VNIVERSITAT DE VALÈNCIA

Faculty of Medicine and Dentistry — Department of Physiology

Study of metabolic profile, oxidative stress, mitochondrial function and autophagy in diabetes and their relationship with the development of cardiovascular complications: effects of a nutritional intervention and empagliflozin treatment

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Doctoral Program in Physiology

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CERTIFICAN:

Que Don. Francisco Gerardo Canet Suquillo, Master en Investigación y Uso Racional del Medicamento por la Universidad de Valencia, ha realizado bajo su dirección el trabajo titulado "Study of metabolic profile, oxidative stress, mitochondrial function and autophagy in diabetes and their relationship with the development of cardiovascular complications: effects of a nutritional intervention and empagliflozin treatment" y autorizan su presentación como Tesis Doctoral para la obtención del grado de Doctor en Fisiología por la Universidad de Valencia.

Y para que así conste a todos los efectos oportunos, firman el presente certificado en Valencia en Septiembre de 2023.

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“The nitrogen in our DNA, the calcium in our teeth, the iron in our blood, the carbon in our apple pies were made in the interiors of collapsing stars."

Carl Sagan

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FIGURES AND TABLES

REGULATION AND STRUCTURE

According to the regulations approved by the Doctoral School and the Academic Committee of the PhD Programme in Physiology of the Universitat de València:

1. The present PhD thesis has been structured in the form of a compendium of publications:
    - The core of the thesis is composed of three original first-author articles published in Q1 journals according to the Journal Citation Reports database (Annex I, II and III: Publications).

2. Based on the present PhD thesis Mr. Francisco Gerardo Canet Suquillo would like to apply for the International Doctorate Mention:

    - The core of this PhD thesis is written in English and includes an abstract in English. Additionally, it includes summary in Spanish (Resumen) since it is one of the official languages of the Universitat de València.
    - During the training period the PhD candidate has spent four months in a research centre outside Spain (University of Oslo, Department of Nutrition, Institute for Basic Medical Sciences, Medical Faculty).
    - The thesis will be defended in Spanish, except for the conclusion, which will be defended in English.

ABBREVIATIONS

ACS: acute coronary syndrome

ADA: American Diabetes Association

AGEs: Advanced glycation end products

AMPK: AMP-activated protein kinase

ApoA: apolipoprotein A

ApoB: apolipoprotein B

ApoE: apolipoprotein E

ATG: Autophagy-related

ATP: Adenosine triphosphate

BCCAs: branch chain amino acids

BMI: body mass index

BNIP3: BCL-2/adenovirus E1B 19 kDa protein-interacting protein 3

CKD: chronic kidney disease

CRP: C-reactive protein

CVD: Cardiovascular diseases

DAMPs: damage-associated molecular patterns

DASH: Dietary Approaches to Stop Hypertension

DCFH: 2', 7'-dichlorodihydrofluorescein diacetate

DFCP1: zinc-finger FYVE domain-containing protein 1

DHA: Docosahexaenoic acid

DPP4: dipeptidyl peptidase 4t

ECAR: extracellular acidification rate

eNOS: endothelial nitric oxide synthase

EPA: eicosapentaenoic acid

ER: Endoplasmic reticulum

FCCP: Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone

G3P: glyceraldehyde 3-phosphate

GABARAPS: gamma-aminobutyric acid receptor-associated proteins

GAD65: 65 kDa glutamic acid decarboxylase

GAPDH: glyceraldehyde-3-dehydrogenase

GlcNAc: N-acetylglucosamine

GLP1: glucagon-like peptide 1

GSH: reduced glutathione

GSSG: glutathione disulphide (oxidized form)

HbA1c: glycated haemoglobin

HBSS: Hank`s Balance Salt Solution

HDL: High-density lipoprotein

HDL-C: High-density lipoprotein cholesterol

HE: hydroethidine

HLA: Human Leukocyte Antigens

HOMA-IR: homeostatic model assessment of insulin resistance

hs-CRP: high-sensitivity C-reactive protein

HUVEC: human umbilical vein endothelial cells

IA-2: insulinoma-associated protein 2

iAUC: incremental Area Under the Curve

ICAM1: Intercellular Adhesion Molecule 1

IFG: Impaired fasting plasma glucose

IFNγ: Interferon-gamma

IGT: Impaired glucose tolerance

IL-1: Interleukin-1

IL-10: interleukin-10

IL-1RA: IL-1 receptor antagonist

IL-6: Interleukin-6

IRS: insulin receptor substrates

iSGLT2: sodium-glucose cotransporter 2 inhibitors

JNK: c-JUN N-terminal kinase

LC3: microtubule-associated protein light chain 3

LDL: Low-density lipoprotein

LDL-C: Low-density lipoprotein cholesterol

LIRs: LC3-interacting motifs

LP-IR: Lipoprotein Insulin Resistance index

MCP-1: Monocyte chemotactic protein-1

MG-H1: methylglyoxal hydroimidazolone 1

MitoSOX: mitochondrial superoxide indicator

MMPs: matrix metalloproteinases

mTOR: mammalian Target of Rapamycin

MUFA: Monounsaturated fatty acids

MUFA %: ratio of monounsaturated fatty acids (MUFA) to total fatty acids

NAD+: nicotinamide adenine dinucleotide (oxidized form)

NADH: nicotinamide adenine dinucleotide (reduced form)

NDUFC2: NADH:Ubiquinone Oxidoreductase Subunit C2

NF-κB: nuclear factor kappa B

NHE: Sodium/Hydrogen exchanger

NMR: nuclear magnetic resonance

OCR: oxygen consumption rate

OGTT: Oral glucose tolerance test

PARP-1: poly (ADP-ribose) polymerase 1

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline solution

PE: phosphatidylethanolamine

PI3K: phosphatidylinositol 3-kinase

PI3P: phosphatidylinositol 3-phosphate

PKC: Protein Kinase C

PKR: double-stranded RNA dependent protein kinase

PMNs: polymorphonuclear leukocytes

PUFA: polyunsaturated fatty acid

RCT: randomised controlled trials

ROS: Reactive oxygen species

SCFAs: Short-chain fatty acids

SFA: saturated fatty acids

SGLT2: sodium-glucose cotransporter 2

SMC: smooth muscle cell

SOD: Superoxide dismutase

SQSTM1: sequestosome 1

T1D: Type 1 diabetes

T2D: Type 2 diabetes

TH1: T Helper 1

TH2: T Helper 2

TLR: Toll-like receptor

TNF-α: Tumour necrosis factor alpha

TRX: thioredoxin

UCP2: Uncoupling Protein 2

ULK1: Unc-51-like kinase 1

VCAM1: vascular cell adhesion protein 1

VLDL: very low-density lipoproteins

WC: Waist circumference

WIPIs: WD repeat domain phosphoinositide-interacting proteins.

ZNT8: zinc transporter 8

ABSTRACT

Introduction

Definition and classification

Diabetes is a chronic disease in which blood glucose levels rise because the body cannot produce enough insulin or is unable to use the insulin it produces effectively to modulate glucose levels. Most cases fall into two categories: type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is caused by absolute deficiency in insulin secretion due to an autoimmune and pathological process occurring in the pancreas. Whereas T2D is due to a combination of resistance to insulin action and an inadequate compensatory capacity for insulin secretion.

There is also an intermediate category of people whose blood sugar levels are higher than normal, but not yet high enough to be diagnosed as diabetics. This group of people are known as pre-diabetics, and may manifest impaired fasting glucose or glucose intolerance, both of which are intermediate stages in the progression to any type of diabetes and a risk factor for developing cardiovascular disease (CVD). It is important to note that the term prediabetes is mainly used to refer to people at risk of developing T2D.

Epidemiology and clinical impact

T1D accounts for 5-10% of cases, and is the most common type of diabetes in children. Although its aetiology is not yet fully understood, it is known to be related to genetic and environmental risk factors. T2D, on the other hand, is much more common, accounting for more than 90% of cases, and is a health problem that is expanding worldwide and is closely linked to the obesity epidemic.

Diabetes can lead to health problems, decreased quality of life and increased risk of premature death. Diabetes can result in long-term complications, such as heart attack, stroke, kidney failure, vision loss, among others. In addition, there is an increased incidence of CVD in diabetic patients.

Pathophysiology

T2D is primarily caused by insulin resistance and progressive loss of insulin secretion in the pancreas, which occurs due to pre-existing insulin resistance in muscle, liver, and adipose tissue. There are at least eight pathological processes that contribute to impaired glucose homeostasis, all of which were well established early in the natural history of T2D. According to Ralph DeFronzo, these eight "actors" are: insulin resistance in muscle and liver, impaired

insulin secretion in pancreatic β-cells (β-cell dysfunction), increased glucagon secretion in pancreatic α-cells, accelerated lipolysis in adipose tissue, decreased incretin effect in gastrointestinal tissues, increased glucose reabsorption in the kidney, and insulin resistance in the brain. In addition, two further processes can play a part: activation of inflammatory pathways and impairment of insulin-mediated vasodilation. It should be remembered that pre-diabetes is an early stage in all these metabolic derangements.

T1D, on the other hand, is characterised by autoimmune destruction of β-cells. During the natural history of the disease, autoantibodies first appear and are not pathogenic, but rather markers of the development of autoimmunity. Autoimmunity is likely to have developed long before the first clinical manifestations. The impaired ability to secrete insulin is due to a combination of destruction of β-cells and inhibition of their function (due to inflammation), leading to metabolic changes and eventually to diabetes.

Metabolic disturbances beyond glucose in diabetes

Insulin plays a key role in glucose, glycogen, lipid, and protein metabolism, as well as inflammation and vasodilation, so diabetes can disrupt any of these metabolic pathways.

With the application of metabolomics technologies — such as nuclear magnetic resonance (NMR) spectroscopy — it is possible to obtain a detailed snapshot of systemic metabolism and to better understand the aetiology and progression of metabolic diseases, as well as to detect early markers of disease and monitor response to treatment.

Several metabolites associated with insulin resistance, pre-diabetes and diabetes have been identified. For example, multiple studies have shown a positive association between circulating concentrations of branched-chain amino acids and insulin resistance and the development of T2D; as well as the inflammatory marker GlycA — a composite marker integrating signals from several acute-phase inflammatory proteins, identified by NMR — which has been associated with increased cardiometabolic risk.

Molecular mechanisms involved in the pathophysiology of diabetes

Inflammation is crucial in the pathogenic processes that occur in diabetes, and in this sense, innate immunity is particularly implicated in the development of diabetic complications. In T1D, inflammation is a primary event in which autoimmunity towards pancreatic β-cells triggers the disease; in T2D, chronic inflammation is thought to result from insulin resistance and alterations in glucose metabolism. There is ample evidence to suggest that T2D is a chronic inflammatory disease.

Many metabolic stressors (like obesity or the excess of nutrients, such as glucose or fatty acids) can cause immune and non-immune cells to release large amounts of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α and the activation of inflammation- and stress-induced kinases like IKKβ (also known as inhibitor of nuclear factor Kappa B kinase subunit beta (NF-κB)), all of which together promote the development of insulin resistance and the recruitment of leukocytes to the sites of inflammation.

It is now known that hyperglycaemia causes the production of reactive oxygen species (ROS) in diabetic patients via the electron transport chain in the mitochondria, lipoxygenases, and peroxidases, among other sources. When levels of ROS are in excess, they develop an oxidative stress situation, which plays a key role in the development of diabetes and diabetic complications. In this regard, the focus has been on mitochondrial ROS, as they directly stimulate insulin resistance and the production of proinflammatory cytokines, and activate mechanisms by which hyperglycaemia induces vascular damage.

Mitochondria perform the oxidation of glucose, fatty acids, and amino acids through oxidative phosphorylation, and during this process ATP and ROS are generated. When there is mitochondrial dysfunction, an excessive amount of ROS is produced, a deficit in energy supply occurs and several mitochondrial signalling pathways are altered, resulting in a pathological environment that affects different biological processes. Mitochondrial dysfunction has been observed in liver, muscle, adipose tissue and even brain in animal models and patients with obesity, T2D or metabolic syndrome.

In the context of metabolic disturbances in diabetes, autophagy plays a key role in maintaining cellular homeostasis. Autophagy is a catabolic process that removes cellular components (e.g., organelles, proteins, etc.) and aids their recycling; for example, autophagy can remove dysfunctional mitochondria or misfolded proteins. The aim of autophagy is to reduce cellular stresses, which, if left unresolved, promotes the development of metabolic disorders. In the context of atherosclerosis, autophagy seems to play a protective role by protecting atheroma plaque cells against oxidative stress. Autophagy is normally a protective process; however, under certain conditions, excessive autophagy can be detrimental, as it induces apoptosis.

