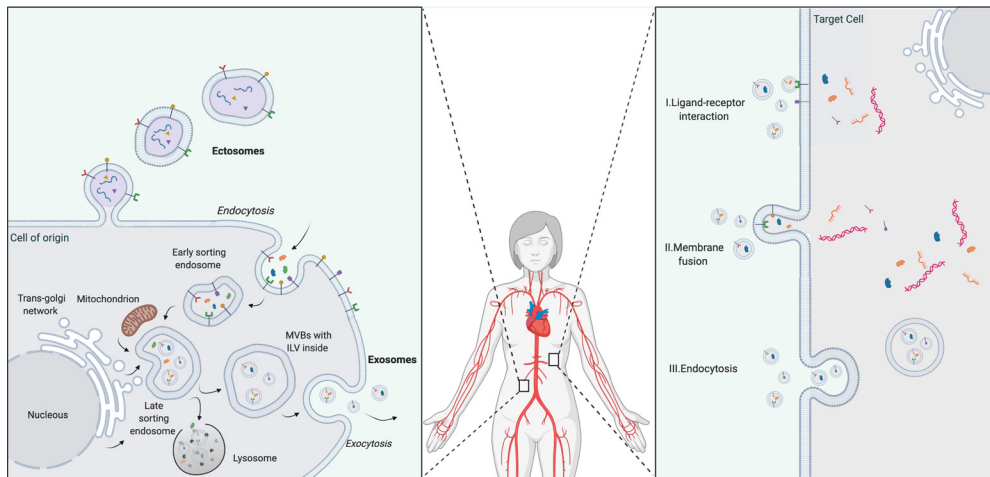


Figure 13. How EVs are produced and secreted (left) and how EVs are received by the target cells (right). (Left) Large extracellular vesicles (EVs) are formed by budding of plasma membrane and are called ectosomes or microvesicles. Exosomes are smaller EVs that result from the invagination of plasma membrane, collecting extracellular components, forming in a first stage, an early sorting endosome, that may fuse with other endosomal compartments and inclusively interact with endoplasmic reticulum (ER), trans-Golgi network and mitochondria, developing to late sorting endosomes; a second invagination happens at this stage generating intraluminal vesicles (ILVs); the volume and dimension of these invaginations will determine the future exosome cargos; late sorting endosomes will give rise to multivesicular bodies (MVBs), that can either fuse with autophagosomes and ultimately be degraded by lysosomes, or they can follow the pathway of exocytosis, through the cytoskeletal and microtubule network; exocytosis will result in the release of exosomes. The EVs may be delivered in a proximal cell or in the blood flow to reach a distant target cell. (Right) At the target cell, EVs can deliver its cargoes by three general mechanisms: I. Direct interaction ligand-receptor between EV membrane and cell membrane, activating the correspondent signaling pathways; II. Membrane fusion, when fluidity level of both membranes allows it; III. Endocytosis such as phagocytosis, micropinocytosis or receptor-mediated endocytosis. (adapted from Maia et al. 2018; Van Niel, D'Angelo, and Raposo 2018; Kalluri and LeBleu 2020).

6.2. Extracellular vesicles: delivery

According with their composition and surface protein profile, EVs can be delivered to a proximal cell or, on the other hand, to a very distal cell. Thus, protein content and especially membrane composition are responsible for biodistribution.

The way EVs are up taken appears to depend, at least in part, on the target cell. Upon arrival, EVs trigger three major deliver routes; the first is through the direct interaction between surface receptor or ligands of target cells, activating intracellular signaling. The second, is by membrane fusion. The third is by endocytosis, that can happen in three different ways, by phagocytosis, by micropinocytosis or by receptor-mediated endocytosis (Maia et al. 2018; Kalluri and LeBleu 2020) (Fig. 13). There is an interesting point regarding membrane fusion – both membranes need to display similar fluidity and that only is possible at a pH~5, limiting this mechanism to acidic environments, that are characteristic of tumor cells. This also calls the attention for the electrostatic behavior of EVs that may be critical determining its physiological role. A certain molecule carried in the EV may be more active in the membrane rather than its soluble form, or vice-versa (Yáñez-Mó et al. 2015).

The basal level of EVs in circulation results from the balance between their production and clearance. This requires a tight regulation of EV biogenesis by the parent cell and availability of target cells to internalize them. It is still not well understood how this organization is achieved but there are some cargoes in the vesicles that participate in this process.

Importantly, besides delivering biomolecules, EVs can also act as decoys sequestering circulation factors and targeting them for degradation. In particular, EVs can scavenge multiple toxins, acting as a defense mechanism (Keller et al. 2020). It is still unclear, however, whether the decoy effect of EVs play a role in the progression of metabolic diseases.

6.3. Extracellular vesicles: heterogeneity

EVs are quite heterogeneous. They can vary in origin, size and importantly in cargoes. On top of that, they differ on the functional impact on the recipient cells. Nevertheless, extensive literature exists about the effect of some specific components they transport in a variety of pathological contexts. Namely, in cancer (Costa-Silva et al. 2015), neurodegeneration (Jan et al. 2017), and interestingly to us, also in metabolic disorders (Chelakkot et al. 2018).

The microenvironment of cells determines the content of EVs and their surface markers. Noteworthy, the extracellular environment is an important determinant factor in this equation, the composition of EVs reflects their interaction with the extracellular milieu and not merely their intracellular biogenesis, which will ultimately influence their fate. EVs contain membrane proteins, cytosolic and nuclear proteins, extracellular matrix proteins, metabolites and nucleic acids, namely mRNA, noncoding RNA, and DNA. Figure 14 represents a classic view of an EV (Kalluri and LeBleu 2020; Colombo, Raposo, and Théry 2014).

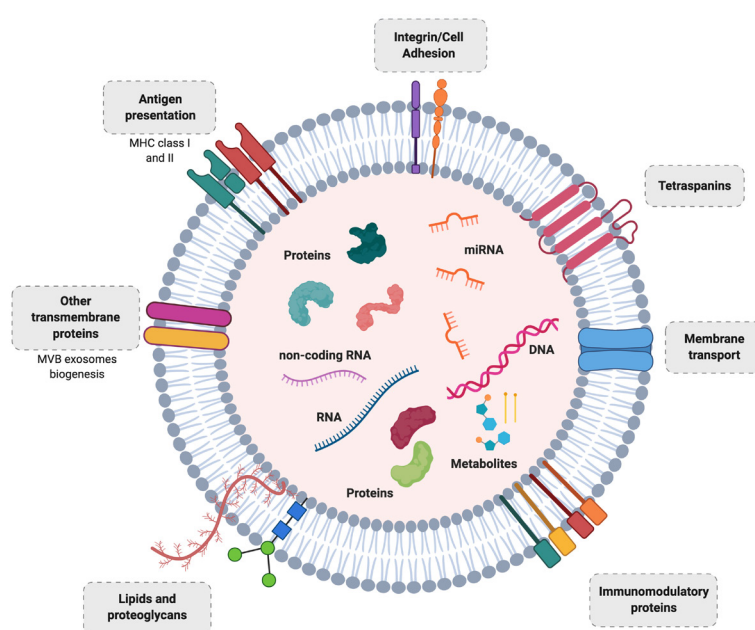


Figure 14. Schematic representation of an EV in terms of composition and membrane orientation. Common membrane proteins in EVs are tetraspanins; antigen presenting factors; cell adhesion-related proteins; immunomodulatory components; lipids and other proteins related with biogenesis. Inside, cargoes can go from proteins, to lipids, to metabolites, and to nucleic acids (DNA to RNA) (adapted from Kalluri and LeBleu 2020; Colombo, Raposo, and Théry 2014).

The most common luminal proteins among EVs are cytoskeletal, cytosolic, heat shock and plasma membrane proteins, likewise trafficking proteins. One category of proteins that are less abundant are the intercellular organelle ones. Characterization of EVs protein have been done by proteomics, immunoblotting, immune gold labelling combined with electron microscopy and antibody-coupled

bead flow cytometry. Proteins identified to be enriched in exosome-EVs are used as markers; and we can divide them in two groups: the ones present in the membrane and the luminal ones. Concerning the first ones, we have the tetraspanins (CD9, CD63 and CD81), integrins and major compatibility complex (MHC) molecules. Regarding the cytosolic proteins, stress proteins (heat shock proteins (HSPs)), tumor susceptibility gene 101 (Tsg101), and the endosomal sorting complex required for transport (ESCRT-3) binding protein ALIX (Yáñez-Mó et al. 2015; Kalluri and LeBleu 2020; Van Niel, D'Angelo, and Raposo 2018; Hoshino et al. 2015). Also, several glycan-binding proteins are present in exosome-EV and they appear to be fundamental for the targeting of these vesicles. Likewise, active lipolytic moieties, such as phospholipases, that lead to the formation of lipid mediators, like the case of FAs and prostaglandins, that have the ability to interact with peripheral G-proteins coupled to receptors on the cell membrane, are also present in these vesicles (Yáñez-Mó et al. 2015; Van Niel, D'Angelo, and Raposo 2018). Cytokines are another category of bioactive proteins found in EVs, that are normally produced as intermediaries of an immunological response (Maia et al. 2018).

Extracellular RNA is a curious occurrence, it can exist bound to a protein complex, freely in circulation or well protected inside EVs. Inclusively, there are evidence that certain populations of RNA are enriched in EVs compared with the parental cells where they were formed (Nolte' T Hoen et al. 2012). Even in the case that this enrichment happens because of size restriction, there is a specific repertoire of miRNAs selectively exported to EVs, while other miRNAs are excluded, indicating the presence of an active sorting mechanism (Mittelbrunn et al. 2011). It is documented functional mRNA in EVs that are internalized and translated by the target cells, however it is still challenging to understand the extent of the effect of each individual mRNA that is carried (Koppers-Lalic et al. 2014).

When protected in EVs miRNAs can avoid degradation during circulation in blood. While we know that the miRNAs carried inside EVs play an important role in intercellular communication, the amount of miRNA required to elicit a paracrine or endocrine effect is an open question (Squadrito et al. 2014). DNA was also found in EVs, but less explored. Nevertheless there are studies evidencing the presence of mitochondrial DNA, single-stranded DNA, double-stranded DNA and also oncogene amplifications (Lázaro-Ibáñez et al. 2014).

Another component identified, through metabolomic analyses in EVs, are the lipids; these are key players in their physiological role, not only in its formation, but also in its function. Compared with the parent cells, EVs seem to be enriched in sphingomyelin, cholesterol, polysaccharides and glycosphingolipids. These are particularly important in the membrane to confer stability in different environments. Also, it was observed that, similarly to other EVs components, they are not randomly included in the vesicles (Choi et al. 2013; Record et al. 2014).

EVs, due to their advantage of having a lipid membrane encapsulating the cargoes and protecting its content of degradation, carry information from one organ to another using majorly the blood flow, importantly EVs are present in every fluid of the body: urine, saliva, synovial fluid, bile, cerebrospinal fluid, bronchoalveolar fluid, nasal fluids, uterine fluid, amniotic fluid, breast milk, seminal plasma and also feces (Yáñez-Mó et al. 2015).

6.4. Extracellular vesicles: isolation

In the last decade the number of papers studying EVs rose exponentially, consequently the debate regarding isolation methods gained particular attention. Isolation methods are quite diverse, thus substantially affecting yield, purity and quality. The most commonly used methods for EV isolation are: differential ultra-centrifugation; size-exclusion chromatography; and commercially available

kits (polymer-based precipitation or immunocapture by antibody-coated beads). Additionally to ultracentrifugation, in order to acquire a more enriched exosomal sample, by separating membrane-enclosed vesicles from proteins aggregates, a sucrose gradient can be added – as protein aggregates deposit in the sucrose layer, whereas lipid-containing vesicles float on top of an equilibrated gradient, allowing to filtrate better the vesicles population (Colombo, Raposo, and Théry 2014).

In total, given the diversity of isolation methods, when comparing EVs findings across the literature, we should be cautious and pay particular attention to EV characterization. When looking at EVs as potential disease biomarkers, we have to keep in mind that methods developed for research purposes are time-consuming and labor intensive and are not yet adequate to be used in clinical laboratories.

6.5. Extracellular vesicles in metabolic disorders

With the ubiquitous nature of EV secretion by most cells in the human body, and their role in cell-to-cell communication, it is expectable to also have EVs participation in regulating the complex multiorgan interaction with insulin function and glucose homeostasis. Numerous studies have explored the involvement of EVs in metabolism, especially in the context of metabolic disorders such as T2D. Accumulative evidence shows that EV levels are higher in those conditions and with effects on pathophysiology, including vascular complications and inflammation (Lakhter and Sims 2015).

Several papers have reported EVs being released by the insulin-producing pancreatic β -cell. One interesting study demonstrated that increased β -cell EV secretion was stimulated by glucose or calcium treatment (Hyo, Jeong, and Lee 2009). Also β -cell EV secretion and cargo seems to be modified by contact with inflammatory cytokines (Hospital and Diseases 1990). Furthermore, proteomic studies revealed significant disparities among EV contents of cytokine-stimulated and non-stimulated rat β -cells. The first ones presented increased protein levels from tumor necrosis factor- α (TNF- α) signaling pathways, suggesting EVs with a role in inflammatory response (Palmisano et al. 2012).

Adipose tissue has also been greatly explored from EV perspective studies, since it revealed to be involved in glucose homeostasis. Adiponectin, an adipokine associated to IR, was described in circulating EVs, and EV adiponectin content is reduced in HFD-fed mice compared with control (Palmisano et al. 2012). Additionally, visceral-adipose tissue (VAT)-derived EVs confirmed to have the capacity to trigger systemic inflammation and IR; after VAT-EVs intravenous injection into wild-type (WT) mice, serum IL-6 and TNF α levels were increased and glucose intolerance was induced, the same way as IR (Deng et al. 2009). Adipose tissue EVs inclusively showed to have influence on other metabolic syndrome-related tissues; in obese patients, adipose tissue-EVs were able to modulate insulin responses in hepatocytes and muscle cells (Kranendonk et al. 2014).

Effect of skeletal muscle-derived EVs in obesity and IR has also been explored. Data showed EVs derived from lipotoxic skeletal muscle with ability to transfer lipids and impact gene regulation of recipient muscle cells, not directly inducing IR (Aswad et al. 2014).

Other tissue focus of great attention on this field was the liver, where the role of EVs have been explored over the years. EV release from hepatocytes have demonstrated to activate inflammation through the induction of pro-inflammatory cytokines and macrophage activation (Lakhani 2019). Also, liver-EVs are quite relevant in liver diseases, such as NAFLD and metabolic syndrome, since they are able to modulate and regulate drug resistance and even hepatocellular carcinoma (Chen et al. 2018).

In the case of the gut, curiously, the study on EVs was done mostly on microbiota EVs that showed to be quite relevant in scenarios of disease. Exosomes released into the mucosa demonstrated

to participate on local innate responses to invading bacteria through microbicidal activity (Hu et al. 2013) and also to mediate gut permeability through the regulation of tight junctions (Chelakkot et al. 2018). EVs definitely play an important role in the interaction of the microbiota with intestinal cells and even more distant cells in the body (Chang et al. 2020; Smythies and Smythies 2014).

Plasma EVs lipid content such as FAs, prostaglandins and cholesterol, also demonstrated to affect endogenous cell lipid metabolism and trafficking, having this a great relevance on T2D field (Record et al. 2014).

III

Chapter

Aims and Hypothesis

Aims and Hypothesis

Maintenance of metabolic homeostasis in response to nutritional cues requires the coordinated action of multiple organs. Therefore, higher organisms developed diverse communication systems through which one organ can affect metabolic pathways in distant ones. Impairment of systemic communication contributes to human pathologies, namely obesity and diabetes. Recently, EVs emerged as an important mean of organismal communication and were implicated in the pathophysiology of diabetes. Furthermore, technical advances such as proteomics and associated data-driven bioinformatics help us to a better understand of the complex metabolic crosstalk going on in the organism. The complex and vast network of tissues involved in the pathophysiology of prediabetes led us to look for an intermediary of such demanding communication, the EVs, small vesicles capable of transporting specific cargo and inducing functional changes in recipient cells. From the impressive outcomes of metabolic surgery, the gut holds the key for the treatment of metabolic disorders, such as prediabetes and T2D. Importantly, epithelial cells in the gut secrete EVs. Thus, focusing our attention on that tissue, we aimed at studying gut derived EVs (GDE) in the context of prediabetes.

In the present thesis, we explored the hypothesis that prediabetes has a gut specific trigger, which in turn communicates with the liver, by secreting GDE, which are capable of imprinting a hepatic dysmetabolic phenotype.

The main objectives of this thesis are:

AIM 1. To characterize the impact of prediabetes in the protein cargo of GDE.

AIM 2. To elucidate the gut communication with other organs mediated by GDE; by identify the target organ(s) and cell type of GDE action.

AIM3. To shed light into the pathophysiological role of GDE in modulating metabolism.

Our approach to study GDE in prediabetes is schematized in Figure 15. To achieve the proposed goals, we used the routinely used in the field diet (high fat and sugar) induced mouse model of prediabetes. First, GDEs were isolated from prediabetic mice, then analyzed and characterized. Consequently, GDE were administered in lean healthy mice so that we could understand their biodistribution and metabolic function in the organism.

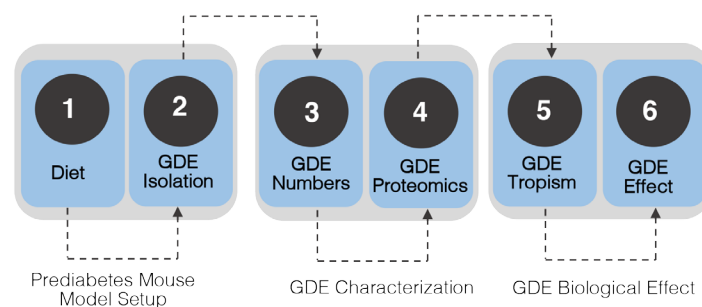


Figure 15. Schematic representation of the experimental design in chronologic order. This project consisted in the following phases. 1) Prediabetic mouse model: mice fed with high fat and normal chow diet for 12 weeks; 2) Gut-derived EV (GDE) isolation by sequential ultracentrifugation and sucrose gradient; 3) GDE numbers – EVs isolated from both groups were quantified in terms of size, concentration and total protein; 4) GDE proteomics – EVs protein cargo was analyzed by mass spectrometry; 5) GDE tropism – GDE were injected in healthy mice and traced; 6) GDE effect – GDE were injected in healthy mice for several weeks to evaluate its pathophysiological effect. Two first points compose the first task of obtaining GDEs (prepare and extract the material), the following two were the second task of GDE characterization (in terms of amount and content), the last two were GDE application to analyze the behavior in the organism (evaluation of tropism and metabolic impact).

Chapter III

General Methodology

General Methodology

Animal studies

1. Animals producers of extracellular vesicles

1.1. Diet-induced prediabetic model

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. High-fat diet (HFD) (OpenSource Diet, D12331) composition is 16.5% protein, 25.5% carbohydrate, and 58% fat with 700 kcal of sucrose. The control normal chow diet (NCD) (Special Diets Services, RM3) composition is 26.51% proteins, 62.14% carbohydrates and 11.35% fat. All studies were performed in male mice. All animals were treated according with National (Portaria 1005/92) and European Union Directive for Protection of Vertebrates.

1.2. Glucose tolerance test (GTT)

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.).

1.3. Anesthesia and sacrifice

At week 12 of diet mice were sacrificed. Mice were anesthetized using isoflurane 1.5-3%. Mice were scarified under 12 hours of fasting, with a previous feeding period of 2 hours, with the previous 8 hours of fasting. Blood, liver and gut were collected from prediabetic mice and respective controls.

1.4. Tissue preparation in OCT for histology

After sacrifice, the small intestine and the liver were well-washed and fixated for histology. A small portion of the tissues were processed in the following manner: gut was placed in 2% Paraformaldehyde (PFA) with 20% Sucrose, overnight at 4°C; washed three times in Phosphate Buffered Saline (PBS) , 10 min each; transferred to 30% sucrose for 2 hours at 4°C; left overnight at 4°C in Optimal Cutting Temperature compound (OCT) with 30% Sucrose (1:1). In the following day, tissues were embbeded in molds in 20% Sucrose (dilution of 1:4) + (OCT) (dilution of 3:4) in a box with dry ice and immediately stored -80°C. Consequently, tissues were sliced into 6 µm thin sections in the cryostat and stored at -80°C for following applications. Gut samples were used for CD81 staining and liver samples for Hematoxylin-Eosin staining.

1.4.1. Immunohistochemistry

Gut coverslips were fixed with 4% PFA for 10 min. In a covered dark humidified chamber, the slides with the cells were incubated for 20 min with Phosphate Buffered Saline with Tween-20 (PBS-T) and after with 5%Goat Serum in PBS-T for 1 hour. Cells were washed and then incubated with primary antibody CD81 (Santa Cruz) diluted in 1%Bovine Serum Albumin (BSA)/PBS-T overnight, at 4°C. In the following day, slides are washed with PBS-T, three times, 10 min, and incubated with the secondary antibody anti-mouse Alexa-Fluor 488-conjugated, at 1:500 (Invitrogen). Coverslips were mounted in mounting media

Pro-Long Gold Antifade Mountant with DAPI (ThermoFisher) to visualize the nuclei.

Fluorescence was observed using a Zeiss AxioImager M2 motorized upright widefield fluorescence microscope. Images were taken adjusting the levels of fluorescence of each channel up to a maximum threshold defined by the absence of signal in the negative control and processed using ImageJ software.

1.4.2. Hematoxylin-eosin staining

Histological analysis of liver sections were performed in the Histopathology Unit of Instituto Gulbenkian de Ciência. Liver cells were stained with hematoxylin-eosin and examined under light microscope (Leica DM LB2, Leica Microsystems).

2. Animals receptors of extracellular vesicles

2.1. Biodistribution experiments

Mice were anesthetized using isoflurane 1.5-3% for few seconds, then injected retro-orbitally, specifically in the orbital sinus of the mice, with 5 µg of near infra-red (nIR)-labelled GDE derived from mice fed with HFD and NCD, and PBS as control, in a total volume of 100 µl. 24 hours post-injection mice were sacrificed and the following tissues were collected: brain, gut, liver, fat, muscle, kidney and lung. Tissues were washed in PBS and signal emission was recorded in Odyssey imager system (LI-COR Biosciences). Settings of acquisition and analysis used were in accordance to manufacturer's instructions.

2.2. Education experiments

Male C57Bl/6J mice at 8 weeks of age were injected with gut EV, in different experiments, that were optimized along time. To the method of prolonged injections of gut EV we called education, this concept is further explored in the second part of Chapter IV.

5, 10 and 20 µg of gut EV protein was injected directly into the blood flow, through the orbital sinus in a volume of 100 µl PBS. Mice were injected every other day, 3 times a week. 5 µg dosage was injected for 3 weeks. 10 µg and 20 µg for 6 weeks. Retro-orbital injection of PBS was used as control. Mice were fed with NCD with unlimited access to water and food, with the exception of mice of education 5 that were fed with HFD. Animals were injected under anesthesia using isoflurane 1.5-3% for few seconds until no sensory perception.

ITT was performed to mice of education 5 two weeks before sacrifice and GTT performed one week before sacrifice (according to previous section 1.2).

2.3. Insulin tolerance test (ITT)

ITT performed on educated mice two weeks prior to sacrifice. Mice were fasted for 4 hours and weighed. Mice were then given an intra-peritoneal injection of human insulin at 0.75U/kg body weight (Humulin, Eli, Lilly). Serum blood glucose levels were measured at 15, 30, 60, 90 and 120 min after injection. Blood glucose levels were measured using OneTouch Ultra glucose meter (LifeScan Inc.).

2.4. Tissue harvesting

At the end of each education and prior to sacrifice, mice were weighed. Blood was collected through orbital sinus under anesthesia through a heparinized capillary. The liver was divided in two parts, one

part was snap frozen in liquid nitrogen and the other one fixed for histology. The small intestine was carefully removed, washed with PBS with a syringe and a needle.

2.5. Liver lipid assay

Hepatic lipids were extracted as previously described (Matyash et al. 2008). Briefly, approximately 250 mg of frozen tissue was rapidly mixed with high performance liquid chromatography (HPLC)-grade methanol (4.6 mL/g) followed by methyl-tert-butyl ether (MTBE) (15.4 mL/g). The mixture was placed in a shaker for 4 h and then centrifuged at 13.000g for 10 min. The liquid fraction was collected, and phase separation was induced by adding 1 mL of distilled water and letting it rest at RT for 10 min. The liquid was then centrifuged for 10 min at 1000g. The organic phase, which contained the lipids, was separated and dried under nitrogen gas in a glass vial protected from light. It was then dissolved in butanol/(Triton X-100:methanol [2:1]).

2.6. Liver cell suspension non-parenchymal cells purification

Non-parenchymal cells (NPC) were isolated from liver lobes by perfusion with Collagenase H (Sigma) and density centrifugation as described by (Duarte et al. 2018). To enrich for KCs, NPC were placed on top of 4 layers of a Percoll (GE Healthcare) gradient of 80%, 60%, 40% and 20% Percoll and centrifuged for 20min at 400g. The interface between the 60% and 40% Percoll layer containing the KCs was collected. To sort purify KCs, the enriched fraction was re-suspended in PBS with 2%Fetal Calf Serum (FCS) (Life Technologies).

2.7. Flow cytometry analysis

2.7.1 Liver

For the biodistribution experiments, after one single-injection of labelled-EV, the liver was collected and placed in media 2% Fetal Bovine Serum (FBS) in PBS, followingly is smashed against a 70 µm cell strainer with the help of a syringe. Cells were centrifuged at 500g for 5 min. Red blood cells were depleted from the pellet by incubation with ammonium chloride-potassium buffer (ACK) for 3 min at RT and add 10 ml of 2% FBS. Cells were centrifuged at 500g for 5 min, supernatant is discharged and cells were resuspended in PBS. The whole liver cells are next blocked for flow cytometry staining.

Livers of educated mice were treated as described in the previous point 2.6.

2.7.2. Bone marrow

In educated mice, femurs were cutted at both ends and with a needle and syringe filled with ice-cold 0.5%BSA+2mM EDTA PBS bone marrow was flushed, cell suspension was collected. Cell suspension is filtered in a 70µm cell strainer and washed with same media. Cells were centrifuged at 500g for 10 min at 4°C, and pellet was collected. Pellet was resuspended with ACK (Life Technologies) and incubated for 5 min at RT. Cells were centrifuged at 4°C, supernatant was discharged and pellet resuspended in PBS.

After blocking with Fc Receptor Blocking Solution (anti-CD16/CD32 antibodies) (BioLegend) (dilution 1:50), cells were stained with specific antibodies CD45 APC-Cy7 (BD, Biosciences) (dilution 1:100); F4/80 PE (BioLegend) (dilution 1:200); CD11b PE-Cy7 (BioLegend) (dilution 1:400); Ly6c BV421 (BioLegend) (dilution 1:200). Flow cytometry analysis was carried out on LSR Fortessa X20 (BD, Beckman Coulter). All data were acquired with DIVA software (BD) and analyzed by FlowJo software (TreeStar).

Extracellular vesicles studies

1. Extracellular vesicles isolation

1.1. Extracellular vesicles isolation from blood

Blood (600-1000 µL from each animal) was collected with a heparinized capillary from anesthetized mice to a 1,5 mL tube with 100 µL of heparin. Blood was centrifuged at 500g for 10 min; supernatant was collected and centrifuged at 3000g for 20 min; in order to obtain plasma. Plasma was stored at -80°C for future EV isolation or continue to ultracentrifugation (section 1.3).

1.2. Extracellular vesicles isolation from small intestine

After sacrifice the small intestine was removed, washed with PBS and cultured overnight in RPMI medium (Sigma) supplemented with 1% PenStrep and 10% Fetal Bovine Serum EV-free, to avoid contaminations from serum EVs. In the following day medium was collected and centrifuged at 500g for 10 min; supernatant was collected and centrifuged at 3.000g for 20 min. Supernatant was stored at -80°C for later EV isolation or continue to ultracentrifugation (next section 1.3).

1.3. Ultracentrifugation

The supernatant stored at -80°C was transferred to ultracentrifuges tubes and centrifuged at 12.000g for 20 min at 10°C; supernatant was collected and another ultracentrifugation was done at 100.000g, for 140 min at 10°C; at this step, the supernatant was discharged and the pellet was collected.

At this step EV can be labelled how it is described in the next section and continue to sucrose cushion; or continue immediately for sucrose cushion.

Sucrose Cushion: The pellet was resuspended in 1 mL of filtered PBS and transferred to a new tube and it was added filtered PBS until a total volume of 16 ml. Next, sucrose solution was prepared with 30g of protease-free sucrose (Sigma), 2.4g of Tris-base (Sigma) and 50 ml of heavy water (Sigma) to a total volume of 200 ml, and pH was adjusted to 7.4 with HCl; solution was then filtered with 0.22 µm filter vacuum inside the laminar flow. In a medium ultracentrifuge tube it was transferred 4 mL of the sucrose solution and, on top of that, the 16 ml of sample was added in a very careful way. The sucrose cushion and the EV pellet were centrifuged at 100.000g, for 70 min at 10°C. The fraction of interest (which is the sucrose cushion with the EV) was collected and added to a new tube with 16 mL of PBS that was centrifuged at 100.000g overnight. The next day the pellet was collected and resuspended in 200 µl of PBS.

Isolation of EV was done by ultracentrifugation (Beckman Ti70, rotor 70Ti).

2. Extracellular vesicles labelling

2.1. Extracellular vesicles labelling for odyssey image system

At the step that was mentioned in the previous section, after the first three ultracentrifugations, to the pellet that was collected was added 1 mL of diluent C from the CellVue NIR815 Kit for Membrane Labeling (Polysciences, Inc.) into a new tube. To that mixture of EV and diluente C it was added 4 µL Cell Vue NIR815 dye (Polysciences, Inc.) (excitation of 786 nm and emission of 814 nm). Then 2 mL 0.5% BSA

was added and labeled EV were washed at 100.000g for 70 min.

Labelling of EV is normally done during the isolation but can also be done afterwards, with the drawback of losing some EVs during the extra ultra centrifugation that need to be done if the labelling is post-isolation.

In that case, to the EV sample it was added 20 ml of filtered PBS and ultracentrifuged at 100.000g overnight. In the next day, the pellet is collected and the procedure is the same of the one just mentioned.

2.2. Extracellular vesicles labelling for fluorescence

For fluorescence microscopy and flow cytometry analysis, purified EV were fluorescently labelled using PKH67 Fluorescent Cell Linker Kit with excitation (490 nm) and emission (504 nm) similar to fluorescein (Sigma). The protocol is exactly the same of the previous section.

3. Nanoparticle tracking analysis (Nanosight)

The concentration and size of EVs were analyzed in NanoSight NS300 (NS3000) system equipped with a blue laser (405 nm), according to the manufacturer's instructions. EVs were diluted 1:1000 in filtered sterile PBS. 1 mL of sample is collected with a 1 mL syringe that was inserted in the equipment flow system. Each sample analysis was conducted for 90 seconds and measured 5 times using Nanosight automatic analysis settings. The software calculated the size distribution in nanometers and the concentration in number of particles per mL.

4. Protein extracts, SDS-PAGE and western blot

Protein extracts for western blot analysis were obtained using cold lysis buffer (20 mM Tris- HCl pH 7.4, 5 mM EDTA pH 8.0, 1% Triton-X 100, 2 mM Na₃VO₄, 100 mM NaF, 10 mM Na₄P₂O₇) in the presence of protease inhibitors (completeTM, Mini, EDTA-free Protein inhibitor cocktail tablets, Roche, Sigma). Gut tissue and gut EVs were homogenized in the lysis buffer and sonicated three time for 30 seconds each at 10 µm amplitude with 1 minute incubation on ice between each sonication step. Lysates were centrifuged at 18.000 g for 10 min at 4°C. Soluble fraction was collected and protein concentration was determined using the PierceTM BCA Protein Assay kit (Thermo Fisher). For protein expression analysis, equal amounts of total protein (15 – 20 µg) were mixed with sample buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 8% β-mercaptoethanol, bromophenol), before being denaturated at 95°C for 10 min. Samples were then resolved by an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were subsequently electrotransferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P Membrane, PVDF, Millipore) for 22 min at 25 mA and 2,5 mV in a Trans-Blot Turbo TM Transfer System (BioRad). Membranes were blocked for 1 hour at RT with 5% non-fat milk (Molico, Nestlé) in tris-buffered saline with 0,1% Tween (TBS-T) (blocking solution). Membranes were incubated overnight at 4°C, with CD63 antibody (Santa Cruz Biotechnology) prepared in 3% BSA in TBS-T, diluted 1:1000. Membranes were washed three times with TBS-T and incubated for 1 hour at RT with the secondary antibody anti-rabbit antibody diluted 1:5000 (Santa Cruz Biotechnology). Blots were developed with ECL (ECL Prime, GE Healthcare, Enzymatic), according to the manufacturer's instructions, and a Chemidoc touch equipment (Bio-Rad) was used to detect chemiluminescence. Band intensities were quantified using ImageLab software and normalized using β-actin as loading control.

5. Proteomic analysis of extracellular vesicles

Mass spectrometry of gut EV were performed by Rune Mathiessen, Principal Investigator of Computational and Experimental Biology in CEDOC, Lisbon.

5.1. Nano-LC-MSMS analysis

Peptide samples were analysed by nano-LC-MSMS (Dionex RSLCnano 3000) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Briefly, 5 μ L of sample was loaded onto a custom made fused capillary pre-column (2 cm length, 360 μ m OD, 75 μ m ID) with a flow of 5 μ L per min for 7 min. Trapped peptides were separated on a custom made fused capillary column (20 cm length, 360 μ m outer diameter, 75 μ m inner diameter) packed with ReproSil Pur C18 3- μ m resin (Dr. Maish, Ammerbuch-Entringen, Germany) with a flow of 300 nL per minute using a linear gradient from 92 % A (0.1% formic acid) to 28 % B (0.1% formic acid in 100 acetonitrile) over 93 min followed by a linear gradient from 28 % B to 35 % B over 20 min at a flowrate of 300 nL per minute. Mass spectra were acquired in positive ion mode applying automatic data-dependent switch between one Orbitrap survey MS scan in the mass range of 400 to 1200 m/z followed by HCD fragmentation and Orbitrap detection of the 15 most intense ions observed in the MS scan. Target value in the Orbitrap for MS scan was 1,000,000 ions at a resolution of 70,000 at m/z 200. Fragmentation in the HCD cell was performed at normalized collision energy of 31 eV. Ion selection threshold was set to 25,000 counts and maximum injection time was 100 ms for MS scans and 300 and 500 ms for MSMS scans. Selected sequenced ions were dynamically excluded for 45 seconds.

5.2. 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 and VEMS.

6. Statistical Analysis

Data are presented as means $\pm$ 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 $p < 0.05$.