Impact of diabetes in cardiovascular diseases

People with prediabetes and diabetes are at increased risk of developing CVD; indeed, in terms of macrovascular function, it manifests mainly as atherosclerosis. Patients with diabetes display an accelerated atherosclerotic process associated with several risk factors, such as insulin resistance and hyperinsulinaemia, activation of inflammatory pathways, oxidative

stress, endothelial dysfunction, and traditional cardiovascular risk factors (such as hypertriglyceridaemia, hypertension, low levels of HDL (high-density lipoproteins), elevated levels of LDL (low-density lipoproteins) especially small and dense particles, etc.).

Atherosclerosis is a chronic, progressive disease characterised by the accumulation of lipids and inflammatory cells underneath the intima (the deepest layer of the arteries). Advanced atherosclerotic plaques can block blood flow or rupture and form a thrombus, leading to serious health complications. Inflammation is a central process in the development of atherosclerosis and is considered a chronic inflammatory process of the arterial wall.

During the onset of atherosclerosis, LDL particles accumulate in the intima, where they oxidise and become pro-inflammatory, while circulating monocytes bind to adhesion molecules expressed by the activated endothelium and migrate into the intima, where they bind to LDL particles and transform into foam cells. Plaque formation continues to progress as more lipids and foam cells accumulate and trigger the production of extracellular matrix molecules (such as collagen and elastin) by smooth muscle cells, contributing to lesion thickening.

Leukocytes as biomarkers in translational diabetes research

Several studies have recognised circulating leukocytes — peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) — as suitable biomarkers to represent metabolic status at the systemic and cardiovascular levels. As leukocytes circulate in the bloodstream, they can incorporate metabolic signals from different sites in the body. Leukocyte metabolism is intricately linked to their activation state and function; for example, circulating monocytes encountering a site of inflammation must rapidly activate and migrate to sites of injury, where they can differentiate into proinflammatory (M1) phenotypes and increase their glycolysis, or anti-inflammatory (M2) phenotypes and increase their oxidative phosphorylation. In addition, circulating leukocytes play a key role in the initiation and progression of CVD such as atherosclerosis. All this, together with the fact that their isolation is minimally invasive, makes them a valuable tool for the study of diabetes and associated CVD.

Clinical management of diabetes

Adequate management of diabetes requires healthy lifestyle habits and medication for glycaemic control. Dietary habits influence numerous cardiometabolic risk factors, such as obesity, blood pressure, glucose-insulin homeostasis, lipoprotein concentrations and function, oxidative stress, inflammation, endothelial health, cardiac function, and microbiome composition.

Many evidence-based beneficial dietary patterns (like the Mediterranean diet) emphasise fish consumption, as fish contains several healthy components, including specific proteins, unsaturated fats, vitamin D and long-chain omega-3 polyunsaturated fatty acids (found mainly in oily fish), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have been widely attributed with reducing cardiovascular risk.

The consumption of lean fish has also been linked to positive cardiometabolic effects, suggesting that components of fish other than fatty acids are also beneficial; namely, fish protein. Clinical trials with fish protein supplements mainly derived from lean fish have shown cardiometabolic benefits, but very few human studies have been carried out to date. In a recent study (FishMeal study), Hustad et al. investigated the effect of a salmon protein supplement in people at increased risk of T2D over a period of 8 weeks. The results of the study indicated that the intervention had no effect on cardiometabolic markers in the participants.

In terms of pharmacological treatment, T1D requires mainly insulin administration, whereas in T2D the treatment is more complex, as no single drug alone reverses the multiple disorders underlying T2D. In this context, combination therapy has gained wide acceptance. The main classes of oral antidiabetic drugs are biguanides (metformin), sulphonylureas, meglitinide, thiazolidinediones, DPP4 inhibitors, GLP1 receptor agonists, sodium-glucose cotransporter 2 inhibitors (iSGLT2) and alpha-glucosidase inhibitors.

iSGLT2s (dapagliflozin, canagliflozin and empagliflozin) are a new class of antidiabetic drugs that improve blood glucose by reducing glucose reabsorption in the kidney. The mechanism of action of iSGLT2 is independent of insulin secretion and the presence of insulin resistance. As a result, except in the presence of moderate to severe chronic kidney disease, these drugs can be used as glucose-lowering agents in all phases of T2D.

In addition to their anti-hyperglycaemic effects, iSGLT2 exerts cardiovascular and renal protection. The EMPA-REG OUTCOME study showed that empagliflozin treatment reduced cardiovascular events in patients with T2D and high cardiovascular risk.

There are several potential mechanisms by which iSGLT2 exerts its cardioprotective effects, including an influence on traditional risk factors (improvement of glycaemic control, reduction of weight and blood pressure, etc.) and a reduction of chronic low-grade inflammation and oxidative stress. All these mechanisms probably operate together, though at present they are hypothetical and need to be confirmed by further studies.

Objectives

Based on that presented in the introduction, the aim of this thesis is to evaluate cellular, molecular and redox status alterations in hyperglycaemic conditions — from patients at an increased risk of developing diabetes to patients with a diagnosis of T1D or T2D — and to determine whether any of these alterations can be modulated by nutritional intervention or pharmacological treatment.

The three main objectives are to:

1. Characterise the plasma metabolomic and PBMCs' transcriptomic profiles of individuals at increased risk of developing diabetes and identify biomarkers that can predict progression to T2D; and further, to determine whether any of these can be modulated by a nutritional intervention with salmon protein.

2. Evaluate the bioenergetic and redox state of PBMCs from T1D patients compared to healthy controls and assess the autophagy state and leukocyte-endothelium interactions.

3. Evaluate the potential cardioprotective effects of empagliflozin treatment on leukocyte-endothelium interactions, adhesion molecules, mitochondrial ROS production, and inflammatory markers in T2D patients.

Materials and methods

Study population

For the present thesis, people at risk of developing diabetes, patients diagnosed with T1D or T2D and healthy volunteers were recruited.

The University of Oslo (Oslo, Norway) provided anonymised clinical data as well as metabolomics and transcriptomics data for the population at risk of developing diabetes. This population had previously participated in a randomised controlled clinical trial (FishMeal study) with the aim of evaluating the effect of a nutritional intervention with salmon protein on markers of cardiometabolic risk. Participants in this study received capsules containing salmon protein or placebo for 8 weeks. The daily dose in the experimental group was 5.2 g of salmon protein. Study participants were non-diabetic, but with elevated glucose levels: fasting glucose ≥ 5.6 mmol/L (100.9 mg/dL), 2-h glucose during an oral glucose tolerance test (OGTT) ≥ 6.5 mmol/L (117.1 mg/dL) or HbA1c ≥40 mmol/mol (≥5.8%).

On the other hand, patients with T2D who had begun treatment with empagliflozin (10 mg/day), patients with T1D, and healthy volunteers were recruited from the Endocrinology Service of the University Hospital Doctor Peset (Valencia, Spain). Diagnosis of T1D or T2D was confirmed according to the criteria of the American Diabetes Association.

Sample collection and biochemical determinations

Patients with T1D or T2D and healthy controls were examined in the Endocrinology Service and anthropometric measurements were taken. Other essential information, such as drug treatment received, was also recorded. After 12-h fasting, blood samples were collected from the patients in EDTA tubes and a serum isolation tube. They were subsequently analysed by the hospital's Clinical Analysis Service to measure routine biochemical parameters (serum glucose, cholesterol, triglycerides, insulin, etc.).

Plasma metabolomics

The University of Oslo managed these experiments. In the population of patients at risk of developing diabetes, circulating metabolites were quantified in plasma both before and after the nutritional intervention, using an NMR metabolomics platform at the Nightingale facility in Finland. This method allows the quantification of 250 metabolites (14 lipoprotein subclasses and their lipid compositions, apolipoproteins, cholesterol, fatty acids, glycolysis-associated metabolites, amino acids, ketone bodies, water balance markers and inflammatory markers) in a single plasma sample.

Measurements of all these metabolites provide a comprehensive picture of systemic metabolism and can be used as markers for pathological processes, as they extend our knowledge beyond traditional risk factors.

Soluble cytokines and adhesion molecules in serum

Serum samples from T2D patients were used to measure levels of proinflammatory markers (TNF-α, IL-1β, and IL-6) and cell adhesion molecules (P-Selectin, ICAM1, VCAM1) using a Luminex® 200 system. This is a bead-based assay system in which beads are encoded with different combinations of fluorescent dyes and antibodies specific for different biomarkers. In this way, it measures multiple biomarkers (such as proteins and cytokines) in a single biological sample.

Blood antioxidant capacity

Blood antioxidant capacity was measured in both healthy controls and T1D patients as a reflection of their redox status. An electrochemical method was employed for these assays, as it allowed us to distinguish the amounts of fast- and slow-acting antioxidants.

Functional analysis

Isolation of leukocytes

Leukocytes from T1D and T2D patients were isolated using different methodologies. In T1D patients, neutrophils and PBMCs were isolated by immunomagnetic removal of non-target cells from blood collected in EDTA tubes using commercial kits. In T2D patients, PBMCs and PMNs were isolated using Ficoll density gradient centrifugation from blood in citrate tubes. After centrifugation, PBMCs were obtained from the buffy-coat and PMNs from the sediment.

In both cases, fresh PMNs and PBMCs were used immediately after extraction for functional analyses (cytometry, leukocyte-endothelium interaction, and mitochondrial function) and the remaining cells were stored at -80°C for further molecular analyses.

Static cytometry

Static cytometry was employed to measure mitochondrial superoxide production in leukocytes. For this purpose, freshly extracted PMNs were seeded in a 48-well plate and incubated with MitoSOX indicator and nuclear staining. Fluorescence visualisation and measurement was performed with a fluorescence microscope coupled to static cytometry software.

Flow cytometry

Flow cytometry was used to quantify ROS production in whole blood leukocytes. To do this, blood samples were incubated with anti-human CD45 antibody to label CD45+ cells corresponding with the leukocyte population. The labelled sample was diluted and incubated with DCFH (2',7'-Dichlorodihydrofluorescein) to measure total free radical production or with HE (Hydroethidine) for superoxide production. Fluorescence was measured in a flow cytometer and data were taken for the PMN population.

Leukocyte-endothelium interaction assay

We used an *ex vivo* model that simulates the interaction of leukocytes with endothelial cells inside blood vessels under physiological conditions. This assay requires two cell types: a

confluent monolayer of human umbilical vein endothelial cells (HUVECs) and PMNs. A solution of freshly isolated PMNs is perfused onto the HUVEC monolayer using a parallel flow chamber, and real-time images of the region of the monolayer exposed to the flow for 5 minutes are recorded using a video camera attached to a microscope.

During video analysis, three parameters were assessed: 1) rolling leukocyte flow, which is the number of PMNs rolling in 1 minute; 2) rolling velocity, calculated as the time for 20 PMNs to travel 200 μm; and 3) adhesion, calculated as the number of PMNs that firmly adhere to the endothelium and resist the flow for at least 30 seconds.

Real-time metabolic flux analysis of leukocytes

We analysed mitochondrial function in real time in PBMCs using a Seahorse XFp system and the XFp Cell Mito Stress kit (both from Agilent). This method measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in live cells as respiration-modulating drugs are injected, thereby calculating important parameters of mitochondrial function, such as basal respiration, respiration linked to ATP production, maximal respiratory capacity, reserve capacity and non-mitochondrial respiration.

Gene and protein expression in leukocytes

Real-time PCR and nCounter

Gene expression was measured by reverse transcription quantitative PCR (RT-qPCR) of frozen PBMC samples. RNA was first isolated, cDNA was then generated, and real-time PCR was performed using primers specific for Superoxide dismutase 1, Glutathione peroxidase 1, RELA proto-oncogene, NF-κB subunit, and Actin beta genes (Table 1 for details). The 2-ΔΔCT method was employed to analyse the data and the expression of β-actin as an endogenous control.

In addition, we analysed transcriptomics data from the FishMeal study. The University of Oslo performed the experiments in question using a NanoString nCounter system. The nCounter is a high throughput multiplex nucleic acid hybridisation technology which uses optical barcodes that hybridise to each target oligonucleotide to allow digital counting of individual RNA molecules without any enzymatic steps. This system was used in conjunction with the nCounter Metabolic Pathways Panel, which allows the quantification of 768 genes annotated in different metabolic pathways (including 20 reference genes and positive and negative controls). In addition, this panel was customised to measure 30 additional genes (Table 2) related to immune and lipid metabolism.

Western blot

The relative expression of protein markers of inflammation, oxidative stress, and autophagy in PBMCs was performed by Western blotting. Protein was first extracted from frozen samples of PBMCs, the obtained protein concentration was quantified, the samples were separated by electrophoresis in polyacrylamide gels and transferred to a nitrocellulose membrane using the wet method. Proteins of interest were detected using specific primary antibodies (Table 3) together with peroxidase-conjugated secondary antibodies. Chemiluminescent signals were analysed by densitometry.

Statistical analysis and bioinformatics tools

The normality of all variables was assessed using the Shapiro-Wilk test. Normally distributed variables are shown as mean ± standard deviation, while non-normal variables are shown as median and interquartile range.

Comparisons between two groups were performed using a t-test (normal variables) or Mann-Whitney (non-normal variables). Comparisons between three groups were performed by ANOVA (normal variables), Kruskal-Wallis (non-normal variables) or the Friedman test (non-normal variables with repeated measures), in both cases followed by tests to correct for multiple comparisons.

Characterisation of the metabolic profile (metabolomics and transcriptomics) of the population at risk of developing diabetes required additional steps. Patients were divided into tertiles based on their insulin incremental area under the curve (iAUC) after a 2h OGTT. The iAUC was calculated using two measurements of insulin concentration (fasting and 2h after receiving a 75g glucose load). Individuals in the lowest tertile were classified as the low insulin iAUC group (n = 24), while those in the highest tertile were classified as the high insulin iAUC group (n = 24).

First, the metabolic profiles of the low vs. high insulin iAUC groups were compared before beginning the nutritional intervention (i.e. their baseline values). Next, changes in metabolic profile after the intervention were analysed and compared among individuals in the same insulin iAUC group who received the salmon protein capsules or placebo; that is, changes in metabolic profile were compared between patients in the high iAUC insulin group who received salmon protein vs. those who received the placebo, and the same for individuals in the low iAUC insulin group. Transcriptomics and metabolomics analyses were performed using

linear models corrected for age, sex, body mass index (BMI) and, in the case of transcriptomics analyses, for smoking and non-smoking.

To understand patterns in gene expression that may have changes between the low and high insulin iAUC groups, and would subsequently have been affected by the salmon protein intervention, we performed gene set tests. A gene set test is a differential expression analysis in which a p-value is assigned to a set of genes as a unit. We performed a gene set enrichment tests (using Gene Ontology (GO) terms and KEGG pathways), and a competitive test called Camera (which identifies the most relevant gene sets for differentiating between the low and high insulin iAUC phenotype).

Results and discussion

Detailed characterisation of the population at risk of diabetes and effect of nutritional intervention

Differences in glucose metabolism and insulin sensitivity were found between patients in the low and high insulin iAUC group. The low iAUC group displayed better glycaemic regulation by having lower postprandial glucose concentrations, fasting insulin, postprandial insulin and HOMA-IR, and a higher Matsuda index.

Comparing the metabolomic profile of these two groups with different glycaemic regulation, we found that the high insulin iAUC group had higher plasma concentrations of the inflammatory marker GlycA, a higher ratio of monounsaturated fatty acids (MUFAs) to total fatty acids, and lower concentrations of the amino acid glycine and short-chain fatty acid (SCFA) acetate, compared to the low insulin iAUC group.

In line with our results, several epidemiological studies have reported an association between GlycA and insulin resistance, BMI, markers of metabolic syndrome and inflammatory markers such as hs-CRP (high-sensitivity C-reactive protein). Therefore, GlycA has been proposed as a new marker of cardiometabolic risk and T2D and may be a more accurate marker than hs-CRP.

Regarding glycine levels, other studies have obtained equivalent results to ours, finding an inverse association between circulating glycine concentration and the development of T2D. Of note, it is not yet clear whether low glycine levels play a key role in the development of T2D, but interventions that delay or prevent the development of T2D, such as physical exercise or bariatric surgery, have been shown to increase glycine concentration. In addition, glycine supplementation improves glucose tolerance. In this regard, there is evidence that glycine can

improve glycaemic control by reducing oxidative stress and inflammation at the systemic level and increasing insulin secretion in the pancreas.

Regarding the fatty acid profile, we found that patients in the high insulin iAUC group had a higher proportion of MUFAs. Similarly, studies in large cohorts of patients have observed that higher plasma MUFA concentrations are associated with higher HbA1c and fasting glucose and an increased risk of heart disease.

In relation to the transcriptomic results, the gene with the highest differential expression (in terms of p-value) was CPT1A, whose expression was increased in PBMCs from the high iAUC insulin group compared to the low iAUC group. This gene codes for the CPT1A protein, an enzyme that is in the outer membrane of the mitochondria and is key for the β-oxidation of fatty acids. In line with our results, other studies have reported a positive association between CPT1A expression in blood samples and fasting glucose levels, triglycerides, and BMI.

In the competitive gene set analysis, we observed a lower expression of Myc pathways in PBMCs from the high insulin iAUC group and hypothesised that this indicated reduced mitochondrial biogenesis, as the Myc pathway induces biogenesis and increases mitochondrial function through multiple pathways. Altered mitochondrial function contributes to the development of insulin resistance through various mechanisms (such as overproduction of ROS), and mitochondrial dysfunction has been observed in multiple tissues (such as adipose and muscle tissue, leukocytes, among others) in people with obesity, T2D or metabolic syndrome.

As mentioned at the beginning of this section, the group with high insulin iAUC showed reduced levels of acetate, a SCFA generated in the liver under fasting conditions, but also produced in the gut by the microbiota. Acetate production by the microbiota may play a role in glycaemic regulation; for example, by stimulating the secretion of GLP1 (a hormone that aids glycaemic regulation).

Having characterised the metabolic profile of patients in the low and high insulin iAUC groups, we assessed possible changes after 8 weeks of receiving a salmon protein supplement versus placebo. The intervention did not affect any markers of glycaemic regulation in either patient group. However, we did observe an increase in plasma acetate concentration in the low insulin iAUC group that received the supplement compared to the placebo group, but this increase did not occur in the high iAUC group. We hypothesised that this was due to differences in the composition of the microbiota between the two groups. Recent research suggests that the composition of the microbiota can help identify which individuals may benefit from nutritional

intervention. Apart from changes in acetate, the intervention in our study did not affect any other metabolites or produce major changes in gene expression.

The results of this study suggest that GlycA, MUFA-to-total fatty acid ratio, glycine and acetate are markers for predicting progression to T2D, and that salmon protein supplementation has no effect patients at increased risk of developing T2D.

Redox state, mitochondrial function, and autophagy status of T1D leukocytes and their implications in atherosclerosis

For this part of the study, a population of healthy volunteers was compared to a population of T1D patients. There were no differences in age and body composition between the two groups, and T1D patients had higher fasting glucose and HbA1c levels, as expected.

We observed that leukocytes from T1D patients produced higher amounts of ROS and superoxide radicals compared to controls. We also assessed the antioxidant capacity of the blood in both groups as a reflection of their systemic redox status, and found that T1D patients had lower amounts of fast-acting antioxidants and total antioxidants. Similarly, other studies have shown that monocytes from T1D patients produce more superoxide and are in a proinflammatory state (producing more IL-1β and IL-6), and that there is a higher serum concentration of ICAM1 (cell adhesion molecule) than in controls, highlighting the relationship between inflammation and oxidative stress.

The lower blood antioxidant capacity in the T1D group of patients may reflect a higher utilisation of circulating antioxidants due to increased ROS production. Similar studies have observed that T1D patients and their non-diabetic relatives have lower serum antioxidant capacity compared to unrelated controls. Moreover, as diet did not vary between the 3 groups, the differences in antioxidant capacity were probably the result of a higher utilisation due to ROS production and not the result of lower antioxidant intake.

Since chronic hyperglycaemia can saturate the electron transport chain, which increases ROS production and impairs mitochondrial function, we analysed mitochondrial function in T1D patients. We observed that basal respiration and respiration linked to ATP production were similar in leukocytes from the T1D group and in those from controls. However, T1D leukocytes showed a trend towards lower maximal respiratory capacity and a significant reduction in reserve respiratory capacity.

Reserve capacity estimates the health and flexibility of mitochondria and determines the ability of cells to adapt to stressful situations. In this respect, insufficient reserve capacity has

been shown to be linked to the development of cardiovascular diseases and diabetic complications.

Together with mitochondrial dysfunction and increased ROS production, we observed a classic pattern of autophagy activation in T1D PBMC consisting of higher expression of P-AMPK and Beclin-1 (both markers of autophagy induction), lower amounts of the cargo proteins P62 and NBR1 and higher LC3-II/I ratio. AMPK has been suggested as a protein that may activate autophagy in response to mitochondrial ROS production.

When analysing the expression of the LC3-II/I ratio as a function of HbA1c we observed a positive correlation, and saw that this ratio increased when HbA1c exceeded 5.7% (the threshold for normal values), thus supporting the association between increased autophagy and poor glycaemic control and suggesting a situation of cellular stress due to chronic hyperglycaemia.

Leukocyte adhesion to the endothelium is a hallmark of the inflammatory process at the onset of atherosclerosis, so we assessed leukocyte-endothelium interaction in these two groups. We observed that T1D leukocytes exhibited more interactions with the endothelium than those from controls; the former displayed a lower rolling velocity, higher rolling leukocyte flux (number of leukocytes that interact with the endothelium) and higher leukocyte adhesion (number of leukocytes that adhere to the endothelium and remain stationary).

Cell adhesion molecules (such as P-Selectin, VCAM1 and ICAM1) are important for binding circulating leukocytes to the vascular endothelium at sites of inflammation and mediating leukocyte transmigration from blood vessels to adjacent inflamed tissues. Several cytokines, such as TNF-α and IL-1β, considerably increase the expression of many adhesion molecules. Consistent with this pro-inflammatory state, other studies have shown that patients with T1D have higher levels of pro-inflammatory cytokines in serum, and that even leukocytes isolated from these patients spontaneously produce higher amounts of these cytokines compared to controls.

There is an interaction between increased ROS production, mitochondrial dysfunction, inflammation, and autophagy. Activation of inflammatory pathways (such as the JNK or NF-κB pathway) in response to ROS production can activate autophagy. In the context of atherosclerosis, various stimuli (inflammation, oxidative stress, hypoxia, lack of nutrients) can activate autophagy in different cell types, including leukocytes, endothelial cells, and smooth muscle cells.

This study supports the idea that T1D affects mitochondrial function and promotes oxidative stress and autophagy in leukocytes, and that these mechanisms enhance the risk of atherosclerosis by increasing leukocyte-endothelium interaction.

Potential molecular mechanisms responsible for the cardioprotective effect of empagliflozin treatment

This study was conducted in a population of T2D patients who had initiated treatment with empagliflozin 10mg/day and continued the treatment for 24 weeks. Participants were evaluated at the start of treatment (baseline), and then at 12 and 24 weeks. We observed a progressive reduction in weight, BMI, and waist circumference, as well as an improvement in glycaemic control, manifested by lower fasting glucose and HbA1c levels from 12 weeks of treatment onwards.

We evaluated leukocyte-endothelium interactions to determine possible beneficial effects of empagliflozin treatment. After 24 weeks, we observed a reduced level of interaction of patients' leukocytes due to an increase in their rolling velocity, which reduced the flow of rolling leukocytes and their adhesion to the endothelium compared to baseline. These results suggest that empagliflozin exerts a protective effect against the initial stages of atherosclerosis. In parallel to this reduction in leukocyte-endothelium interactions, we observed a reduction in the concentration of the adhesion molecules P-Selectin and ICAM1 in serum at 24 weeks of treatment. It has been suggested that iSGLT2 exerts multiple beneficial effects at the cardiovascular level, including positive effects on endothelial function, inflammation (e.g., by reducing the expression of ICAM1, VCAM1, TNF-α and IL-6) and oxidative stress.

Hyperglycaemia is a key factor in the process of atherosclerosis and is related to impaired mitochondrial function and oxidative stress. Our data confirmed the positive effect of empagliflozin treatment on glycaemia, as we observed that mitochondrial superoxide production in leukocytes was reduced at 24 weeks of treatment. In addition, SOD1 (antioxidant enzyme) mRNA expression in PBMCs was increased at 24 weeks. These results are in line with those of a previous study by our group in which we saw that empagliflozin treatment promoted an antioxidant response in leukocytes from patients with T2D. The antioxidant effect of empagliflozin may be modulated by other cellular mechanisms beyond its positive effect on glycaemia; e.g., reduction of mitochondrial overload or activation of antioxidant response pathways such as Nrf2/ARE.

Chronic low-grade inflammation is characterised by reduced adiponectin levels and simultaneous secretion by adipocytes and immune cells of various adipokines, cytokines and chemokines, such as TNF-α and IL-6, which thereby promotes oxidative stress and endothelial dysfunction. This chronic low-grade inflammation is associated with increased total body fat and visceral fat, and, thus, with the development of cardiovascular disease.