IV

Chapter

Results

1. **Extracellular vesicles in prediabetes: a proteomic characterization**

1.1. **Messages from the intestine carried by extracellular vesicles**

The escalating incidence of T2D as a consequence of sedentary lifestyle and caloric-unbalanced diets, imposes a substantial burden to the health care systems explaining why it is considered a major public concern. Prediabetes or intermediate hyperglycemia is a high-risk state for diabetes and is defined by glycemic levels higher than normal, but lower than diabetes limits. Furthermore, IR at the level of the liver and peripheral tissues is observed. Metaphorically, this stage can be seen as a fork in the road, when left untreated, the likelihood to progress to diabetes is high. Additionally, when timely diagnosed and with patients compliance to lifestyle intervention, this condition is reversible to normoglycemia (Tabák et al. 2012). Over the past, scientists studied each organ as an individual part of a complex metabolic network. However, recent data suggest that inter-organ communication is crucial, and it can be mediated by EV. These small vesicles are important vehicles of systemic communication in multiple physiologic and pathologic contexts. They 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 (Zhang et al. 2015; Théry, Zitvogel, and Amigorena 2002). Epithelial cells in the gut secrete EVs and their content may reflect the cell environment (Mallegol, Van Niel, and Heyman 2005). In fact, metabolic surgery is the only known intervention that leads to diabetes remission (Evers, Sandoval, and Seeley 2017). Interestingly, gut dysbiosis recently emerged as a possible player in the development of T2D. (David et al. 2014). Despite the abundant literature on the impact of microbiota in T2D onset and development, the role of the intestine independent of its biota is still elusive. While gut-derived EV (GDE) are considered to play an important role in intestinal diseases (Burcelin et al. 2011) it is not known whether GDE protein content is modulated in prediabetes. Therefore, the present chapter aims at characterizing GDE proteome.

Since we wanted to study prediabetes, we used a mouse model that recapitulates many features of prediabetes (Kowalski and Bruce 2014). High fat and sugar feeding can lead to obesity, hyperinsulinemia and altered glucose homeostasis. So, we used HFD which composition is 58%fat and sucrose, in contrast with normal-chow diet (NCD) that is 11%fat. Since obesity is induced by environmental manipulation rather than genes, it is considered the best model to mimic the human condition. By administration of diet for 12 weeks, HFD mice demonstrated significantly increased body weight (Fig.16B) and liver steatosis (Fig.16C). They also developed glucose intolerance that was evaluated by a glucose tolerance test (GTT) in the previous week of sacrifice and correspondent area under the curve (AUC) for each group (Fig.16D; E).

After 12 weeks of diet, with confirmed glucose intolerance, mice were sacrificed, and gut was removed. Small intestine was washed with saline solution and placed in culture media, EV-free, at 37°C overnight. Post-incubation EVs were isolated according to well established protocol, consisting on a sequence of high-speed centrifugations, where fraction by fraction, the particles were separated by size and an intermediate step of sucrose gradient was added in order to exclude free lipid compounds, which were separated based on density. In the last step of the isolation, EVs were labelled with a dye that intercalates with the lipids present on their membranes. Note that from now on the tissue will always be referred as gut or intestine, but specifically, EVs were isolated from small intestine.

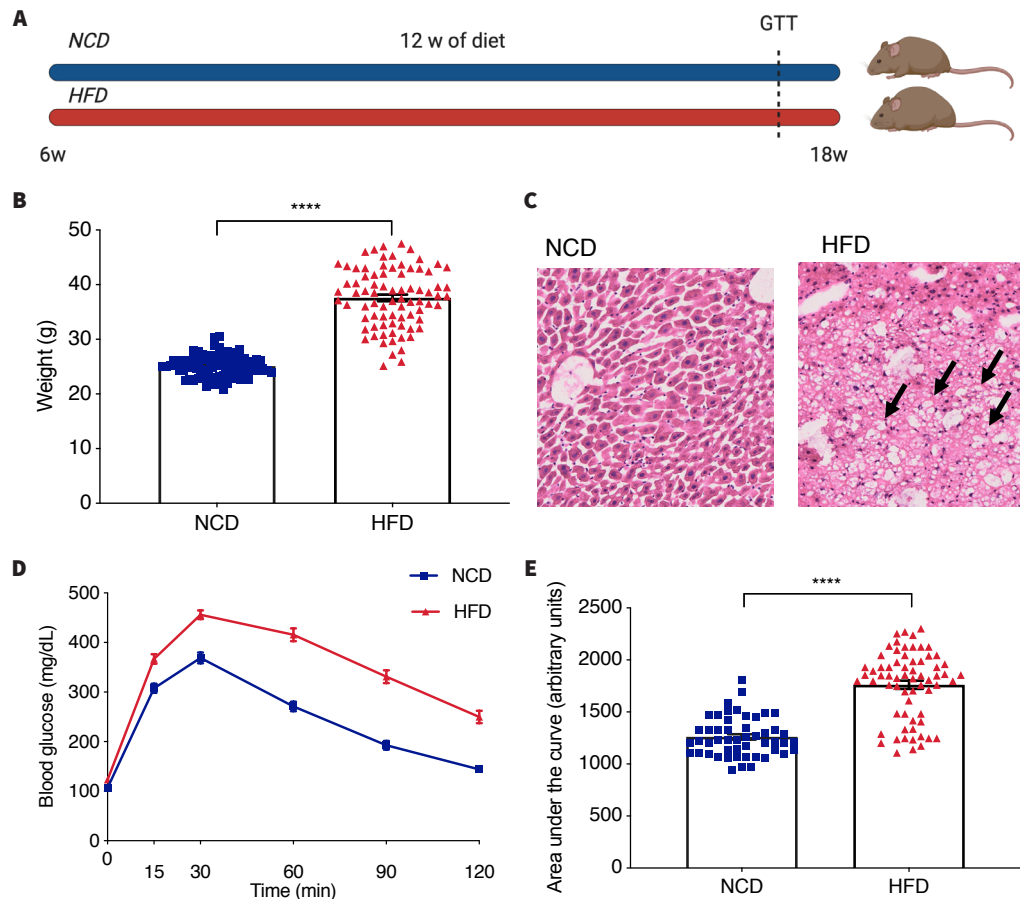


Figure 16. High fat diet is an efficient model for prediabetes. Metabolic characterization of prediabetic mice. **A.** Diet plan schematic representation. **B.** Statistical analysis of mice body weight. **C.** Hematoxylin-Eosin staining in liver, histological sections of NCD and HFD mice. White circles (black arrows) are lipid droplets, revealing steatosis in the liver of mice subjected to HFD. **D.** Glucose tolerance test (GTT) at different time points after glucose bolus (0, 15, 30, 60, 90, 120 minutes). **E.** Analysis of the area under the curve of the GTT. Results are expressed as mean $\pm$ SEM; $n=60$ for NCD and $n=70$ for HFD. Unpaired t test with Welch's correction for B and D; **** $p<0.0001$.

To validate the presence of EVs in the gut of the mouse model in study, we stained gut cross sections for CD81 as a commonly used EV-marker and the presence of EVs was detected in the intestine lumen in both conditions (Fig.17A). Moreover, western blot was performed to detect the enrichment of CD63 in GDE, in fact, CD63 was only detected in the GDEs, in comparison with gut tissue (Fig.17B). Both markers did not show differences in terms of amounts between the groups. Inclusive, we did not find any particular topographic profile looking to the CD81 signal in the gut, but that is something we would like to look into detail in the future.

Gut EVs were characterized by nanoparticle tracking analysis (NTA) and total amount of protein was quantified by BCA. The mean size of the particles obtained by NTA, in both conditions, was around 100 nm, what is consistent with the expected size of small EVs, specifically exosomes (Fig.17C). The only reason why we do not call our EVs exosomes is because we did not investigate their intracellular origin, namely endosomal origin. To be conservative we opted for referring to it as EVs. Moreover, it should be noted the presence, although in much smaller proportion, of larger vesicles (around 200 nm in diameter). While the number of particles did not change with diet (Fig.17D), protein cargo was higher in GDEs isolated from HFD-fed mice (HFD-GDE) than in the ones isolated from NCD-fed mice (NCD-GDE) (Fig.17D; E). The ratio of the two parameters gives the amount of protein per EV. Interestingly, GDEs isolated from prediabetic mice are packed with more protein when compared to controls (Fig.17F).

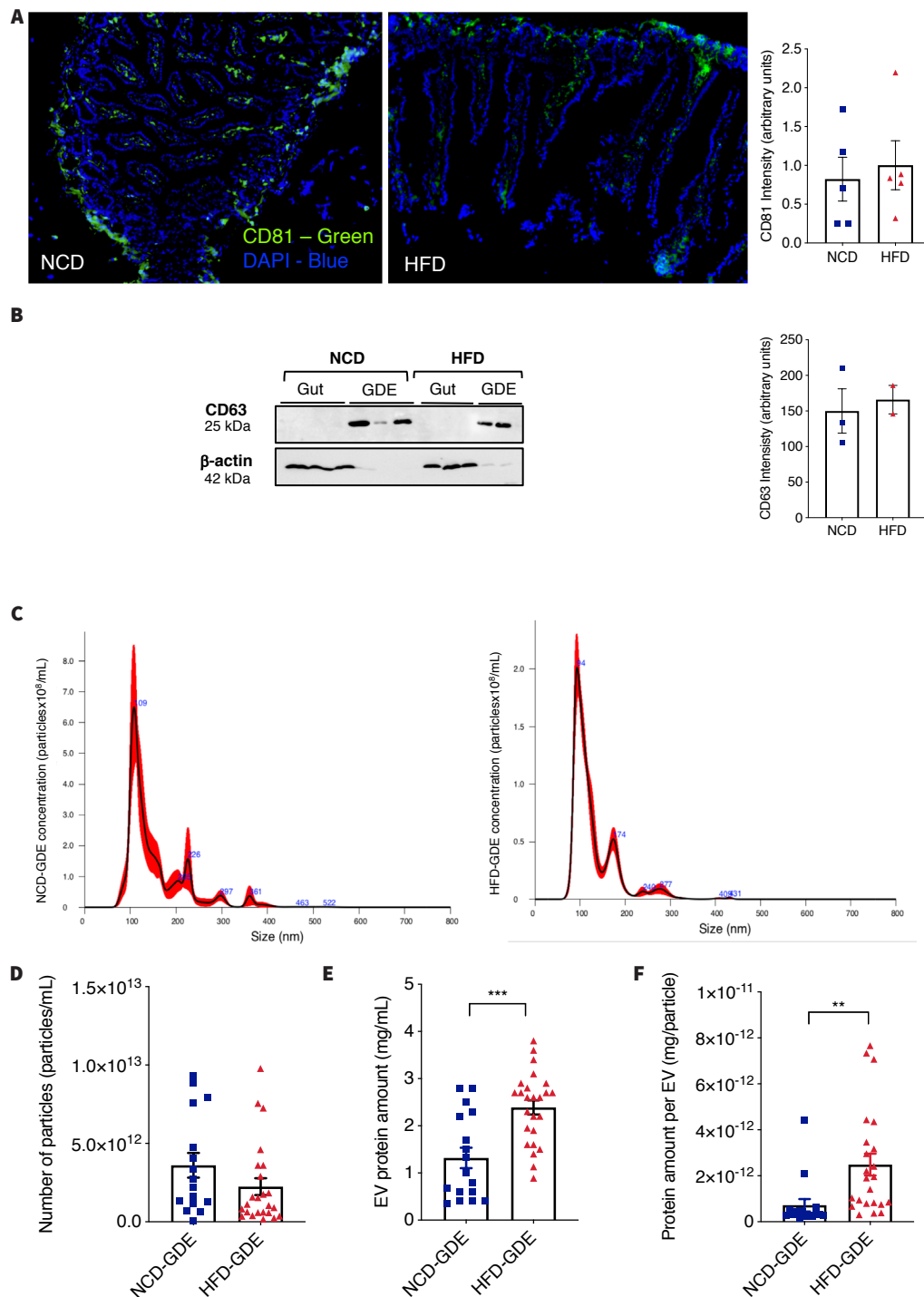


Figure 17. Prediabetes increases protein content/cargo in gut-derived EV. **A.** EVs labelled in green (CD81) and nuclei in blue (DAPI). NCD gut tissue in the left and HFD gut tissue in the right. Analysis of the CD81 quantification in the gut of injected animals. **B.** Immunoblotting of gut tissue and gut-derived EV (GDE) in NCD conditions and HFD. Protein extracts prepared from mice gut (10 µg) and from mice GDE (15 µg) were analyzed by western blotting using antibodies against EV marker CD63 or protein marker of other subcellular compartments (β-actin). Analysis of CD63 quantification of immunoblotting. **C.** Size and concentration distribution of GDE determined by nanoparticle tracking analysis (NTA). A representative NTA graph of GDE of mice fed with NCD (NCD-GDE) (left) and HFD (HFD-GDE) (right). **D.** Analysis of the number of particles per mL of sample. **E.** Protein quantification in GDE in mg per mL of sample, obtained by BCA. **F.** Protein content per each GDE, represented in mg of protein per particle. Results are expressed as mean ± SEM; n=17 for NCD-GDE and n=25 for HFD-GDE. Unpaired t test with Welch's correction (A, B, D, E, F); ***p<0.0005, **p<0.005.

The technological advance in equipment and in methods analysis are leading to a more precise understanding of multiple pathophysiological mechanisms. One of the research approaches that have been flourishing is proteomics. Importantly, EVs protein cargo provides innumerable information regarding the cellular environment where EVs were produced (Kalra, Drummen, and Mathivanan 2016). EVs proteomic studies have been applied to dig into cell biological and pathophysiological functions and demonstrated to be a rich source for identification of novel biomarker candidates for diseases (Brown 2008; Raimondo et al. 2011). The gold-standard in proteomics is mass spectrometry (MS) and, it is vastly used in EV studies with great success (Conde-Vancells et al. 2008). Essentially, the proteome is digested into peptides, followed by chromatographic separation and MS-based analysis, yielding a list of detected peaks characterized by their retention time (Matthiesen 2013).

We proposed that a diet induced dysmetabolism environment influences the protein composition of GDEs. Thus, to identify the impact of this milieu to GDE protein cargo, we isolated EVs from the guts of 20 mice under HFD and respective controls. GDEs were pooled in order to obtain enough material for further analysis by MS. The EVs pool was divided in three. Each technical sample was run three times, giving us a total of nine technical replicates. Obtained data was quantified and analyzed with bioinformatic tools (Fig. 18A).

Because we wanted to study the impact of gut in particular, without the effect of microbiota, we first evaluated if the isolated EVs were indeed coming from gut cells, and not from gut colonizing bacteria. In fact, we observed that almost every protein identified were from *Mus musculus*, with the other bars counting for homologous proteins (Fig.18B). In addition, by streaking an agar petri dish, in the absence of antibiotics, with the culture media where the guts were deposited overnight, we did not observe any bacterial growth, indicating that the isolated EVs were GDEs and not biota derived EVs (Fig.18C).

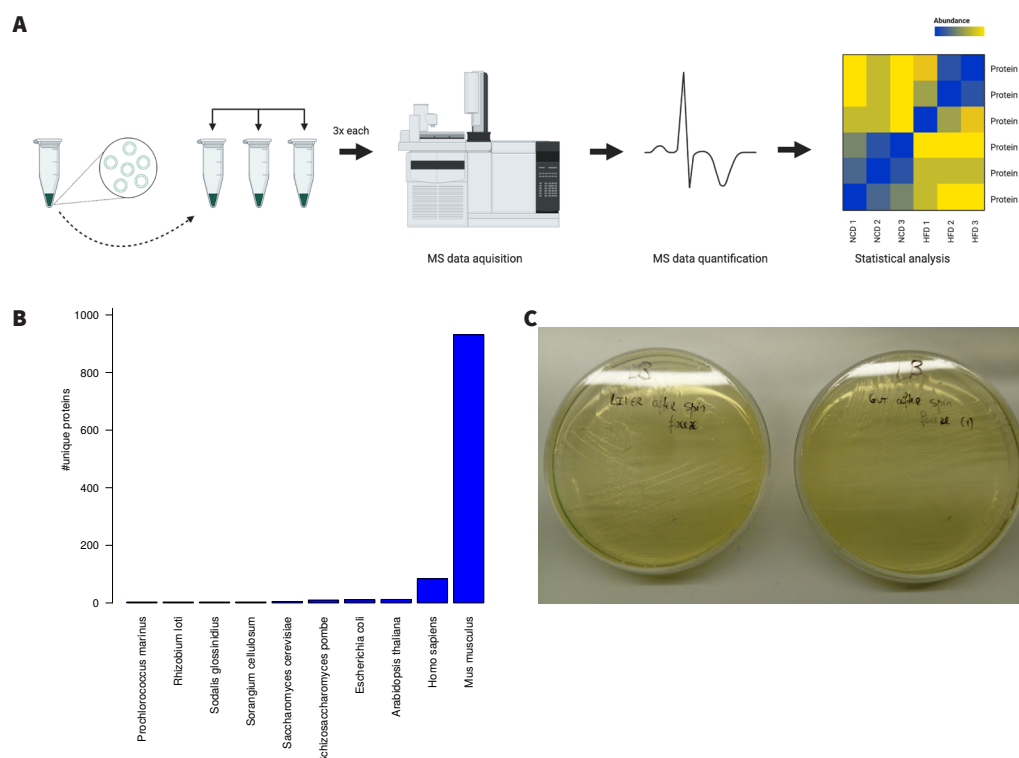


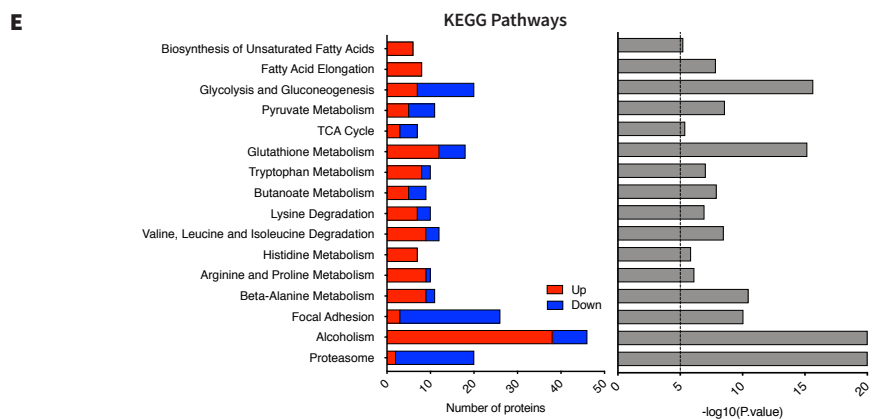
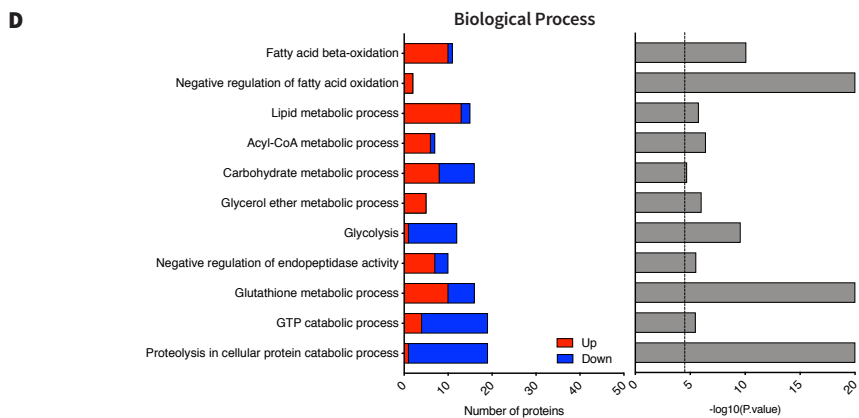
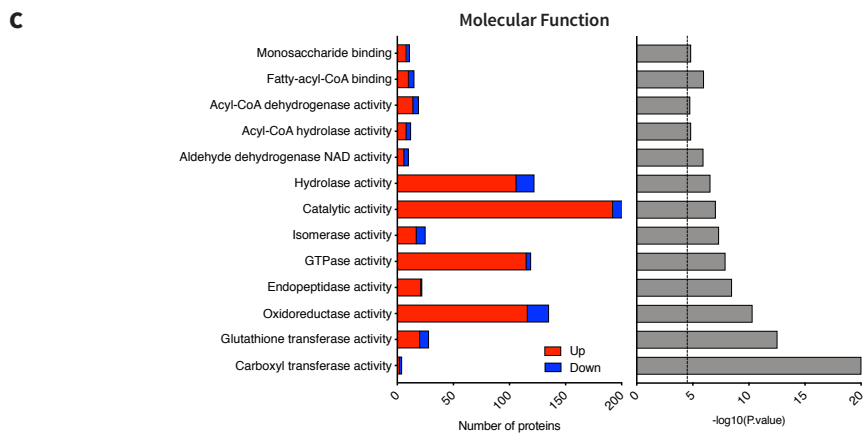
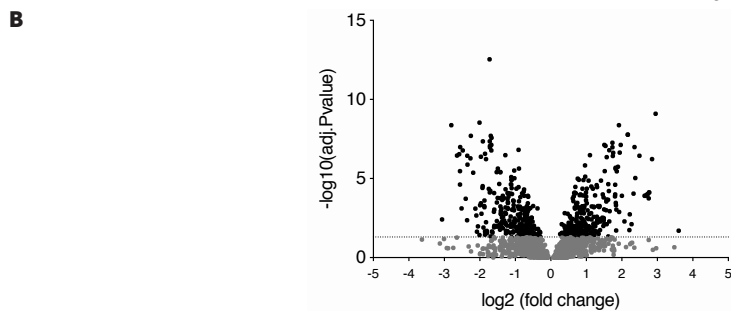
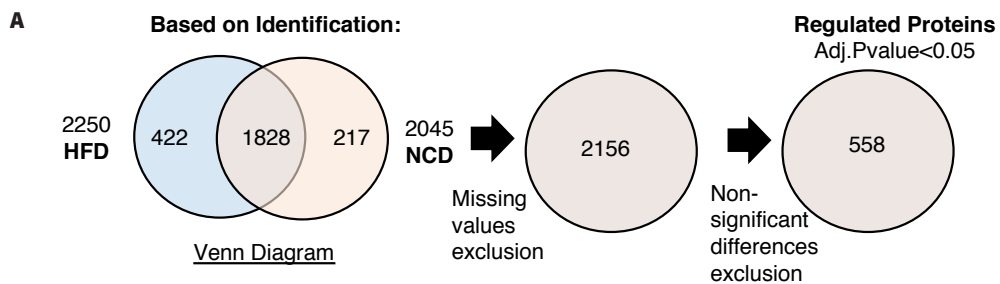
Figure 18. Study flow of proteomic approach for characterization of gut-derived EV protein cargos.

A. Schematic representation of the experimental process of analysis of gut-derived EV (GDE). **B.** Bar plot indicating the number of proteins uniquely identified from different species when searched against all proteins from all species in UniProt. **C.** Picture of LB-agar (without antibiotics) petri dish after 24 after hours of inoculation with media where guts were deposit overnight at 37°C prior to EVs isolation.

After filtrating the identified proteins from reverse proteins and possible contaminating proteins lead to a total of 2467 identified proteins, with 217 proteins predominantly identified in the NCD group and 422 in the HFD group. Excluding proteins with missing quantification values resulted in 2156 proteins. The raw data was then submitted to normalization (Fig.19A). We started by excluding every protein with missing values. The missing values are acquisition values of zero, among the same replica. In addition, we evaluated every protein in each group, and established that a protein is classified as unique in one group, based on abundance, importantly it does not exclude its expression in the other group. The Venn Diagram on Figure 19A represents the number of protein identification from each of the two sample conditions. After excluding proteins with missing quantitative values all the remaining proteins were identified in both conditions, so the two circles of the Venn Diagram culminated in one with a final list of 2156 identified proteins (Appendix Document). Subsequently, we distinguished between identified and regulated proteins. In the present study, we defined regulated proteins which reflect the difference between the two groups, as the ones that are at least two-fold of regulated and with an adj. p-value < 0.05. After applying the aforementioned criteria to our data set, we obtained a list of robustly regulated 558 proteins, suggesting that diet-induced prediabetes alters the abundance of many proteins in GDEs (Fig.19A). All data, before applying the filtering criteria, is represented in a volcano plot that describes the level of significance and magnitude of changes observed, comparing the HFD with the NCD group (Fig.19B). The dotted line in the plot represents the threshold of adj.p-value<0.05, and from that line above each individual dot represents a protein within the 558 regulated ones. Moreover, on the left the down-regulated proteins are grouped and, on the right, the up-regulated ones. We observed a similar number of down and up regulated proteins.

Afterwards, bioinformatics analysis was performed on the 588 regulated proteins. Gene Ontology (GO) and Kyoto Encyclopedia for Genes and Genomes (KEGG) enrichment analysis revealed that altered proteins are involved on distinct molecular functions, and consistently the vast majority are closely related with metabolism. In terms of molecular function, enzyme activity shows up in the top of list, isomerases for example catalyze reactions such as glycolysis and carbohydrate metabolism, while hydrolases are very common in lipid metabolism. Furthermore, oxidoreductase proteins are also up-regulated, these are mediators of an oxidation-reduction reaction, where the oxidation state of an atom within a molecule is altered (Fig.19C). Most of the enriched pathways for GO biological process emphasized lipid metabolism, highlighting FA β -oxidation. Curiously, glycolysis-related and proteolysis in cellular protein catabolic processes-related proteins are mostly down-regulated (Fig.19D). Considering KEGG pathways enrichment analysis, the affected proteins belong primarily to FA, carbohydrate and amino acid metabolism. Interestingly, alcoholism-related proteins show up with great significance (Fig.19E). In terms of down-regulate proteins, based on the KEGG analysis the proteasome and focal adhesions are markedly down-regulated. Finally, to assess sub-cellular localization of the GDE protein cargo regulated by diet we used GO cellular component analysis and found that the majority of altered proteins are mitochondrial, followed by the cytosolic ones. Once again, proteasome related proteins show up as down-regulated (Fig.19F).

For a more detailed characterization of GDE proteome, we looked into the subset of proteins that were overly altered among groups. For that, we established an adj.p-value<0.0001 as cut-off (Fig.20A). The proteins with that significance are represented in the heatmap. We found a variety of proteins that are up-regulated and others down-regulated in GDEs from prediabetic mice compared with control animals, suggesting that altogether these proteins can change the metabolism of the recipient cells upon EV uptake (Fig.20B).



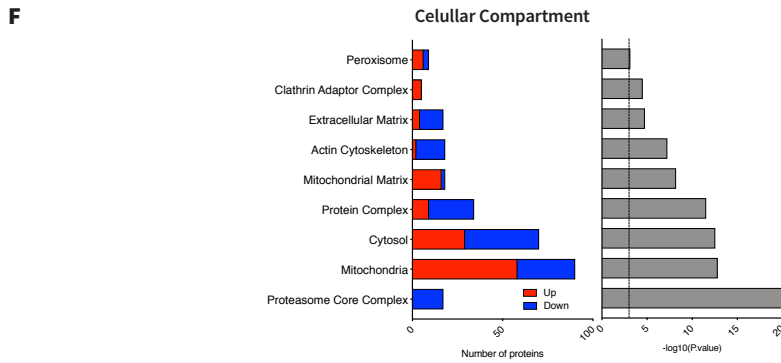
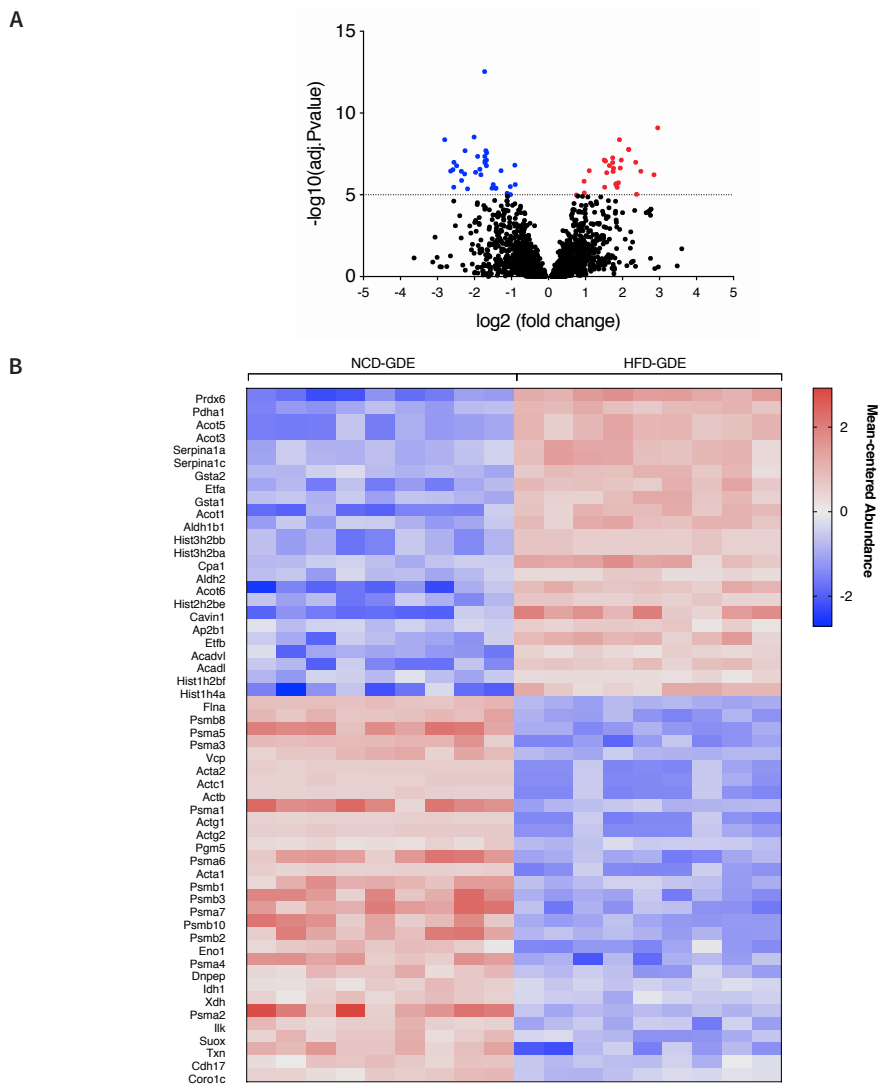


Figure 19. Characterization of prediabetic gut-derived EV by GO and KEGG enrichment analysis directly against mouse annotation, according to GO terms. A. Strategy used for selection of proteins to be analyzed with confidence. **B.** Volcano plot representing the fold change and adj.p-value in the gut-derived EV (GDE) isolated from prediabetic mice and control groups. x-axis represents fold change, and y-axis adj.p-value. Dotted line sets the threshold of adj.p-value<0.05. In grey are the non-regulated proteins, in black the 558 regulated proteins. **C.** Molecular function analysis of the GDE identified proteins. GO enrichment analysis according to Molecular Function. **D.** Analysis of the main biological regulated by the proteins identified in the GDE. GO enrichment analysis according to Biological Process. **E.** KEGG enrichment analysis for KEGG pathway. **F.** GO enrichment analysis according to Cellular Compartment. For C, D, E and F; on the left panel, changes are displayed as the number of proteins with increased (red) or decreased (blue) levels (horizontal axis). On the right panel, the horizontal axis indicates the significance $-\log_{10}(p\text{-value})$ of the functional association, which is dependent on the number of submitted proteins in the class, the number of proteins annotated in the class, the total number of submitted proteins and the total number of mouse proteins.



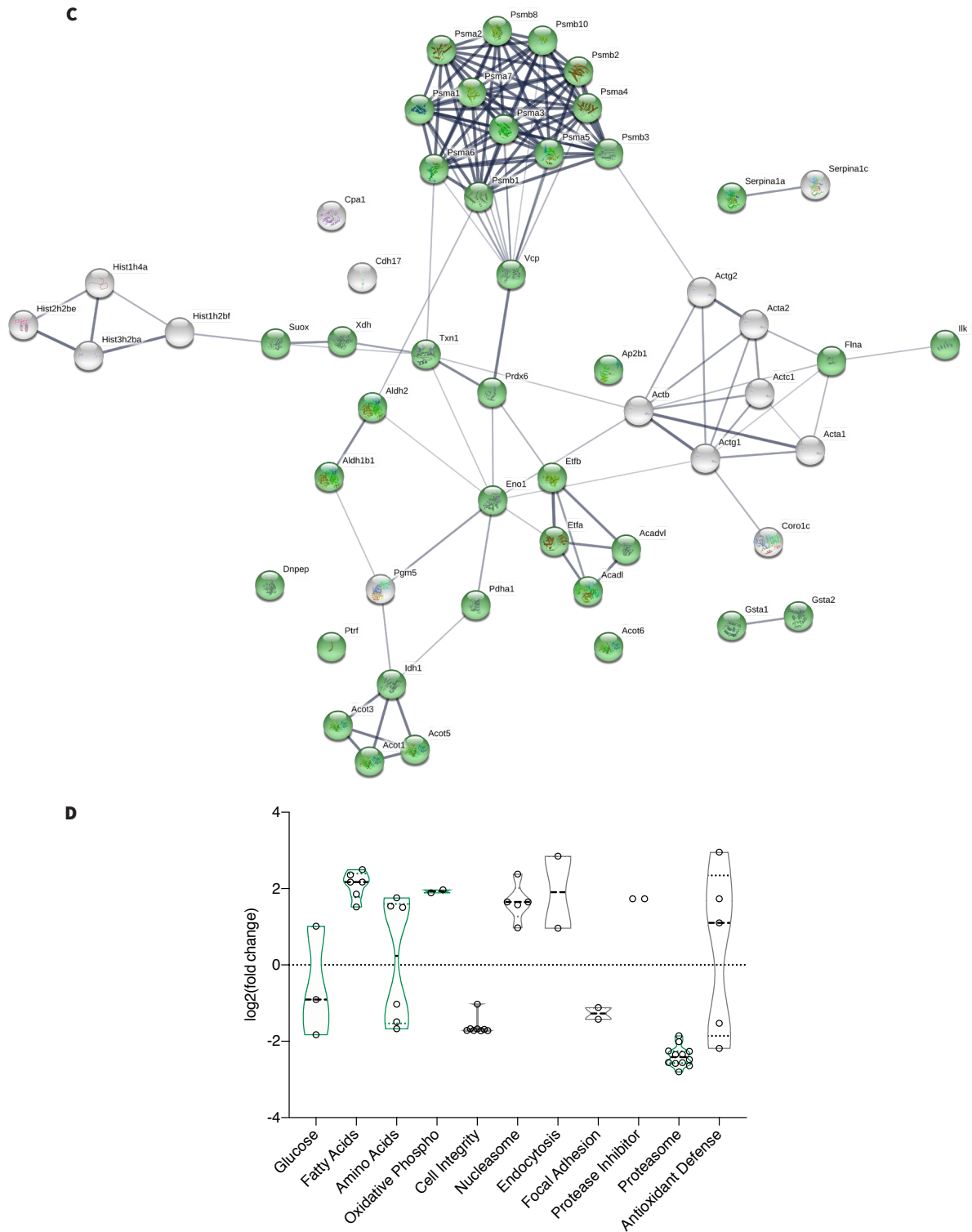
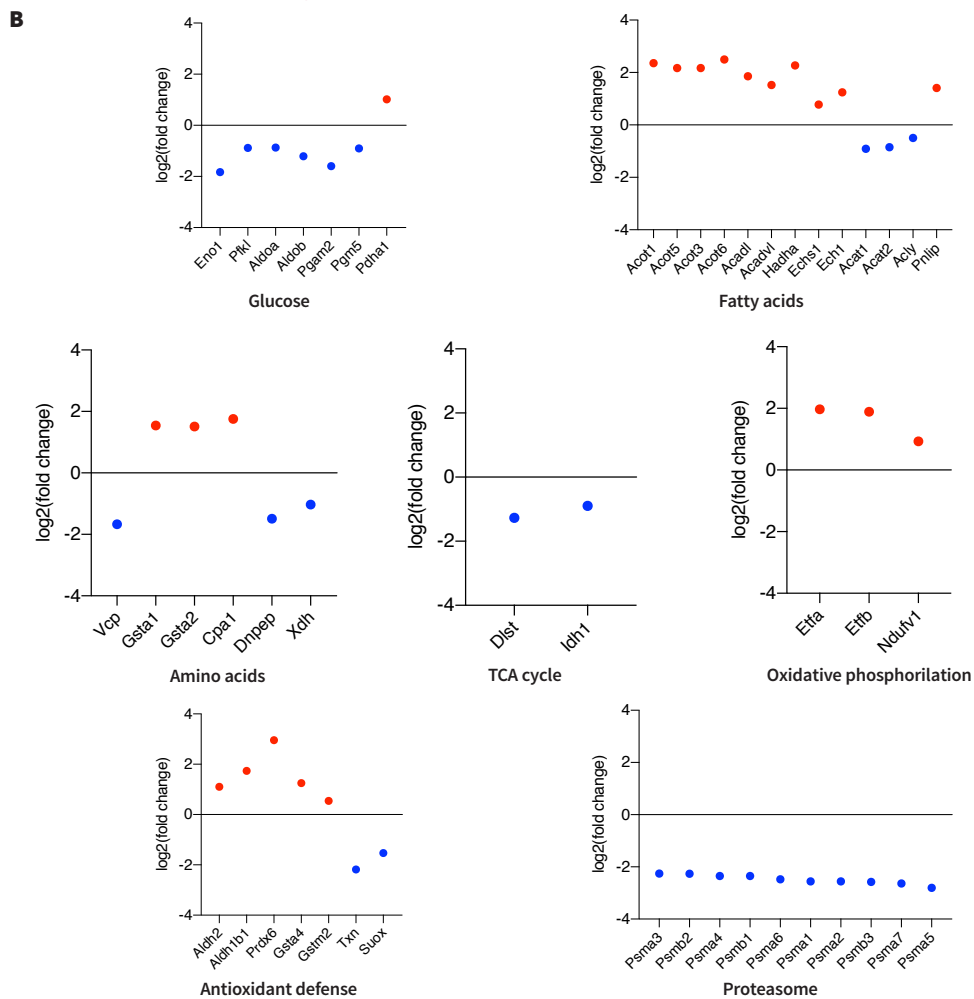
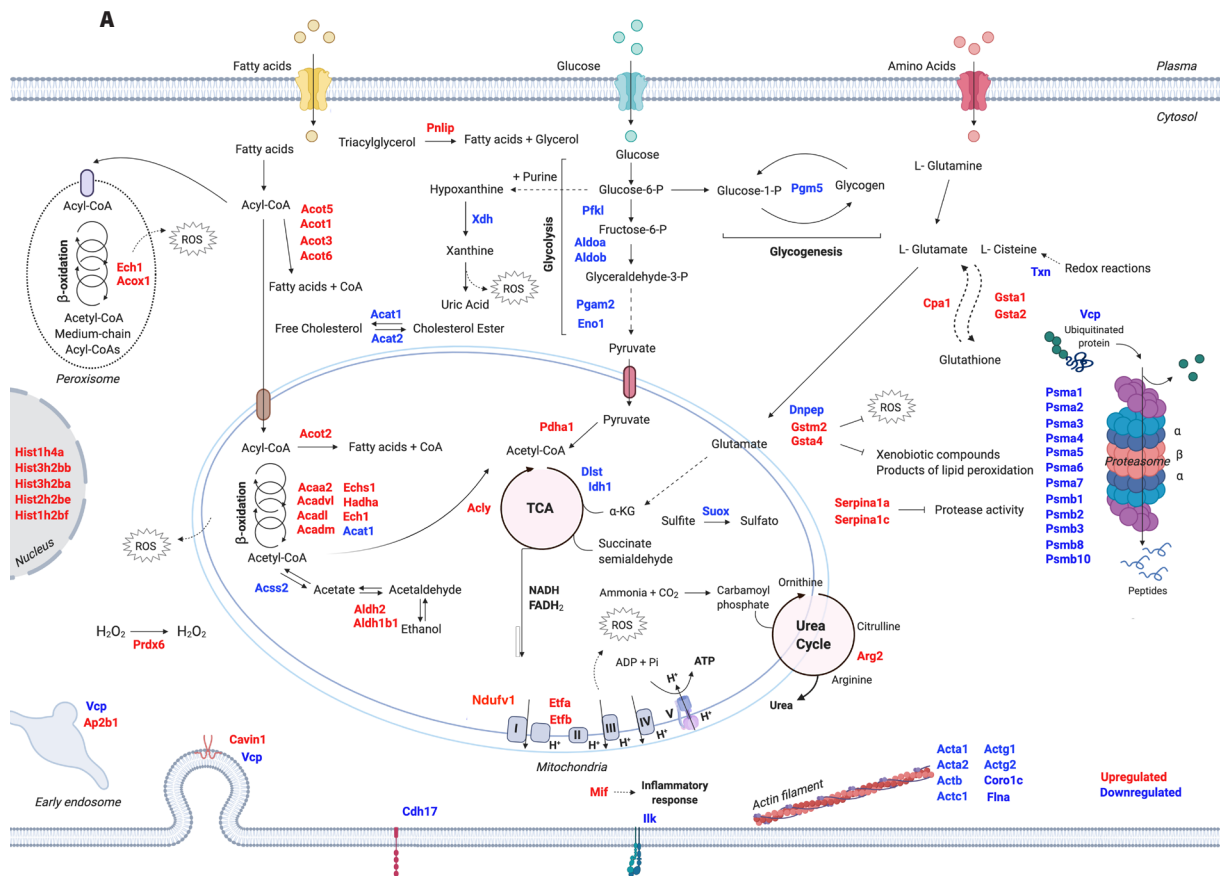


Figure 20. Cluster analysis of the top50 altered proteins of gut-derived EV in prediabetic mice. A. Volcano plot representing the fold change and adj.p-value of gut-derived EV (GDE) proteins isolated from prediabetic mice and control groups. x-axis represents fold change, and y-axis adj.p-value. Dotted line sets the threshold of adj.p-value<0.0001, highlighting the most altered proteins in GDE from HFD comparing with GDE from NCD. In blue are the most down-regulated proteins and in red the most up-regulated. **B.** Heatmap representing mean-centered abundance of the nine technical replicas for the most altered proteins decorated in the previous volcano plot. **C.** Protein-protein interaction network. The network was built using the STRING online software with a medium confidence level (0.4). Each circle represents one protein. The line thickness indicates the strength of the evidence, with thicker connections indicating higher confidence in the protein-protein interaction. Green circles represent the proteins belonging to cellular metabolic process according to GO enrichment analysis for biological processes. **D.** Violin plot demonstrating the fold changes of the most altered proteins and correspondent cellular functions. Cellular metabolic process (GO) represented in green.

To gain insight into protein-protein interaction of GDE identified protein cargo, we used the STRING software to link some of the altered proteins and to display the level of relationship between them. The proteins that demonstrate higher level of interaction are the ones related with the proteasome, both α subunits (PSMA) and β (PSMB). Actin related proteins which function is to maintain cellular integrity (ACTA1, ACTA2, ACTB, ACTC1, ACTG1, ACTG2, CORO1C, FLNA, MYL6, MYL6B) also display relevant interaction. Moreover, network analysis highlights strong bias towards metabolic-related proteins, which are represented by green circles (Fig.20C). The fold change of all altered proteins varied up to an approximately 2-fold difference in abundance when comparing HFD with NCD EVs content. Importantly, looking into metabolic pathways, the altered proteins are mainly involved in nutrient metabolic pathways, such as glucose, FA and amino acid, being the FA pathway represented only by up-regulated proteins, on the contrary, cell integrity, focal adhesion and proteasome-related proteins are all down-regulated (Fig.20D).

For proper visualization of subcellular origin of the key proteins regulated by the diet and packaged inside GDE, Figure 21A illustrates the most altered ones according to their fold change. Data is displayed in terms of HFD compared with NCD, meaning that when a certain protein is up or down regulated, this is in the HFD-GDE versus NCD-GDE. These observations demonstrate that, in fact, GDEs mirror the environment of the cells of origin. In Figure 21B it is represented the significant fold change for each protein in the image, according to its most associated pathway. Naturally, there is some level of functional homology, since the same protein can play role in diverse pathways. Noteworthy to mention, carbohydrate metabolism, which ensures the supply of energy to the cells, has several proteins affected but mainly down-regulated (PFKL, ALDOA, ALDOB, PGAM2, ENO1, PGM5). Contrary to the proteins related with lipid homeostasis (ACOT1, ACOT2, ACOT3, ACOT5, ACOT6), FA mitochondrial β -oxidation (HDHA, ACADVL, ACADL, ECHS1, ACAA2) and very-long chain FA peroxisomal β -oxidation (ECH1, ACOX1) that are all up-regulated. On the other hand, proteins involved in TCA cycle are down-regulated (DLST, IDH1). Moreover, cholesterol homeostasis related proteins are down-regulated (ACAT1, ACAT2). Metabolic pathways of amino acids are also greatly affected, namely glutathione (GSTA1, GSTA2). In the trafficking front, endocytic proteins are being up-regulated, namely AP2B and CAVIN1. Proteasomal proteins are consistently down-regulated, with a big portion of proteins altered, and all of them significantly down-regulated (PSMA1, PSMA2, PSMA3, PSMA4, PSMA5, PSMA6, PSMA7, PSMB1, PSMB2, PSMB3, PSMB8, PSMB10). Finally, proteins related with antioxidant defense are majorly up-regulated (ALDH2, ALDH1B1, PRDX6, GSTA4, GSTM2). Table 1 describes all the proteins up- and down-regulated according to its biological function.

In summary, our proteomics approach revealed that in GDEs isolated from a diet-induced prediabetic mouse model, the top regulated pathways are the metabolic, particularly pathways related with nutrient sensing and utilization.



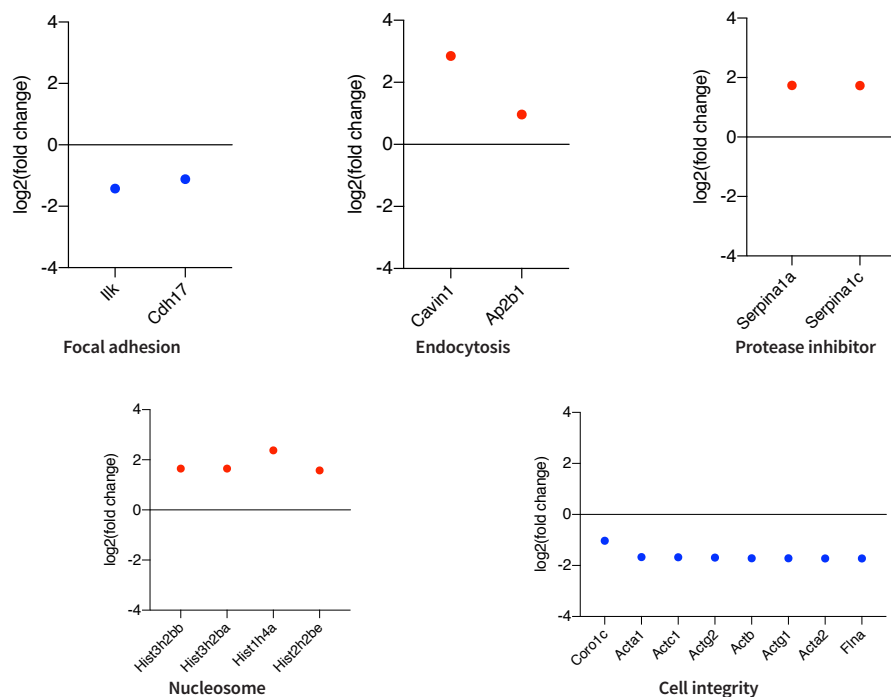


Figure 21. Overview of prediabetes impact on gut-derived EV proteome. A. The identified proteins with higher differential expression are represented in the image according to their function and estimated subcellular localization. Red color for proteins up-regulated and blue color for proteins down-regulated in gut-derived EV of mice fed with HFD compared with controls, mice fed with NCD. ROS, reactive oxygen species; α -KC, α -ketoglutarate; TCA, tricarboxylic acid cycle; I, II, IV and V, complexes of mitochondrial oxidative phosphorylation (I-IV: respiratory chain complexes; V: ATP synthase complex); Pi, inorganic phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; α and β , proteasome subunits. The detailed description of protein names is shown in Abbreviations List. **B.** Data plots demonstrating the log fold changes of each protein in the image in accordance to its related pathway.

Biological Function	Up	Down
Glucose metabolism		
Glycolysis		PFKL; ALDOA; ALDOB; PGAM2; ENO1; PGM5
Conversion of pyruvate to acetyl-CoA	PDHA1	
Amino acid metabolism		
Glutathione metabolism	GSTA1; GSTA2	
C-terminal amino acid release	CPA1	
Protein degradation		VCP
Peptide metabolism		DNPEP
Purine metabolism		XDH
Lipid metabolism		
Mitochondrial β -oxidation	HADHA; ACADVL; ACADL; ECH1; ECHS1	
Peroxisome β -oxidation	ECH1; ACOX1	
Lipid homeostasis	ACOT1; ACOT2; ACOT3; ACOT5; ACOT6	ACSS2
Lipid biosynthesis	ACLY	
Cholesterol metabolism		ACAT1; ACAT2
Triglyceride metabolism	PNLIP; ACOT	
Tricarboxylic acid cycle		DLST; IDH1
Oxidative phosphorylation	ETFA; ETFB; NDUFV1	

Biological Function	Up	Down
Sulfur oxidative degradation		SUOX
Urea cycle	ARG2	
Antioxidant defense	PRDX6; GSTA4; GSTM2; ALDH2; ALDH1B1	TXN
Proteasome complex		PSMA1; PSMA2; PSMA3; PSMA4; PSMA5; PSMA6; PSMA7; PSMB1; PSMB2; PSMB3; PSMB8; PSMB10
Protease inhibitor	SERPINA1A; SERPINA1C	
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
Inflammatory response	MIF	

Table 1. Biological function of the main up- and down- regulated proteins in HFD-GDE compared with NCD-GDE by proteomics. The detailed description of protein names is shown in Abbreviations list.

1.2. Proteomic comprehensions on plasma EV of prediabetic mice

Once the route used by EV for mobilization is the circulatory system, next we studied how much of the proteome of EV traveling in the blood meet the one from GDEs. Therefore, we isolated plasma EVs from the same prediabetic mice model and respective controls. Blood is first fractioned into plasma (Fig.22A). Plasma EVs were analyzed in the NTA, that revealed increased number of particles in HFD mice (Fig.22B), and the BCA protein quantification also demonstrated that the same mice had increased amount of protein (Fig.22C), that culminated in similar average amount of protein per EV in both groups (Fig.22D).

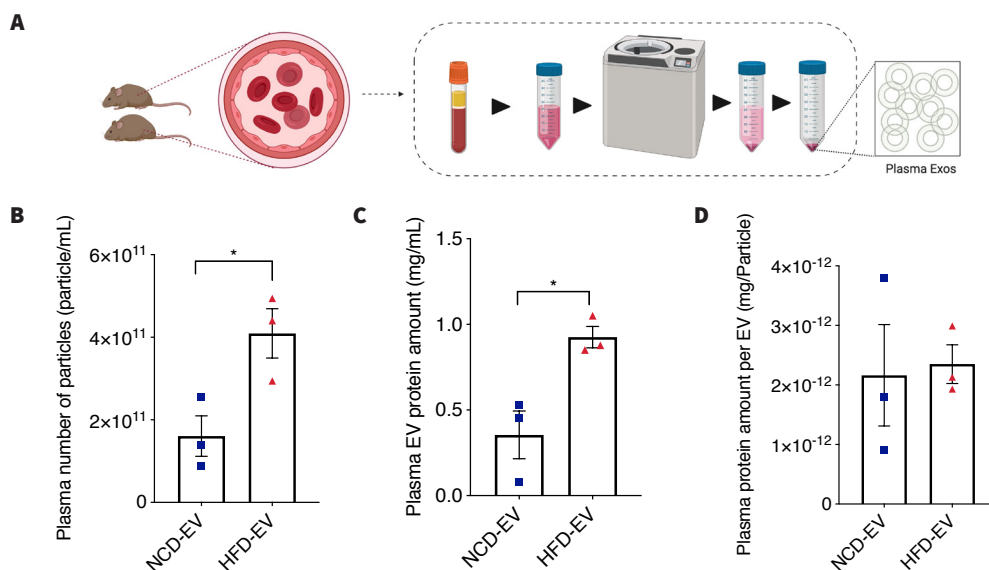


Figure 22. EV isolation and quantification in plasma of mice fed with NCD and HFD. **A**. Schematic representation of plasma EVs isolation. **B**. Quantification of the number of EVs per mL of plasma. **C**. Quantification of amount of protein in plasma EVs, in mg per mL of sample. **D**. Representation of the amount of protein per EVs of plasma, resulting from the ratio of B and C. Results are expressed as mean $\pm$ SEM; Each n is a pool of 20, n=3 for NCD and HFD. Unpaired t test with Welch's correction. *p<0.05

MS studies were performed in plasma EV in order to trace the proteome profile and link with gut observations. Using the same exclusion criteria of gut EV, 161 proteins were identified in plasma EV. From those, 129 proteins were in common with gut (Fig.23A). The co-expressed proteins are highlighted in red in the volcano plot (Fig.23B).

An analysis of the distribution of biological processes related with all proteins identified was done by PANTHER software, based on GO annotations, for plasma EV (Fig.23C) and for gut EV (Fig.23D), in order they could be compared side-by-side. We can see that in both tissues the EV proteins are predominantly related with metabolic processes, with a small increment in the case of gut EVs (Fig.23C). The level of significance for our plasma EV data was much inferior to the gut. One of the reasons is because we only had two replica of plasma EVs. Therefore, we established a threshold of $p\text{-value} < 0.9$ for regulated proteins. By studying the overlap between regulated proteins in plasma and gut, the list of proteins with similar variance are plotted in Figure 23D.

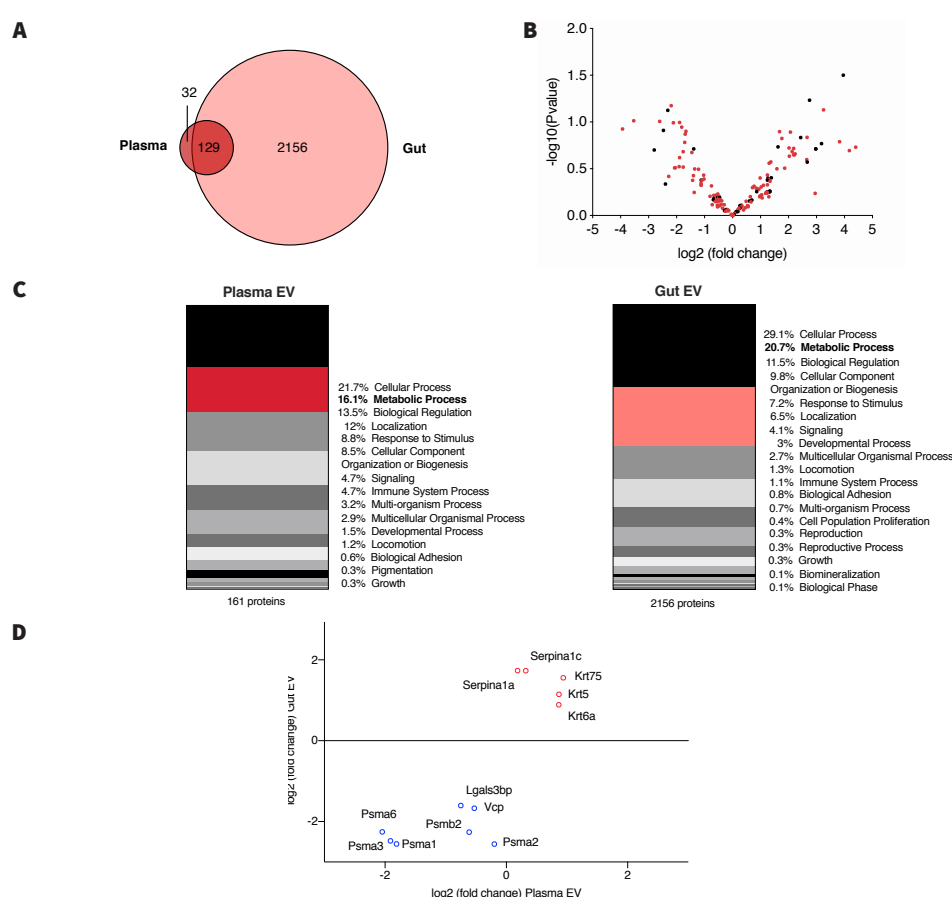


Figure 23. Proteome analysis of plasma EV. A. Venn diagram demonstrating the intersection of plasma EV-identified proteins with gut EV-identified proteins. B. Univariate, significance (p-value) vs. fold change analyses representing all identified proteins in plasma EVs. Each dot symbolizes a protein. The red dots represent the proteins identified in both tissues. C. Percental distribution of biological processes related with proteins identified in plasma EV (left). Percental distribution of biological processes related with proteins identified in gut EV (right). Analysis performed using the PANTHER online tool, based on GO annotations for biological processes. D. Relation of fold change variation of regulated proteins in plasma EVs and gut EVs. Red circles highlight up-regulated proteins on EV of both tissues in HFD scenario and blue circles highlight down-regulated proteins on EV of both tissues in the same condition. The detailed description of protein names is shown in Abbreviations list.

Discussion

Today's modern society has witnessed a profound change in lifestyle and diet. In fact, changes have been seen towards the over-consumption of processed fatty and sugary foods, combined with a decreased intake of fruits and vegetables. These changes in dietary habits are widely accepted as the so-called western diet, viewed as one of the main culprits in the exponentially increased of obesity rates. Moreover, western diet consumption is strongly associated with a general pro-inflammatory state with deleterious consequences in organismal homeostasis (Losacco et al. 2018). Therefore, the relationship between dietary habits and the incidence of multiple metabolic disorders is one of the main public health concerns. One of the first organs to contact with food and its derivatives is the gut, as a consequence it is where the first organismal response should be found. Fundamentally, the gut is the first sensing organ to exogenous energy sources (Abumrad and Davidson 2012). The impact of diet on gut microbiota and consequently in its metabolites is well described in the literature (Zhang and Yang 2016). Likewise, western diet-induced obesity considerably alters intestinal flora (David et al. 2014) resulting in IR and metabolic diseases (Saad, Santos, and Prada 2016). How western diet-induced obesity mechanistically trigger prediabetes, partially independent of gut microbiota, is still elusive. Here, we report, for the first time, that GDE isolated from animals fed with HFD have a distinct proteome that strongly reflects the intestinal dysmetabolic milieu.

The present findings show that the GDE proteome reflects the capability of the intestine to sense dietary regimens and mirrors its metabolic alterations. Interestingly, in GDE from prediabetic animals, we found numerous pathways associated with macronutrients altered. Several lines of evidence support a model in which the metabolic alterations induced by the HFD are translated into alterations in GDE protein cargo. For simplicity, we will discuss it by relevant groups in metabolic profiles.

Carbohydrate metabolism

Our data revealed that glucose metabolism is disturbed, in particular glycolysis, the enzymes participating in the first steps of the pathway were found downregulated, namely phosphofructokinase (PFKL); phosphoglycerate mutase (PGAM2), aldolase A and B (ALDOA and ALDOB), β -enolase (ENO1) (Tarnopolsky 2018) (Fig.21). However, down the glycolysis pathway, pyruvate dehydrogenase (PDHA1) was upregulated, indicating a possible compensation in the pathway given the importance of acetyl-CoA for the TCA cycle and in many other biochemical reactions in protein and lipid metabolism. From the enrichment analysis we observed that both glycolysis-gluconeogenesis pathway and pyruvate pathway appear in the top of the most affected routes (Fig.19D; E).

The decreased in glycolysis might be explained by the low amount of fibers provided by the diet; since the percentage of fibers in HFD is considerably reduced and in part replaced by sucrose. In accordance, fibers have a beneficial effect on NCD mice, by improving intestinal integrity, enhancing energy expenditure and reducing inflammation (Jangra et al. 2019). In fact, because HFD has increased sucrose, we envisage that sucrose is being directed to an alternative pathway, such as DNL, known to also occur in the gut (Hoffman, Alvares, and Adeli 2019).

Amino acid metabolism

The gut is the place where amino acids are digested from dietary protein. Even though GDE were released exclusively from intestinal cells (Fig.18B; C), we cannot exclude the impact of gut microbiota

metabolites in the cell. Thus, this might explain why we see proteins classically related with bacterial metabolites pathways being affected in the GDE (Gu et al. 2019). That is the case of short-chain fatty acids (SCFA) and amino acids that derive from bacterial activity in the gut (Neis, Dejong, and Rensen 2015). The KEGG analysis revealed several pathways associated to essential and non-essential amino acids to be affected (Fig.19E). Amino acids are particularly important in the survival and growth of bacteria, but also controlling energy and protein homeostasis (Neis, Dejong, and Rensen 2015). Upon uptake by bacteria, amino acids can be directly assimilated into bacterial cells or catabolized. Lysine, whose pathway we see affected in HFD-GDE, is greatly used by bacteria (Stoll et al. 1998); alternatively it intermediates protein biosynthesis, such as carnitine, a key factor in FA metabolism (Azevedo and Saiardi 2016). Interestingly, carnitine deficiency due to poor lysine diet induces triglycerides accumulation (Khan and Bamji 1979).

The amino acids derived from microbial protein fermentation can also serve as precursors of SCFA synthesis, for example lysine fermentation pathway leads to the production of butanoate (Van Nielen et al. 2014; Kreimeyer et al. 2007) that we also see, in our analysis, to have its related metabolic proteins greatly affected. Butanoate is absorbed by the intestinal epithelium and is used by liver cells for gluconeogenesis and cholesterol synthesis (Neis, Dejong, and Rensen 2015).

In HFD-induced obesity and T2D there is a marked increase in the portal concentrations of several essential amino acids, particularly the branched-chain amino acids (BCAA), valine, leucine and isoleucine (Lian et al. 2015; J. Wang et al. 2019). From our data we cannot ascertain amino acids and SCFA levels; however their pathways are definitely affected in HFD mice, one more evidence of diet impact on intestinal microenvironment (Oliphant and Allen-Vercoe 2019) (Fig.19E).

Lipid metabolism

In accordance with lipidic diet content, we observed that the lipid metabolism is massively altered, especially with many proteins being upregulated. Not only GO and KEGG enrichment analysis revealed a dominant number of pathways related with lipid metabolism, such as biosynthesis of unsaturated FA and FA elongation, but also among the top 50 most affected proteins are many lipid related-ones.

The excess of fat in the diet is known to cause increased uptake of FA into the cells, which can be either metabolized for the production of energy or, when in excess, will be stored. Our findings that several proteins inside the HFD-GDE involved in mitochondrial β -oxidation (HADHA, ACADVL, ACADL, ECH1, ECHS1) and peroxisome β -oxidation (ECH1, ACOX1) are upregulated, suggest that fatty acids are favored as energy source over glucose. Over-activation of those lipid metabolism related pathways is a common feature of obesity and T2D, leading to a state of hyperglycemia (Deng et al. 2010; Kanuri et al. 2018; Knebel et al. 2015)(Fig.21). Additionally, we identified a family of acyl-CoA thioesterases (ACOTs) particularly up-regulated. The FA, upon entry in the cell, can be directed to the TCA cycle, and produce ATP, or alternatively can be stored. ACOT are intermediaries in that decision, directing FA to be stored (Wang et al. 2018; Steensels et al. 2020). In this crossroad, ACSS2 is the protein that conducts FA to the TCA cycle pathway, and that we found downregulated in HFD-GDE. In addition, we saw that PDHA1, responsible for pyruvate degradation and sending it to TCA cycle, is upregulated. Altogether, this fits the idea that HFD regimen shifts the burden of energy provision from carbohydrates toward fat derived from diet, since ACSS2 is downregulated and PDHA1 is upregulated in order to ensure the energetic need of the cell. Based on recent literature, obesity-induced activation of ACOTs directs FA towards TG synthesis for incorporation into chylomicrons or VLDL particles (Alves-Bezerra et al. 2019; Ersoy et al. 2018). Indeed, from our results we conclude that intestinal cells are facing an overload of FA leading

to over-activation of lipid related metabolic pathways. Interestingly, some studies consider activation of ACOT protective against FA over-supply, slowing the flux of FA to downstream metabolic pathways (Yamaguchi et al. 2007; Fujita et al. 2011; Franklin, Sathyanarayan, and Mashek 2017). Whether this is the case or not needs to be further explored.

Additionally, intestinal cells in the HFD model appear to prioritize production of energy from FA, instead of carbohydrates. This is shown by the upregulation of ATP-citrate lyase (ACLY), an enzyme responsible for the conversion of citrate to acetyl-CoA – the first step in DNL.

Oxidative milieu

Increased FA oxidation promotes mitochondrial dysfunction, as well as FA peroxidation and glucose dysmetabolism, altogether leading to reactive oxygen species (ROS) accumulation to pernicious levels (Kuschner 2017). The excessive release of ROS, superior to the endogenous antioxidant capacity, is an indicator of oxidative stress. Oxidative stress causes macromolecular damage and is implicated in various disease states such as atherosclerosis, cancer, neurodegeneration, and diabetes (Luo et al. 2017). Coincidentally we observed several proteins involved in antioxidant defense mechanisms such as removal of free radicals (PRDX6) and elimination of xenobiotic compounds and products of lipids peroxidation (GSTA4, GSTM2) up-regulated in HFD-GDE (Fig.21). It is widely recognized that intracellular hyperglycemia, which we speculate to occur in gut cells due to the diet, promotes production of ROS (Volpe et al. 2018), therefore the observed activation of an antioxidant response to fight ROS elevation in such cells.

Surprisingly, by KEGG analysis it was possible to observe profound alterations in alcohol related pathways, namely the upregulation of the numerous isoforms of the aldehyde dehydrogenase (ALDH) family. ALDHs are a family of oxidizing enzymes responsible for cellular detoxification, differentiation and drug resistance by oxidation of biogenic and xenogenic aldehydes (Yang et al. 2017), commonly associated with ethanol consumption. Consistent with our model, ALDHs are responsible for detoxifying aldehydes that accumulate through metabolism, to which we are exposed from the environment or in certain disease states, such as diabetes (Chen et al. 2014). In particular, ALDH1B1 role was highlighted since its ability to metabolize acetaldehyde enables the maintenance of glucose homeostasis (Singh et al. 2015). Of interest, ALDH2 has been explored as a pharmacological target, due to its role in cellular damage defense against oxidative stress induced by pathological conditions, for instances high glucose (Chen et al. 2014). This piece of information led us to disclose that, in fact, food choices may be as toxifying as alcohol, and the organism uses the same weapons to fight both damaging effects. Besides, it is known that in absence of oxygen and as a result of yeast fermentation, production of ethanol from glucose is observed (Aslankoohi et al. 2015). Whether the biota has a role in the modulation of ALDH family needs to be unveiled.

Proteasome

Lastly, evidence that the HFD not only induced obesity, but also a pathological state arises from the massive alteration of proteasome-related proteins. In fact, these proteins were all indisputably down-regulated (Fig.20). The proteasome recognizes unfolded and damaged proteins eliminating them by degradation, therefore gained attention as an important target in many diseases (Amir, Weber, Beard, Bomyea 2008). By degrading several cellular proteins, the proteasome controls many other processes,

e.g. cell cycle, transcription, signaling, trafficking, protein quality control and amino acid homeostasis (Rousseau and Bertolotti 2018). Normally, dietary amino acids constitute 20% of the amino acid supply to build the proteins an adult requires, and the remaining 80% of amino acids comes from the recycling of amino acids following protein degradation (Kaushik and Cuervo 2018). Our data clearly shows the proteasome to be impaired what results in a defective degradation of proteins, an hallmark of disease (Rousseau and Bertolotti 2018). However, it is necessary to further validate if the alterations in GDE are truly mirrored in the cell.

We conclude that diet composition is definitely a game-changer on the cell's background. Furthermore, diet composition will be mirrored on metabolic/proteomic profile of the GDE that are continuously being produced. Overall, from the observed alteration in GDEs we speculate that in the gut upon HFD glucose metabolism is jeopardized in favor of FA metabolism; there is an impairment on amino acid synthesis that results in increased oxidative stress inside the cell; and to counteract this oxidative stress one would expect the proteasome to be upregulated but this is not the case, probably if we had analyzed an early time point of diet we would have observed an up-regulation of proteasome components. The down regulation of proteasome proteins suggests an inability to detoxify the cell of misfolded proteins, leading to increased accumulation of toxic molecules and disease progression.

Plasma EVs

Parallel to GDE proteome characterization, we tested the possibility of using plasma EVs as liquid biopsies in prediabetes. This would allow us to predict gut malfunction, thus providing an early biomarker of the disease. Considering EVs' role as communication vehicles, we hypothesize that enterocytes sense and translate the energetic clues given by the diet into alterations in GDE protein cargo. Changes in GDE protein cargo will propagate the diabetogenic message to other organs, thus promoting disease progression. Therefore, we evaluated which of the proteins present in GDE overlap with the ones in plasma EVs. In a preliminary approach, we searched for a biomarker of dysmetabolic features by performing a comprehensive and comparative analysis of the proteome of HFD plasma EVs to the ones derived from control animals. Interestingly, we observed an altered metabolic footprint in plasma EVs from HFD mice, when compared with NCD plasma EVs (Fig.25).

We found a small group of proteins that were similarly altered in GDE and plasma EVs. The Serpin family (SERPINA1A, SERPINA1C) and Keratin family (KRT5, KRT6A, KRT75) were both upregulated. Serpins are protease inhibitors, with some of its family members presenting proteolytic activity against insulin. In particular serpinB1 was proposed to mediate hyperinsulinemia, an hallmark of prediabetes (Glicksman et al. 2017; Xu et al. 2019), highlighting the idea that EV mirror the metabolic state of the organism.

Keratins are fibrous structural proteins giving stability and protection from cell stress (Xu et al. 2019). Keratins' upregulation in EVs may be counteracting the oxidative stress to which cells are exposed, helping to reshape the cell. While the particular keratins found altered in the present study have not been associated to diabetes, keratin 8 is known to be responsive to glucose and participates in the regulation of pancreatic β cells, in T2D phenotypes (Alam et al. 2013).