As mentioned above, we observed that empagliflozin treatment reduced BMI and waist circumference (a surrogate marker of visceral fat) at 24 weeks. In parallel, we found that serum IL-6 levels were reduced in these patients. In this regard, our group had previously shown that empagliflozin treatment promotes an anti-inflammatory response by reducing levels of the inflammatory enzyme myeloperoxidase and increasing concentrations of the anti-inflammatory cytokine IL-10 in patients with T2D.

To explore the molecular mechanisms behind the anti-inflammatory effect of empagliflozin, we measured protein and gene expression of the pro-inflammatory transcription factor NF-κB in patient leukocytes and found that both were reduced after 24 weeks of treatment.

This study suggests that empagliflozin treatment reduces leukocyte-endothelium interactions, oxidative stress, and inflammation, and that these mechanisms underlie its cardioprotective effect.

Conclusions

1) The metabolic characterization of individuals at increased risk of T2D suggests that several plasma metabolites (GlycA, MUFA %, glycine, and acetate) serve as biomarkers to predict the progression of T2D; however, their usefulness requires further testing and validation in future prospective studies. Whether these new biomarkers are more sensitive to dietary interventions in people with an increased risk of T2D is still to be determined.

2) Inflammation and oxidative stress are key mechanisms contributing to atherosclerosis, and leukocytes play a pivotal role in this process. Our characterization of leukocytes from T1D patients reveals augmented ROS, impaired mitochondrial respiration rate, and activated autophagy. These alterations occur in parallel to an overall reduction in antioxidant defences and increased interaction between leukocytes and endothelial cells. These findings support the idea that T1D impairs mitochondrial function and promotes oxidative stress and autophagy in leukocytes, and that these mechanisms contribute to an increased risk of atherosclerosis by augmenting leukocyte-endothelial interactions.

3) Empagliflozin decreases leukocyte–endothelium interactions, adhesion molecules, mitochondrial ROS, IL-6, and NF-κB expression in T2D and enhances antioxidant activity. This highlights the value of this drug for preventing the atherosclerotic process, inflammation, and, consequently, possible cardiovascular events in T2D patients.

RESUMEN

Introducción

Definición y clasificación

La diabetes es una enfermedad crónica en la cual los niveles de glucosa en sangre aumentan porque el organismo no puede producir suficiente insulina o es incapaz de utilizar la insulina que produce de forma eficaz para modular los niveles de glucosa. La mayoría de los casos se pueden clasificar en dos categorías, diabetes tipo 1 (T1D) y tipo 2 (T2D). La T1D está causada por la deficiencia absoluta en la secreción de insulina debido a un proceso autoinmune y/o patológico que ocurre en el páncreas. Mientras que la T2D se debe a una combinación de resistencia a la acción de la insulina y una inadecuada capacidad compensatoria de secreción de insulina.

También se reconoce una categoría intermedia de personas que tiene los niveles de azúcar sanguínea más alta de lo normal, pero aún no lo suficientemente alto para recibir el diagnóstico de diabetes. A este grupo de personas se les conoce como prediabéticos, y pueden manifestar alteración de la glucosa en ayunas o intolerancia a la glucosa; ambas situaciones son estadios intermedios en la progresión hacia cualquier tipo de diabetes y también un factor de riesgo para desarrollar enfermedades cardiovasculares (CVD). Es importante resaltar que el término prediabetes se usa principalmente para referirse a las personas en riesgo de desarrollar T2D.

Epidemiología e impacto clínico

La T1D representa del 5-10% de los casos, siendo el tipo más común de diabetes en niños y aunque su etiología aún no se conoce completamente, está relacionada con factores de riesgo genéticos y ambientales; por otro lado, la T2D es mucho más común llegando a representar más del 90% de casos, es un problema de salud que se está expandiendo a nivel mundial y está estrechamente ligado a la epidemia de la obesidad.

Cualquier tipo de diabetes puede provocar problemas de salud, descenso en la calidad de vida y aumento del riesgo de morir prematuramente. La diabetes puede resultar en complicaciones a largo plazo como ataque al corazón, infartos, fallo renal, pérdida de visión, entre otras. Además, hay una mayor incidencia de CVD en estos pacientes.

Fisiopatología

La T2D es causada principalmente por la resistencia a la insulina en músculo esquelético, hígado y tejido adiposo, así como por la pérdida progresiva de secreción de insulina en el páncreas. Existen al menos ocho procesos patológicos que contribuyen al deterioro de la homeostasis de la glucosa, todos ellos bien establecidos en la definición y desarrollo de T2D. Según Ralph DeFronzo, estos ocho "actores" son los siguientes: resistencia a la insulina en el músculo y el hígado, alteración en la secreción de insulina en las células β pancreáticas (disfunción de las células β), aumento de la secreción de glucagón en las células α pancreáticas, aceleración de la lipólisis en el tejido adiposo, disminución del efecto incretina en los tejidos gastrointestinales, aumento de la reabsorción de glucosa en el riñón y resistencia a la insulina en el cerebro. Además, podrían incorporarse dos procesos más: la activación de vías inflamatorias y el deterioro de la vasodilatación mediada por insulina. Hay que recordar que la prediabetes es una etapa temprana en todos estos desajustes metabólicos.

Por otro lado, la T1D se caracteriza por la destrucción autoinmune de las células β. Durante la historia natural de la enfermedad, primero aparecen autoanticuerpos, los cuales no son patogénicos sino más bien marcadores del desarrollo de autoinmunidad. La autoinmunidad es probable que se haya desarrollado mucho tiempo antes de las primeras manifestaciones clínicas. El deterioro en la capacidad de secretar insulina se debe a una combinación de destrucción de las células β e inhibición de su función (debido a la inflamación), lo que conduce a cambios metabólicos y, finalmente, a la diabetes.

Alteraciones metabólicas más allá de la glucosa en la diabetes

La insulina tiene un papel clave en el metabolismo de la glucosa, el glucógeno, los lípidos y las proteínas, así como en la inflamación y la vasodilatación, por lo que la diabetes podría alterar cualquiera de estas vías metabólicas.

Con la aplicación de tecnologías de metabolómica — como la espectroscopía de resonancia magnética nuclear (NMR) — es posible obtener una fotografía instantánea y detallada del metabolismo sistémico y comprender mejor la etiología y la progresión de las enfermedades metabólicas, así como detectar marcadores tempranos de enfermedad y realizar un seguimiento de la respuesta al tratamiento.

Se han identificado varios metabolitos asociados con la resistencia a la insulina, la prediabetes y la diabetes. Por ejemplo, en múltiples estudios se ha observado la asociación positiva entre las concentraciones circulantes de aminoácidos de cadena ramificada y resistencia a la insulina

y el desarrollo de T2D; así como también el marcador inflamatorio GlycA — marcador compuesto que integra las señales de varias proteínas inflamatorias de la fase aguda, identificado mediante NMR — que se ha visto asociado a mayor riesgo cardiometabólico.

Mecanismos moleculares implicados en la fisiopatología de la diabetes

La inflamación es crucial en los procesos patogénicos en la diabetes, y en este sentido, la inmunidad innata está particularmente implicada en el desarrollo de complicaciones diabéticas. En la T1D, la inflamación es un evento primario en el cual la autoinmunidad hacia las células β pancreáticas desencadenan la enfermedad; en la T2D se piensa que la inflamación crónica es el resultado de la resistencia a la insulina y alteraciones en el metabolismo de la glucosa. Existe amplia evidencia que sugiere que la T2D es una enfermedad inflamatoria crónica.

Muchos de los factores de estrés metabólico (como la obesidad o el exceso de nutrientes en la dieta como la glucosa o los ácidos grasos) puede provocar que células inmunitarias y no inmunitarias liberen grandes cantidades de citocinas proinflamatorias como IL-1β, IL-6 y TNF-α y la activación de quinasas inducidas por la inflamación y estrés como IKKβ (también conocida como inhibidor de la subunidad beta de la quinasa del factor nuclear Kappa B (NF-κB)), y todo esto en conjunto favorecer el desarrollo de resistencia a la insulina y el reclutamiento de leucocitos a los focos de inflamación.

Actualmente se conoce que la hiperglicemia aumenta la producción de especies reactivas de oxígeno (ROS) en pacientes diabéticos a través de la cadena de transporte electrónico mitocondrial, lipoxigenasas, peroxidasas, entre otras fuentes. Cuando los ROS se encuentran en exceso se genera una situación de estrés oxidativo, el cual tiene un papel clave en el desarrollo de la diabetes y de las complicaciones diabéticas. En este sentido, se ha puesto el foco principalmente en los ROS mitocondriales, ya que estos estimulan directamente la resistencia a la insulina, la producción de citocinas proinflamatorias y activan mecanismos por los cuales la hiperglicemia induce daño vascular.

Las mitocondrias realizan la oxidación de la glucosa, ácidos grasos y aminoácidos mediante la fosforilación oxidativa y durante este proceso se genera ATP y ROS. Durante una situación de disfunción mitocondrial se produce una excesiva cantidad de ROS, un déficit en el suministro de energía y se alteran varias rutas de señalización mitocondrial, resultando un entorno patológico que afecta a distintos procesos biológicos. Se ha observado disfunción mitocondrial

en el hígado, el músculo, el tejido adiposo e incluso el cerebro en modelos animales y pacientes con obesidad, T2D o síndrome metabólico.

En el contexto de las alteraciones metabólicas en la diabetes, la autofagia tiene un papel importante para mantener la homeostasis celular. La autofagia es un proceso catabólico por el cual se eliminan componentes celulares (como orgánulos, proteínas, etc.) y ayuda a que sean reciclados; por ejemplo, se pueden eliminar mediante autofagia mitocondrias disfuncionales o proteínas mal plegadas. La autofagia tiene como objetivo reducir las situaciones de estrés celular que, de no ser limitadas, promueven los trastornos metabólicos. En el contexto de la aterosclerosis, la autofagia probablemente tiene un papel protector al proteger a las células de la placa de ateroma frente al estrés oxidativo y evitar su ruptura. La autofagia normalmente es un proceso protector; sin embargo, en determinadas condiciones el exceso de autofagia puede ser perjudicial al inducir apoptosis.

Impacto de la diabetes en las enfermedades cardiovasculares

Las personas con prediabetes y diabetes presentan un mayor riesgo de desarrollar CVD, y centrándonos a nivel macrovascular, se manifiestan principalmente como aterosclerosis. Los pacientes con diabetes muestran un proceso aterosclerótico acelerado, asociado a varios factores de riesgo como la resistencia a la insulina e hiperinsulinemia, activación de vías inflamatorias, estrés oxidativo, disfunción endotelial y también factores de riesgo cardiovascular tradicionales (como hipertrigliceridemia, hipertensión, bajos niveles de HDL (high-density lipoproteins), altos niveles de LDL (low-density lipoproteins), etc.).

La aterosclerosis es una enfermedad crónica y progresiva caracterizada por la acumulación de lípidos y células inflamatorias bajo la íntima (la capa más profunda de las arterias). Las placas ateroscleróticas avanzadas pueden bloquear el flujo sanguíneo o romperse y formar un trombo, dando lugar a complicaciones graves de la salud. La inflamación es un proceso central en el desarrollo de la aterosclerosis y se considera un proceso inflamatorio crónico de la pared arterial.

Durante el inicio de la aterosclerosis, las partículas de LDL se acumulan en la íntima donde se oxidan y se vuelven proinflamatorias, mientras que los monocitos circulantes se unen a las moléculas de adhesión expresadas por el endotelio activado y migran hacia la íntima, donde se unen a partículas de LDL y se transforman en células espumosas. La formación de la placa continúa avanzando a medida que se acumulan más lípidos y más células espumosas y se desencadena la producción de moléculas de matriz extracelular (como colágeno y elastina) por las células musculares lisas, lo que contribuye al engrosamiento de la lesión.

Leucocitos como biomarcadores en la investigación traslacional en diabetes

Numerosos estudios han reconocido a los leucocitos circulantes — células mononucleares de sangre periférica (PBMCs) y leucocitos polimorfonucleares (PMNs)— como biomarcadores adecuados para representar el estado metabólico a nivel sistémico y cardiovascular. Al circular por el torrente sanguíneo, los leucocitos pueden ir incorporando señales metabólicas de distintos sitios del organismo. El metabolismo de los leucocitos está muy ligado a su estado de activación y función; por ejemplo, los monocitos circulantes al encontrar un sitio de inflamación deben activarse rápidamente y migrar a los sitios de lesión, donde pueden diferenciarse en fenotipos proinflamatorios (M1) y aumentar su glicolisis, o antiinflamatorios (M2) y aumentar su fosforilación oxidativa. Además, los leucocitos circulantes tienen un papel importante en el inicio y progreso de las CVD como la aterosclerosis. Todo esto, junto con que su aislamiento es mínimamente invasivo, hace que se consideren como una buena herramienta para el estudio de la diabetes y las CVD asociadas, así como para las enfermedades que estén asociadas con estrés oxidativo y cambios en el estado redox.

Manejo clínico de la diabetes

El adecuado manejo de la diabetes requiere de hábitos de vida saludables y de fármacos para el control glucémico. Los hábitos alimentarios influyen en numerosos factores de riesgo cardiometabólico, como la obesidad, presión arterial, la relación glucosa-insulina, concentraciones y función de las lipoproteínas, el estrés oxidativo, la inflamación, la salud endotelial, la función cardiaca y la composición del microbioma.

Muchos patrones alimenticios respaldados por evidencia científica (como la dieta mediterránea) hacen énfasis en el consumo de pescado, ya que el pescado contiene múltiples componentes saludables, como proteínas específicas, grasas insaturadas, vitamina D y ácidos grasos poliinsaturados omega-3 de cadena larga (presentes principalmente en el pescado graso), como el ácido eicosapentaenoico (EPA) y el docosahexaenoico (DHA), a los cuales se les ha atribuido en gran medida la reducción en el riesgo cardiovascular.

El consumo de pescado magro también se ha relacionado con efectos positivos cardiometabólicos; lo que sugiere que no solo estos ácidos grasos son saludables, sino también otros componentes como la proteína del pescado. Ensayos clínicos con suplementos de proteína de pescado, principalmente derivados de pescado magro, han demostrado beneficios cardiometabólicos, pero hay muy pocos estudios en humanos al respecto. En un estudio reciente (FishMeal study), Hustad et al., investigaron el efecto de un suplemento de proteína de salmón en personas con mayor riesgo de T2D durante un período de 8 semanas. Los

resultados del estudio indicaron que la intervención no tuvo ningún efecto en los marcadores cardiometabólicos de los participantes.

En cuanto al tratamiento farmacológico, la T1D requiere principalmente la administración de insulina, mientras que en la T2D el tratamiento es más complejo. Ningún fármaco por sí solo revierte las múltiples alteraciones subyacentes a la T2D, por lo que la terapia combinada ha ganado una amplia aceptación. Las principales clases de fármacos antidiabéticos orales son biguanidas (metformina), sulfonilureas, meglitinida, tiazolidinedionas, inhibidores de la DPP4, agonistas del receptor GLP1, inhibidores del cotransportador 2 de sodio-glucosa (iSGLT2) e inhibidores de la alfa-glucosidasa.

Los iSGLT2 (dapagliflozina, canagliflozina y empagliflozina (entre otros)) son una nueva clase de fármacos antidiabéticos que mejoran la glucemia al reducir la reabsorción de glucosa en el riñón. El mecanismo de acción de los iSGLT2 es independiente de la secreción de insulina y de la presencia de resistencia a la insulina. Como resultado, excepto en presencia de enfermedad renal crónica severa o moderada, estos fármacos pueden utilizarse como agentes reductores de la glucosa en todas las fases de la T2D.

Además de sus efectos antihiperglucémicos, los iSGLT2 ejercen protección cardiovascular y renal. El estudio EMPA-REG OUTCOME demostró que el tratamiento con empagliflozina reducía los eventos cardiovasculares en pacientes con T2D y con alto riesgo cardiovascular.

Existen varios mecanismos potenciales por los cuales los iSGLT2 ejercen sus efectos cardioprotectores como: efecto sobre factores de riesgo tradicionales (mejora de control glucémico, reducción de peso y presión sanguínea, etc.), reducción de la inflamación crónica de bajo grado y reducción del estrés oxidativo. Todos estos mecanismos operarían en conjunto, aunque por el momento son hipotéticos y necesitan ser confirmados por estudios adicionales.

Objetivos

En base a lo presentado en la introducción, el objetivo de esta tesis fue evaluar las alteraciones celulares, moleculares y el estado redox en condiciones de hiperglicemia — desde pacientes con mayor riesgo de desarrollar diabetes hasta pacientes con un diagnóstico de T1D o T2D — y determinar si alguna de estas alteraciones puede ser modulada por una intervención nutricional o tratamiento farmacológico.

Los tres objetivos principales son:

1. Caracterizar el perfil metabolómico en plasma y transcriptómico en PBMCs de individuos en riesgo de desarrollar diabetes, e identificar biomarcadores que puedan predecir el progreso hacia T2D, y posteriormente determinar si estos pueden ser modulados por una intervención nutricional con proteína de salmón.

2. Evaluar el estado bioenergético y redox de PBMCs de pacientes con T1D comparado con el de controles sanos y evaluar el estado de la autofagia y las interacciones leucocito-endotelio.

3. Evaluar los potenciales efectos cardioprotectores del tratamiento con empagliflozina sobre las interacciones leucocito-endotelio, moléculas de adhesión, producción de ROS mitocondrial y marcadores inflamatorios en pacientes con T2D.

Materiales y métodos

Población de estudio

Para la presente tesis se reclutaron personas en riesgo de desarrollar diabetes, pacientes con diagnóstico de T1D o T2D y voluntarios sanos.

La Universidad de Oslo (Oslo, Noruega) proporcionó los datos clínicos anónimos, así como también los datos de metabolómica y transcriptómica de la población en riesgo de desarrollar diabetes. Esta población había participado previamente en un ensayo clínico controlado y aleatorizado (FishMeal study), con el objetivo de evaluar el efecto de una intervención nutricional con proteína de salmón sobre marcadores de riesgo cardiometabólico. Los participantes de este estudio recibieron durante 8 semanas cápsulas que contenían proteína de salmón o placebo. La dosis diaria en el grupo experimental fue de 5,2 g de proteína de salmón. Los participantes del estudio eran personas no diabéticas, pero con niveles elevados de glucosa: glucosa en ayunas ≥ 5,6 mmol/L (100.9 mg/dL), glucosa a las 2-h durante una prueba de tolerancia a la glucosa oral (OGTT) ≥ 6,5 mmol/L (117.1 mg/dL) o HbA1c ≥40 mmol/mol (≥5,8%).

Por otro lado, los pacientes con T2D que habían comenzado el tratamiento con empagliflozina (10 mg/día), los pacientes con T1D y los voluntarios sanos se reclutaron en el Servicio de Endocrinología del Hospital Universitario Doctor Peset (Valencia, España). El diagnóstico de T1D o T2D se hizo acorde a los criterios de la American Diabetes Association.

Colección de muestras y determinaciones bioquímicas

Los pacientes con T1D, T2D y controles sanos fueron examinados en la enfermería del Servicio de Endocrinología y se tomaron mediciones antropométricas. También se registró otra información importante como el tratamiento farmacológico que recibían. Después de 12 h en ayunas, se recolectaron muestras de sangre de los pacientes en tubos EDTA y un tubo para aislamiento de suero. Las muestras de sangre fueron analizadas por el Servicio de Análisis Clínico del hospital para medir parámetros bioquímicos de rutina (glucosa en suero, colesterol, triglicéridos, insulina, etc.).

Metabolómica de plasma

La Universidad de Oslo gestionó estos experimentos. En la población de pacientes en riesgo de desarrollar diabetes, se cuantificaron los metabolitos circulantes en el plasma tanto antes como después de la intervención nutricional, mediante una plataforma de metabolómica de NMR en las instalaciones de Nightingale en Finlandia. Este método permite cuantificar 250 metabolitos (14 subclases de lipoproteínas y sus composiciones lipídicas, apolipoproteínas, colesteroles, ácidos grasos, metabolitos asociados a la glicolisis, aminoácidos, cuerpos cetónicos, marcadores de balance hídrico y marcadores inflamatorios) a partir de una sola muestra de plasma.

Las mediciones de todos estos metabolitos dan una imagen bastante precisa del metabolismo sistémico y pueden ser usadas como marcadores de procesos patológicos ya que extienden nuestro conocimiento más allá de los factores de riesgo tradicionales.

Citocinas solubles y moléculas de adhesión en suero

Se utilizaron muestras de suero de los pacientes con T2D para medir los niveles de marcadores proinflamatorios (TNF-α, IL-1β, e IL-6) y moléculas de adhesión celular (P-Selectina, ICAM1, VCAM1) usando un sistema Luminex® 200. Este es un sistema de análisis basado en microesferas que están codificadas con diferentes combinaciones de colorantes fluorescentes y anticuerpos específicos para diferentes biomarcadores, y así medir múltiples biomarcadores (como proteínas y citocinas) en una sola muestra biológica.

Capacidad antioxidante de la sangre

Tanto en los controles sanos y los pacientes con T1D se midió la capacidad antioxidante de la sangre como reflejo de su estado redox. Se utilizó un método electroquímico que permite distinguir la cantidad de antioxidantes de acción rápida y lenta.

Análisis funcionales

Aislamiento de leucocitos

Los leucocitos de pacientes T1D y T2D se aislaron utilizando diferentes metodologías. En los pacientes T1D, los neutrófilos y PBMCs se aislaron usando kits comerciales mediante la eliminación inmunomagnética de células no deseadas en sangre recolectada en tubos con EDTA. En el caso de los T2D, los PBMCs y PMNs fueron aislados utilizando centrifugación de gradiente de densidad con Ficoll a partir de sangre en tubos con citrato. Después de la centrifugación los PBMCs se obtuvieron a partir de la capa leucoplaquetar (buffy-coat, en inglés) y los PMNs del sedimento.

En ambos casos, después de la extracción los PMNs y PBMCs frescos fueron utilizados inmediatamente en análisis funcionales (citometría, interacción leucocito-endotelio y función mitocondrial) y las células restantes se almacenaron a -80°C para análisis moleculares posteriores.

Citometría estática

Se utilizó citometría estática para medir la producción de superóxido mitocondrial en los leucocitos. Para ello, los PMNs recién extraídos se sembraron en una placa de 48 pocillos y se incubaron con la sonda fluorescente MitoSOX y una tinción nuclear. La visualización y medición de la fluorescencia se hizo en un microscopio de fluorescencia acoplado a un software de citometría estática.

Citometría de flujo

Se utilizó citometría de flujo para cuantificar la producción de ROS en leucocitos de sangre completa. Para ello, se incubaron las muestras de sangre con el anticuerpo anti-CD45 humano para marcar las células CD45+ que corresponden a la población de leucocitos. La muestra marcada se diluye y se incuba con DCFH (2',7'-Dichlorodihydrofluorescein) para medir la producción de radicales libres totales o con HE (Hydroethidine) para la producción de superóxido. La fluorescencia se mide en un citómetro de flujo y se toman los datos correspondientes a la población de PMNs.

Ensayo de interacción leucocito-endotelio

Se utilizó un modelo *ex vivo* que simula la interacción de los leucocitos con las células endoteliales en el interior de los vasos sanguíneo en condiciones fisiológicas. Este ensayo requiere de dos tipos de células: una monocapa confluente de células endoteliales de vena umbilical humana (HUVECs) y PMNs. Se perfunde una solución de PMNs recién aislados sobre

la monocapa de HUVECs usando una cámara de flujo paralelo y se toman imágenes en tiempo real de la región de la monocapa expuesta al flujo durante 5 minutos utilizando una cámara de video acoplada a un microscopio.

Durante el análisis del video se evalúan tres parámetros: 1) flujo de leucocitos en rodamiento, que es el número de PMNs que ruedan en 1 minuto; 2) velocidad de rodamiento, calculada como el tiempo en que 20 PMNs recorren 200 µm; y 3) adhesión, calculada como el número de PMNs que se adhieren firmemente al endotelio y que resistieron el flujo durante al menos 30 segundos.

Análisis de flujo metabólico en tiempo real de leucocitos

Analizamos la función mitocondrial en tiempo real de los PBMCs mediante un sistema Seahorse XFp y utilizando el kit XFp Cell Mito Stress (ambos de Agilent). Este método mide la tasa de consumo de oxígeno (OCR) y la tasa de acidificación extracelular (ECAR) en células vivas a medida que se inyectan fármacos que modulan la respiración; así se calculan parámetros importantes del funcionamiento mitocondrial como la respiración basal, respiración ligada a la producción de ATP, capacidad respiratoria máxima, capacidad de reserva y respiración no mitocondrial.

Expresión génica y proteica en leucocitos

PCR en tiempo real y nCounter

Se midió la expresión génica mediante PCR cuantitativa de transcripción inversa (RT-qPCR) a partir de muestras congeladas de PBMCs. Primero se aisló el RNA, se generó el cDNA y se realizó una PCR en tiempo real utilizando cebadores específicos para los genes *Superoxide dismutase 1, Glutathione peroxidase 1, RELA proto-oncogene, NF-κB subunit* y *Actin beta* (Tabla 1 para más detalles). Se utilizó el método $2^{-\Delta\Delta CT}$ para analizar los datos y la expresión de la β-actina como control endógeno.