On the contrary, we found down-regulated, valosin containing protein (VCP), galectin 3 binding protein (LGALS3BP) and several proteasome proteins (PSMA1, PSMA2, PSMA3, PSMA6, PSMB2). VCP is related

with vesicle budding and ubiquitin-dependent sorting to the proteasome. Interestingly, evidences link it to diabetes, for being an apoptotic regulator and fundamental target of Akt signaling, and activating the insulin-like growth factor receptor (IGF-R) signaling; also, it was demonstrated that the loss of VCP causes polyubiquitinated cellular proteins accumulation (Vandermoere et al. 2006; Lu et al. 2008) that, together with inhibited proteasome, increases the accumulation of proteins to be degraded and ultimately also increases oxidative stress. LGALS3BP promotes integrin-mediated cell adhesion that is thought to be activated in immune response to cell toxicity (Xu et al. 2019), which in fact matches the GDE general behavior in terms of focal adhesion. Overall, the downregulation of proteasome components appears to be a common feature among plasma EV and GDE.

In summary, we have found that protein composition of plasma EVs does not fully match the one seen in GDEs. However, plasma EVs derived from diet induced obese mice had a disturbed metabolic setting. It is not surprising that plasma EVs do not fully correspond to gut EVs, since plasma EVs carry information from many tissues and systems. Nonetheless, more detailed information still needs to be addressed in future studies.

In toto, our study highlights the importance of understanding the intestine as a metabolically active organ that may be targeted in the future and the importance of GDE as mediators of prediabetes.

2. Gut extracellular vesicles follow gut-liver axis towards macrophages leaving a metabolic footprint

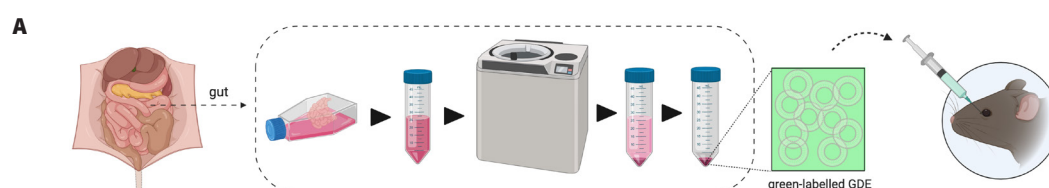
2.1. Gut extracellular vesicles biodistribution

Metabolic homeostasis is achieved by a complex, multidirectional crosstalk between key metabolic tissues, inclusively gut and liver. This crosstalk, conventionally intermediated by hormones and metabolites, becomes dysregulated in diseases such as obesity and diabetes. A recently discovered alternative way of cells communicate is by secreting EVs, which became well-known for mediating local and systemic cell communication through horizontal transfer of genetic, protein and lipidic information. EVs carry on their surface proteins that are exposed to the extracellular environment, targeting them to specific recipient cells (Are 2016). The function of these cells is modulated upon EVs content incorporation; for instance some cells may even differentiate or become activated, according to the material delivered by the EVs (Rana et al. 2012).

In the previous chapter we examined the effect of prediabetes in GDEs protein cargo, revealing a significant change in 588 GDEs proteins due to high fat and sugar diet and the concomitant glucose intolerance and fat accumulation in the liver, hallmarks of prediabetes (Campbell et al. 2020). Interestingly, we observed alteration in glucose and lipid pathways related proteins. Our results suggest that the prediabetic environment in the gut induces marked alterations in the GDEs proteome. However, it remains unknown how prediabetic GDEs modulate disease progression. To test the biological role of GDEs in insulin and glucose sensitivity in the periphery, as well as in cholesterol and TG levels in liver, we started by studying the uptake of labelled-GDEs in different organs and to study the metabolic changes induced by the prediabetic GDEs in healthy animals.

In summary, in the present chapter we addressed whether GDEs from prediabetic mice can induce some of the disease hallmarks on a healthy animal and on the other side, can healthy GDEs alleviate or even revert some of the prediabetic hallmarks. To do so, we developed a model, based on the prosperous discovery of EV function in cancer metastasis (Costa-Silva et al. 2015), and adapted it to answer our question. Detailed optimization will be exposed in the next section.

First, to explore which organs capture the GDEs and thus evaluate biodistribution, near-infrared (nIR) labelled GDEs were retro-orbitally injected into *wild-type* (WT) mice and PBS was used as control. Equal amount of protein of GDE from NCD-fed mice and HFD-fed mice were injected in WT mice and tissues were collected 24h after. Quantification of nIR signal revealed that GDEs were preferentially localized in the liver (Fig.24B). To evaluate whether biodistribution is altered by time, we evaluated it at different times after injection, 1, 3, 6 and 24 hours post-injection and no difference was observed (Fig.24C; D and Supplementary Fig.1).



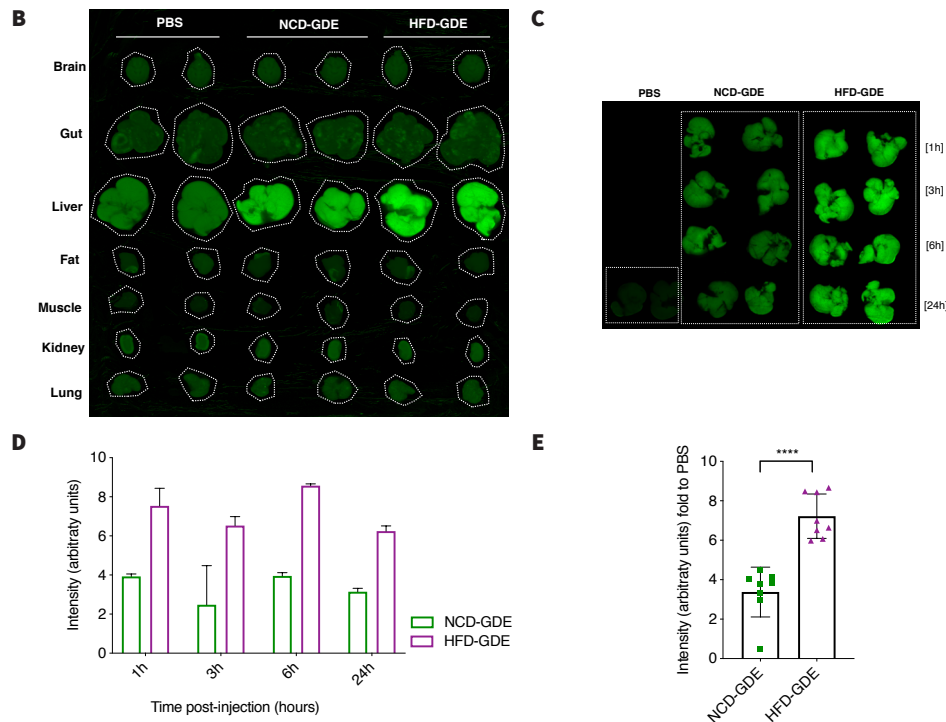


Figure 24. Gut-derived EV are preferentially captured by the liver. Biodistribution after retro-orbital injection in WT mice. **A.** Schematic representation of gut-derived EV (GDE) isolation and subsequent retro-orbital injection in mice. **B.** Intensity of near infra-red signal detection in several tissues from the injected mice, acquired in Odyssey imaging system (LI-COR Biosciences). Groups: mice injected with PBS, mice injected with GDE from NCD-fed mice (NCD-GDE) and mice injected with GDE from HFD-fed mice (HFD-GDE). **C.** Liver signal detection at different time-points after injection (1, 3, 6 and 24 hours). **D.** Analysis of liver signal detection quantification at different time-points after injection (1, 3, 6 and 24 hours), n=2 for PBS, NCD-GDE and HFD-GDE. **E.** Statistical analysis of liver signal detection, independently of time-point. Values normalized to PBS. n=2 for PBS and n=8 for NCD-GDE and HFD-GDE. Results are expressed as mean $\pm$ SEM. Unpaired t test with Welch's correction. ****p<0.0001

Being the liver a particularly heterogeneous organ in terms of cell types, we next aimed to evaluate the specific cells that take up GDE. The macrophages, for its phagocytic ability, have been already described as cells that uptake EVs (Feng et al. 2010; Costa-Silva et al. 2015) so we started by looking at Kupffer cells (KC).

For this purpose, we used fluorescently labelled GDE from NCD-fed mice and HFD-fed mice and injected retro-orbitally in WT mice. 24 hours after injection the frequency of EV+ cells in liver slides was assessed (Fig.25A); we found that over 70% of liver cells that take up EVs were F4/80+ by immunofluorescence, indicating KC to be the potential targets of EVs, confirming our prospects (Fig.25B).

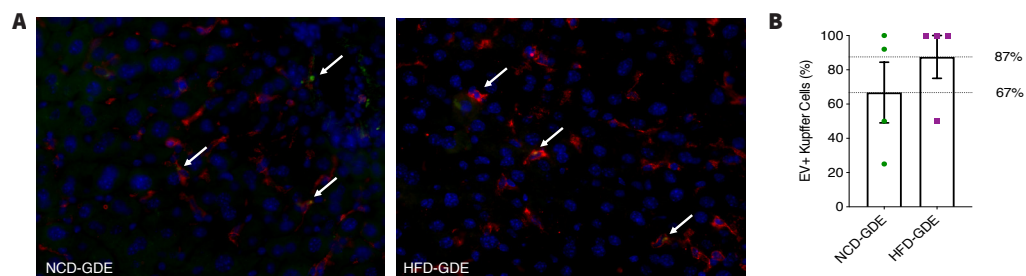


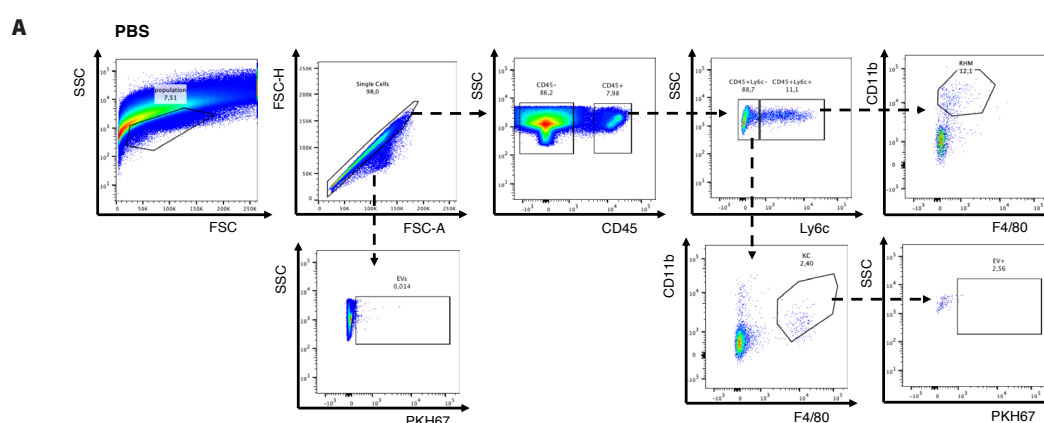
Figure 25. Co-localization of gut-derived EV with Kupffer cells. 24 hours after injection with green-labelled gut-derived EV (GDE) from different diets into WT mice animals were sacrificed and livers were fixed and then processed for immunohistochemistry. Kupffer cells (KC) are stained in red with anti-F4/80 antibody; GDE are labelled in green with PKH67; nuclei with DAPI in blue. **A.** Liver cross-section of a mice injected with GDE from NCD mice (NCD-GDE) (left). Liver cross-section of a mice injected with GDE from HFD-mice (HFD-GDE) (right). Arrows indicate KC EV positive. **B.** Statistical analysis of EVs co-localized with KC; n=4 for NCD-GDE and HFD-GDE. Results are expressed as mean $\pm$ SEM. Unpaired t test with Welch's correction.

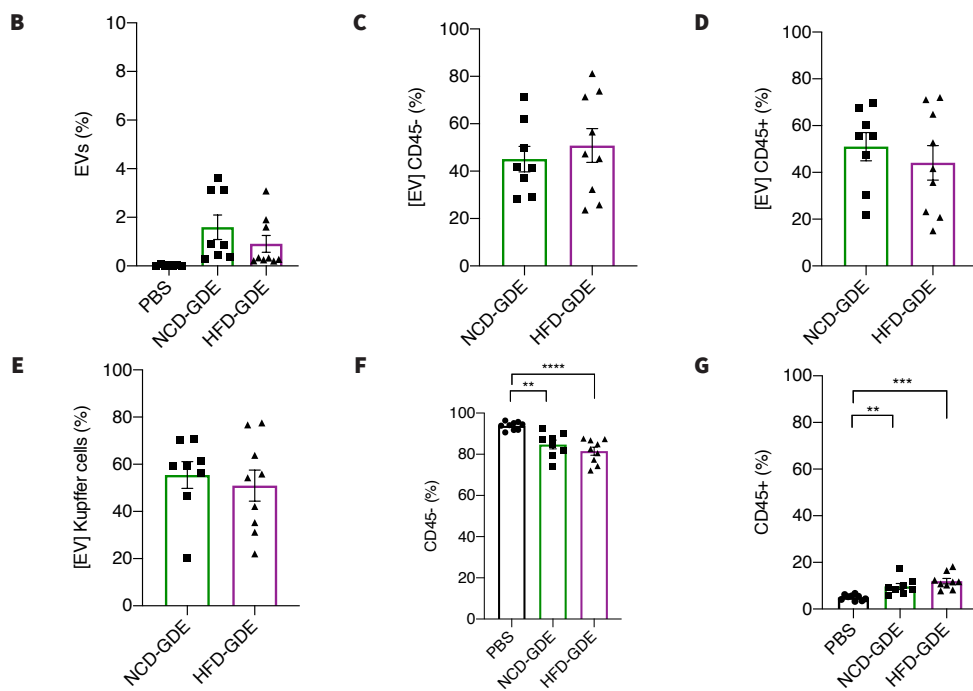
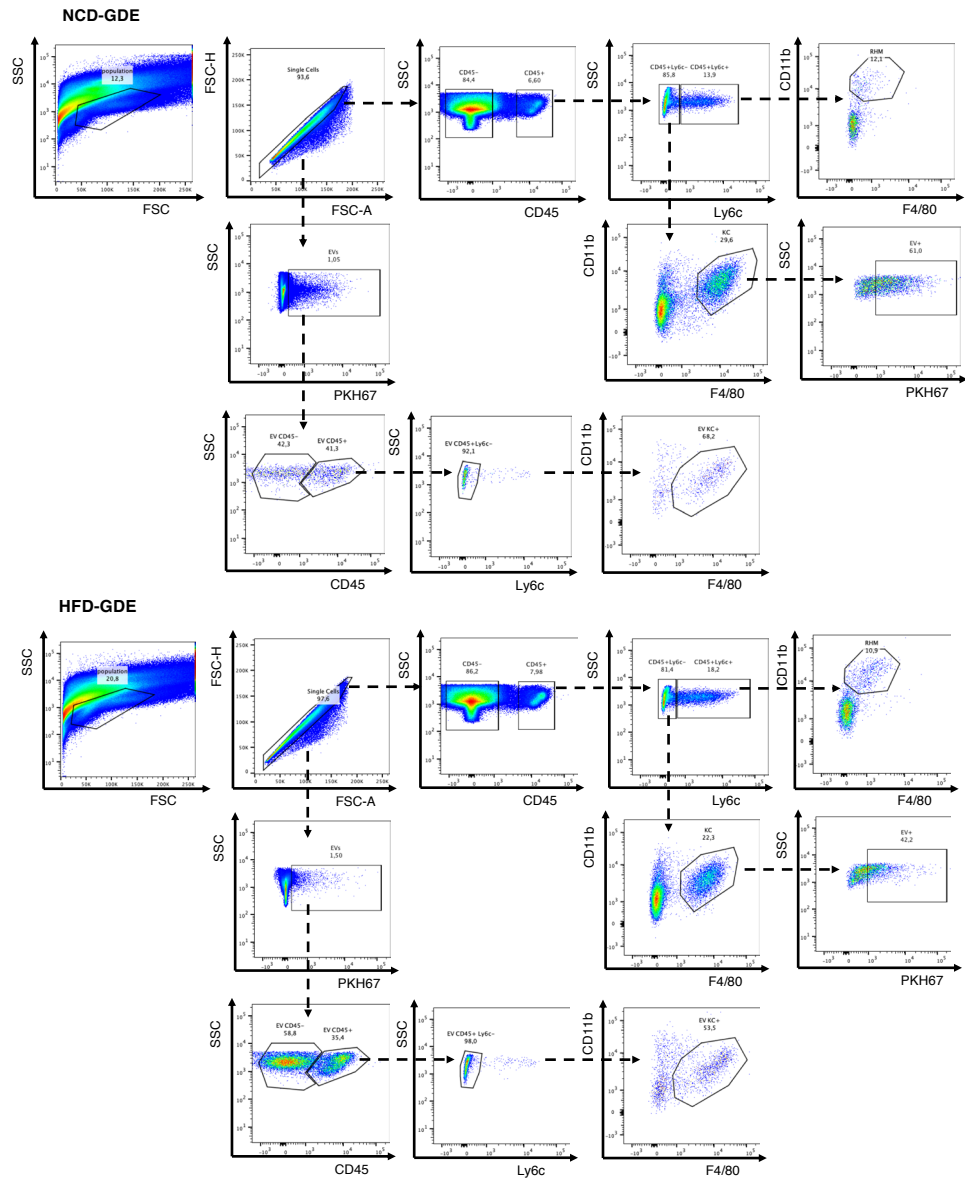
To further confirm the results obtained in the immunohistochemistry experiments, next we performed flow cytometry analysis using the same animal protocol as before. The liver of green-labelled injected mice was analyzed by flow cytometry, stained with CD45, Ly6c, CD11b and F4/80. CD45 is the leukocyte common antigen, typically used to identify immune cells (Altin and Sloan 1997). Ly6c is a marker of recruitment of macrophages (Ramachandran et al. 2012). CD11b or Mac-1 (macrophage-1 antigen) is other common marker of macrophages, amongst other immune cells, it is an integrin that mediates inflammation by regulating adhesion and migration of the cell (Ye and Boulay 1997). Finally, F4/80 is a specific marker for KC, that are CD45+ Ly6c- CD11b+ F4/80+.

From this experiment, we observed that over 50% of EV+ cells were CD11b+F4/80+, inside the CD45+Ly6c- population, a phenotype consistent with KC (Fig.26E). The abundance of CD45- (Fig.26F) in these cells can be explained by the protocol we used, where cells were isolated from the whole liver through mechanical smashing, without any previous treatment, only red blood cells were depleted, so we were expecting to find excess of debris.

When looking to the white blood cell population (CD45+) they seem to increase in EV-injected mice (Fig.26G), the same way as KC (Fig.26H), which increment is more relevant in the mice injected with GDE from NCD mice (NCD-GDE mice). The percentage of KC that take up EV in NCD-GDE mice are around 30% and 15% in mice injected with GDE from HFD mice (HFD-GDE mice) (Fig.26I). Ly6c enables us to distinguish the resident macrophages, the KC (CD45+ Ly6c- CD11b+ F4/80+), from the recruited (recruited hepatic macrophages, RHM), that are known for promoting inflammation (CD45+ Ly6c+ CD11b+ F4/80low) (Holt, Cheng, and Ju 2008). RHM are not very frequent and seem to be increased with EV-injection, but with statistical difference (Fig.26J).

By microscopy we observed that 70-90% of KC were receiving EV while by flow cytometry the numbers were slightly decreased, in an average of 50-60%, that we believe to be related with processing of the samples that must be different for each protocol. Also, by flow cytometry we were able to use more markers specific of KC. However, it is clear that KC are predominantly engulfing GDE, in comparison to remainder cells. That evidence corroborates the literature also for the crucial role that KC have demonstrated in several aspects of metabolic syndrome (Dixon et al. 2016).





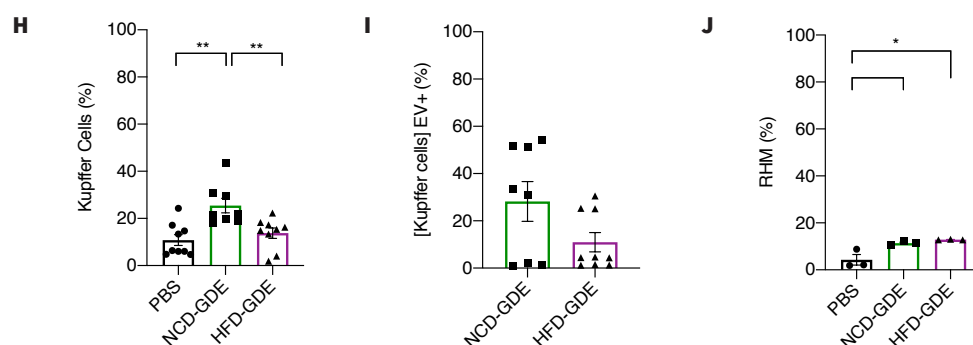


Figure 26. Kupffer cells favorably uptake gut-derived EV. 24 hours post injection with green-labelled gut-derived EV (GDE) animals were sacrificed and liver cells were isolated and prepared for flow cytometry staining. **A.** Flow cytometry plots with the gating strategy used for the analysis of mice injected with PBS (PBS), mice injected with GDE from NCD fed mice (NCD-GDE) and mice injected with GDE from HFD fed mice (HFD-GDE). **B.** Analysis of the percentage of EVs. **C.** Analysis of the percentage of CD45-cells among EVs. **D.** Analysis of the percentage of CD45+ cells among EVs. **E.** Analysis of the percentage of Kupffer Cells (KC) (CD45+ Ly6c-CD11b+ F4/80+) among EVs. **F.** Analysis of the percentage of CD45-cells. **G.** Analysis of the percentage of CD45+ cells. **H.** Analysis of the percentage of KC. **I.** Analysis of the percentage of EV+ cells among KC. **J.** Analysis of the percentage of recruited hepatic macrophages (RHM) (CD45+Ly6c+CD11b+F4/80low). Results are expressed as mean $\pm$ SEM; n=8 for PBS, NCD-GDE and HFD-GDE. Unpaired t test with Welch's correction (C, D, E); ANOVA multiple comparisons (B, F, G, H, I, J); *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001.

2.2. Gut extracellular vesicles biological effect

Next, we evaluated the biological impact of GDEs isolated from our prediabetic mice model, by studying whether those play a role in the induction of the disease. First, we implemented a model, based on the prosperous discovery of EV function in cancer metastasis (Costa-Silva et al. 2015) and adapted it to our setting of metabolic disorders. Briefly, this model is based in the “education” of WT mice with our EVs of interest, achieved by injecting WT mice with GDE isolated from prediabetic animals. In the dictionary education is defined as the process of receiving or giving systematic instruction. In our case the instructions are given by the EVs cargo and then we evaluate if the animal is following those instructions by analyzing the diabetic phenotype of the animals after education. In summary, we intend to test whether the phenotype of the EV producing animal can be induced in the recipient one. We knew that GDEs were carrying many metabolism related proteins altered in prediabetes. We started by determining the GDEs dosage and frequency, the education length and assessing the diabetic phenotype. All technical adjustments and phenotype characterization are represented in Figure 27.

As before, WT mice were retro-orbitally injected with GDE from NCD fed mice and GDE from HFD fed mice for a prolonged period, in a process defined as education. Our first approach was to evaluate if GDEs had any biological effect on healthy mice. Based on the evidence that GDE protein amount was altered in prediabetic mice (Fig. 17B), we calculated that 5 μ g of NCD-GDE were equivalent to 3 μ g of HFD-GDE, by number of EV. All doses were diluted to a final volume of 100 μ l. In Education 1, WT mice, fed *ad libitum* with NCD, were retro-orbitally injected with 5 μ g of NCD-GDE, 3 μ g of HFD-GDE, and 100 μ l of PBS, every other day, for three weeks. After three weeks of education, we did not observe any differences neither in the glucose tolerance, that we evaluated through the GTT, nor in terms of lipid accumulation in the liver (Supplementary Fig.2).

Since we did not see any effect of either NCD-GDE or HFD-GDE education of WT mice, next we fixed the dose to 5 μ g of both NCD-GDE and HFD-GDE and increased the time of the education to six weeks. The GTT was performed at week 5 and lipids in the liver were measured after sacrifice. We did not observe any differences in glucose tolerance (Fig.27B). While cholesterol levels in the liver were not affected by the education, mice injected with HFD-GDE had significantly increased levels of TG in the liver (Fig.27D).

For the third education we duplicated the dosage and kept the six weeks duration, which we considered, at the time, to be the minimum time required to alter the metabolism of mice. Education 3 consisted on a dosage of 10 μ g of NCD-GDE and HFD-GDE, for six weeks, injected in WT mice fed *ad libitum* with NCD. We observed that glucose tolerance of educated mice was altered only in the post-prandial state at 90-minutes post glucose bolus (Fig.27E). In terms of lipids in the liver, consistent with education 2, cholesterol did not change upon education and TG showed a trend to increase from PBS to GDE injected animals, however it did not reach statistical significance (Fig.27F; G). Noteworthy to mention, the distribution of the cholesterol values is much more disperse than in education 2.

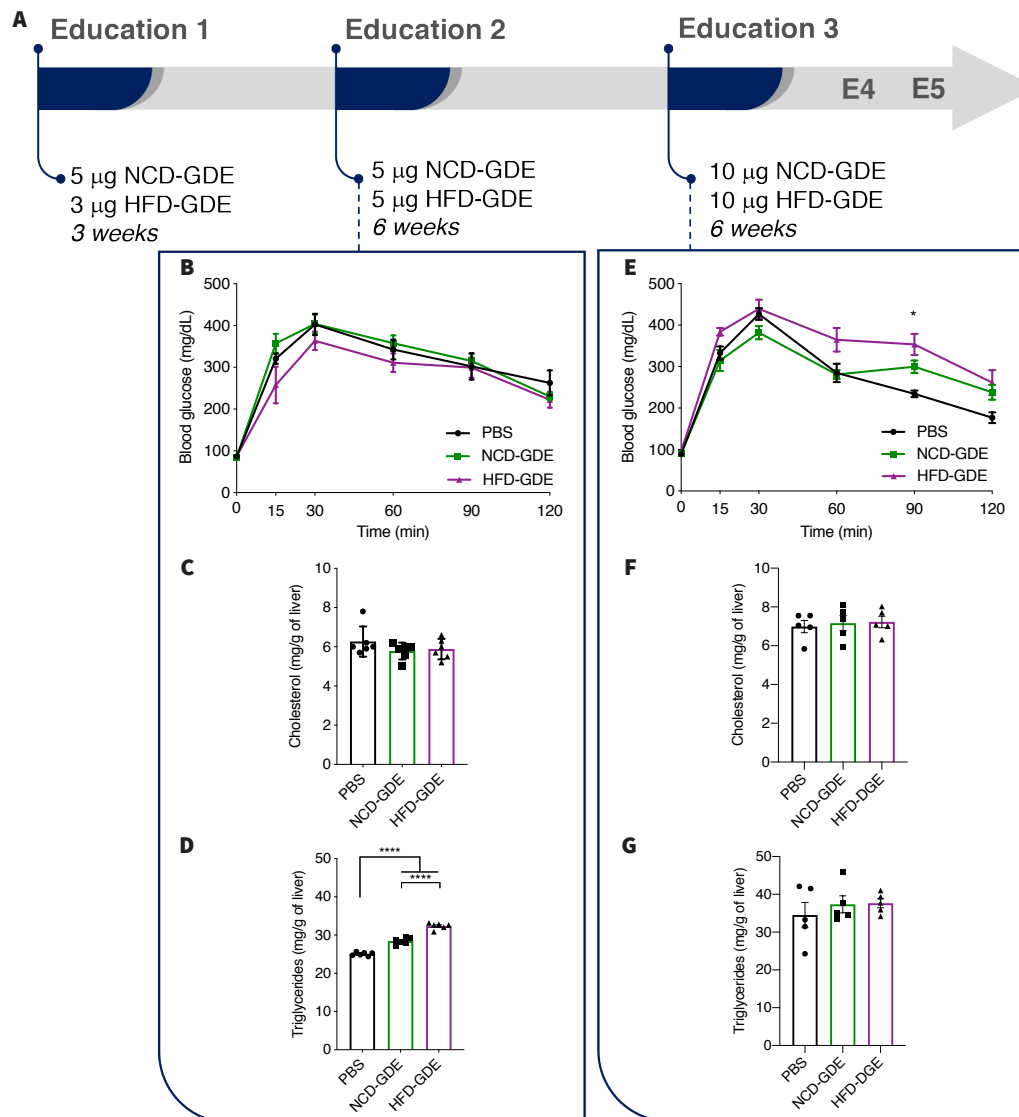
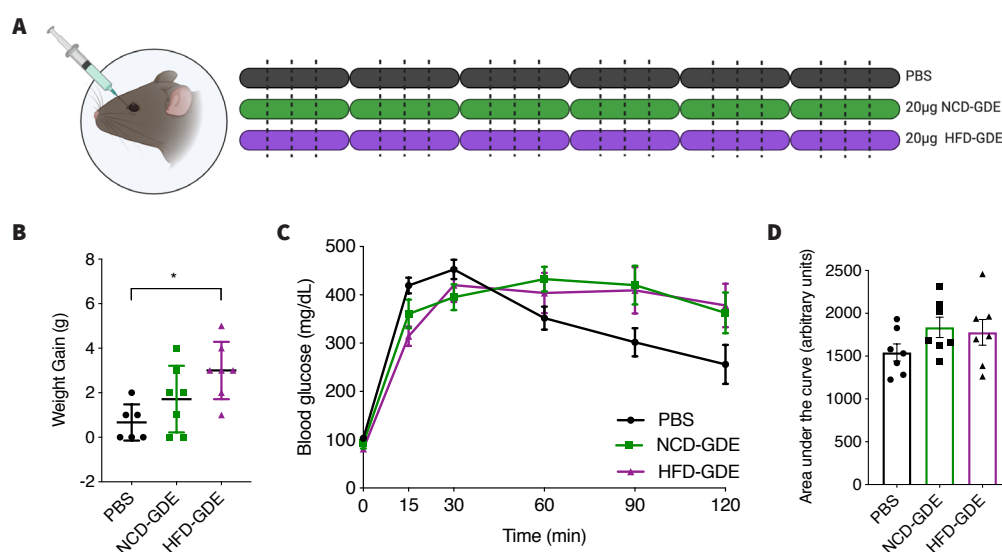


Figure 27. Scheme of the education optimization process. **Education 1** - Mice were injected with 5 μ g of gut-derived EV (GDE) from NCD-fed mice (NCD-GDE) and 3 μ g of GDE from HFD-fed mice (HFD-GDE) for three weeks. **Education 2** - Mice were injected with 5 μ g of NCD-GDE and 5 μ g of HFD-GDE for six weeks. **Education 3** - Mice were injected with 10 μ g of NCD-GDE and 10 μ g of HFD-GDE for six weeks. **A.** Time-line of education's evolution. **B.** Glucose tolerance test (GTT) at different time points (0, 15, 30, 60, 90, 120 minutes) of mice from education 2. **C.** Analysis of cholesterol levels in the liver of mice from education 2. **D.** Analysis of triglyceride (TG) levels in the liver of mice from education 2. **E.** GTT at different time points (0, 15, 30, 60, 90, 120 minutes) of mice from education 3. **F.** Analysis of cholesterol levels in the liver mice from education 3. **G.** Analysis of TG levels in the liver of mice from education 3. Cholesterol and TG represented in milligram per gram of liver tissue. Results are expressed as mean $\pm$ SEM; n=4 for PBS, n=7 for NCD-GDE and HFD-GDE. ANOVA multiple comparisons. *p<0.05, ***p<0.0005.

Despite of not a complete recapitulation of the GDE producer mice phenotype upon education, our data was indicating at least some metabolic alteration induced by the prediabetic GDE. Anyhow, we were not expecting with our model a 100% mimicking of the prediabetic phenotype, relying uniquely on GDE the transmission of disease.

Given the route of administration of the GDE, retro-orbital injection into the blood flow, we wanted to make sure that we were giving enough EVs to overtake the normal levels in circulation. Next, the dosage was calculated based on the number of EVs in systemic circulation and we kept the six weeks duration. Plasma EV analysis showed that NCD mice had an average of 0.35 μg of EV protein per μL of plasma (Fig.22 and Supplementary Fig.3). This means that in 100 μL of plasma, mice have about 35 μg of EV protein. 100 μL is the maximum volume that can be injected in the orbital sinus. Ideally, we would inject a dose of 35 μg of GDE, but due to limitation of GDE availability, we established the six weeks as a favorable time window for the metabolism alterations, and we fixed the dosage to 20 μg . Therefore, WT mice fed *ad libitum* with NCD were injected with 20 μg of NCD-GDE and HFD-GDE, every other day, for six weeks (Fig.28A). We observed that HFD-GDE mice had significantly higher weight gain, in comparison with the PBS group (Fig.28B). GTT glycemic curves did not show overall glucose tolerance alterations upon education (Fig.28C). Regarding the AUC, while it did not reach statistical relevance, it revealed a slightly increase from the PBS to the groups educated with the GDEs (Fig.28D). In contrary to what we did in previous educations, where GDE from the same diets were all pooled together and then divided into the defined doses to be injected; in this education GDE were pooled just enough to educate a single recipient mouse. This way, we could track the donor of the recipient mice and correlate phenotypes. Importantly, we observed a positive correlation of the GTT AUC of donor mice with the GTT AUC of the correspondent recipient mice, i.e. injected with GDE from that donor (Fig.28E). Insulin tolerance test (ITT) was performed at week 4 of education, the ITT informs us about insulin resistance. We perceived that HFD-GDE educated mice were displaying some degree of resistance, however it was not statically significative. In addition, in terms of insulin resistance quantified by the area above the curve, mice injected with NCD-GDE showed a trend to be more resistant than PBS ones, and the ones injected with HFD-GDE, also showed a trend to be even more resistant than the NCD-GDE injected ones; however, ANOVA of multiple comparisons did not show any statistical differences (Fig.28F, G). Finally, cholesterol levels did not change either in NCD-GDE or HFD-GDE educated mice in relation to controls (Fig.28H). Consistent with the previous educations, TG are increased in educated mice, in a step-wise manner from PBS to HFD-GDE injected animals (Fig.28I).