Adicionalmente, analizamos datos de transcriptómica del estudio FishMeal. Estos experimentos fueron realizados por la Universidad de Oslo utilizando un sistema NanoString nCounter. El nCounter es una tecnología de hibridación multiplex de ácidos nucleicos de alto rendimiento que utiliza códigos de barras ópticos que se hibridan con cada oligonucleótido diana para permitir el recuento digital de moléculas individuales de RNA sin ningún paso enzimático. Este sistema se utilizó junto con el nCounter Metabolic Pathways Panel, que permite cuantificar 768 genes anotados en diferentes vías metabólicas (incluidos 20 genes de

referencia y controles positivos y negativos). Además, este panel se personalizó para medir 30 genes adicionales (Tabla 2) relacionados con el metabolismo inmunitario y lipídico.

Western blot

La expresión relativa de marcadores proteicos de inflamación, estrés oxidativo y autofagia en PBMCs se realizó mediante Western blot. Primero se extrajo proteína a partir de muestras congeladas de PBMCs, se cuantificó la concentración de proteínas obtenida, las muestras se separaron mediante electroforesis en geles de poliacrilamida y se transfirieron a una membrana de nitrocelulosa utilizando el método húmedo. Las proteínas de interés fueron detectadas utilizando anticuerpos primarios específicos (Tabla 3) junto con anticuerpos secundarios conjugados con peroxidasa. Las señales quimioluminiscentes se analizaron mediante densitometría.

Análisis estadísticos y herramientas bioinformáticas

Se evaluó la normalidad de todas las variables utilizando la prueba de Shapiro-Wilk. Las variables con distribución normal se muestran como la media ± desviación estándar, mientras que las no normales como mediana y rango Inter cuartil.

Las comparaciones entre dos grupos se realizaron mediante prueba t (variables normales) o Mann-Whitney (variables no normales). Las comparaciones entre tres grupos se realizaron mediante ANOVA (variables normales), Kruskal-Wallis (variables no normales) o test de Friedman (variables no normales con mediciones repetidas), en ambos casos seguidos de pruebas *post hoc* para corregir por comparaciones múltiples.

La caracterización del perfil metabólico (metabolómica y transcriptómica) de la población en riesgo de desarrollar diabetes necesitó de pasos adicionales. Se dividió a los pacientes en tertiles en función de su área incremental bajo la curva (iAUC) de insulina después de realizarse una prueba OGTT de 2h. El iAUC fue calculado utilizando dos mediciones de la concentración de insulina (en ayunas y 2h después de recibir una sobrecarga oral de 75g de glucosa). Los individuos en el tertil más bajo se clasificaron como grupo bajo iAUC de insulina (n = 24), mientras que los que se encontraban en el tertil más alto se clasificaron como grupo alto iAUC de insulina (n = 24).

Primero se compararon los perfiles metabólicos de los grupos bajo vs. alto iAUC de insulina antes de comenzar la intervención nutricional (es decir sus valores de referencia o línea base). Posteriormente se analizaron cambios en el perfil metabólico después de la intervención, los cambios se compararon entre individuos del mismo grupo iAUC de insulina que recibieron las

cápsulas con proteína de salmón o el placebo. Es decir, se compararon cambios en el perfil metabólico entre los pacientes del grupo alto iAUC de insulina que recibieron la proteína de salmón vs. los que recibieron el placebo, y lo mismo para los individuos en el grupo bajo iAUC de insulina. Los análisis de transcriptómica y metabolómica se realizaron utilizando modelos lineales corregidos por edad, sexo, índice de masa corporal (BMI) y en el caso de los análisis de transcriptómica, también por si era fumador o no.

Para entender patrones en la expresión génica que puedan ser distintos entre el grupo bajo y alto iAUC de insulina y, posteriormente, afectados por la intervención con proteína de salmón, realizamos análisis de conjuntos de genes (gene set test). Una prueba de conjunto de genes es un análisis de expresión diferencial en el que un valor p se le asigna a un conjunto de genes como una unidad. Realizamos pruebas de enriquecimiento de conjuntos de genes (utilizando los términos de Gene Ontology (GO) y rutas KEGG), y una prueba competitiva llamada Camera (que permite identificar los conjuntos de genes más relevantes para diferenciar en el fenotipo bajo y alto iAUC de insulina).

Resultados y discusión

Caracterización detallada de la población en riesgo de diabetes y efecto intervención nutricional

Primero encontramos diferencias en el metabolismo de la glucosa y sensibilidad a la insulina entre los pacientes del grupo bajo y alto iAUC de insulina. El grupo con bajo iAUC tenía mejor regulación glucémica al tener menores concentraciones de glucosa postprandial, insulina en ayunas, insulina postprandial, HOMA-IR y un mayor índice de Matsuda.

Al comparar el perfil metabolómico de estos dos grupos con distinta regulación glucémica, encontramos que el grupo con alto iAUC de insulina tenía mayores concentraciones plasmáticas del marcador inflamatorio GlycA, mayor proporción de ácidos grasos monoinsaturados (MUFA) con relación a ácidos grasos totales (MUFA %) y, por otro lado, menor concentración del aminoácido glicina y el ácido graso de cadena corta (SCFA) acetato, en comparación con el grupo con bajo iAUC de insulina.

En línea con nuestros resultados, varios estudios epidemiológicos han reportado una asociación entre GlycA y resistencia a la insulina, BMI, marcadores del síndrome metabólico y varios marcadores inflamatorios como la hs-CRP (high-sensitivity C-reactive protein). Por lo tanto, se ha propuesto a GlycA como un nuevo marcador de riesgo cardiometabólico y T2D, y que puede ser un marcador más preciso que la hs-CRP.

En cuanto a los niveles de glicina, otros estudios han obtenido resultados similares a los nuestros, encontrando una asociación inversa entre la concentración de glicina en la circulación y el desarrollo de T2D. Es de destacar que aún no está claro si niveles bajos de glicina tienen un papel importante en el desarrollo de T2D, pero se ha visto que intervenciones que retrasan o evitan el desarrollo de T2D como el ejercicio físico o la cirugía bariátrica, incrementan la concentración de glicina. Además, la suplementación con glicina mejora la tolerancia a la glucosa. En este sentido, existe evidencia de que la glicina puede mejorar el control glucémico al reducir el estrés oxidativo y la inflamación a nivel sistémico, y aumentar la secreción de insulina en el páncreas.

En referencia al perfil de ácidos grasos, encontramos que los pacientes en el grupo alto iAUC de insulina tenían mayor proporción de MUFA (MUFA %). De forma parecida, estudios en grandes cohortes de pacientes han observado que mayores concentraciones plasmáticas de MUFA se asocian con más HbA1c y glucosa en ayunas y mayor riesgo de enfermedad cardíaca.

En relación con los resultados de transcriptómica, el gen con mayor expresión diferencial (en términos de valor p) fue *CPT1A*, cuya expresión se encontraba incrementada en los PBMCs del grupo con alto iAUC de insulina en comparación al grupo con bajo iAUC. Este gen codifica para la proteína CPT1A, una enzima que se localiza en la membrana externa de la mitocondria y es clave en la β-oxidación de ácidos grasos. De forma similar a nuestros resultados, otros estudios han encontrado una asociación positiva entre la expresión de *CPT1A* en muestras de sangre y los niveles de glucosa en ayunas, triglicéridos y también el BMI.

En el análisis competitivo de conjunto de genes, encontramos una menor expresión de las rutas Myc en los PBMCs del grupo con alto iAUC de insulina, e hipotetizamos que esto puede indicar una menor biogénesis mitocondrial ya que la ruta Myc induce biogénesis y aumenta la función mitocondrial a través de múltiples vías. La función mitocondrial alterada contribuye al desarrollo de resistencia a la insulina a través de varios mecanismos (como la sobreproducción de ROS) y se ha observado disfunción mitocondrial en múltiples tejidos y células (como en tejido adiposo y muscular, leucocitos, entre otros) en personas con obesidad, T2D o con síndrome metabólico.

Como mencionamos al inicio de esta sección, el grupo con alto iAUC de insulina mostraba niveles reducidos de acetato. El acetato es un SCFA que se genera en el hígado en condiciones de ayuno, pero también se produce en el intestino por acción de la microbiota. La producción de acetato por la microbiota puede tener un papel en la regulación glucémica, por ejemplo, al

estimular la secreción de GLP1 (hormona con efecto incretina y papel fundamental en la regulación dela glucemia).

Una vez caracterizado el perfil metabólico de los pacientes en los grupos bajo y alto iAUC de insulina, evaluamos posibles cambios después de 8 semanas recibiendo un suplemento de proteína de salmón comparado con placebo. La intervención no afectó ningún marcador de regulación glucémica en ninguno de los grupos de estudio. Sin embargo, sí observamos un incremento en la concentración de acetato en el plasma del grupo con bajo iAUC de insulina, que recibió el suplemento en comparación al placebo, pero este cambio no se dio en el grupo con alto iAUC. Hipotetizamos que esto se debe a diferencias en la composición de la microbiota entre ambos grupos. Investigaciones recientes sugieren que la composición de la microbiota puede ayudar a identificar qué personas pueden beneficiarse de una intervención nutricional. A parte de los cambios en el acetato, la intervención no afectó a ningún otro metabolito ni provocó cambios importantes en la expresión génica.

Los resultados de este estudio sugieren que GlycA, la proporción MUFA contra ácidos grasos totales, glicina y el acetato pueden ser marcadores para predecir el progreso hacia T2D, y que la suplementación con proteína de salmón no tuvo efecto en el grupo de pacientes con mayor riesgo de desarrollar T2D.

Estado redox, función mitocondrial y estado de autofagia de los leucocitos T1D y sus implicaciones en la aterosclerosis

Para esta parte del estudio se comparó una población de voluntarios sanos con una de pacientes con T1D. No hubo diferencias en cuanto a edad y composición corporal entre ambos grupos, y como se esperaba, los pacientes con T1D tenían mayores niveles de glucosa en ayunas y HbA1c.

Se observó que los leucocitos de pacientes con T1D producen mayor cantidad de ROS totales y radical superóxido en comparación a los controles. También evaluamos la capacidad antioxidante de la sangre en ambos grupos como reflejo de su estado redox a nivel sistémico, y encontramos que los T1D tienen menor cantidad de antioxidantes de acción rápida y antioxidantes totales. De forma similar, otros estudios han demostrado que los monocitos provenientes de pacientes con T1D producen mayor cantidad de superóxido y presentan un mayor estado proinflamatorio (producen más IL-1β e IL-6), y mayor concentración de ICAM1 (molécula de adhesión celular) en suero, comparando con pacientes control, resaltando la relación entre inflamación y estrés oxidativo.

La menor capacidad antioxidante de la sangre en el grupo de pacientes T1D puede reflejar una mayor utilización de los antioxidantes en circulación debido a la mayor producción de ROS. Estudios similares han observado que los pacientes con T1D y sus familiares no diabéticos, tienen una menor capacidad antioxidante en el suero en comparación a controles no emparentados; y que, al no encontrar diferencias en la alimentación de estos 3 grupos, sus diferencias en la capacidad antioxidante se deben a una mayor utilización debido a producción de ROS y no por una menor ingesta de antioxidantes.

Ya que la hiperglucemia crónica puede saturar la cadena de transporte electrónico mitocondrial, lo que aumenta la producción de ROS y deteriora la función mitocondrial, analizamos la función mitocondrial en la T1D. Observamos que los leucocitos del grupo T1D poseen una respiración basal y respiración ligada a la producción de ATP similar a la de los controles. Sin embargo, los leucocitos de T1D mostraban una tendencia hacia una menor capacidad respiratoria máxima y una reducción significativa de la capacidad respiratoria de reserva.

La capacidad de reserva estima la salud y flexibilidad de las mitocondrias y determina la capacidad de las células para adaptarse a situaciones de estrés. En este sentido, se ha demostrado que una capacidad de reserva insuficiente está relacionada con el desarrollo de enfermedades cardiovasculares y complicaciones diabéticas.

Junto a la disfunción mitocondrial y la mayor producción de ROS, observamos un patrón clásico de activación de la autofagia en los PBMCs de T1D, que consiste en mayor expresión de P-AMPK y Beclin-1 (ambos marcadores de inducción de la autofagia), menor cantidad de las proteínas cargo P62 y NBR1 y mayor ratio LC3-II/I. Se ha sugerido a AMPK como una proteína que puede activar la autofagia en respuesta a la producción de ROS mitocondrial.

Al analizar la expresión del cociente LC3-II/I en función de la HbA1c observamos una correlación positiva, y que este cociente aumentaba cuando la HbA1c excedía el 5,7% (el umbral para los valores normales), apoyando la asociación entre un aumento en la autofagia y el pobre control glucémico y sugiriendo que existe una situación de estrés celular debido a la hiperglicemia crónica.

La adhesión de los leucocitos al endotelio es un sello distintivo del proceso inflamatorio al inicio de la aterosclerosis, por lo que evaluamos la interacción leucocito-endotelio en estos dos grupos. Observamos que los leucocitos de T1D presentan mayores interacciones con el endotelio en comparación a los de controles, ya que los leucocitos de T1D tienen una menor velocidad de rodamiento, mayor flujo de leucocitos en rodamiento (número de leucocitos que

interaccionan con el endotelio) y mayor adhesión leucocitaria (número de leucocitos que se adhieren al endotelio y permanecen estacionarios).

Las moléculas de adhesión celular (como P-Selectina, VCAM1 e ICAM1) son importantes para fijar los leucocitos circulantes al endotelio vascular en los focos de inflamación y mediar en la transmigración de leucocitos desde los vasos sanguíneos hacia los tejidos inflamados adyacentes. Varias citocinas, como el TNF-α y IL-1β, aumentan fuertemente la expresión de muchas moléculas de adhesión. En concordancia con este estado proinflamatorio, otros estudios han demostrado que los pacientes con T1D presentan mayores niveles de citocinas proinflamatorias en suero, y que incluso los leucocitos aislados de estos pacientes producen de forma espontánea mayores cantidades de estas citocinas, en comparación a controles.

Existe una interacción entre el aumento de la producción de ROS, la disfunción mitocondrial, la inflamación y la autofagia. La activación de rutas inflamatorias (como la ruta JNK o NF-κB) en respuesta a la producción de ROS pueden activar la autofagia. En el contexto de la aterosclerosis existen varios estímulos (inflamación, estrés oxidativo, hipoxia, falta de nutrientes) que pueden activar la autofagia en diferentes tipos celulares entre los cuales se pueden incluir, leucocitos, células endoteliales y células musculares lisas.

Este estudio apoya la idea de que la T1D afecta la función mitocondrial y promueve el estrés oxidativo y la autofagia en los leucocitos, y que estas alteraciones podrían aumentar el riesgo de aterosclerosis al incrementar la interacción leucocitos-endotelio.

Potenciales mecanismos moleculares responsables del efecto cardioprotector del tratamiento con empagliflozina

Este estudio se realizó en una población de pacientes con T2D que habían comenzado un tratamiento con 10mg/día de empagliflozina y lo continuaron durante 24 semanas. Se evaluó a los participantes al comenzar el tratamiento (línea base), y después a las 12 y 24 semanas. Observamos una progresiva reducción en el peso, BMI y circunferencia de cadera; así como también una mejora en el control glucémico de los pacientes al observar menores niveles de glucosa en ayunas y HbA1c, desde las 12 semanas de tratamiento en adelante.

Evaluamos la interacción leucocito-endotelio para determinar posibles efectos beneficiosos del tratamiento con empagliflozina. Encontramos que, tras 24 semanas, los leucocitos de los pacientes reducían sus interacciones con el endotelio ya que aumentaron su velocidad de rodamiento, se redujo el flujo de leucocitos en rodamiento y se redujo su adhesión al endotelio en comparación a la línea base. Estos resultados sugieren que la empagliflozina

ejerce un efecto protector frente a las etapas iniciales de la aterosclerosis. Paralelamente a esta reducción de las interacciones leucocito-endotelio, observamos una reducción en la concentración de las moléculas de adhesión P-Selectina e ICAM1 en suero a las 24 semanas de tratamiento. Se ha sugerido que los iSGLT2 ejercen múltiples efectos beneficiosos a nivel cardiovascular que incluyen efectos positivos sobre la función endotelial, la inflamación (por ejemplo, al reducir la expresión de ICAM1, VCAM1, TNF-α e IL-6) y el estrés oxidativo.

La hiperglicemia es un factor clave en el proceso de aterosclerosis y está relacionada con el deterioro de la función mitocondrial y el estrés oxidativo. Nuestros datos confirmaron el efecto positivo del tratamiento con empagliflozina sobre la glucemia y, en consonancia, observamos que la producción de superóxido mitocondrial en los leucocitos se redujo a las 24 semanas de tratamiento. Además, la expresión del mRNA de *SOD1* (enzima antioxidante) en PBMCs aumentó a las 24 semanas. Estos resultados concuerdan con un estudio previo de nuestro grupo que reportó que el tratamiento con empagliflozina promueve una respuesta antioxidante en los leucocitos de pacientes con T2D. El efecto antioxidante de la empagliflozina puede estar modulado por otros mecanismos celulares más allá de su efecto positivo sobre la glucemia, por ejemplo, la reducción de la sobrecarga mitocondrial o la activación de vías de respuesta antioxidante como Nrf2/ARE.

La inflamación crónica de bajo grado se caracteriza por la reducción en los niveles de adiponectina y al mismo tiempo los adipocitos y las células inmunitarias secretan varias adipocinas, citocinas y quimiocinas, como TNF-α e IL-6, de tal manera que promueven el estrés oxidativo y la disfunción endotelial. Esta inflamación crónica de bajo grado está relacionada con el aumento de la grasa corporal total y la grasa visceral y por lo tanto con el desarrollo de enfermedades cardiovasculares.

Como se mencionó antes, observamos que el tratamiento con empagliflozina redujo el BMI y la circunferencia de la cintura (marcador subrogado de grasa visceral) a las 24 semanas. Paralelamente, encontramos que se redujeron los niveles de IL-6 en el suero de estos pacientes. En este sentido, nuestro grupo previamente había demostrado que el tratamiento con empagliflozina favorece una respuesta antiinflamatoria al reducir los niveles de la enzima inflamatoria mieloperoxidasa e incrementar la concentración de la citocina antiinflamatoria IL-10 en pacientes con T2D.

Para entender posibles mecanismos moleculares detrás del efecto antiinflamatorio de la empagliflozina, medimos la expresión proteica y génica del factor de transcripción

proinflamatorio NF-κB en los leucocitos de pacientes, y encontramos que ambas se reducían a las 24 semanas de tratamiento.

Este estudio sugiere que el tratamiento con empagliflozina reduce las interacciones leucocito-endotelio, el estrés oxidativo y la inflamación, y que estos mecanismos pueden estar detrás del efecto cardioprotector de la empagliflozina.

Conclusiones

1) La caracterización metabólica de los individuos con mayor riesgo de padecer T2D sugiere que varios metabolitos plasmáticos (GlycA, MUFA %, glicina y acetato) sirven como biomarcadores para predecir la progresión de la T2D; sin embargo, su utilidad requiere más pruebas y validación en futuros estudios prospectivos. Aún está por determinar si estos nuevos biomarcadores son más sensibles a las intervenciones dietéticas en personas con un mayor riesgo de T2D.

2) La inflamación y el estrés oxidativo son mecanismos clave que contribuyen a la aterosclerosis, y los leucocitos desempeñan un papel fundamental en este proceso. Nuestra caracterización de los leucocitos de pacientes con T1D revela un aumento de ROS, un deterioro de la tasa de respiración mitocondrial y una autofagia activada. Estas alteraciones se producen en paralelo a una reducción general de las defensas antioxidantes y un aumento de la interacción entre los leucocitos y las células endoteliales. Estos hallazgos apoyan la idea de que la T1D deteriora la función mitocondrial y promueve el estrés oxidativo y la autofagia en los leucocitos, y que estos mecanismos pueden contribuir a un mayor riesgo de aterosclerosis al aumentar las interacciones leucocito-endotelio.

3) La empagliflozina disminuye las interacciones leucocito-endotelio, las moléculas de adhesión, los ROS mitocondriales, la IL-6 y la expresión de NF-κB en la T2D y promueve la actividad antioxidante. Estos efectos destacan el valor de este fármaco para prevenir el proceso aterosclerótico, la inflamación y, en consecuencia, posibles episodios cardiovasculares en pacientes con T2D.

1 INTRODUCTION

1.1 Prediabetes and Diabetes

1.1.1 Definition and diagnosis

Diabetes is a long-term serious condition that can occur when blood glucose levels rise because the body cannot produce any or enough insulin, or cannot efficiently use the insulin for regulating glucose levels (International Diabetes Federation, 2021). Insulin is a hormone produced in the pancreas and is essential for controlling the levels of glucose that move from the bloodstream into the body`s cells, where it is used for energy metabolism or stored. Moreover, insulin is necessary for the metabolism of proteins and fat. In this sense, a lack of insulin production, or an impaired cell response to it, leads to increased levels of blood glucose, known as hyperglycaemia, which is the one of the main clinical indicators of diabetes (International Diabetes Federation, 2021).

According to the World Health Organization and International Diabetes Federation, diabetes should be diagnosed if patients meet one or more of the following criteria: fasting plasma glucose ≥ 126 mg/dL, two-hour plasma glucose ≥ 200 mg/dL after an oral glucose tolerance test (OGTT, 75 g glucose load), glycated haemoglobin (HbA1c) ≥ 6.5% or/and random plasma glucose ≥ 200 mg/dL (International Diabetes Federation, 2021; World Health Organization, 2016).

Almost all patients have prediabetes before developing diabetes; in other words, higher-than-normal blood glucose levels, but not high enough to be diagnosed with diabetes (International Diabetes Federation, 2021). These patients are defined as having Impaired Fasting Glucose (IFG, fasting plasma glucose between 110-125 mg/dL), or Impaired Glucose Tolerance (IGT, 2-h values in the OGGT between 140-200 mg/dL) (World Health Organization, 2016). Moreover, the ADA (American Diabetes Association) recommends diagnosing prediabetes with HbA1c values between 5.7-6.4 % and IFG when fasting glucose is between 100-125 mg/dL (American Diabetes Association, 2020).

The threshold levels for the diagnosis of prediabetes and diabetes are shown in Figure 1.

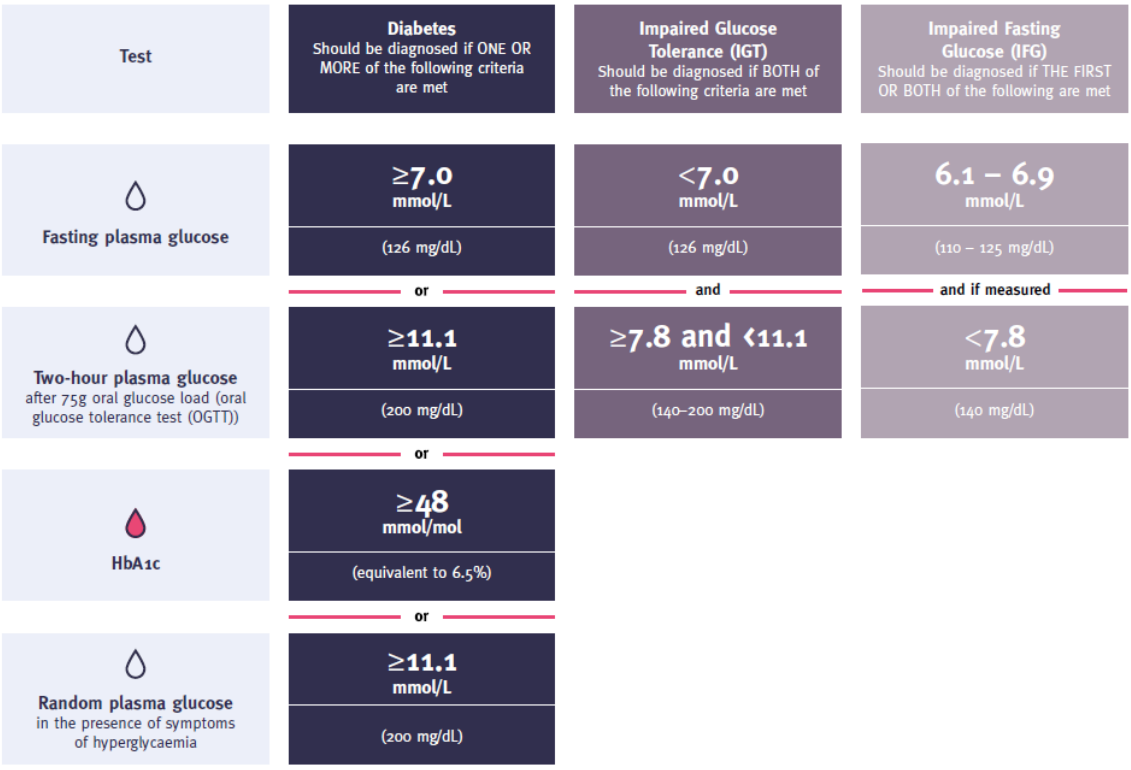

Figure 1: Diagnostic criteria for diabetes.
Abbreviations: HbA1c: glycated haemoglobin. Obtained from (International Diabetes Federation, 2021).