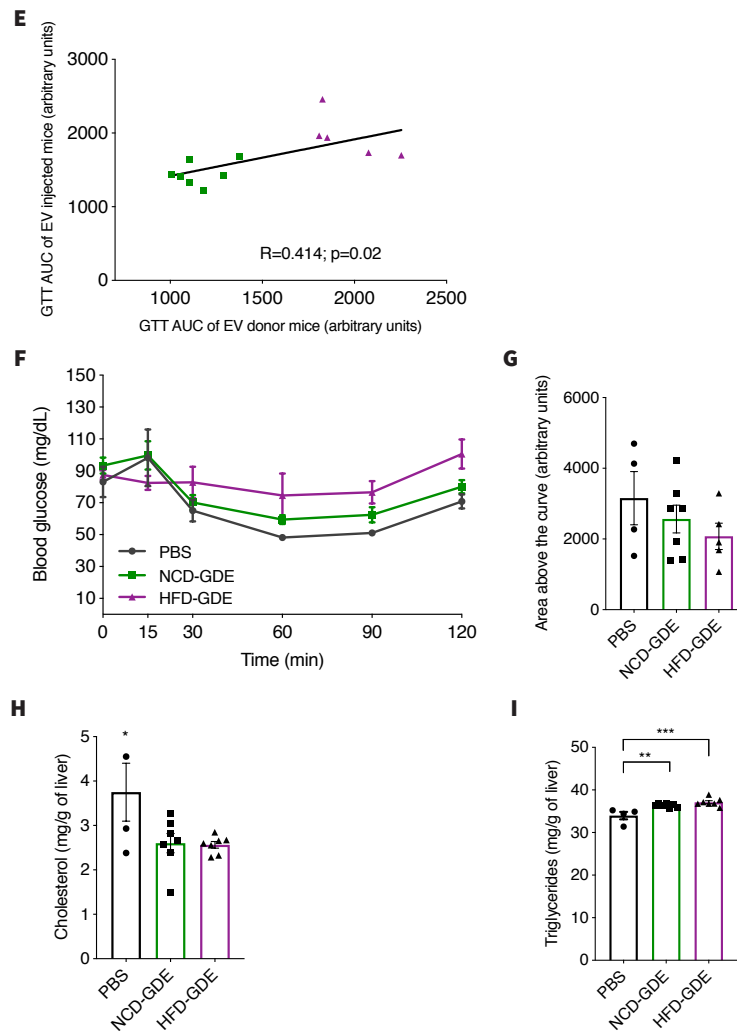


Figure 28. Gut-derived EV induce an increase in liver triglycerides when administered to WT mice under a control diet. **A.** Schematic representation of Education 4: Mice were retro-orbitally injected, every other day, with 20 μ g of gut-derived EV (GDE) from NCD-fed mice (NCD-GDE), 20 μ g of gut-derived EV from HFD-fed mice (HFD-GDE) and PBS, for six weeks and fed with NCD. Individual doses were calculated for 100 μ l of volume. **B.** Analysis of weight gain of treated mice after education. **C.** Glucose tolerance test (GTT) at different time points (0, 15, 30, 60, 90, 120 minutes). **D.** Analysis of the area under the curve (AUC) of the GTT. **E.** Positive correlation between the EV-donor mice GTT AUC and correspondent EV-recipient mice GTT AUC. **F.** Insulin tolerance test (ITT) of mice at different time points (0, 15, 30, 60, 90, 120 minutes). **G.** Analysis of the area above the curve of the ITT. **H.** Analysis of cholesterol levels in the liver of educated mice. **I.** Analysis of triglyceride (TG) levels in the liver of educated mice. Cholesterol and TG are represented in milligram per gram of liver tissue. Results are expressed as mean $\pm$ SEM; $n=4$ for PBS, $n=7$ for NCD-GDE and HFD-GDE. ANOVA multiple comparisons. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$.

Altogether, our results indicate that GDEs are carrying a metabolic message with particular impact on FA synthesis. Hypothesizing that GDEs are auxiliary in the organismal adjustment to signals from the environment – the diet, in our case – we added a stimulus to the education. Assuming GDEs are mediators of the complex system that diabetes is, we wanted to see their behavior with the HFD background. Therefore, we followed exactly the same protocol as education 4, WT mice injected, as before, with 20 μ g of NCD-GDE and 20 μ g of HFD-GDE and simultaneously fed with HFD for six weeks duration of the education (Fig. 29A). In terms of weight gain, we did not see any differences (Fig. 29B). GTT was done at week 5 of education and, despite not significantly different, we observed a trend for glucose intolerance in the HFD-GDE educated mice, as expected from our hypothesis (Fig. 29C; D). ITT was performed at week 4, again the differences did not reach statistical relevance, however HFD-GDE injected mice appear to be more resistant to insulin than PBS and NCD-GDE (Fig. 29E; F). Considering cholesterol levels, no

statistical differences were observed, while there is a trend for gradual increase from PBS, to NCD-GDE and HFD-GDE (Fig.29G). Importantly, TG levels consistently demonstrate significant increased in NCD-GDE mice and even more in HFD-GDE mice (Fig.29H). Nevertheless, liver histology analysis of these mice did not show any evidence of steatosis at this point yet (Supplementary Fig.4).

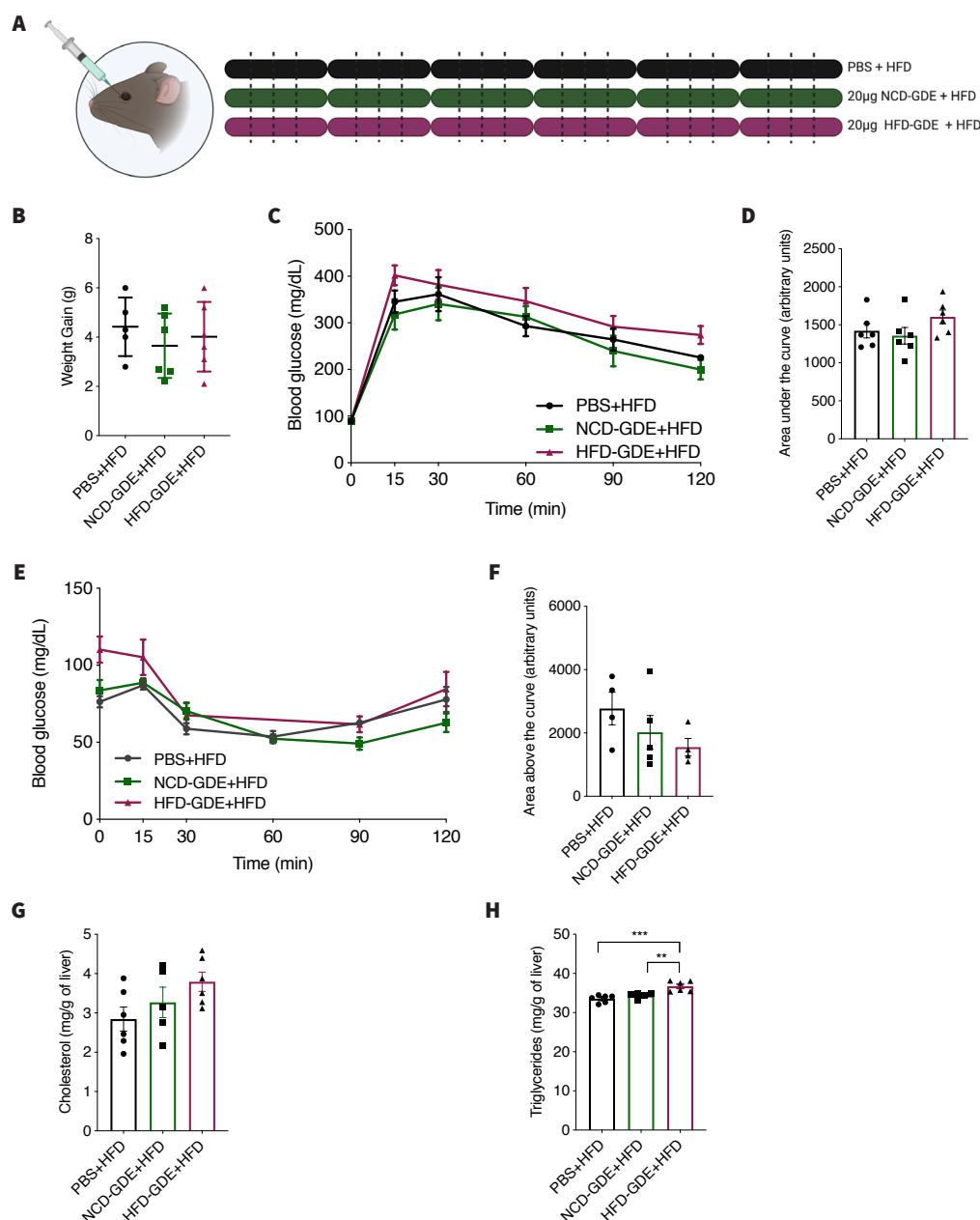
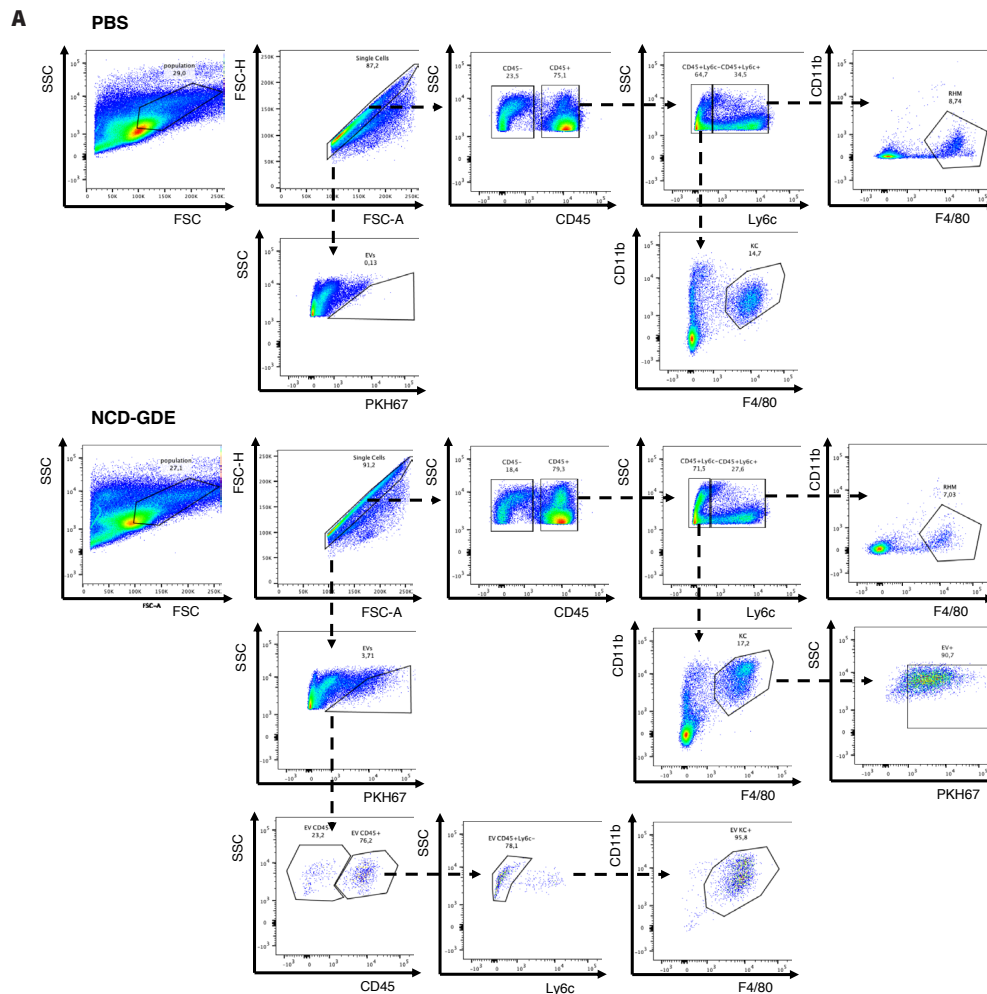


Figure 29. Gut-derived EV induce an increase in liver triglycerides when administered to WT mice under an HFD. **A.** Schematic representation of Education 5: mice were retro-orbitally injected with 20 µg of gut-derived EV (GDE) from NCD-fed mice (NCD-GDE) and 20 µg of gut-derived EV from HFD-fed mice (HFD-GDE) every other day for six weeks and fed with HFD. Every dose was calculated for a total volume of 100 µl. **B.** Weight gain of injected mice after education. **C.** Glucose tolerance test (GTT) of mice at different time points (0, 15, 30, 60, 90, 120 minutes). **D.** Analysis of the area under the curve of the GTT. **E.** Insulin tolerance test (ITT) of mice at different time points (0, 15, 30, 60, 90, 120 minutes). **F.** Analysis of the area above the curve of the ITT. **G.** Analysis of the cholesterol levels in the liver of injected mice. **H.** Analysis of triglyceride (TG) levels in the liver of injected mice. Cholesterol and TG are represented in milligram per gram of liver tissue. Results are expressed as mean ± SEM; n=4 for PBS, n=7 for NCD-GDE and HFD-GDE. ANOVA multiple comparisons. *p<0.05, **p<0.005, ***p<0.0005.

From the single retro-orbital injection of GDE in WT mice we observed that those GDE were directed preferentially to the KC (Fig.26). However, we also observed that GDE provoked alterations in the livers of injected mice, after six weeks of injections, specifically on the amount of fat stored, what have effects on tissue morphology and probably in the cellularity. So, aiming to learn about the distribution of these EVs in the different hepatic cell populations after six weeks of every other day injections, we performed flow cytometry analysis of liver tissue (Fig.30A). To that end, livers of educated mice (education 5) were perfused, non-parenchymal cells were enriched through a Percoll gradient (Duarte et al. 2018) and stained for CD45, Ly6c, CD11b and F4/80. The uptake of EVs is visible from the differences between the PBS-injected group and the other two groups, but the levels were not very expressive, about 3% of the cells seemed to take up EV. Anyway, HFD-GDE injected mice showed up with increased EVs than NCD-GDE (Fig.30B). Replicating the previously observed pattern of only one injection of GDE, EVs were predominantly going to the KC (Fig.30E). Meanwhile, the population of KC looked to be diminished in educated mice, with no statistically difference (Fig.30H) importantly almost every KC was EV+, indicating great activation of these cells by EV (Fig.30I). The RHM (CD45+ Ly6c+ CD11b+ F4/80low), arise has being decreased in injected mice compared with PBS (Fig.30J).

Tissue-resident macrophages adapt to perform specific functions vital for tissue homeostasis (Davies et al. 2014). Besides, KC are particularly dynamic, recruited when necessary from monocytes, normally derived from bone marrow (Scott et al. 2016). In order to have more information about the effect of GDE on cell recruitment, we analyzed the bone marrow (Supplementary Fig.5) of the educated mice. However, the proportions of GDE positivity in that tissue was very few, the observed frequency of EV was about ~0.5%.



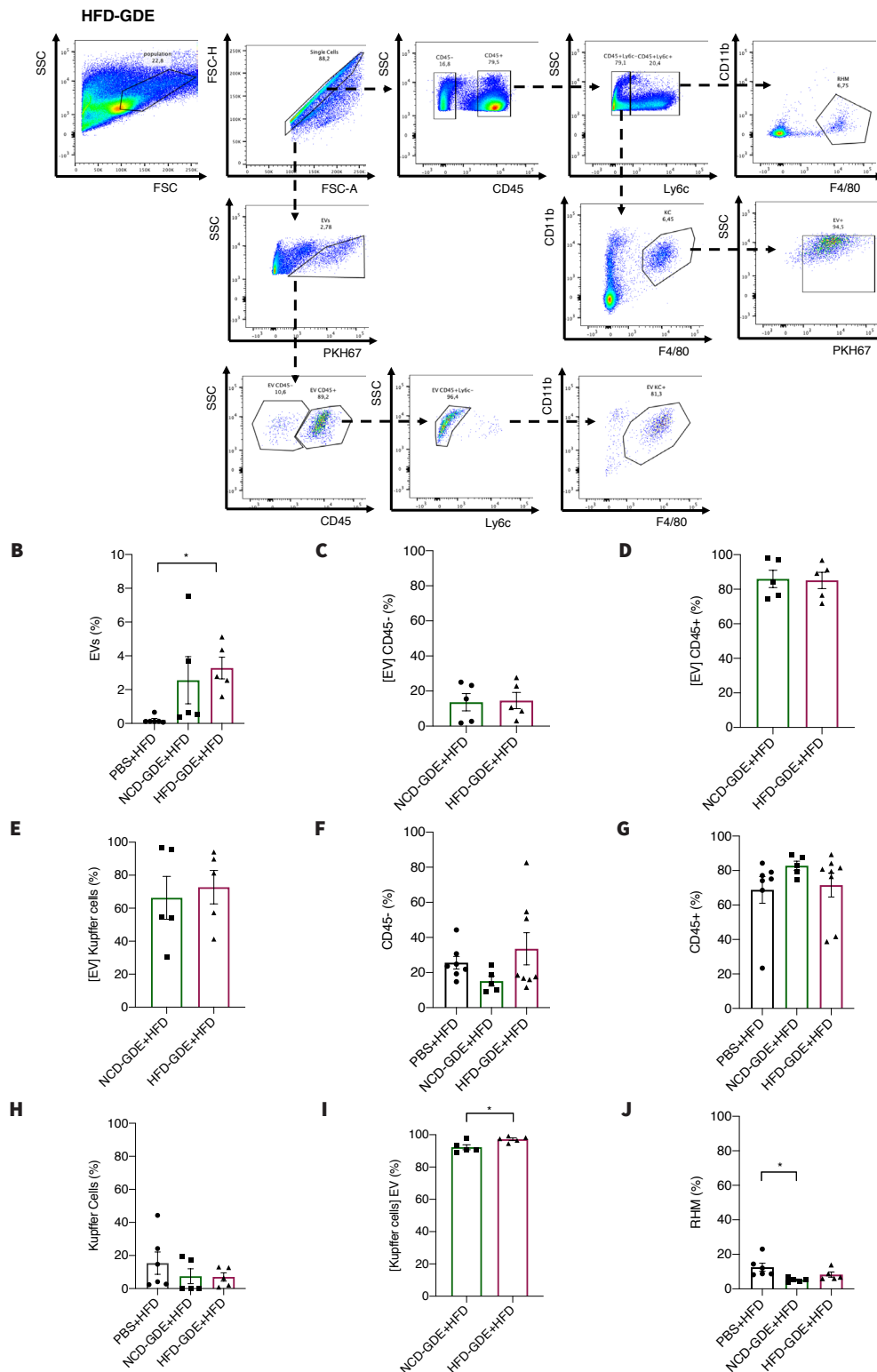


Figure 30. Kupffer cells favorably take up gut-derived EV after six weeks of injections. Mice were fed with HFD for 6 weeks and during the same period were injected with 20 μ g of fluorescent-labelled gut-derived EV (GDE) from NCD-fed mice (NCD-GDE) and 20 μ g of fluorescent-labelled gut-derived EV from HFD-fed mice (HFD-GDE). **A.** Flow cytometry plots with gating strategy for the analysis of PBS-injected group, NCD-GDE and HFD-GDE. **B.** Analysis of the percentage of EVs. **C.** Analysis of the percentage of CD45 negative (CD45-) cells among EVs. **D.** Analysis of the percentage of CD45 positive (CD45+) cells among EVs. **E.** Analysis of the percentage of Kupffer cells (KC) among EVs, KC are CD45+Ly6c-CD11b+F4/80. **F.** Analysis of the percentage of CD45- cells. **G.** Analysis of the percentage of CD45+ cells; **H.** Analysis of the percentage of KC. **I.** Analysis of the percentage of EV+ cells among KC. **J.** Analysis of the percentage of recruited hepatic macrophages (RHM). RHM are CD45+Ly6c+CD11b+F4/80low. Results are expressed as mean $\pm$ SEM, n=7 for PBS and n=5 for NCD-GDE and HFD-GDE; Unpaired t test with Welch's correction (C, D, E); ANOVA multiple comparisons (B, F, G, H, I, J); *p<0.05.

Discussion

The concept of tissue-derived EVs playing a role in metabolic diseases arose in recent years (Huang-Doran, Zhang, and Vidal-Puig 2017). Deng et al. observed that by administering EVs isolated from the adipose tissue of obese mice into healthy ones, glucose intolerance and insulin resistance developed (Deng et al. 2009). In humans, distinct EV miRNA profiles for metabolic syndrome, T2D, hypercholesterolemia and hypertension were identified (Karolina et al. 2012). From mice to humans we find great relevance for EVs in metabolism and whole-body homeostasis. However, the role of GDE in metabolic disorders has not been fully addressed. We previously described that GDE proteome is highly altered by high-fat diet. Here we investigated the fate of HFD-GDE in non-obese WT mice, both in terms of organ as well as cellular population; and whether they trigger dysmetabolism in healthy mice. Our findings clearly show that GDE were directed to the liver (Fig.24), and in great extent, specifically to the KC (Fig.25;26). In terms of metabolic alterations, non-obese WT mice injected with HFD-GDE display elevated levels of hepatic TG. In contrast, glucose and insulin homeostasis was not affected.

The bidirectional relationship between the gut and the liver classical described by the anatomy, is here for the first time further highlighted by the uptake of GDE exclusively by the liver (DeSesso and Jacobson 2001). Most interestingly, HFD-GDE mice livers' display a more intense signal than NCD-GDE (Figure 24C), suggesting HFD-GDE to have higher specificity for the liver or increased charge, or even both. It is noteworthy that KC are the primary innate immune defense against foreign components from the diet absorbed in the intestinal tract (Parker and Picut 2005; Remmerie and Scott 2018). Along with our observation that GDE are preferentially going to KC, these results suggest that GDE derived from healthy animals may work as a detoxifying system by carrying toxic products from the diet and targeting them to KC in order to trigger a defense response. In addition, when animals are fed for prolonged time with an HFD regimen, KC can no longer detoxify the excess of lipids coming from the diet and dysmetabolism arises in the liver. Metabolomics studies over the near future may shed light on this important question.

We proceeded to test the hypothesis that chronic exposure of healthy mice to HFD-GDE triggers a prediabetic obese phenotype. The complexity of optimizing a protocol within the metabolic field, since there are innumerable parameters to take into account, is quite challenging; nevertheless we started with the protocol described in the cancer field, where EVs content is particularly aggressive (Costa-Silva et al. 2015). While this was a starting point, we have to keep in mind that the metabolic system is predefined to be highly adaptative and to be continuously under adjustments, well-illustrated by the intermittent feeding regimen.

According to Heydemann, who did an overview on the development of T2D in the HFD model, mice start to become glucose intolerant and IR around 8 weeks post HFD feeding (Heydemann 2016). Taken into account we treated healthy lean mice only with GDE, in a dose that does not surpass plasma EVs concentration evaluated in HFD mice, it is not surprising that no differences were observed in glucose and insulin resistance.

It is thus likely that, at six weeks of injections every other day with GDE, the animals' immune and antioxidant systems are still capable to counteract the pro-diabetogenic and pro-inflammatory effects carried by the HFD-GDE from the gut to the KC. Therefore, one would not expect to see marked changes in glucose tolerance and insulin resistance, at this age and time of treatment, yet we observed a difference on the GTT curves from PBS to GDE injected mice, but with no statistical meaning (Fig.27B, E;

28C). These data raise the possibility that by increasing the time of treatment with GDE, the observed trend would become more pronounced and gain statistical power. In addition, ITT from education 4 (Fig.28F; G) shows a trend to increased insulin resistance from PBS to NCD-GDE, and from those to HFD-GDE. In the same line of evidence, again, with no statistical meaning, it is curious to observe that animals educated with NCD-GDE and simultaneously fed an HFD display decreased glucose tolerance in relation to PBS, indicating that “healthy” GDE might also have a protective effect in a scenario of disease (Fig.29D). Nevertheless, to fully understand the impact of HFD-GDE on whole-body glucose and insulin homeostasis further studies are of the uttermost importance. We anticipate that by administering HFD-GDE to mice previously exposed to an HFD we would be able to observe a fastest progression of metabolic disorders, such as diabetes and obesity. Furthermore, even in lean healthy mice, if the treatment with GDE from diet induced obese animals would be prolonged a pathophysiological effect of those on liver and peripheral glucose metabolism would be expected.

Interestingly, increased levels of hepatic TG in HFD-GDE versus NCD-GDE, and PBS (Fig.27D; 28; 29) were observed, further strengthening the GDE proteomics characterization, that revealed an upregulation of lipid metabolism related proteins in HFD-GDE. Considering GDE tropism to KC, and the role of KC in hepatic lipid accumulation and inflammation, it is acceptable that hepatic lipid content is altered upon administration of HFD-GDE. This result and the fact that overload of specific lipid species derived from diet activates KC in models of NAFLD and steatohepatitis indicates that GDEs might be vehicles of lipid transport to the KC, where these lipids suffer remodeling and are posteriorly sent to the hepatocytes (Dixon et al. 2016).

The previous idea is further supported by the fact that KCs gene expression profile is enriched in genes involved in the uptake, processing and export of excess cholesterol to be transported to hepatocytes (Scott et al. 2016). It would be interesting to test whether KC also mediate the export of excessive TG or their building blocks, the FFAs, via EVs or other alternative pathways. In the case of NAFLD, the characteristic inflammatory state is triggered by the activation of KC that rapidly secretes cytokines and chemokines such as IL-1 β and TNF α contributing to a paracrine activation of protective or apoptotic signaling pathways in hepatocytes and the recruitment of other immune cells (Duarte et al. 2015). A different study also showed that, under HFD or upon FFA treatment, KCs are activated producing high levels of proinflammatory cytokines (Leroux et al. 2012). Interestingly, KCs from mice exposed to HFD were shown to accumulate increased amount of DAG and FA as consequence of activated lipolysis (Gordon and Plüddemann 2017; Huang et al. 2014). Thus, lipid sensing by KCs may condition its sensitivity to proinflammatory triggers. Most importantly, McGettigan demonstrated that dietary lipids alter specifically the transcriptome of KC in NAFLD (McGettigan et al. 2019). Not only, McGettigan, but also Tabas and Bornfeldt demonstrated KC to have a transcriptional profile highly specialized on lipid metabolism, that are acquired by dealing with lipids of dying cells they phagocytize (Tabas and Bornfeldt 2016; Scott et al. 2016). Further analysis needs to be done to test the molecular effects of GDE in KC, particularly at the inflammatory level in order to identify potential early steps of disease.

Briefly, GDEs promote changes in lipid metabolism, seen in vivo by the increase hepatic TG. Thus, we conjecture that intestinal cells are overloaded with lipids coming from the diet, hindering a message that is encapsulated in GDEs which in turn are delivered to the liver. No doubt, obesity is associated with a spectrum of liver alterations, being one of them increased hepatic TG. This idea is supported by the increase in weight gain in HFD-GDE mice. However, we need to understand if there is lipid accumulation

in other tissues, namely in white adipose tissue, and/or a hormonal dysregulation controlling their eating behavior; for that it would be important to monitor food intake as well. Additionally, our preliminary lipidomics data comparing liver derived EV with GDE showed that the latter had an enrichment in ceramides, which are highly pro-inflammatory (results not shown) (Meeusen et al. 2018).

The fact that we observed an increase in hepatic TG induced by HFD-GDEs expands our understanding of how EVs modulate body metabolic homeostasis and opens new doors for future studies in the applicability of using healthy EV to revert metabolic diseases phenotype.

V

Chapter

Final Discussion and Future Perspectives

Final Discussion and Future Perspectives

Prediabetes precedes T2D, which is a multisystemic disease and represents the end stage of several metabolic impairments (e.g. hyperglycemia, insulin resistance) being associated with obesity and dyslipidemia (Hinder et al. 2017). The vast network of metabolic organs participating in the disease suggests the existence of more than one mode of communication. EVs emerged in the last few years as novel mediators of cell-to-cell and organ-to-organ interaction. Owing to their ubiquitous presence and stability in several human biofluids they demonstrate to be extremely relevant targets in multiple diseases (Feng et al. 2010). Recent studies show that in obesity, insulin resistance, NAFLD and diabetes, the number of circulating EVs increases, suggesting an important role for these vesicles in pathophysiological mechanisms (Huang-Doran, Zhang, and Vidal-Puig 2017).

We have explored the role of GDE in prediabetes, since dysbiosis has emerged as a strong diabetogenic factor (Shen, Obin, and Zhao 2013). In fact, metabolic surgery is the only known intervention that leads to diabetes remission, prior to and largely independent of weight loss (Batterham and Cummings 2016). Therefore, our aim was to understand if GDE proteome was affected by dysmetabolism, specifically in a setting of diet induced obesity and prediabetes. Additionally, we aimed at understanding if GDE could be used as surrogate markers of disease progression and prognosis. With these goals in mind, we started by analyzing the effect of HFD feeding in the proteome of GDE. We observed, for the first time, that GDE protein cargoes are altered both in terms of concentration and profile from a healthy balanced diet to a western diet mimicking one. Consistent with previous reports, our results suggest that GDE mirrored the milieu of parent cells (Vileigas et al. 2019). We clearly observed variations in the expression of proteins related with carbohydrate, fat and protein metabolism. Plus, we observed the up-regulation of some protector proteins in ROS responsiveness pathways for food derived metabolites. Furthermore, the fact that many proteasomal proteins were down-regulated, indicates that we are dealing with an initial pathologic state.

In order to understand if the unique cargoes in NCD-GDE vs. HFD-GDE impact on the development of metabolic disorders in vivo we administered GDE from NCD or HFD fed mice to lean healthy mice. First, we aimed at determining the tropism of these vesicles; secondly, we studied the effect of a prolonged exposure to these vesicles on the development of metabolic dysfunction. One single injection of GDEs in lean healthy mice showed that GDE are mainly target to the liver, in particular to its resident macrophages, Kupffer cells (Fig.25;26). KCs are particularly relevant in certain pathologies, such as NAFLD that is closely associated to the development of T2D. NAFLD is triggered by fat accumulation in the liver due to LPS uptake from blood by KC (Wenfeng et al. 2014). Cardiovascular diseases, such as atherosclerosis, are another example where the interaction of lipids with KC worsens a pathophysiological state (Remmerie and Scott 2018). The specific mechanisms through which lipids activate macrophages are not completely known (Méndez-Sánchez et al. 2020), data gathered in this dissertation is thought-provoking. Observing that GDE derived from obese animals are favorably going to KC raises the question of a potential inflammatory activation. If this is the case, how do HFD-GDE activate the inflammatory response? Although the proteomics characterization did not reveal any relevant alterations in inflammation related proteins, other studies showed that microRNA delivered by EVs modulate the inflammatory response (Karolina et al. 2012). In order to clarify this point, first, one would expose primary KC to GDE and assess whether or not there is induction of inflammation. At that point, we would be ready to dissect the underlying molecular mechanism.

Do HFD-GDE per se induce prediabetic hallmarks? Indeed, we saw an accumulation of TG in the

liver of HFD-GDE injected mice. Note that in the liver, the production and secretion of TG is closely linked to nutrient availability and insulin levels. Interestingly, in our experimental settings those animals which displayed increased accumulation of hepatic TG were not exposed either to an HFD, or to hyperinsulinemia, thus suggesting that HFD-GDE carry lipids from the gut to the liver and are per se the inducers of the observed phenotype. TG constitute the biggest store of lipids from diet (Jensen-Urstad and Semenkovich 2012), again those mice were not under an HFD regimen, they were uniquely injected with HFD-GDE. And yet, elevated levels of TG are observed in the liver while the EV were isolated from gut. Other than that, these data is consistent with the proteomic analysis where many proteins related with lipid pathways were impressively up-regulated. We highlight a particular family of proteins considerably up-regulated in HFD-GDE (Fig.20B), the ACOTs enzymes, which are described in the literature to be directly associated with the synthesis of TG, directing FFA to be stored into the form of TG (Alves-Bezerra and Cohen 2018). In summary, we suggest a role for GDE in promoting ectopic lipid accumulation, a hallmark of NAFLD pathogenesis (Xia, Bian, and Gao 2019).

Finally, regarding other prediabetic hallmarks, every other day injections of GDE throughout 6 weeks, did not have a clear impact on glucose tolerance or insulin resistance; however as discussed on the second section, we see a trend for that phenotype alongside with educations (Fig.27B, E; Fig.28C). Moreover, we were not expecting to observe such alteration, reasons exposed in detail on the second section. Importantly, in a very clear and consistent way, educated mice showed increase levels of hepatic TG (Fig.27D, G; 28I).

Keeping on the lipid front, hepatic cholesterol levels in educated mice did not show alterations (Fig.27C,F; 28H). We can related it with the fact that acyl-coenzyme A: cholesterol acyltransferase enzymes (ACAT1 and ACAT2) are down-regulated in HFD-GDE (Fig.21; Table 1). Those proteins are described as responsible for storage of cholesterol in cytosolic droplets (Cohen and Fisher 2013). According to the literature ACAT2 demonstrated to be a major cholesterol-esterification enzyme in mouse small intestine and liver by observing that in ACAT2-deficient mice, fed with HFD, profound resistance to hypercholesterolemia and cholesterol store formations were evident. Hence, inhibition of ACAT by pharmacological intermediation or deletion of ACAT2 significantly demonstrated to reduce the rate of cholesterol absorption (Chen et al. 2001). This might be a feature of the mild phenotype window that we are targeting, where the cell is still able to develop some counteracting behaviors to the excess of fat.

Linking proteomic data with the outcomes from the education we have gathered strong evidence supporting the idea that GDEs are carriers of diet derived lipids from the gut to the liver to be stored in the form of TG. The idea of specific lipids as main components of EVs, is not new and there are studies demonstrating that they play an important role in their biology (Zhang et al. 2019). Inclusively, EVs exhibit particular lipids ordered in its membrane and carry FA binding proteins, meaning that they are transporting bioactive lipids (Record et al. 2014; Skotland et al. 2019). Nevertheless, in preliminary results we found that GDE are enriched in a specific types of ceramides (data not shown), which are known for being pro-inflammatory lipids (Hait and Maiti 2017), indicating that GDE might be an alternative mean of inflammation activation.

The scientific interest in EVs has been growing, considering their importance in cell-to-cell and cell-to-organism communication and their potential in therapeutics, due to efficient transfer of components into selective targets. With this study we highlight an important cargo that has not been well noticed, the lipids. The characteristics of EV such as stability and capacity to protect its loads from degradation, with the ability to target specific cells, made EVs good candidates for delivery tools both in health and

disease (Johnsen et al. 2014). The present dissertation has revealed crucial information for the role of extracellular vesicles in the gut-liver axis, in the context of metabolic disorders such as obesity, diabetes and NAFLD. However, a number of questions remain to be addressed:

1. How do GDE propagate diet alterations? On one side, we learned that their proteome is largely altered upon high fat and high sucrose feeding. In addition, preliminary results show that certain ceramides, toxic lipids, are enriched in HFD-GDE. It remains to be explored their miRNA content. In terms the route taken by the GDE, since chylomicrons transport exogenous lipids in the lymph, should we expect GDE transporting dietary lipid shipments to use that route as well? That could be one possible explanation for the lack of FA-related proteins in plasma EVs from HFD mice. Whether GDE can be found in the lymph is an interesting question to be addressed in the future.

2. Why do GDE mediate the accumulation of TG in particular? Since there is no alteration in terms of hepatic cholesterol, nor hepatic lipid droplets accumulation in HFD-GDE educated mice, the detailed mechanism regarding the accumulation of TG remains to be disclose. For that, we plan to validate, initially by western blot, the levels of ACOT and ACAT enzymes, in the tissues, both gut and liver.

3. What kind of relationship have KC and GDE? The interaction between KC and GDE is another aspect we want to dissect in an upcoming project, specifically the exact mechanisms by which KC are activated upon uptake of HFD-GDEs.

Perhaps the most important and intriguing question that rests is whether GDE per se are effectively able to diffuse all the prediabetic hallmarks and ultimately induce diabetes. Our results strongly suggest that we are in the right path to observe such induction.

We believe that by educating the animals for longer time and on a daily frequency will likely induce a more pronounced phenotype. In addition, injecting GDE from frank diabetic mice, would be an alternative approach to achieve diabetes solely by GDE education. In terms of treatment, we would explore the fact that healthy GDEs could revert some of the prediabetic hallmarks.

However, the challenge for the future will be to apply our findings in a more translational setting, namely in patients. In particular, it will be very interesting to analyze GDE from samples of patients that were submitted to bariatric surgery. I

In any case, from today's findings to tomorrow's applications a lot needs to be understood. The idea of using EVs as liquid biopsies is still one of our long term achievements. In the immediate future, we plan to do lipidomics analysis on plasma EVs and GDE. Additionally, with the help of scientific advance we would like to track in vivo in real time GDE travelling in the blood. Moreover, there are some examples of high-tech approaches to assay multiple EV markers at the same time such as microfluidics biochips (Shao et al. 2015) and proximity barcoding assays, designed to analyze surface proteins of individual EVs, employing a combination antibody-DNA conjugates with next-generation sequencing (Wu et al. 2019).

In conclusion, we set a new viewpoint of prediabetes that underlines on GDEs and lipid metabolism giving a great contribute for the field of EVs in the diabetogenic scene and, thus providing a base for future studies

VI

Chapter

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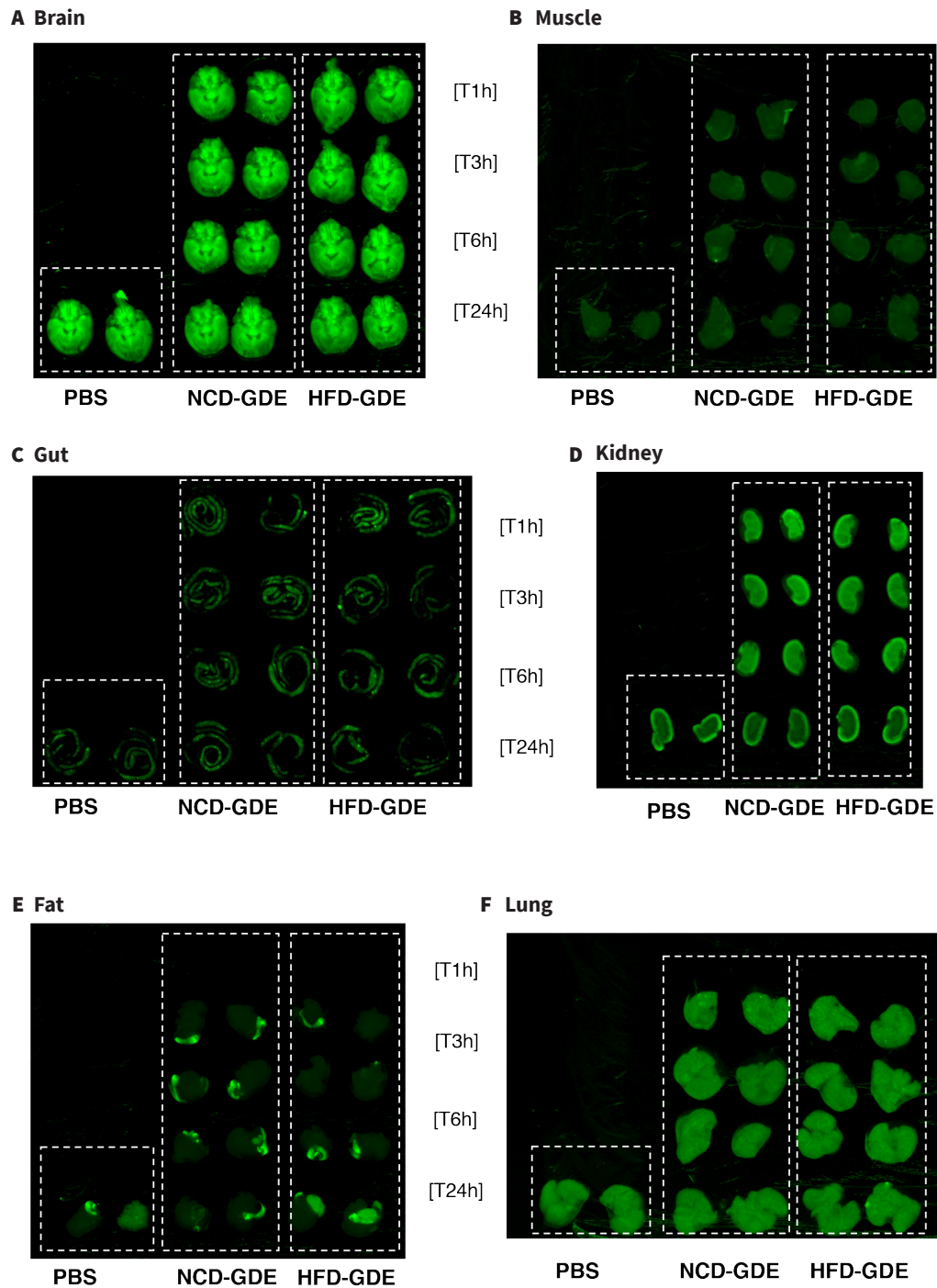
VII

Chapter

Supplementary Data

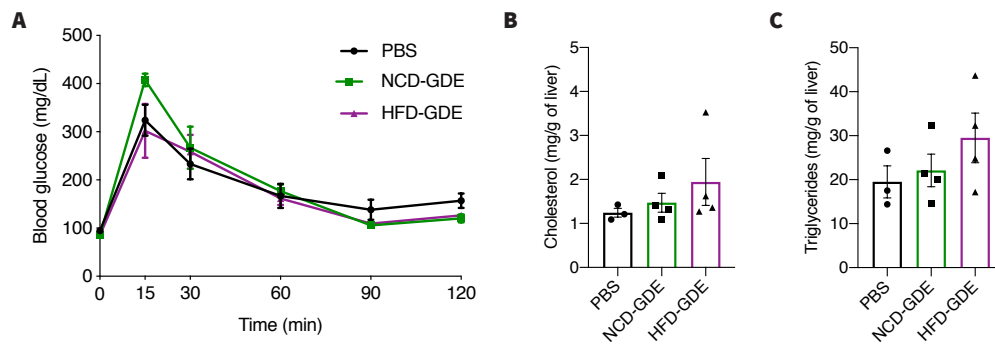
Supplementary Data

- Odyssey images of gut-derived EV biodistribution for all tissues separately



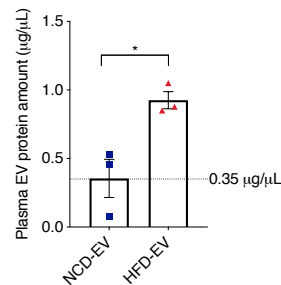
Supplementary Fig.1. Gut-derived EV tracking after retro-orbital injection in healthy mice. Intensity of near infra-red signal detection of several tissues from mice with PBS (PBS), injected with gut-derived EV (GDE) from NCD-fed mice (NCD-GDE) and mice injected with GDE from HFD-fed mice (HFD-GDE), in different time-points after injection (1, 3, 6 and 24 hours). **A.** Infra-red signal detection of brain. **B.** Infra-red signal detection of muscle. **C.** Infra-red signal detection of gut. **D.** Infra-red signal detection of kidney. **E.** Infra-red signal detection of fat /white adipose tissue. **F.** Infra-red signal detection of lung. Images obtained from Odyssey Image System (LI-COR).

2. Education 1



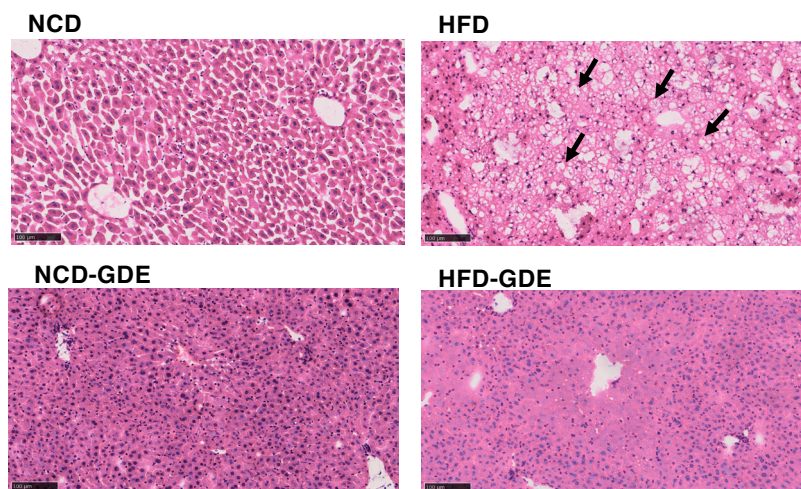
Supplementary Fig.2. Education 1: Mice were injected with 5 μ g of gut-derived EV from NCD-fed mice (NCD-GDE) and 3 μ g of gut-derived EV from HFD-fed mice (HFD-GDE) for three weeks. **A**. Glucose tolerance test of mice at different time points (0, 15, 30, 60, 90, 120 minutes); **B**. Analysis of cholesterol levels in the liver of injected mice. **C**. Analysis of triglyceride (TG) levels in the liver of injected mice. Cholesterol and TG are represented in mg per gram of liver tissue. Results are expressed as mean $\pm$ SEM; n=3 for PBS, n=4 for NCD-GDE and HFD-GDE. ANOVA multiple comparisons.

3. Plasma EV protein amount in healthy and prediabetic mice



Supplementary Fig.3. EV quantification in plasma of mice fed with NCD and HFD. Quantification of amount of protein in plasma EVs, in mg per mL of sample, by Nanosight (NTA). Dotted line highlighting the protein concentration in the plasma of a WT mice, in normal conditions. Results are expressed as mean $\pm$ SEM; Each n is a pool of 20, n=3 for NCD and HFD. Unpaired t test with Welch's correction. *p<0.05

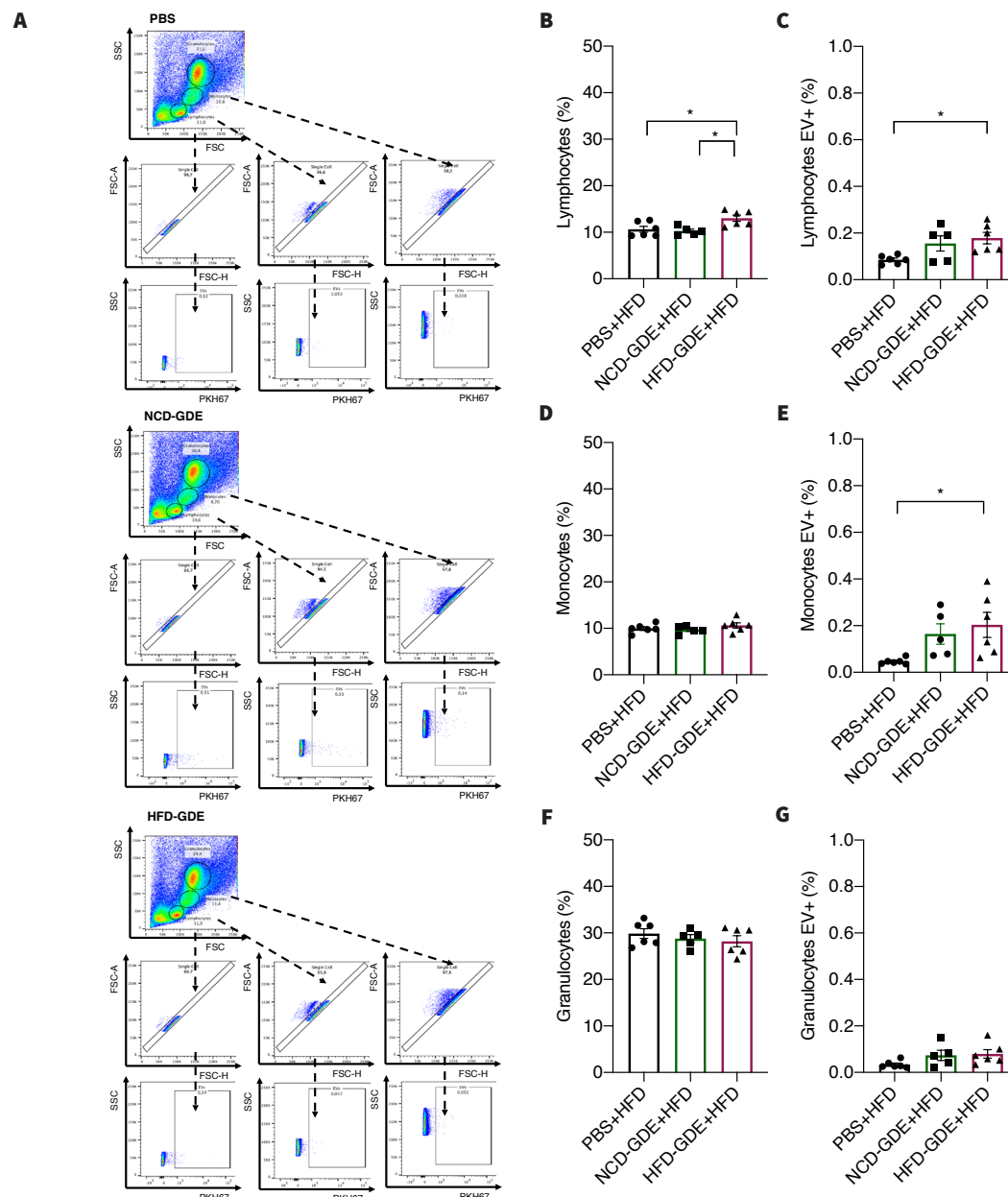
4. Liver histology: diet mice versus educated mice fed with NCD



Supplementary Fig.4. Liver hematoxylin-eosin staining of NCD-fed and HFD-fed mice (top) and NCD-GDE and HFD-GDE injected mice (bottom). Liver of HFD mice reveals steatosis, evidenced by the dominance of the lipid droplets (black arrows) compared with the NCD mice liver (top). Mice were retro-orbitally injected, every other day, with 20 μ g of gut-derived EV from NCD-fed mice (NCD-GDE), 20 μ g of gut-derived EV from HFD-fed mice (HFD-GDE) and PBS for six weeks and fed with NCD (bottom). There is no steatosis observed in the livers of injected mice after six weeks of education.

5. Flow cytometry analysis of bone marrow of educated mice fed with HFD

Considering our preliminary studies on bone marrow cells and gut-derived EV potential interaction, and despite the low frequencies of GDE detected in the tissue, there are some aspects to mention. We observed that lymphocytes were increased in HFD-GDE mice (Supplementary Fig.5B), in opposite to monocytes and granulocytes that did not show any alteration in their population (Supplementary Fig.5D; F), what might indicate an initial phase of an adaptive inflammatory response, since lymphocytes are the first ones to be activated (Burger and Dayer 2002). In terms of EV in this tissue, we could see some traces of EV in the lymphocytes and monocytes but not in the granulocytes, what is in accordance with literature, since there is no record, so far, of granulocytes act as recipient of EV from other cell types, while lymphocytes and macrophages are demonstrated to react to EVs (Chan et al. 2019) (Supplementary Fig.5C; E; G).



Supplementary Fig.5. Bone marrow analysis of educated mice fed with HFD. Bone marrow, collected from femur of mice fed with HFD and injected with PBS, 20 μ g of GDE from NCD-fed mice (NCD-GDE) and 20 μ g of GDE from HFD-fed mice (HFD-GDE) every other day, for six weeks, was analyzed by flow. **A**. Flow cytometry plot with gating strategy for EV tracking in the bone marrow. **B**. Analysis of the percentage of lymphocytes. **C**. Analysis of the percentage of EVs among lymphocytes. **D**. Analysis of the percentage of monocytes. **E**. Analysis of the percentage of EV among monocytes. **F**. Analysis of the percentage of granulocytes. **G**. Analysis of the percentage of EV among granulocytes. Results are expressed as mean $\pm$ SEM; n=6 for PBS and HFD-GDE and n=5 for NCD-GDE. ANOVA multiple comparisons, *p<0.05.

VIII

Chapter

Appendix Document

Proteomic raw data with all the proteins identified in gut-derived EV. In red is highlighted the up-regulated proteins; in blue the down-regulated proteins and in purple the missing values; when two colors are present is because one protein was identified as up or down-regulated but it had an acquisition of zero, therefore it was excluded. Description by columns: Pro - protein headers; Sample names - 18 columns with quantitative values named with sample names (9 columns of NCD sample replicas, 9 columns of HFD sample replicas); mean_C - mean expression for controls (NCD-GDE); mean_T - mean expression for treated (HFD-GDE); GN - gene name; logFC - log2fold change; AveExp - average expression; t - t value; P.Value - Pvalue; adj.P.Val - adjusted P value using BH; B - B-value.

pro	G.data:1	G.data:2	G.data:3	G.data:4	G.data:5	G.data:6	G.data:7	G.data:8	G.data:9	G.data:10	G.data:11	G.data:12	G.data:13	G.data:14	G.data:15	G.data:16	G.data:17	G.data:18	G.data:19	G.data:20	mean_C	mean_T	GN	logFC	AveExp	t	P.Value	adj.P.Val	B
rsp Q06185 ATP5L	0	0	0	0	0	0	0	0	0	0	19.5	20	19	19.9	19.8	19	19	19.4	17.4	0.00000	19.2605	Atp5i	19.3	9.63	76.77	2E-22	7E-19	36	
rsp Q8BTM8 FLNA	23.86	23.9	23.9	23.8	23.9	23.8	24	24	24	22.3	22	22	22.3	22.2	22	22	22	22.1	23.872	22,1496	Flna	-1,7	23	-33,3	2E-16	3E-13	27		
rsp Q08709 PRDX6	22,71	22,6	22,1	22,1	23	22,5	23	23	23	25,5	25	26	26	25,7	26	25	25,3	25,7	22,652	25,6068	Prdx6	2,95	24,1	19,76	8E-13	8E-10	20		
rsp P28063 PSB8_N	25,1	24,8	25,2	24,8	24,9	24,9	25	25	25	23,3	23	23	23,4	22,8	23	23	23,3	22,8	25,016	23,0073	Psmb8	-2	24	-17,9	4E-12	3E-09	18		
rsp Q9Z2U1 PSA5_M	28,33	28,1	28,2	27,1	28,1	27,7	28	28	28	25,4	25	25	25,1	25,5	25	25	25,1	25,4	28,01	25,2089	Psa5	-2,8	26,6	-17,1	7E-12	4E-09	18		
rsp Q8BW13 THIM_M	22,94	23,2	23,1	23,4	23,8	23,5	23	24	24	25,4	25	26	25,2	25	25	25	25,5	25,1	23,415	25,3358	Acaa2	1,92	24,4	16,99	8E-12	4E-09	17		
rsp Q9DCG6 PBLD1_N	19,27	19,7	19,1	19,5	19,3	19,2	19	20	19	21,6	22	21	21,6	21,6	22	21	21,1	21,7	19,336	21,523	Pbl1	2,19	20,4	15,18	5E-11	2E-08	16		
rsp Q6Q2Z6 ACOT5_I	16,25	16,2	16,2	17,2	16,2	16,9	17	17	17	18,8	18	19	19,1	18,8	19	18	18,6	18,9	16,582	18,7521	Acot5	2,17	17,7	15,15	5E-11	2E-08	16		
rsp Q9QNR7 ACOT3_I	16,25	16,2	16,2	17,2	16,2	16,9	17	17	17	18,8	18	19	19,1	18,8	19	18	18,6	18,9	16,582	18,7521	Acot3	2,17	17,7	15,15	5E-11	2E-08	16		
rsp P63268 ACTH_N	28,76	28,8	28,8	28,8	28,8	28,8	29	29	29	26,9	27	28	26,8	26,8	27	28	27,3	27	28,801	27,1108	Actg2	-1,7	28	-14,8	6E-11	2E-08	15		
rsp Q70435 PSA3_N	27,65	27,6	27,7	27,6	27,7	27,7	28	28	27	25,2	25	26	24,8	25,6	26	25	25,4	25,7	27,692	25,4363	Psa3	-2,3	26,6	-14,8	7E-11	2E-08	15		
rsp Q01853 TERA_M	25,05	25,3	25,3	25,6	25,8	25,9	25	26	26	23,9	24	24	24,1	23,8	24	24	23,8	23,8	25,489	23,8189	Vcp	-1,7	24,7	-14,4	1E-10	3E-08	15		
rsp Q60605 MYL6_N	23,54	23,9	24,3	24,2	24,3	23,6	25	24	24	21,9	22	22	22,1	22,5	22	22	21,8	22,3	23,993	22,0779	Myf6	-1,9	23	-13,8	2E-10	4E-08	14		
rsp P62737 ACTA_N	28,76	28,7	28,8	28,8	28,8	28,8	29	29	29	26,8	27	28	26,7	26,8	27	28	27,1	26,9	28,75	27,0282	Acta2	-1,7	27,9	-13,8	2E-10	4E-08	14		
rsp P58404 STRN4_I	14,62	15,7	16,6	18,7	16,4	16	15	18	15	21,8	22	22	22,6	22,6	23	22	22,1	22	16,288	22,2578	Strn4	5,97	19,3	13,75	2E-10	4E-08	14		
rsp P97315 CSR1_N	23,46	23,9	23,8	23,9	24,3	23,8																							

>sp Q8CIE6 COPA_Mi	21,6	21,7	20,2	20,9	20,9	20,6	21	21	21	21,2	21	21	20,8	21	21	21	21,1	20,8	20,899	20,9596	Copa	0,06	20,9	0,375	0,713	1	-7
>sp Q9CQ80 VPS25_	19,36	19,8	23,7	19,1	24,1	23,9	19	24	18	22	18	19	22,7	22,5	23	22	22,2	22,3	21,203	21,5696	Vps25	0,37	21,4	0,373	0,714	1	-7
>sp Q03249 GALT_M	17,27	15,2	14,8	19,1	0	15,8	16	15	0	14,9	15	15	15,3	0	16	16	15,5	15,9	12,535	13,6204	Galt	1,09	13,1	0,372	0,715	1	-7
>sp Q8QZ1 EIF3_LMC	23,02	22,9	22,3	22,3	22,4	22,7	23	22	22	22,4	22	22	22,7	23,2	22	22	22,7	23	22,471	22,5375	Elf3l	0,07	22,5	0,371	0,715	1	-7
>sp Q9DCC4 P5CR3_	21,78	21,6	21,8	22,2	22,3	22,2	22	22	22	21,6	22	22	21,9	22,5	23	22	21,9	22	22,035	22,0889	Pycr3	0,05	22,1	0,371	0,716	1	-7
>sp Q92111 TRFE_MO	20,3	20,5	21,2	21	20,8	21	21	21	20	20,4	21	21	21	20,5	21	20	20,6	20,5	20,758	20,7071	Tf	-0,1	20,7	-0,37	0,716	1	-7
>sp P08775 RPB1_A	18,33	17,1	18,3	18,6	17	17,4	18	17	18	17,2	17	17	18,3	18,6	18	18	17	16,9	17,715	17,5997	Polr2a	-0,1	17,7	-0,37	0,717	1	-7
>sp Q60967 PAP51_	20,66	20,9	21,9	22,3	20	22	20	20	20	22,8	23	23	19,6	23,4	20	20	19,9	19,3	20,981	21,211	Papss1	0,23	21,1	0,366	0,719	1	-7
>sp Q8K274 KT3K_IV	17,51	17,4	16,2	17,4	17,4	16,1	17	17	18	16,2	17	17	16,7	16,5	17	17	17,8	17,1	17,037	16,9459	Fn3knp	-0,1	17	-0,36	0,721	1	-7
>sp Q921M3 SF3B3_	19,41	19,9	18,9	20,1	18,9	18,8	19	19	19	19	19	19	19,9	19,6	20	20	19,7	18,7	19,267	19,3556	Sf3b3	0,09	19,3	0,363	0,721	1	-7
>sp Q8K4Z3 JNRE_M	17,66	19,6	19,3	18	19,2	18,6	19	19	19	19,8	19	18	18,8	19,1	18	19	18,8	18,4	18,81	18,7012	Naxe	-0,1	18,8	-0,36	0,721	1	-7
>sp P47955 RLA1_IV	15,99	14,5	13,7	13,6	13,7	0	0	19	18	19,4	0	0	21,7	20,9	21	0	0	12,3	12,064	10,5603	Rplp1	-1,5	11,3	-0,36	0,721	1	-7
>sp Q8CHW4 B2BE_A	14,67	14,5	13,7	14,7	14,2	13,3	14	14	14	15,2	0	16	15,1	15,2	15	16	14,5	15,3	14,184	13,5742	Ef2b5	-0,6	13,9	-0,36	0,722	1	-7
>sp Q8CEC6 PPWD1_	18,63	18,8	0	0	0	0	18	18	18	9,02	15	0	0	13,7	0	20	0	19,8	10,067	8,528594	Ppwd1	-1,5	9,3	-0,36	0,723	1	-7
>sp O54962 BAF_MC	22,15	22,4	19,9	23,6	23,6	22,8	23	23	22	23,3	22	22	22,8	22,9	22	23	23,1	23,2	22,549	22,7017	Banf1	0,15	22,6	0,361	0,723	1	-7
>sp Q9CWU9 NUP37	20,72	21,9	21,5	21,6	22	21,5	22	20	22	21,5	21	21	21,7	20,8	22	22	21,9	22,1	21,419	21,5246	Nup37	0,11	21,5	0,359	0,724	1	-7
>sp Q80X76 SPA3F_I	19,6	19,8	18,2	19	18	18,8	18	17	18	18,6	19	19	19,4	18,7	18	19	18,5	19	18,613	18,7181	Serpina3f	0,11	18,7	0,356	0,726	1	-7
>sp Q99P31 HPBP1_	20,03	18,6	20	19,7	17,2	20,1	19	19	19	20,2	20	20	16,5	20,3	16	19	19,3	18,4	19,14	18,9228	Hspbp1	-0,2	19	-0,36	0,727	1	-7
>sp Q99M74 KRT82_	19,36	20,1	17,3	18,2	18,6	16,7	19	17	17	18,1	17	17	17,8	18,3	19	20	18,6	18,1	18,129	18,3124	Krt82	0,18	18,2	0,354	0,728	1	-7
>sp Q6ZQ88 KDM1A_	17,51	17,5	18,7	17,1	16	17,1	17	17	19	17,3	17	17	18,2	17,7	18	17	17,4	17,5	17,425	17,5277	Kdm1a	0,1	17,5	0,351	0,73	1	-7
>sp Q9D312 K1C20_	20,77	21,7	21,3	21,2	21,1	19,9	21	20	21	19,7	21	21	20,9	21,1	21	22	20,4	20,4	20,857	20,7673	Krt20	-0,1	20,8	-0,35	0,73	1	-7
>sp Q80X41 VRK1_A	18,61	18,8	18	19,2	19,3	17,5	19	19	19	19	19	18	18,3	17,4	19	19	19,2	19	18,693	18,589	Vrk1	-0,1	18,6	-0,35	0,731	1	-7
>sp P0C872 JIMD7_	16,23	16,2	18,3	15,9	14,3	16,9	15	15	19	16,5	21	18	16	16,2	18	12	17,5	16,4	16,385	16,7237	Jimd7	0,34	16,6	0,349	0,731	1	-7
>sp Q922V4 PLRG1_	17,46	17,9	16,4	17,5	17,3	14,1	18	18	18	19,7	19	17	17,5	16,7	16	17	17,5	16,7	17,203	17,3963	Plrg1	0,19	17,3	0,348	0,732	1	-7
>sp P67984 RL22_IV	20,36	21	20,1	19,9	20,6	21,2	21	21	21	19,9	21	21	20,4	20,8	21	21	20,9	20,8	20,706	20,7783	Rp122	0,07	20,7	0,348	0,732	1	-7
>sp Q8VCM7 FBG_M	20,24	20,9	21,1	21,3	21,2	21,3	21	21	21	21,1	21	21	21,3	20,8	22	21	21,5	21,2	21,122	21,1762	Fbg	0,05	21,1	0,347	0,733	1	-7
>sp Q921F4 JHNRLL_A	14,8	15,1	13,1	16,4	14,7	15,7	17	16	16	16,5	15	14	16,3	15,5	15	16	15,9	15,8	15,424	15,5797	Hnrrpll	0,16	15,5	0,347	0,733	1	-7
>sp P14434 HA2B_A	18,49	18,2	18,5	18	18	18,5	17	19	19	18,4	19	17	17,7	19,8	19	18	18,1	18,2	18,309	18,4038	H2-Aa	0,09	18,4	0,346	0,734	1	-7
>sp P70265 F262_IV	14,04	13,8	13,8	13,5	13	12,7	14	13	12	13,3	12	13	13,6	13,3	13	12	13,3	14	13,229	13,1332	Pf1fb2	-0,1	13,2	-0,34	0,735	1	-7
>sp Q91WU0 CES1F_I	22,65	22,7	16,6	23,8	23,6	16,7	23	19	18	20,2	20	20	20,3	20,6	18	22	19,8	20,4	20,582	20,2215	Ces1f	-0,4	20,4	-0,34	0,736	1	-7
>sp P56371 RAB4A_	20,42	16,8	20	20,5	20,4	21	20	15	20	20	20	20	20,5	20,3	16	20	20,3	20,1	19,48	19,7547	Rab4a	0,27	19,6	0,341	0,737	1	-7
>sp Q9Z0Z4 HEPH_MK	20,68	19,7	20,4	19,7	19,5	19,7	20	20	20	19,5	20	21	19,3	21,2	19	19	21,5	19,7	19,937	20,0497	Heph	0,11	20	0,339	0,739	1	-7
>sp P99029 PRDX5_	22,52	23,2	23,3	22,6	24,4	23,7	24	24	24	24,5	24	24	22,5	23,6	23	23	23,4	22,2	23,462	23,3579	Prdx5	-0,1	23,4	-0,34	0,74	1	-7
>sp Q9CYA6 ZCHC8_I	17,92	20,7	21,4	17,5	17,5	17,5	17	16	16	9,61	22	22	18,8	22,3	20	18	17,7	17,4	18,078	18,5402	Zchc8	0,46	18,3	0,33	0,745	1	-7
>sp Q922E4 PCY2_M	18,27	23,3	20,9	20,4	19,4	19,9	23	22	22	20,1	20	20	20	20,9	20	23	20,3	22,7	21,061	20,8389	Pcyt2	-0,2	21	-0,33	0,746	1	-7
>sp Q61151 2A5E_IV	16,54	19,9	19,5	19,5	19,6	19,6	20	17	19	18,8	17	19	19,5	19,4	20	19	17,8	18,7	19,019	18,862	Ppp2r5e	-0,2	18,9	-0,33	0,746	1	-7
>sp P24549 AL1A1_	25,01	25	25,2	25,6	25,6	25,7	26	26	26	25,3	25	25	25,7	25,6	26	25	25,5	25,6	25,485	25,5222	Aldh1a1	0,04	25,5	0,326	0,748	1	-7
>sp Q00898 A1AT5_	24	23,9	23,3	21,6	21,2	24,1	24	24	24	24,1	23	22	23,2	22,7	24	23	24,2	22,2	23,358	23,2097	Serpina1e	-0,1	23,3	-0,33	0,749	1	-7
>sp Q921W4 QORL1_	18,15	18,3	19,1	19	18,6	18,8	20	21	20	18,6	19	19	18,7	18,9	19	20	20,1	18,9	19,19	19,0794	Cryz1l	-0,1	19,1	-0,33	0,749	1	-7
>sp Q8CG72 ARHL2_	17,61	22,2	22	21,9	22	21,9	22	17	16	19	21	21	21,3	20,8	21	21	20,7	20,4	20,378	20,656	Adprhl2	0,28	20,5	0,325	0,749	1	-7
>sp Q9DC61 MPPA_I	21,64	22,3	21,5	21,9	22,1	22,5	22	22	22	21,4	21	20	23,1	23	23	22	22,1	19,9	21,933	21,7994	Pmpca	-0,1	21,9	-0,32	0,752	1	-7
>sp Q3UW53 NIBAN_	21,24	20,4	19,9	20	20,3	19,8	21	20	20	20	20	20	20,4	20,4	20	20	19,9	20,2	20,337	20,2721	Fam129a	-0,1	20,3	-0,32	0,754	1	-7
>sp Q9R0Q6 ARC1A_	19,29	19,6	19,4	19,2	19	20,3	19	20	20	19,5	20	19	21	19	19	19	19	21,1	19,577	19,6814	Arcp1a	0,1	19,6	0,319	0,754	1	-7
>sp Q6P8X1 SNX6_IV	18,99	18,8	17,9	18,9	17,3	17,4	18	18	17	18,1	18	17	18,7	17,8	18	19	18,2	16,7	18,044	17,9454	Snx6	-0,1	18	-0,31	0,757	1	-7
>sp Q9D9C6 CC182_	17,32	16,9	18,7	17,2	18,5	16,8	17	19	18	17,5	16	18	17,7	18,3	19	18	17,4	18	17,701	17,8262	Ccdcl82	0,13	17,8	0,314	0,758	1	-7
>sp Q07076 ANKA7_	18,47	19,1	21,6	18,2	17,8	17,7	18	19	19	18,3	22	22	18	17,5	18	19	18,7	17,6	18,738	18,9669	Anxa7	0,23	18,9	0,313	0,758	1	-7
>sp P09405 NUCL_IV	18,08	17,8	18	18	17,5	16,5	18	18	18	18,1	18	18	17,3	17,2	17	18	18,1	18,1	17,743	17,8114	Ncl	0,07	17,8	0,313	0,758	1	-7
>sp P40142 TKT_MO	25,2	25	25,7	25,7	25,6	25,8	26	26	26	25	26	25	25,6	25,4	25	26	25,8	25,8	25,559	25,5193	Tkt	-0	25,5	-0,31	0,759	1	-7

>sp Q49714 KRT35_A	20,49	16,3	16,4	16,5	16,3	15,6	16	19	19	8,48	20	17	16,6	20,7	17	22	17,3	22,1	17,364	17,8344	Krt35	0,47	17,6	0,312	0,759	1	-7
>sp A6X935 ITH4_M	22,64	22,4	22	22,2	22,9	22,2	22	22	21	21,6	23	23	23,1	22,9	23	18	22,7	21,8	22,218	22,0426	Ith4	-0,2	22,1	-0,31	0,761	1	-7
>sp Q9QZE5 COPG1_I	23,61	23,5	23,5	20,2	23	19,1	22	22	19	23,3	23	20	23,5	23,6	24	23	19	19,1	21,781	22,0759	Copg1	0,3	21,9	0,308	0,762	1	-7
>sp P60867 RS20_IV	23	20,7	20,5	20,6	20,7	21,2	20	20	21	20,6	21	21	20,5	20,2	21	20	21,2	21	20,847	20,7515	Rps20	-0,1	20,8	-0,31	0,762	1	-7
>sp P29341 PABP1_I	21,72	20,5	20,7	20,3	20,6	21	20	20	21	20,3	21	21	20,7	20,8	21	21	20,8	20,7	20,652	20,7025	Pabpc1	0,05	20,7	0,307	0,763	1	-7
>sp P53994 RAB2A_I	20,51	20,4	20,2	20,1	19,6	20,5	20	21	21	20,7	20	20	20,5	20,3	20	21	20,5	20,3	20,374	20,4211	Rab2a	0,05	20,4	0,307	0,763	1	-7
>sp P59108 CPNE2_I	16,96	14,9	15,9	15,5	16,2	15,7	17	16	17	15,5	16	16	15,6	15,7	18	18	15,6	16,5	16,168	16,2984	Cpne2	0,13	16,2	0,305	0,764	1	-7
>sp Q88939 ZBT7A_I	16,48	17,5	14	13,9	18	14,2	16	16	16	16,3	15	16	14,3	14,5	16	18	18,4	16,9	15,903	16,1099	Zbtb7a	0,21	16	0,305	0,764	1	-7
>sp P97346 NXN_MK	14,3	13,7	13,5	14,1	13,2	14,7	13	11	12	8,31	12	12	16,8	11,9	17	12	10,9	15,3	13,266	12,9511	Nxn	-0,3	13,1	-0,3	0,765	1	-7
>sp Q8R164 BPHL_IV	19,76	21	24,2	19	18,9	19,4	20	23	24	23,1	24	23	19,2	23,4	19	19	19,1	21,6	21,01	21,3105	Bphl	0,3	21,2	0,304	0,765	1	-7
>sp P45591 COF2_IV	23,08	23,1	25	22,8	23,2	23,2	23	24	25	22,7	24	23	23,5	24,3	24	23	24,5	23,1	23,517	23,6176	Cfl2	0,1	23,6	0,304	0,765	1	-7
>sp P82198 BGH3_A	21,68	22	23	22	23	22,6	23	22	23	22,2	22	22	22,9	23,4	23	22	22,3	22,5	22,441	22,5124	Tgfb1	0,07	22,5	0,303	0,766	1	-7
>sp Q8CG03 PDE5A_I	19,27	19,2	19,1	18,5	19,2	19	19	19	19	18,7	19	19	19	19,2	19	19	18,4	19	19,029	18,9913	Pde5a	-0	19	-0,3	0,767	1	-7
>sp Q8BYM8 SYCM_IV	14,58	15,2	16,5	14,8	17	17,1	16	16	17	15	14	15	16,9	16,2	19	15	16	15,9	16,033	15,8676	Cars2	-0,2	16	-0,3	0,768	1	-7
>sp Q99N89 SF3B1_I	14,6	13,8	16,8	14,6	14,8	14,1	18	15	16	15,3	17	17	15,3	14,5	15	15	14,7	14,5	15,18	15,3367	Sf3b1	0,16	15,3	0,3	0,768	1	-7
>sp Q7TMB8 CYFP1_I	18,79	18,3	18,4	18,1	18,1	18	17	18	17	18,9	18	18	18,4	17,3	18	18	17,9	17,5	18,046	17,9734	Cyfp1	-0,1	18	-0,3	0,77	1	-7
>sp B1AQ75 KRT36_I	20,42	20,4	16,9	17,1	16,7	16,4	16	19	19	13,6	20	17	16,9	20,7	17	22	17,3	22	18,099	18,4257	Krt36	0,33	18,3	0,297	0,77	1	-7
>sp Q8CGA0 PPM1F_I	16,76	18	18	16,7	17,8	18,1	18	18	18	17,5	18	18	17,9	17,6	17	17	17,6	17,7	17,72	17,6581	Ppm1f	-0,1	17,7	-0,29	0,772	1	-7
>sp P63073 IF4E_MC	20,22	21,8	22,2	20,9	20,6	21,2	21	21	21	21,1	21	20	20,9	20,7	21	21	21,6	20,6	21,039	20,9631	Ef4e	-0,1	21	-0,29	0,773	1	-7
>sp Q61646 HPT_MC	16,1	16,3	14,3	23,9	15,7	24	24	16	16	22,4	23	15	16,5	16,7	23	14	15,3	15,2	18,373	17,8311	Hp	-0,5	18,1	-0,29	0,774	1	-7
>sp P62849 RS24_IV	18,35	16	14,8	15	15,7	15,7	16	17	0	18,5	18	0	16,5	16,1	0	15	16,8	18,1	14,203	13,3057	Rps24	-0,9	13,8	-0,29	0,775	1	-7
>sp Q9D518 F227B_I	18,82	0	18,8	18,6	17,6	19,1	18	17	17	17,3	18	18	14,5	18,7	0	17	17,8	16,9	16,168	15,3591	Fam227b	-0,8	15,8	-0,29	0,776	1	-7
>sp Q80YX1 TENA_MK	18,11	18,2	18	18,3	17,8	18,2	18	17	18	17,7	20	20	17,8	18,1	18	17	17,8	17,1	17,982	18,075	Tnc	0,09	18	0,287	0,778	1	-7
>sp Q9CQ65 MTAP_A	20,9	21,3	25,7	24,7	24,8	21,1	22	25	22	23,7	21	24	24,2	20,1	24	24	24	23,9	22,973	23,2102	Mtap	0,24	23,1	0,283	0,781	1	-7
>sp Q62418 DBNL_IV	0	0	0	16	15,2	0	15	0	0	0	12	0	0	0	0	0	14,2	12,2	5,1854	4,24932	Dbnl	-0,9	4,72	-0,28	0,782	1	-7
>sp Q9Z1Q9 SVC_MK	22,37	21,7	21,5	21,7	21,7	21,6	22	22	22	21,7	21	22	21,6	21,8	22	22	21,6	21,8	21,736	21,7087	Vars	-0	21,7	-0,28	0,782	1	-7
>sp Q8VE47 UBA5_IV	16,36	16,1	16,9	16,9	16,6	15,1	16	16	16	14,5	16	17	16,8	17,1	17	16	16,8	15,9	16,285	16,3785	Uba5	0,09	16,3	0,28	0,783	1	-7
>sp Q9D826 SOX_MC	17,34	17,3	17	20,6	20,8	21,6	21	18	17	17,9	18	18	19,4	20,6	20	21	17,2	17,1	18,989	18,7669	Pipox	-0,2	18,9	-0,28	0,783	1	-7
>sp Q9ESB3 HRG_MO	19,59	19,9	19,4	19,6	19,4	19,1	20	19	19	19,3	19	19	20,6	19,1	20	19	19,4	19,7	19,507	19,554	Hrg	0,05	19,5	0,278	0,784	1	-7
>sp Q70624 MYOC_A	14,88	15,7	15,2	15,2	15,4	15,6	15	14	15	14,3	15	16	15,5	15,2	15	15	14,9	15,2	15,16	15,1031	Myoc	-0,1	15,1	-0,28	0,785	1	-7
>sp Q1RLI3 CPNE9_A	16,63	14,7	15,7	15,2	15,9	15,4	17	16	17	15,1	16	15	15,4	15,4	17	18	15,3	16,2	15,892	16,0097	Cpne9	0,12	16	0,277	0,785	1	-7
>sp Q8JZW4 CPNE5_I	16,63	14,7	15,7	15,2	15,9	15,4	17	16	17	15,1	16	15	15,4	15,4	17	18	15,3	16,2	15,892	16,0097	Cpne5	0,12	16	0,277	0,785	1	-7
>sp P46471 PRS7_IV	19,03	19,4	20,3	19,1	19,5	19	19	21	21	19,1	19	20	20,8	20,4	20	19	19	19,5	19,703	19,6058	Psmc2	-0,1	19,7	-0,28	0,785	1	-7
>sp Q35098 DPYL4_I	19,69	19,9	18,8	19,9	18,5	19	19	19	18	18,2	19	19	18,6	19,3	19	20	19	19,2	19,121	19,0387	Dpyl4	-0,1	19,1	-0,28	0,786	1	-7
>sp Q61107 GBP4_A	0	0	16,3	16,6	16,4	16,6	17	16	16	14,6	0	15	15,8	15,9	16	15	14,9	15,3	12,819	13,6274	Gbp4	0,81	13,2	0,276	0,786	1	-7
>sp Q5U5V2 HYKK_IV	17,53	17,2	17,1	17,3	17,3	16,5	17	17	17	15,8	17	17	17,1	17,7	17	17	16,4	17,6	17,111	17,0501	Hykk	-0,1	17,1	-0,27	0,787	1	-7
>sp Q91W10 S39A8_I	24,6	24,5	17,6	24,6	14,8	24,7	19	19	19	16,8	16	17	25	17	25	25	25	17,1	20,912	20,3915	Slc39a8	-0,5	20,7	-0,27	0,788	1	-7
>sp Q9CYR6 AGM1_A	23,45	23,3	21,8	21,9	22,1	21,9	23	22	23	21,3	21	21	21,7	21,2	24	24	24,3	24,2	22,512	22,6681	Pgm3	0,16	22,6	0,273	0,788	1	-7
>sp Q9D7B6 ACAD8_I	18,33	18,1	18,8	19,2	19	18,9	19	19	18	18,9	19	19	18,8	18,2	19	18	18,4	18,4	18,65	18,6987	Acad8	0,05	18,7	0,271	0,79	1	-7
>sp Q8JZR0 ACSL5_IV	15,38	15	14,9	14,2	16,1	16,1	16	16	15	14,7	20	14	14,9	15,3	14	14	14,5	15,6	15,436	15,2679	Acs15	-0,2	15,4	-0,27	0,79	1	-7
>sp Q88543 CSN3_IV	19,16	18,8	18,8	18,1	18,3	18,5	18	19	21	20,2	19	19	18,7	19	18	18	18,9	18,5	18,869	18,9527	Cops3	0,08	18,9	0,269	0,791	1	-7
>sp Q99KV1 DI11_I	15,04	14,5	14,1	16,7	13,3	14,1	16	19	20	19,6	18	15	15	14,9	14	15	14,8	14,1	15,812	15,5497	Dnagb11	-0,3	15,7	-0,27	0,792	1	-7
>sp Q9DC53 CPNE8_I	16,59	14,6	15,6	15,1	15,8	15,4	17	16	17	15,1	16	15	15,3	15,4	17	18	15,2	16,2	15,835	15,9481	Cpne8	0,11	15,9	0,267	0,793	1	-7
>sp Q9Z140 CPNE6_I	16,59	14,6	15,6	15,1	15,8	15,4	17	16	17	15,1	16	15	15,3	15,4	17	18	15,2	16,2	15,835	15,9481	Cpne6	0,11	15,9	0,267	0,793	1	-7
>sp Q0QV82 CPNE7_I	16,7	14,8	15,7	15,2	16	15,5	17	16	17	15,2	16	15	15,4	15,5	17	18	15,4	16,2	15,96	16,0723	Cpne7	0,11	16	0,265	0,794	1	-7
>sp Q8BLR2 CPNE4_I	16,7	14,8	15,7	15,2	16	15,5	17	16	17	15,2	16	15	15,4	15,5	17	18	15,4	16,2	15,96	16,0723	Cpne4	0,11	16	0,265	0,794	1	-7
>sp Q9JL8 SART3_MK	16,49	16,4	15,6	16,3	18,7	19,4	19	19	16	14,7	18	18	17,5	17,6	17	17	17,4	17,3	17,411	17,2472	Sart3	-0,2	17,3	-0,26	0,795	1	-7
>sp P16110 LEG3_M	24,34	24,3	23,7	23,8	25,1	25	25	23	23	22,6	23	25	24,7	24,5	24	24	24,1	24,3	24,096	23,9957	Lgals3	-0,1	24	-0,26	0,795	1	-7

sp P59113 FERM1_	16,18	15,8	14,9	14,1	17,3	16,7	16	16	16	14	18	18	15,5	15	14	14	15	17,2	15,814	15,6467	Fermt1	-0,2	15,7	-0,26	0,795	1	-7
sp Q99MN9 PCCB_I	23,39	21,6	21,5	22,6	20,7	20,8	22	21	22	22,1	22	23	21,9	23,2	23	20	21,6	19,3	21,723	21,8575	Pccb	0,13	21,8	0,263	0,796	1	-7
sp Q9JMA2 TGT_MO	19,47	19,1	20,9	19,3	19,6	19,9	20	20	21	18,9	19	20	20,1	20,1	20	20	19,7	19,5	19,794	19,729	Qtrt1	-0,1	19,8	-0,26	0,796	1	-7
sp P63328 PP2BA_	20,34	20,1	19,6	20,7	20,6	20,2	20	20	21	19,8	21	20	20,3	20,3	21	20	20,8	20,3	20,367	20,4137	Ppp3ca	0,05	20,4	0,262	0,796	1	-7
sp O55142 RL35A_	21,94	21,1	22	24,2	22,2	21,7	21	21	22	21,4	22	22	22,4	22,2	23	22	21,5	21,3	21,944	21,8554	Rpl35a	-0,1	21,9	-0,26	0,797	1	-7
sp Q9R099 TBL2_M	16,52	18,2	19	18	17,9	17,9	17	18	17	19,1	16	20	17,6	16,1	17	18	18	17,5	17,734	17,609	Tbl2	-0,1	17,7	-0,26	0,797	1	-7
sp Q3V0K9 PLS_MC	23,66	23,1	23	22,8	23,2	23,1	23	23	23	22,8	23	23	23,5	22,9	23	23	23,2	23,3	23,002	22,9666	Pls1	-0	23	-0,26	0,8	1	-7
sp Q6A4J8 UBP7_IV	17,99	18,2	17,5	18,4	17,1	17,7	18	16	17	17,5	17	17	18	17,5	18	17	18,8	17,5	17,61	17,6781	Usp7	0,07	17,6	0,255	0,802	1	-7
sp P05202 AATM_A	21,1	21,1	20,1	20	19,9	23,8	20	20	24	20,8	22	21	20,8	23,1	21	21	21,3	20,7	21,061	21,214	Got2	0,15	21,1	0,255	0,802	1	-7
sp P17156 HSP72_	24,57	24,8	25	24,8	24,8	25,7	25	25	25	25	25	25	24,8	25	25	25	25,4	25,4	25,055	25,0902	Hspa2	0,04	25,1	0,253	0,803	1	-7
sp P23492 PNPH_A	23,36	23	22,1	23	22,6	22,5	23	23	23	23,3	22	22	23,3	23,3	23	23	23,5	23,1	22,935	22,9935	Pnp	0,06	23	0,253	0,804	1	-7
sp P46935 NEDD4_	19,42	17,7	18,5	18,9	18,5	18,5	19	19	19	18,7	18	19	19,6	19,6	17	19	19,3	19	18,816	18,895	Nedd4	0,08	18,9	0,253	0,804	1	-7
sp Q8K0B8 FIBB_MC	21,36	21	21,1	21,3	21,5	21,4	21	21	21	21,1	23	20	21	20,8	22	21	20,8	20,5	21,149	21,0854	Fgb	-0,1	21,1	-0,25	0,804	1	-7
sp P16294 FA9_MC	19,92	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13,9	2,2134	1,54269	F9	-0,7	1,88	-0,25	0,805	1	-7
sp P97798 NEO1_IV	16,15	15,4	15,3	16	15,4	14,3	16	16	14	15,3	15	15	16,3	15,4	16	15	14,7	15	15,298	15,2228	Neo1	-0,1	15,3	-0,25	0,805	1	-7
sp O35381 AN32A_	22,47	21,8	23,6	21,7	21,4	20,9	21	21	21	22,5	22	23	22,9	22,5	23	20	20,3	20,6	21,741	21,8524	Anp32a	0,11	21,8	0,251	0,805	1	-7
sp Q60994 ADIPO_I	18,86	19,3	19,7	19,6	19,4	20,1	20	19	19	18,9	19	19	19,8	19,9	19	19	20,1	20	19,505	19,454	Adipoq	-0,1	19,5	-0,25	0,805	1	-7
sp Q99LB2 DHRS4_I	19,59	19,7	17,7	17	19,1	19,5	19	18	18	18,4	19	19	18,6	19,3	19	18	18,3	19,3	18,663	18,7526	Dhrs4	0,09	18,7	0,249	0,806	1	-7
sp Q9WV91 FPRP_A	15,12	15,9	16,5	15,8	15,7	15,2	16	14	15	15,5	16	16	15,5	15,6	15	15	15,5	15,3	15,506	15,5647	Ptgrn	0,06	15,5	0,249	0,807	1	-7
sp Q9WTP7 KAD3_A	19,01	20,5	20,1	20,2	20,1	20,3	20	20	21	20,1	20	20	20,4	20	21	20	20,4	19,9	20,195	20,2442	Ak3	0,05	20,2	0,249	0,807	1	-7
sp Q6ZMU9 RS27_IV	19,61	20,1	20,1	18,7	18,9	19,1	19	20	20	19,8	19	20	19,3	19,5	20	19	18,9	19,4	19,438	19,487	Rps27	0,05	19,5	0,247	0,808	1	-7
sp Q6ZW3 RS27_A	19,61	20,1	20,1	18,7	18,9	19,1	19	20	20	19,8	19	20	19,3	19,5	20	19	18,9	19,4	19,438	19,487	Rps27l	0,05	19,5	0,247	0,808	1	-7
sp Q9DCM2 GSTK1_	19,29	19,3	19,6	19,6	15	19	19	19	16	19,9	20	20	19	19	19	17	18,8	14,3	18,387	18,5906	Gstk1	0,2	18,5	0,245	0,81	1	-7
sp Q8R3P0 ACY2_IV	16,66	17,7	17,9	17,4	17,3	17,3	18	18	18	17,3	17	18	16,2	18,1	18	17	17,9	17,8	17,493	17,5546	Aspa	0,06	17,5	0,244	0,81	1	-7
sp P62835 RAP1A_	21,99	22,1	21	22,2	21,8	22,2	22	22	21	21,2	23	22	22,6	22,5	22	21	20,7	22,1	21,741	21,8148	Rap1a	0,07	21,8	0,243	0,811	1	-7
sp P18760 COF1_IV	24,47	24,1	24,1	24,4	24,2	24,3	24	25	24	24,2	24	24	24,7	23,9	24	24	24,7	24,2	24,328	24,2943	Cfi1	-0	24,3	-0,24	0,811	1	-7
sp Q99L13 3HIDH_A	19,2	19,9	20,4	18,1	19,1	18,7	20	20	20	19,5	20	20	18,9	19,8	19	20	19	20	19,359	19,4243	Hibadh	0,07	19,4	0,241	0,813	1	-7
sp Q9WVP1 AP1M2	20,35	20,5	20,1	20,6	20,3	20	20	20	19	20,2	20	20	20	20,4	20	20	19,9	20,1	20,145	20,1781	Ap1m2	0,03	20,2	0,24	0,813	1	-7
sp P45700 MA1A1_	20,63	20,7	19,3	19,4	19,3	18,9	19	18	20	19,4	19	19	17,7	18	18	21	21,7	18,9	19,391	19,2545	Man1a1	-0,1	19,3	-0,24	0,814	1	-7
sp Q8BTZ7 GMPPB_	21,87	22,2	22,5	22,3	23,8	23,5	23	23	23	22,7	23	23	22,4	22,8	23	22	22,7	22,9	22,788	22,7323	Gmppb	-0,1	22,8	-0,24	0,814	1	-7
sp P39039 MBL1_A	17,21	18,3	21,8	17,5	17,6	16,7	18	18	15	17,4	23	17	16,4	17	17	16	17,6	17,3	17,821	17,6175	Mbl1	-0,2	17,7	-0,24	0,815	1	-7
sp Q3TMX7 QSOX2_	15,85	16,4	15,3	18,8	17,2	15	19	19	18	14,4	14	16	14,6	15,6	15	21	20,8	20,5	17,16	16,9023	Qsox2	-0,3	17	-0,24	0,815	1	-7
sp P61965 WDR5_I	21,99	17,8	17,6	18,3	17,7	18,1	18	18	19	18,5	20	19	17,1	18,5	18	19	18,1	17,9	18,575	18,45	Wdr5	-0,1	18,5	-0,24	0,815	1	-7
sp Q99K30 ESL2_A	14,81	14,8	13,6	15,8	15,2	15,7	16	14	14	15,2	15	15	15,5	15	15	15	13,4	14,8	14,796	14,8781	Eps8l2	0,08	14,8	0,237	0,815	1	-7
sp P46660 AINX_M	0	17,2	17	17,3	17,4	18,1	18	18	17	15,3	16	16	16,1	16,1	16	16	16,3	15,8	15,53	15,9856	Ina	0,46	15,8	0,237	0,816	1	-7
sp Q8QZ7 TPRKB_A	15,31	13,5	13,1	0	0	0	0	18	18	18,6	16	16	18,4	0	0	0	0	0	8,6606	7,69308	Tprkb	-1	8,18	-0,24	0,816	1	-7
sp Q64437 ADH7_A	20,29	20,2	16,2	21,4	21	15,8	21	20	20	19,3	20	20	20,8	16,2	17	22	22,2	16,9	19,629	19,3899	Adh7	-0,2	19,5	-0,23	0,817	1	-7
sp O88685 PRS6A_	20,21	19,4	18,8	19,9	20,8	20,1	20	19	19	18,4	21	18	20,5	20,6	20	20	19,7	20,5	19,709	19,8007	Psmc3	0,09	19,8	0,233	0,819	1	-7
sp Q99K51 PLST_MK	21,8	21,7	21,8	20,8	21,3	21,5	21	22	22	21,2	22	21	20,9	21,1	21	21	21,5	22,3	21,447	21,4033	Pls3	-0	21,4	-0,23	0,82	1	-7
sp Q99J6 RAP1B_A	22,35	22,4	21,6	22,4	22,1	22,3	22	22	22	21,9	23	22	22,9	22,6	23	21	20,7	22,1	22,096	22,0371	Rap1b	-0,1	22,1	-0,23	0,82	1	-7
sp O08795 GLU2B_	15,73	15,9	0	16,4	15,8	16,2	16	16	16	14,8	0	15	15,6	15,8	16	15	15,1	15,6	14,244	13,6783	Prkcsb	-0,6	14	-0,23	0,82	1	-7
sp P70694 DHB5_A	18,09	19,1	19,9	19,9	19,9	20	20	20	20	19,9	18	20	19,9	19,9	20	20	19,6	19,4	19,677	19,613	Akr1c6	-0,1	19,6	-0,23	0,82	1	-7
sp P54823 DDX6_A	18,17	18	16,3	16,7	17,3	17,8	18	18	17	17,2	18	17	17	18,5	19	18	16,8	17,3	17,505	17,5804	Ddx6	0,08	17,5	0,228	0,822	1	-7
sp Q9CQV8 1433B_	24,64	26,4	23,5	25,8	24	24,3	25	25	25	24,4	25	25	25,1	24,7	25	25	25	24,4	24,825	24,8971	Ywhab	0,07	24,9	0,228	0,823	1	-7
sp Q8K0Y2 KT33A_I	16,91	14,9	20,2	20	20	19,8	17	20	20	18,1	19	18	18,5	18,8	19	18	19	19	18,689	18,5389	Krt33a	-0,2	18,6	-0,23	0,823	1	-7
sp P08226 APOE_IV	20,77	21	19,9	19,5	20,6	20,8	21	20	20	19,9	21	21	20,1	20,9	21	20	20,4	19,4	20,309	20,371	Apoe	0,06	20,3	0,227	0,823	1	-7
sp P80317 TCPZ_M	20,94	20,8	20,1	20,7	20,9	21,3	19	21	19	20,6	21	21	19,9	19,9	20	20	20,8	20,6	20,478	20,4115	Cct6a	-0,1	20,4	-0,23	0,823	1	-7
sp Q61554 FBN1_IV	19,02	18,5	20,4	18,2	18,4	19,3	18	20	20	18,1	20	20	19,2	20,4	19	18	18,7	18,3	19,068	19,1536	Fbn1	0,09	19,1	0,227	0,824	1	-7

>sp P47962 RL5_MC	20,99	20,9	18,5	20,6	19,6	19,7	21	19	20	18,5	24	20	19,3	19,3	20	21	19,8	19,2	20,001	20,1419	Rp15	0,14	20,1	0,225	0,825	1	-7
>sp Q6X893 CTL1_M	16,13	0	0	16,5	15,4	15,8	16	15	14	0	14	0	14,2	12,4	14	16	15,7	16,4	12,15	11,4459	Slc44a1	-0,7	11,8	-0,22	0,826	1	-7
>sp Q6P817 KCR5_M	23,77	21,6	21,7	21,5	21,9	23,5	23	22	22	23,2	23	22	21,8	19,5	22	24	21,3	22	22,23	22,1157	Ckmt2	-0,1	22,2	-0,22	0,827	1	-7
>sp Q9CR39 WPI3_A	17,36	16,5	20,8	17,4	17,4	17,1	18	18	17	15,5	20	20	17,1	17,1	17	17	16,1	16,7	17,682	17,5292	Wdr45b	-0,2	17,6	-0,22	0,827	1	-7
>sp Q8BI00 SYDM_MK	15,7	16,4	16,7	14,3	16,8	15,1	15	16	16	16,4	16	16	15,1	14,8	17	16	15,5	16,1	15,814	15,8952	Dars2	0,08	15,9	0,22	0,829	1	-7
>sp Q99JX3 GORS2_I	13,88	15,1	17,7	0	0	0	0	0	0	17,8	18	18	0	0	0	0	0	0	5,189	6,05423	Gorasp2	0,87	5,62	0,219	0,829	1	-7
>sp Q91X51 GORS1_I	13,91	15,2	17,8	0	0	0	0	0	0	17,9	18	18	0	0	0	0	0	0	5,2182	6,08538	Gorasp1	0,87	5,65	0,218	0,83	1	-7
>sp Q9WU11 MK11_A	20,88	21,1	21,4	21	20,7	20,8	21	21	14	21,1	22	22	17,6	17,5	18	21	21,2	20,9	20,295	20,092	Mapk11	-0,2	20,2	-0,22	0,832	1	-7
>sp Q9WV54 ASAHI_I	18,32	18,3	19,2	19,6	21,7	22,1	19	13	14	17,8	18	18	18,1	18,5	19	19	18,5	17,4	18,405	18,1918	Asah1	-0,2	18,3	-0,22	0,832	1	-7
>sp P11983 TCPA_IV	22,58	22,6	19,7	20,6	19,6	20,7	20	19	19	19,9	21	21	21,2	20	21	20	20,4	19,2	20,469	20,3687	Tcp1	-0,1	20,4	-0,21	0,835	1	-7
>sp Q3THK7 GUAA_IV	19,01	20,5	20,4	20,3	18,8	20,5	20	20	20	20,1	19	20	19,8	20,3	19	20	19,7	20,1	19,923	19,8714	Gmps	-0,1	19,9	-0,21	0,838	1	-7
>sp P70124 SPB5_IV	16,31	15,9	15,8	16,2	16	16,2	16	17	17	17	17	17	15,8	15,9	17	15	16,5	15,8	16,274	16,3325	Serpinb5	0,06	16,3	0,207	0,839	1	-7
>sp Q8CHH9 SEPT8_A	25,08	20,2	19,2	19,6	19,8	19,5	19	19	20	20	18	20	20,3	20,3	21	20	20,3	20,5	20,201	20,0633	Sept8	-0,1	20,1	-0,21	0,839	1	-7
>sp Q3U3R4 LMF1_IV	0	17,3	17	18,5	18,1	17,2	17	17	18	16,3	0	17	17,8	16,9	17	17	16,7	16,4	15,566	15,0153	Lmf1	-0,6	15,3	-0,21	0,84	1	-7
>sp O08911 MK12_I	20,61	20,9	21,1	20,8	20,5	20,6	21	21	14	20,9	21	21	17,4	17,3	18	21	21	20,7	20,052	19,857	Mapk12	-0,2	20	-0,21	0,84	1	-7
>sp Q5FWK3 RHG01_I	17,98	19,5	19,2	19,5	18,6	18,3	18	20	20	20,8	19	18	19,9	18,6	20	18	18,4	17,9	18,928	19,0157	Arhgap1	0,09	19	0,205	0,84	1	-7
>sp Q9D964 GATM_A	16,27	17,3	18,6	18	18,5	17,9	19	19	17	17,3	18	18	17,7	17,5	18	19	17,8	18,3	17,887	17,9515	Gatm	0,06	17,9	0,204	0,841	1	-7
>sp P13595 NCAM1	16,26	16,3	15,4	17,2	14,8	16,1	19	18	17	15,1	17	17	16	16,5	17	17	17	16,8	16,665	16,5697	Ncam1	-0,1	16,6	-0,2	0,841	1	-7
>sp Q9QZE7 TSNAX_IV	17,8	20	16,7	18,1	17,9	20,4	17	20	17	20,2	18	16	18	18,1	18	18	20	18,7	18,393	18,269	Tsnax	-0,1	18,3	-0,2	0,842	1	-7
>sp P63323 RS12_IV	21,59	20,5	19,5	19,3	19,5	20,1	22	20	20	19,8	21	21	20,5	20,7	20	20	20,2	20,3	20,284	20,35	Rps12	0,07	20,3	0,201	0,843	1	-7
>sp Q8BTF8 RALYL_M	14,92	17,9	17,2	17,9	16,6	15,9	21	17	20	16,4	22	18	16,9	17,5	17	17	17,3	15,6	17,588	17,4151	Raly1	-0,2	17,5	-0,2	0,843	1	-7
>sp P62334 PRS10_I	18,88	18,7	18,7	18,9	17,9	19	19	20	19	20,1	20	18	18,5	18,2	19	18	18,1	18,4	18,854	18,7855	Psmc6	-0,1	18,8	-0,2	0,844	1	-7
>sp Q91VD9 NDUS1_I	21,03	20,6	20,1	20,6	18,6	21,2	21	21	20	20,9	19	21	20,4	20,4	20	21	20,5	20,6	20,363	20,4251	Ndufs1	0,06	20,4	0,196	0,847	1	-7
>sp O70310 NMT1_A	20,5	20,6	19,4	17,3	18,2	18,9	20	20	20	19,3	20	19	19,3	19,2	19	20	18,8	19,8	19,378	19,4557	Nmt1	0,08	19,4	0,196	0,847	1	-7
>sp Q641P0 ARP3B_I	20,97	21,3	22,1	20,3	22,6	20,5	21	22	22	20,9	21	21	20,7	22,2	20	22	22,2	22,2	21,483	21,4072	Actr3b	-0,1	21,4	-0,2	0,847	1	-7
>sp P48722 HS74L_I	20,33	20,9	22,4	20,5	20,7	21	21	21	21	20,8	22	22	20	19,9	20	22	21,6	21,6	20,986	21,053	Hspa41	0,07	21	0,194	0,848	1	-7
>sp P63011 RAB3A_I	20,69	18,8	20,5	20,7	20,6	21,1	21	18	21	20,3	21	20	20,8	20,7	19	21	20,5	20,3	20,252	20,3277	Rab3a	0,08	20,3	0,194	0,848	1	-7
>sp Q9CZT8 RAB3B_I	20,69	18,8	20,5	20,7	20,6	21,1	21	18	21	20,3	21	20	20,8	20,7	19	21	20,5	20,3	20,252	20,3277	Rab3b	0,08	20,3	0,194	0,848	1	-7
>sp O70591 PFD2_IV	18,65	18,5	19	18,7	18,8	18,9	18	17	19	16,5	19	18	19,4	17	20	19	18,5	18,7	18,432	18,3501	Pfdn2	-0,1	18,4	-0,19	0,851	1	-7
>sp P51410 RL9_MC	20,81	20,9	21,8	20,9	20,8	18,1	21	21	21	18,7	22	22	18,8	22	22	20	20,5	19,3	20,686	20,5795	Rp19	-0,1	20,6	-0,19	0,852	1	-7
>sp Q499X9 SYMM_A	19,52	15,3	15,5	18,8	18,4	16	21	16	15	16,6	18	15	17,4	17,5	17	19	17,6	18,3	17,286	17,4289	Mars2	0,14	17,4	0,187	0,854	1	-7
>sp Q08122 TLE3_MK	18,11	18,4	18,8	18,7	18,1	18,3	18	19	19	19,8	18	17	18,5	18,3	19	19	19,4	17,3	18,442	18,5019	Tle3	0,06	18,5	0,187	0,854	1	-7
>sp Q00897 A1AT4_I	24,31	24,3	24	22,9	23,4	24,4	25	25	25	24,6	24	24	24,1	23,5	24	24	24,6	23,5	24,109	24,0656	Serpina1d	-0	24,1	-0,18	0,856	1	-7
>sp Q9DBM2 ECHP_A	0	0	0	0	0	0	16	15	14	0	0	0	16	16,1	19	0	0	0	4,9609	5,65032	Ehhadh	0,69	5,31	0,185	0,856	1	-7
>sp Q8BGW1 FTO_MC	15,56	14,7	13,8	13,2	15,1	12,9	15	13	0	14,8	0	15	13,8	16	16	15	14,6	12,1	12,598	13,0189	Rto	0,42	12,8	0,183	0,857	1	-7
>sp Q9WV68 PACN2_I	21,51	20,1	19,5	19,5	21,4	19,6	19	20	20	20,2	20	20	20,2	20,3	21	19	19,1	20	19,963	19,8986	Pascin2	-0,1	19,9	-0,18	0,858	1	-7
>sp P62331 ARF6_IV	16,43	24,6	19,3	15,2	16,5	16,7	15	18	18	17,7	18	18	17,4	18	18	18	18,3	17,9	17,733	17,9062	Arf6	0,17	17,8	0,181	0,859	1	-7
>sp Q99PE9 ARL4C	16,43	24,6	19,3	15,2	16,5	16,7	15	18	18	17,7	18	18	17,4	18	18	18	18,3	17,9	17,733	17,9062	Ar14d	0,17	17,8	0,181	0,859	1	-7
>sp P45376 ALDR_IV	23,89	23,9	26	23,7	24,5	23,9	24	24	24	23,9	25	25	23,9	24,3	24	24	23,5	24,2	24,234	24,1819	Akr1b1	-0,1	24,2	-0,18	0,859	1	-7
>sp P35123 UBP4_A	14,18	13,7	15,9	15,4	16,1	15,7	14	15	15	15,5	16	15	15	15,6	15	15	14,9	13,7	14,995	15,0604	Usp4	0,07	15	0,178	0,861	1	-7
>sp Q9DCD0 6PGD_A	25,14	25	24,3	24,6	25,5	24,7	26	25	24	25,1	25	25	24,8	25	25	25	25	25,1	24,9	24,927	Pgd	0,03	24,9	0,178	0,861	1	-7
>sp Q8BVE3 VATH_M	18,56	19,2	18,9	19,5	16,5	18,6	19	20	20	17,6	19	18	19,4	17,7	20	19	19,2	19,2	18,867	18,7928	Atp6v1h	-0,1	18,8	-0,18	0,861	1	-7
>sp Q9CQV6 MLP3B_I	18,18	17,3	19,2	17,3	18,3	18,8	17	17	17	16,8	17	19	19,1	17,8	19	17	17,1	17,6	17,814	17,8882	Map11c3b	0,07	17,9	0,175	0,863	1	-7
>sp Q9D8X1 CUTC_IV	19,26	19,3	18,8	19,4	19	18,3	19	19	20	19,6	20	19	19,7	16,5	18	19	19,5	19,7	19,129	19,0637	Cutc	-0,1	19,1	-0,17	0,864	1	-7
>sp P63017 HSP7C_I	26,68	25,5	25,9	25,8	26	26,5	26	27	26	25,9	26	26	25,7	26	26	28	26,3	26	26,195	26,1556	Hspa8	-0	26,2	-0,17	0,865	1	-7
>sp O88512 AP1G2_I	19,29	19,9	20,8	19,7	19,4	19,9	20	21	20	22,2	18	22	21,2	18	22	18	17,7	21,6	19,92	20,0496	Ap1g2	0,13	20	0,173	0,865	1	-7
>sp Q99KB8 GLO2_IV	18,06	18,2	19,2	18,9	18,1	17,9	20	19	19	18	21	19	18,5	18,3	19	18	18,3	17,5	18,663	18,602	Hagh	-0,1	18,6	-0,17	0,866	1	-7
>sp Q6P6M7 SPCS_IV	19,09	18,8	19,2	16,7	16,6	16,9	16	17	17	16,7	21	21	17,4	17,4	17	13	12,1	19,4	17,419	17,2317	Sepsecs	-0,2	17,3	-0,17	0,866	1	-7

>sp Q9Z1Z0 USO1_Mk	18,97	18,5	19	20,4	20,5	18,9	21	21	21	20	20	20	19,7	19,6	20	20	19,7	19,8	19,8	19,8538	Uso1	0,05	19,8	0,171	0,866	1	-7
>sp Q8BU6 SYIM_MOI	20,57	20	19,8	20,3	20,4	20	21	20	20	20,3	21	21	20,4	20,4	20	20	20,6	20,1	20,322	20,345	lars2	0,02	20,3	0,168	0,868	1	-7
>sp Q62470 ITA3_Mk	16,62	15,2	17	16,2	15,4	16,7	16	17	15	15,5	17	17	16,7	15,3	16	16	15,6	16,3	16,148	16,2024	Itga3	0,05	16,2	0,168	0,868	1	-7
>sp Q8R121 ZP1_MOI	18,63	20,5	17,8	17	17,2	18,3	18	18	18	17,4	19	17	19,4	19,2	20	17	17,9	17,3	18,181	18,2649	Serpina10	0,08	18,2	0,167	0,87	1	-7
>sp Q3U5Q7 CMPK2	21,18	20,8	21	23	22,6	21	21	21	21	22,7	21	20	20,9	21,3	22	21	21,3	21,6	21,446	21,3857	Cmpk2	-0,1	21,4	-0,17	0,871	1	-7
>sp Q8C133 C19L1_A	17,85	17,5	16,9	16,1	17,3	17,6	16	17	19	17,1	17	17	17,1	17,3	17	17	18,9	16,8	17,286	17,2233	Cwf19l1	-0,1	17,3	-0,17	0,871	1	-7
>sp P58710 GGLO_IV	0	19,6	20	20,5	22,2	20	20	21	22	20	0	20	20,9	19,4	20	20	20	20	18,326	17,8024	Gulo	-0,5	18,1	-0,16	0,871	1	-7
>sp Q5DA59 G8G12	0	22,7	22,9	18,6	22,9	17,9	18	23	23	23,4	22	23	0	19,8	19	18	18,7	19	18,699	18,147	Gngl2	-0,6	18,4	-0,16	0,872	1	-7
>sp Q3UJ09 RMD3_A	0	14,7	14,7	14,5	0	0	15	14	0	13,1	0	0	13,9	0	14	13	13,8	0	8,126	7,55638	Rmdn3	-0,6	7,84	-0,16	0,872	1	-7
>sp Q99K9 SYHM_IV	15,64	16,1	15,8	15,9	16,3	16,4	14	16	13	17	16	16	14	14,2	14	16	16,1	16,3	15,463	15,5503	Hars2	0,09	15,5	0,164	0,872	1	-7
>sp Q62188 DPYL3_I	21,51	21,7	21,5	21,8	21,7	21,6	22	21	21	21,4	22	22	21,6	21,8	22	21	21,2	21,6	21,582	21,567	Dpysl3	-0	21,6	-0,16	0,873	1	-7
>sp Q9CW46 RAVR1	19,7	19,5	16,1	19,7	16,5	16,4	20	20	19	17	17	18	17,3	17,1	21	20	20,5	20,3	18,5	18,6245	Raver1	0,12	18,6	0,16	0,875	1	-7
>sp P62823 RAB3C_	21,14	19,5	20,6	20,9	20,7	21,2	21	18	21	20,4	21	21	20,9	20,7	19	21	20,5	20,3	20,39	20,4547	Rab3c	0,06	20,4	0,159	0,875	1	-7
>sp Q9D5V5 CUL5_IV	18,79	19,2	20,9	21,3	21,3	20,8	21	19	21	18,8	21	21	21,1	20,7	21	19	20,6	20,7	20,31	20,3841	Cul5	0,07	20,3	0,158	0,876	1	-7
>sp Q99JF8 PSP1_M	17,12	14,6	19	19,3	19,4	18	17	19	19	18,7	19	19	19	18,7	18	18	15,5	17,3	18,022	18,1218	Psp1	0,1	18,1	0,157	0,877	1	-7
>sp Q99L04 DHRS1_I	14,68	15,7	16	16,1	15,7	16,2	0	16	14	13,3	15	0	15,5	15,1	16	15	15,7	14,9	13,83	13,453	Dhrs1	-0,4	13,6	-0,16	0,877	1	-7
>sp P63325 RS10_IV	15,08	14,8	20,4	16,4	15,2	16,4	0	0	0	16,2	16	17	0	16,4	0	0	16,1	11,5	10,917	10,324	Rps10	-0,6	10,6	-0,16	0,878	1	-7
>sp O55029 COPB2_	21,16	22,1	21,2	22	21,8	21,6	22	21	21	21,4	21	21	22,2	21,4	21	22	22	21	21,583	21,5558	Copb2	-0	21,6	-0,16	0,878	1	-7
>sp P59708 SF3B6_I	20,67	20,5	20,8	21,5	21,2	19,9	20	20	21	20,2	19	20	22	22,3	21	20	20,3	20	20,611	20,5524	Sf3b6	-0,1	20,6	-0,16	0,878	1	-7
>sp P47856 GFPT1_I	19,05	19	20	19,5	19,3	19,3	20	20	20	19,7	19	19	19,5	19,5	20	20	19,5	19,5	19,539	19,5596	Gfpt1	0,02	19,5	0,153	0,88	1	-7
>sp P01803 HVMB3A	16,45	16,7	16,3	19,4	14,5	14,8	16	18	17	18	16	17	19,6	15,3	14	19	16,6	15,1	16,592	16,712	>sp P018	0,12	16,7	0,153	0,88	1	-7
>sp Q8VC30 TKFC_M	20,7	21,1	23,7	21,1	21,1	20,7	22	21	24	21,6	20	23	20,7	22,5	23	21	21,1	21,4	21,651	21,5752	Tkfc	-0,1	21,6	-0,15	0,882	1	-7
>sp Q9ERL7 GMFG_M	17,22	17	19,8	17,6	19	19,3	20	21	21	19,4	20	16	19,2	19,2	19	20	19,7	19,8	19,027	19,1215	Gmfg	0,09	19,1	0,15	0,882	1	-7
>sp O70570 PIGR_M	20,39	20,9	21	21,5	21,6	21,9	21	21	21	19,9	20	20	21,9	20,5	22	22	21,6	22	21,165	21,1129	Pigr	-0,1	21,1	-0,15	0,883	1	-7
>sp Q91WK2 EF3H_A	19,3	19,9	19,8	17,4	16,8	18,3	18	18	17	18,1	19	19	17,7	18,9	19	17	16,8	17,3	18,236	18,1647	Ef3h	-0,1	18,2	-0,15	0,884	1	-7
>sp P57746 VATD_IV	18	17,5	0	16,9	18	15,6	0	16	17	17,2	0	0	18,3	17,6	19	16	17,4	17,3	13,214	13,7333	Atp6v1d	0,52	13,5	0,145	0,886	1	-7
>sp Q8BX02 KANK2_	17,19	16,9	23,8	17,5	16,8	23,7	16	17	16	16,8	23	23	16,8	17,9	18	17	15,7	15,6	18,325	18,1294	Kank2	-0,2	18,2	-0,14	0,887	1	-7
>sp Q6PD03 2A5A_A	15,26	16,4	15,7	16,6	16,2	16,3	16	16	16	16,3	16	16	16,5	16,5	16	16	15,6	16,3	16,12	16,1439	Ppp2r5a	0,02	16,1	0,143	0,888	1	-7
>sp Q9CR16 PPID_M	20,45	21,2	23,2	21,8	23,4	20,4	22	23	21	20,8	23	22	20,9	22,1	22	22	21,7	21,7	21,785	21,7266	Ppid	-0,1	21,8	-0,14	0,889	1	-7
>sp P53612 PGTB2_	17,97	18,4	20,7	21,2	21,1	17,8	21	17	18	17,9	18	20	19,6	19,7	20	20	19,9	19,9	19,221	19,3114	Rabggb	0,09	19,3	0,14	0,89	1	-7
>sp P35282 RAB21_	20	21	20,8	21,3	21,1	21,1	21	21	19	21,7	21	19	21,2	20,2	22	20	20,9	19,9	20,709	20,6553	Rab21	-0,1	20,7	-0,14	0,893	1	-7
>sp Q9D0S9 HINT2_IV	18,75	18,8	17,7	18,8	18,5	19,2	19	19	21	19,1	19	19	19,1	19	18	19	19,1	19,3	18,891	18,9308	Hint2	0,04	18,9	0,136	0,893	1	-7
>sp Q8VHY0 CSPG4_I	16,1	16,3	18,4	18,9	18,7	16,7	18	17	18	17,5	17	18	17,6	17,9	18	18	18,1	17,7	17,673	17,6221	Cspg4	-0,1	17,6	-0,13	0,895	1	-7
>sp Q62468 VILI_MO	23,12	23,1	22,9	23	22,9	22,9	23	23	23	24	23	22	22,8	22,6	23	23	24,2	22,5	23,008	23,0363	Vilil	0,03	23	0,134	0,895	1	-7
>sp Q8BT51 COA4_IV	15,21	18,8	15,3	18,6	18,8	19	17	16	21	17,6	19	16	19,8	15,5	20	16	16,1	20,1	17,66	17,7839	COA4	0,12	17,7	0,133	0,896	1	-7
>sp Q6ZWX6 IF2A_Mk	20,12	20,5	21,3	22,4	22,5	22,2	22	22	22	21,6	21	21	21,8	21,6	22	22	21,6	21,8	21,642	21,6029	Ef2s1	-0	21,6	-0,13	0,897	1	-7
>sp Q9CPX6 ATG3_IV	16,68	16,6	16,8	17,9	18,3	20,7	18	18	18	17,4	17	18	17,3	15,2	17	19	19,4	19	17,844	17,7628	Atg3	-0,1	17,8	-0,13	0,897	1	-7
>sp Q9VWA3 BUB3_I	20,48	21,4	21,3	20,7	21,2	20,9	21	21	20	21,2	21	21	20,8	20,8	21	21	20,5	21,2	20,851	20,8759	Bub3	0,03	20,9	0,131	0,898	1	-7
>sp Q8BP40 PPA6_A	15,18	14,6	16,2	14,5	15,4	16,2	15	15	13	12,9	15	15	15,3	15,4	16	15	14,9	15	14,95	14,8978	Acp6	-0,1	14,9	-0,13	0,898	1	-7
>sp O55013 TPPC3_	18,27	19,4	19,2	18,8	19,2	18,2	19	19	19	18,8	20	19	19	18,8	19	18	19,3	18,5	18,9	18,9259	Trappc3	0,03	18,9	0,13	0,898	1	-7
>sp P52825 CPT2_IV	18,17	19,1	18,7	18,2	17,8	19,3	19	19	18	18,9	18	18	18,7	18,4	19	19	19,5	18,4	18,603	18,5722	Cpt2	-0	18,6	-0,13	0,9	1	-7
>sp Q8BGE6 ATG4B_I	0	15,8	14,1	17,1	17,1	16,9	16	17	15	16,7	0	15	18,1	17,2	17	17	15,1	16,4	14,371	14,7001	Atg4b	0,33	14,5	0,127	0,9	1	-7
>sp Q8VEI4 NLE1_MC	15,94	16,3	15,8	16,9	17	17,1	17	17	17	16,6	15	16	16,6	17,4	17	16	17,4	17,2	16,631	16,5948	Nle1	-0	16,6	-0,13	0,9	1	-7
>sp P58252 EF2_MO	24,49	24,5	25,6	25,2	24,2	25	24	24	26	24,9	24	25	25,3	24,2	25	24	25,2	25	24,828	24,796	Eef2	-0	24,8	-0,13	0,902	1	-7
>sp P21278 GNA11_	19,12	20,3	20,8	20,4	20	19,5	20	20	20	20,1	20	20	19,6	20,1	20	20	19,8	19,8	20,057	20,0344	Gna11	-0	20	-0,12	0,904	1	-7
>sp O35103 OMD_M	17,53	17,6	16,7	17	16,3	16,4	17	19	15	16,5	16	16	16,5	17,3	16	17	17,4	17,4	16,883	16,8336	Omd	-0	16,9	-0,12	0,904	1	-7
>sp Q9D892 ITPA_Mk	21,61	22,1	22,5	22,8	21,6	22,7	23	22	23	22,4	22	22	22,4	22,8	23	22	22,4	22,5	22,352	22,376	Itpa	0,02	22,4	0,123	0,904	1	-7
>sp Q8C650 SEP10_I	24,92	19,7	19,4	18,7	18,8	19,4	20	20	22	20,1	19	22	20	20,3	20	20	20,3	20,2	20,289	20,2045	Sept10	-0,1	20,2	-0,12	0,905	1	-7

>sp Q6DTY7 F264_Mk	14,06	14	14	13,6	13,1	13	14	13	12	13,6	13	13	13,7	13,6	13	13	13,6	14,2	13,393	13,3613	PfMfb4	-0	13,4	-0,12	0,907	1	-7
>sp Q8VDJ3 VIGLN_IV	15,53	13,7	15,7	14,4	14,3	16	15	15	15	15	15	16	14,6	15,2	16	13	15,2	15,2	14,965	15,0067	Hdlbp	0,04	15	0,117	0,908	1	-7
>sp P35235 PTN11_	20,54	19,8	19,8	19,9	19,9	19,9	19	20	20	19	21	20	18,7	20,6	20	20	20,2	19,9	19,872	19,9002	Ptpn11	0,03	19,9	0,117	0,908	1	-7
>sp Q9DAF3 DD1_Mk	17,67	17,6	18,2	17,8	17,7	17,5	18	17	17	17,8	18	18	18,5	18,6	19	15	18,5	15,5	17,644	17,59	Dd1	-0,1	17,6	-0,12	0,909	1	-7
>sp O70311 NMT2_	0	0	0	0	0	0	0	16	16	18,6	17	0	0	0	0	0	0	0	3,5579	3,95933	Nmt2	0,4	3,76	0,115	0,91	1	-7
>sp Q6NSR8 PEP11_	20,97	20,8	19,5	21,8	21,1	22,6	23	22	22	21,8	20	22	21,6	22,2	21	22	21,9	22	21,54	21,5834	Npep11	0,04	21,6	0,113	0,911	1	-7
>sp P30416 FKBP4_	21,43	22,4	22,2	24,9	25,2	21,9	25	22	25	23,5	24	24	21	24,3	24	24	24,2	21,7	23,353	23,4268	Fkbp4	0,07	23,4	0,11	0,914	1	-7
>sp P97864 CASP7_	19,28	22,2	21,4	18,3	18,1	18,5	19	19	19	18,8	19	19	19	19,4	20	21	18,8	18,5	19,342	19,2821	Casp7	-0,1	19,3	-0,11	0,915	1	-7
>sp P97927 LAMA4	17,88	17,4	17,6	17,6	17,4	18,1	18	17	17	17,8	18	16	16,4	15,9	18	19	18,4	18,5	17,527	17,4897	Lama4	-0	17,5	-0,11	0,915	1	-7
>sp Q9QXA5 LSM4_IV	22,71	21,7	25,5	21,8	21	20,9	21	21	21	21,3	22	22	22	22,2	22	23	21,5	21,2	21,829	21,8847	Lsm4	0,06	21,9	0,107	0,916	1	-7
>sp Q5I2A0 SPA3G_	19,93	20,1	19	19,3	18,6	19,2	19	19	19	19,1	19	19	19,7	19,2	19	19	19	19,2	19,158	19,18	Serpina3g	0,02	19,2	0,107	0,916	1	-7
>sp Q9JHK4 PGTA_M	19,48	19,7	19,8	21	21,1	22,2	20	21	21	21,4	20	20	20,9	20,1	20	21	20	21,6	20,567	20,6053	Rabgta	0,04	20,6	0,106	0,917	1	-7
>sp Q9WTM5 RUVB2	15,67	15,2	16,3	16,2	14,9	15,3	15	17	17	17,1	17	17	15,2	15,7	15	16	15,8	15,1	15,938	15,9826	Ruvb2	0,04	16	0,106	0,917	1	-7
>sp Q62395 TFF3_Mk	19,88	21,4	0	20,4	19,8	19,2	21	22	22	18	20	19	18,3	19,2	18	18	18,6	17,9	18,279	18,5152	Tff3	0,24	18,4	0,103	0,919	1	-7
>sp P62855 RS26_IV	18,42	17,7	14,6	15,5	19,3	19,1	18	16	17	16,5	19	17	15,6	15,2	15	19	19,6	18,5	17,415	17,3331	Rps26	-0,1	17,4	-0,1	0,919	1	-7
>sp Q7M758 NALDL_	19,16	19,2	19,9	20,1	19	19,3	20	19	19	19,1	19	19	19,8	19,6	20	19	19,2	19,4	19,407	19,4227	Naalad1	0,02	19,4	0,099	0,922	1	-7
>sp P17710 HXK1_	20,57	20,5	20,7	20,6	20,3	20,1	20	21	19	21,6	18	20	19,9	19,9	22	20	19,8	21,4	20,362	20,3227	Hk1	-0	20,3	-0,1	0,922	1	-7
>sp Q922D8 C1TC_IV	19,92	19,8	19,6	19,4	20,2	19,7	20	20	19	19,7	20	19	18,9	20	20	20	20	19,7	19,687	19,7021	Mthfd1	0,02	19,7	0,099	0,922	1	-7
>sp Q8VE95 CH082_	15,28	0	14,9	14,1	17,1	0	16	16	15	16,7	17	15	0	14,7	0	17	16,8	14,8	12,107	12,4252	>sp Q8VE	0,32	12,3	0,098	0,923	1	-7
>sp Q8C1N8 DFA22_	21,74	23,7	22,6	23,2	21	21,7	23	23	23	22,7	24	23	24	20,9	23	22	22,5	21,9	22,567	22,6105	Defa22	0,04	22,6	0,097	0,924	1	-7
>sp Q8C1P2 DFA21_	21,74	23,7	22,6	23,2	21	21,7	23	23	23	22,7	24	23	24	20,9	23	22	22,5	21,9	22,567	22,6105	Defa21	0,04	22,6	0,097	0,924	1	-7
>sp Q8BFU3 RN214_	14,82	18,3	15,2	18,5	18,9	19,5	19	19	19	17,8	18	18	18,3	18,1	19	18	17,8	18,3	17,998	18,0529	Rnf214	0,06	18	0,095	0,925	1	-7
>sp Q91VH6 MEMO1	21,69	21,8	21,9	21	20,7	20,3	21	20	21	21,5	21	21	21,4	20,8	21	21	21	20,6	20,971	20,9942	Memo1	0,02	21	0,094	0,926	1	-7
>sp Q6PAK3 ANMB_I	21,22	19,8	18,6	19,1	20,3	19,8	20	19	19	19,8	19	19	20,5	18,6	19	20	19,4	19,9	19,559	19,5276	Prmt8	-0	19,5	-0,09	0,926	1	-7
>sp Q9C204 CSN7A_I	18,04	19,1	18,2	17,9	17,7	17,9	18	18	18	16,9	18	19	18,3	18,9	18	19	17,6	17,9	18,129	18,1549	Cops7a	0,03	18,1	0,092	0,928	1	-7
>sp P52430 PON1_	19,84	18,6	18,6	20,1	19,8	18,4	20	24	20	20,5	20	20	19	19,5	19	21	20,5	19,7	19,815	19,8665	Pon1	0,05	19,8	0,09	0,929	1	-7
>sp Q6PDY2 AEDO_M	0	15,9	15,9	15,5	15,7	0	15	16	15	14,8	0	0	16,3	16,9	16	15	16,2	15,9	12,116	12,4081	Ado	0,29	12,3	0,09	0,929	1	-7
>sp Q02105 C1QC_	18,46	21,9	0	22,1	22	15	22	21	22	14,9	17	22	16,2	16,2	15	22	21,4	18	18,273	18,0435	C1qc	-0,2	18,2	-0,09	0,93	1	-7
>sp Q9JLF6 TGM1_Mk	16,42	19,2	16,5	18,8	18,8	16,5	17	19	19	18,3	17	17	18,5	17,5	19	18	17,8	18,4	17,833	17,8766	Tgm1	0,04	17,9	0,089	0,93	1	-7
>sp Q9C230 OLA1_M	21,17	22	21,6	21,6	21,7	21,9	22	22	21	21,3	21	21	22	22,4	22	22	21,8	21,3	21,708	21,7239	Ola1	0,02	21,7	0,087	0,932	1	-7
>sp Q61235 SNTB2_I	19,2	19,9	20	19,3	20,4	19	19	20	21	19,3	21	21	20	19,6	19	20	19,4	19,5	19,861	19,8915	Sntb2	0,03	19,9	0,084	0,934	1	-7
>sp P56380 AP4A_	17,31	18,3	19,5	18,3	17,2	18	17	21	21	19,5	20	19	18,8	18,7	18	17	17,6	18,1	18,606	18,5587	Nudt2	-0	18,6	-0,08	0,936	1	-7
>sp P56389 CDD_Mk	22,09	22,3	22,6	22,3	22	22,4	22	23	23	22,8	23	23	21,7	22,4	22	22	22,9	22,4	22,523	22,5066	Cda	-0	22,5	-0,08	0,938	1	-7
>sp O70562 SPR2K_	17,04	17	17,7	18,6	17,7	18,8	16	17	18	19,6	20	20	0	20,4	19	17	20,9	21,1	17,594	17,4196	Sprr2k	-0,2	17,5	-0,08	0,938	1	-7
>sp Q61765 K1H1_	21,51	21	20,5	20,8	20,2	20,1	18	21	21	19,4	21	19	19,7	21,2	19	22	19,3	22,3	20,348	20,3909	Krt31	0,04	20,4	0,078	0,939	1	-7
>sp Q61897 KT33B_	21,51	21	20,5	20,8	20,2	20,1	18	21	21	19,4	21	19	19,7	21,2	19	22	19,3	22,3	20,348	20,3909	Krt33b	0,04	20,4	0,078	0,939	1	-7
>sp P58389 PTPA_IV	22,57	20,7	20,9	21,3	21	21,1	21	21	21	21,4	22	21	20,8	20,6	21	22	21,8	20,9	21,284	21,2628	Ptpa	-0	21,3	-0,08	0,94	1	-7
>sp P27046 MA2A1	15,33	15,5	15,1	0	12,9	0	0	12	15	14,5	16	15	13,3	15,2	14	0	0	0	9,5193	9,7757	Man2a1	0,26	9,65	0,075	0,941	1	-7
>sp P28474 ADHX_	23,43	23,5	24,5	23,9	23,9	24	24	24	24	23,9	24	24	23,7	23,7	24	24	23,9	23,9	23,886	23,8751	Adh5	-0	23,9	-0,07	0,942	1	-7
>sp Q88456 CPNS1_	19,78	19,1	19,2	20	19,7	19,5	20	19	19	18,3	20	20	19	19	20	19	19,9	20	19,435	19,4152	Capns1	-0	19,4	-0,07	0,942	1	-7
>sp Q8JZN5 ACAD9_I	24,51	20,9	21,4	24,4	24,6	24	24	25	24	24,3	22	22	25	22,4	25	25	24,4	24,6	23,696	23,7446	Acad9	0,05	23,7	0,072	0,943	1	-7
>sp Q10738 MMP7_	18,47	20,5	18,8	21,1	18,5	21,1	21	21	19	20,7	20	21	22	21,1	18	18	17,5	20	19,812	19,7655	Mmp7	-0	19,8	-0,07	0,944	1	-7
>sp Q8R0F8 FAHD1_I	19,98	19,7	22,7	22,4	22,8	19,9	20	21	23	21,1	21	21	21	22,8	21	21	21,2	20,8	21,229	21,2635	Fahd1	0,03	21,2	0,069	0,946	1	-7
>sp O70554 SPR2B_	16,73	16,7	17,4	18,3	17,4	18,5	16	17	18	19,3	19	19	0	20,1	19	17	20,7	20,8	17,325	17,1834	Sprr2b	-0,1	17,3	-0,06	0,949	1	-7
>sp O70558 SPR2G_	16,73	16,7	17,4	18,3	17,4	18,5	16	17	18	19,3	19	19	0	20,1	19	17	20,7	20,8	17,325	17,1834	Sprr2g	-0,1	17,3	-0,06	0,949	1	-7
>sp P00493 HPRT_IV	18,74	18,4	19,2	18,5	18,8	18,9	18	19	18	18,8	19	18	18,5	18,9	18	19	18,5	18,4	18,682	18,6916	Hprt1	0,01	18,7	0,064	0,95	1	-7
>sp Q99KH8 STK24_I	19,51	17,9	17,4	17	17,5	18,2	17	17	17	17,1	18	18	17,5	18,3	18	17	17,3	17,7	17,682	17,7002	Stk24	0,02	17,7	0,061	0,952	1	-7
>sp Q9QZN4 FBX6_M	16,67	17,6	16,9	17,3	17,2	17,2	17	0	17	16	14	17	18	17,4	19	17	0	16,9	15,124	14,9665	Fbxo6	-0,2	15	-0,06	0,954	1	-7

>sp O88895 HDAC3_	19,29	19,2	14,9	16,2	15,3	15	15	14	16	16,2	15	17	17,1	17,4	18	15	15,4	15,8	16,158	16,2005	Hdac3	0,04	16,2	0,058	0,954	1	-7
>sp O70555 SPR2D_	16,5	16,5	17,2	18,1	17,2	18,4	16	17	18	19,1	19	19	0	19,9	18	16	20,5	20,5	17,099	16,9747	Sprr2d	-0,1	17	-0,06	0,955	1	-7
>sp P35276 RAB3D_	21,06	19,6	20,5	20,9	20,6	21,1	21	18	21	20,4	21	21	20,8	20,7	19	21	20,5	20,3	20,386	20,4051	Rab3d	0,02	20,4	0,058	0,955	1	-7
>sp Q991C2 CSTF1_N	19,71	19,6	24,6	20,1	19	19,3	19	20	24	20	24	24	19,4	19,2	19	19	19,4	19,4	20,563	20,5057	Cstf1	-0,1	20,5	-0,06	0,955	1	-7
>sp Q80TY0 FNBP1_N	18,5	19,2	18,8	19,2	19,3	19,5	18	19	19	19,1	18	19	19,4	19,4	20	19	18,8	19,1	18,93	18,9438	Fnbp1	0,01	18,9	0,056	0,956	1	-7
>sp Q923B1 DBR1_N	0	15,8	15,4	15,7	16	15	16	14	15	14,9	0	15	15,4	15,5	16	16	15,6	15,4	13,62	13,7523	Dbr1	0,13	13,7	0,055	0,957	1	-7
>sp P03958 ADA_MK	17,6	17,3	16,6	15,4	16,6	17	16	17	16	15,6	16	17	17	17,3	17	17	17,1	16,1	16,66	16,6757	Ada	0,02	16,7	0,05	0,961	1	-7
>sp Q61205 PA1B3_	19,79	17,1	18,3	17,3	18,1	18	18	19	17	18,2	19	18	17	17,5	16	19	19,3	19,4	18,05	18,0751	Pafah1b3	0,02	18,1	0,05	0,961	1	-7
>sp P62869 ELOB_M	20,6	23,6	21,8	20,2	20,5	22,4	20	22	21	21,5	22	22	21,5	21,9	22	21	20,7	20,4	21,411	21,3898	Elob	-0	21,4	-0,05	0,961	1	-7
>sp Q9D0B6 PBDC1_	18,67	19,5	19,5	19,5	18,5	19,4	19	21	21	19,5	21	20	18,9	18,9	21	19	18,6	18,8	19,532	19,511	Pbdc1	-0	19,5	-0,05	0,962	1	-7
>sp Q991G2 TNPO2_I	16,26	18,2	15,6	17,2	16,8	16	16	18	16	17,3	17	17	16,2	16,8	16	15	18	16,3	16,618	16,6375	Tnp02	0,02	16,6	0,048	0,962	1	-7
>sp O88487 DC1I2_I	18,3	16,2	18,5	18	20,4	20,5	18	19	20	19,6	16	18	20	20,3	18	18	20,2	18,8	18,744	18,7748	Dync1i2	0,03	18,8	0,048	0,962	1	-7
>sp Q61147 CERU_N	19,45	17,9	20,1	17,6	18,4	20,3	18	18	19	19,8	19	19	18,2	18,5	18	18	18,5	19,7	18,788	18,8063	Cp	0,02	18,8	0,046	0,964	1	-7
>sp Q9EP82 WDR4_N	17,32	17,3	17,6	17,3	16,9	16,8	17	18	18	17,8	17	17	16,9	17	17	18	16,7	17,7	17,303	17,2936	Wdr4	-0	17,3	-0,05	0,964	1	-7
>sp Q922Q1 MARC2_	17,86	18,3	18	17,5	18,9	18,6	18	18	18	17,8	18	18	18,1	18,7	19	18	18,8	17,7	18,249	18,2403	Marc2	-0	18,2	-0,04	0,965	1	-7
>sp P00920 CAH2_N	16,35	15,8	18,6	17,9	18,2	15,6	15	18	18	18,5	17	19	16,8	16,9	17	16	15,6	17,5	17,109	17,0848	Ca2	-0	17,1	-0,04	0,966	1	-7
>sp Q61730 IL1AP_N	16,42	16,8	14,3	16,3	16,1	16,6	17	17	15	16	16	17	16,5	16,1	16	16	15,9	15,9	16,118	16,1037	Il1rap	-0	16,1	-0,04	0,968	1	-7
>sp O70559 SPR2H_	16,22	16,2	16,9	17,8	16,9	18	16	16	17	18,7	19	19	0	19,6	18	16	20,2	20,2	16,75	16,6656	Sprr2h	-0,1	16,7	-0,04	0,969	1	-7
>sp Q8BX94 OSB12_I	0	0	0	15,7	12,9	0	0	0	0	0	0	18	0	0	0	12	0	0	3,1781	3,29506	Osbp12	0,12	3,24	0,039	0,97	1	-7
>sp Q62448 IF4G2_N	15,57	14,8	16,1	14,8	15,8	15,9	15	15	12	14,4	14	15	15,6	14,7	15	15	16,6	15,5	15,095	15,0795	Eif4g2	-0	15,1	-0,03	0,973	1	-7
>sp Q62465 VAT1_N	19,61	19	19,8	19,3	19,8	19,8	22	20	20	19,7	20	19	18,8	19,6	20	19	19,9	23,1	19,855	19,8386	Vat1	-0	19,8	-0,03	0,973	1	-7
>sp Q9ZZN8 ACL6A_I	21,18	21,4	21,2	21	21,5	21,3	22	22	22	22	22	21	21,3	21,3	22	21	21,1	21,2	21,438	21,4429	Actl6a	0	21,4	0,032	0,975	1	-7
>sp P0C027 NUD10_	17,49	16,4	0	17,6	17,4	18	18	0	17	16,4	0	0	17,2	16,8	18	18	17,5	18	13,555	13,4407	Nudt10	-0,1	13,5	-0,03	0,975	1	-7
>sp P0C028 NUD11_	17,49	16,4	0	17,6	17,4	18	18	0	17	16,4	0	0	17,2	16,8	18	18	17,5	18	13,555	13,4407	Nudt11	-0,1	13,5	-0,03	0,975	1	-7
>sp P63087 PP1G_N	23,51	23,7	23,5	23,5	22,9	23,2	23	24	24	23,7	24	23	22,8	23,5	23	23	23,6	23,8	23,42	23,4241	Ppp1cc	0	23,4	0,029	0,977	1	-7
>sp P63321 RALA_N	19,14	18,4	20,2	18,5	18	18,7	19	20	20	19,2	20	21	18,2	19,4	18	18	19,8	19,1	19,186	19,1742	Rala	-0	19,2	-0,03	0,977	1	-7
>sp O08756 HCD2_N	15,92	18,3	16,7	15,3	15,5	15,2	15	17	17	13,7	15	16	17,9	17,3	21	15	15,1	15,8	16,301	16,3214	Hsd17b1	0,02	16,3	0,028	0,978	1	-7
>sp P63037 DNJA1_	17,98	18,1	19,3	19,3	16,4	17,2	19	18	18	18,2	19	19	17,8	18,3	18	17	17,8	17,5	18,104	18,0941	Dnja1	-0	18,1	-0,03	0,98	1	-7
>sp P00329 ADH1_N	27,61	27,7	23,9	27	27	23,9	28	28	26	26,2	27	26	26,1	26,3	26	28	26,5	26,5	26,505	26,4934	Adh1	-0	26,5	-0,02	0,983	1	-7
>sp Q922B2 SYDC_M	22,06	23,2	21	22,5	22,5	22,1	21	22	20	22,3	21	22	22,1	22,6	22	22	21,2	21,2	21,838	21,8456	Dars	0,01	21,8	0,022	0,983	1	-7
>sp Q3TYL0 YH010_N	14,36	17,3	14	17,3	17,3	17,5	16	14	19	16,1	17	14	18,3	13,9	19	14	14,4	18,5	16,229	16,2097	>sp Q3TYL	-0	16,2	-0,02	0,984	1	-7
>sp P97496 SMRC1_	14,42	14,3	14,2	13,9	14	13,9	14	12	14	13,9	15	14	15,1	11,7	14	14	14	13,4	13,958	13,9659	Smrcc1	0,01	14	0,021	0,984	1	-7
>sp Q8ROW0 EPIPL_N	16	16	14,7	16	15,8	15,4	16	15	16	15,8	16	16	15,5	15,7	15	15	15,9	15,7	15,598	15,5948	Eppk1	-0	15,6	-0,02	0,985	1	-7
>sp Q8JZV7 NAGA_M	18,41	19	20,2	18,8	20	19,1	19	18	19	18,5	19	19	19,6	19,9	20	19	18,6	17,6	19,072	19,0779	Amdhd2	0,01	19,1	0,018	0,986	1	-7
>sp P00755 K1KB1_	19,78	19,7	19,3	19,1	19,7	19,4	19	19	19	19,4	19	19	20	19,5	19	19	19,8	19,8	19,364	19,361	Klk1b1	-0	19,4	-0,02	0,986	1	-7
>sp Q8BFQ4 WDR82_	22,01	19,5	20	20,1	19,9	19,3	20	20	20	19,7	20	20	20,1	20	21	20	19,5	19,6	19,983	19,9784	Wdr82	-0	20	-0,02	0,987	1	-7
>sp Q61390 TCPW_N	19,53	19,2	19,4	16,7	15,8	20,3	16	20	16	19,2	20	20	19,6	18,8	16	18	19,8	15,5	18,094	18,1074	Cct6b	0,01	18,1	0,016	0,988	1	-7
>sp P62270 RS18_N	20,72	20,7	20,8	19,8	20,5	20,3	20	20	20	19,6	21	20	20	20,4	21	20	20,3	20,6	20,328	20,3307	Rps18	0	20,3	0,014	0,989	1	-7
>sp Q9DCS2 MTL26_I	17,64	16,8	19,7	17,3	17,3	18,1	18	17	22	18	22	18	17,7	17,8	18	17	17,4	17,5	18,138	18,1482	Mettl26	0,01	18,1	0,014	0,989	1	-7
>sp P48678 LMNA_N	17,96	20,8	20,4	20,8	20	20,3	20	20	20	19,9	21	20	20,2	20,1	20	20	20,1	19,9	20,106	20,1021	Lmna	-0	20,1	-0,01	0,99	1	-7
>sp P70303 PYRG2_	19,6	19,4	19,6	21,3	21,4	21,3	19	18	21	21,1	20	20	18,8	21,6	19												