According to the ADA: "Diabetes may be identified anywhere along the spectrum of clinical scenarios—in seemingly low-risk individuals who happen to have glucose testing, in individuals tested based on diabetes risk assessment, and in symptomatic patients" (American Diabetes Association, 2020).

1.1.2 Classification

Most diabetes cases fall into two broad etiopathogenetic categories: type 1 (T1D) and type 2 diabetes (T2D). The cause of T1D is an absolute deficiency of insulin secretion; patients at risk of developing this type of diabetes can be identified by serological signs of an autoimmune pathological process occurring in the pancreatic islets and by genetic markers. On the other hand, the cause of T2D is a combination of resistance to insulin's action and an inadequate compensatory insulin secretory response. T2D is the most common type of diabetes (American Diabetes Association, 2013).

Type 1 diabetes

T1D represents about 5-10% of all diabetic individuals. However, it is the most prevalent type of diabetes and the most common chronic disease among children (International Diabetes Federation, 2021; Katsarou et al., 2017). T1D can develop at any age, although it occurs more

frequently in children and young adults, the incidence peaking at 12-14 years of age (Katsarou et al., 2017). The classification of diabetes in adults is still a challenge, as T1D is often misclassified as T2D (Katsarou et al., 2017). The aetiology of T1D is not completely understood, but the risk of developing it is associated with genetic and environmental factors (Serrano-Rìos et al., 1999). Patients with T1D rely on daily and life-long insulin injections to maintain glycaemia within an appropriate range, as without insulin they would not survive (International Diabetes Federation, 2021).

Type 2 diabetes

T2D is far more common than T1D and other types of diabetes, and accounts for more than 90% of all cases. It is an expanding health problem and closely linked to the epidemic of obesity (DeFronzo et al., 2015). In some countries the incidence of T2D is increasing among older children as childhood overweight and obesity are becoming more common (International Diabetes Federation, 2021). The clinical presentation, underlying pathophysiology and disease progression can vary widely among individuals, and atypical presentation of symptoms can make it difficult to diagnose T2D. At the time of diagnosis, many T2D patients are asymptomatic, while others can suffer from a range of symptoms, from severe hyperglycaemia to ketoacidosis (DeFronzo et al., 2015). Because many T2D individuals are symptomless, there is a long pre-diagnostic period and at least 30%-50% of T2D patients remain undiagnosed (DeFronzo et al., 2015; International Diabetes Federation, 2021).

Other types of diabetes

Another major type of diabetes is Gestational diabetes mellitus, defined as the diagnosis of diabetes in the 2nd or 3rd trimester of pregnancy in a woman in whom diabetes was not evident prior to gestation (American Diabetes Association, 2020; World Health Organization, 2019). There are specific types of diabetes due to other causes, such us monogenic diabetes syndromes (monogenic defect of β-cell function or insulin action), diseases of the exocrine pancreas (i.e., pancreatitis), and drug- or chemical-induced diabetes (i.e., use of glucocorticoids or HIV treatment).

Prediabetes

Additionally, there is an intermediate category of individuals whose degree of hyperglycaemia does not meet the criteria for diabetes, yet who have higher-than-normal levels. People in these groups are referred to as prediabetic and have IFG or/and IGT. IFG and IGT should not be viewed as clinical entities on their own, but rather as risk factors for developing diabetes and

cardiovascular diseases (CVD). IFG and IGT are intermediate stages of the disease's progression in any type of diabetes (American Diabetes Association, 2013). Prediabetic individuals are a heterogeneous group regarding pathophysiology and are clinically very diverse (DeFronzo et al., 2015). The term prediabetes is increasingly used to refer to patients with an increased risk of developing T2D (International Diabetes Federation, 2021). The conversion rates of prediabetes to T2D after 5 years of being diagnosed with IGT or IFG are 26% and 50%, respectively (Richter et al., 2018).

1.1.3 Epidemiology

The prevalence of diabetes has risen substantially in developed and developing countries over the past few decades, converting it in an important public health problem (Danaei et al., 2011; World Health Organization, 2016). Prevalence increased worldwide from 108 million in 1980 (World Health Organization, 2016) to 536 million people in 2021, and it is estimated that it will increase to 783 million people by 2045 (International Diabetes Federation, 2021). This steady rise in prevalence is due to population growth and aging, and is increasingly age-specific (Danaei et al., 2011; International Diabetes Federation, 2021; World Health Organization, 2016). The estimates of people having IGT and IFG in 2021 are considerable (10.6 % and 6.2% adults worldwide, respectively) and are also projected to increase by 2045 (International Diabetes Federation, 2021).

1.1.4 Clinical impact of diabetes and global burden

It is estimated that 4.2 million adults aged 20–79 died in 2019 because of diabetes and its complications. This equals to a death every eight seconds. Nearly half (46.2%) of diabetes-related deaths in the 20-to-79-year-old age group occur in those under 60 years of age – in other words, the working population – which means the problem also has a negative economic impact (International Diabetes Federation, 2019).

Diabetes of any type can cause health complications and increase the risk of premature death. Acute complications (diabetic ketoacidosis in both T1D and T2D, or hyperosmolar coma in T2D) contribute significantly to mortality, costs, and poor quality of life. Diabetes can result in long-term complications, such as heart attack, stroke, kidney failure, leg amputation, vision loss, and nerve damage (World Health Organization, 2016). There is an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease in diabetic patients (American Diabetes Association, 2013), discussed in more detail in section 1.5 Diabetes and cardiovascular diseases.

1.1.5 Risk factors for developing diabetes

Type 1 diabetes

The exact causes of T1D are unknown. T1D is a polygenic disease with environmental influences. Genetic risk factors are important but not decisive (Katsarou et al., 2017). Genetic risk factors are related with Human Leukocyte Antigens (HLA) genotypes, and the two main genotypes for developing T1D in the western world are HLA-DR4-DQ8 and HLA-DR3-DQ2 (Krischer et al., 2015; Ziegler et al., 2013).

Type 2 diabetes

T2D risk is determined by a combination of genetic and metabolic factors. Ethnicity, family history of diabetes, and previous gestational diabetes all increase risk, together with older age, overweight and obesity, unhealthy diet (high intake of saturated fatty acids, total fat, sugar-sweetened beverages, and insufficient consumption of dietary fibre), physical inactivity, and smoking. Excess body fat, which is a composite measurement of several aspects of diet and physical activity, is the most significant risk factor for T2D (World Health Organization, 2016).

1.2 Pathophysiology

1.2.1 Prediabetes pathophysiology

The degree of hyperglycaemia (if present) can change over time and depends on the degree and magnitude of the underlying pathological process. A disease process may be present (i.e., β-cell loss, insulin resistance in multiple tissues) but not progressed far enough to produce diabetes. The same pathological process can cause prediabetes (IGT or/and IFG) (American Diabetes Association, 2013). With lifestyle interventions (weight reduction, exercise, diet) and oral glucose-lowering agent, individuals could effectively delay or prevent T2D (DeFronzo et al., 2015). Thus, the disease process can regress, stay the same or progress (American Diabetes Association, 2013). The dynamics of the degree of hyperglycaemia are depicted in Figure 2.

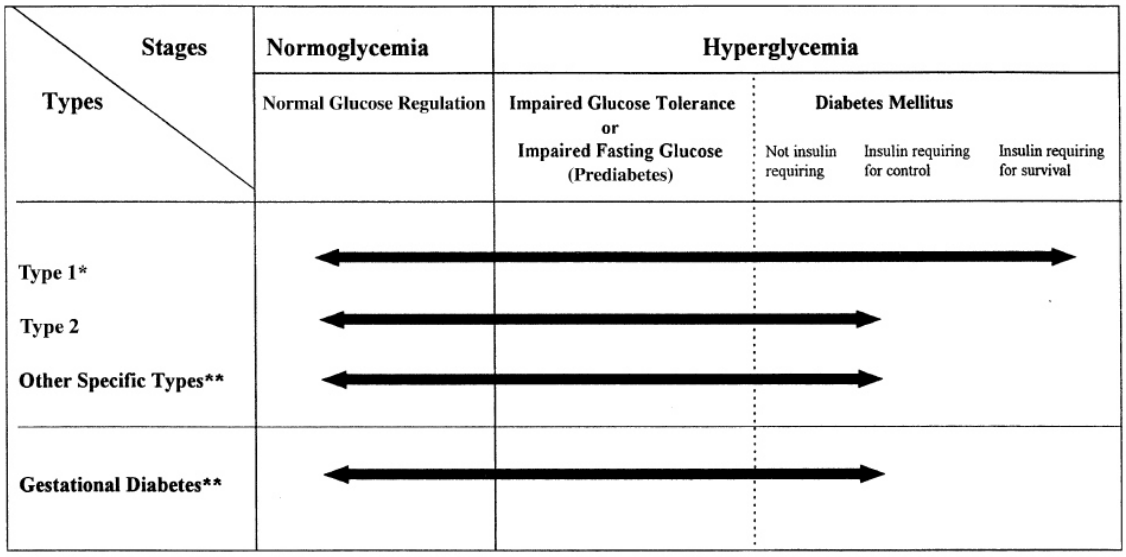

Figure 2: Aetiology and stages of glycaemia disorders.
*The degree of hyperglycaemia may change over time and depends on the extent of the underlying disease process. A disease process may be present but not progressed far enough to cause hyperglycaemia. The severity of the metabolic alteration can progress, regress, or stay the same. *Even after presenting in ketoacidosis, these patients can briefly return to normoglycaemia without requiring continuous therapy (i.e., "honeymoon" remission). **in rare instances, patients in these categories (e.g., Vacor toxicity, type 1 diabetes presenting in pregnancy) may require insulin for survival. Obtained from (American Diabetes Association, 2013).*

As shown in Figure 2, IGT and/or IFG (prediabetes) should not be considered clinical entities in their own right, but intermediate states during the development of any type of diabetes (American Diabetes Association, 2013). However, it is important to note that the term prediabetes is increasingly being used to refer to individuals who are at increased risk of developing T2D (International Diabetes Federation, 2021), and we will use the term in the same sense throughout this thesis.

In prediabetes, pancreatic β-cell dysfunction (which results in impaired insulin secretion) and hepatic and muscle insulin resistance (which results in increased nocturnal production of glucose by the liver and decreased peripheral glucose utilization by the muscles, respectively) are recognized as the principal metabolic impairments for the onset and progression of T2D (Gupta et al., 2018). There are other pathological processes that contribute to a steadily rising glycaemia (Figure 3), and prediabetes is an early stage of this continuum of metabolic impairments (Gupta et al., 2018). These impairments will be discussed in more details section 1.2.2 Pathophysiology of type 2 diabetes.

Prediabetes is directly link with increased insulin resistance; in this sense, it has been shown that prediabetic individuals have increased levels of fasting serum insulin and the homeostatic model assessment of insulin resistance (HOMA-IR) (Gupta et al., 2018). Although individuals with IGT display increased absolute plasma insulin concentrations during an OGTT test, this is

not proof that their β-cells are working normally. In fact, individuals with a 2-h plasma glucose between 120-139 mg/dL have lost two-thirds of their β-cell function; (DeFronzo, 2009).

Prediabetes is influenced by adiposity. Clinical measurements of adiposity, such us body mass index (BMI) and waist circumference (WC), are related with prediabetes. WC is a clinical surrogate of visceral adipose tissue, which when present in excess can contribute to insulin resistance (Rajala & Scherer, 2003) and predispose individuals to prediabetes (Gupta et al., 2018). Adipose tissue is a dynamic endocrine organ and regulates energy homeostasis by secreting adipokines, which in most cases circulate in proportion to body fat mass. Adipokines are essential in regulating appetite (such as leptin), in vascular metabolism (such as plasminogen activator inhibitor-1 and angiotensinogen), in glucose homeostasis and insulin sensitivity (adiponectin and resistin), and in inflammatory processes (such as tumour necrosis factor (TNF-α), interleukin-1 (IL-1) and -6 (IL-6) and monocyte chemotactic protein-1 (MCP-1)) (Moreno-Aliaga et al., 2005).

Moreover, prediabetes alters serum lipid profile (increased triglycerides and decreased high density lipoproteins), and increases cardiac risk ratios (Total cholesterol/HDL-C (High-density lipoprotein cholesterol) and LDL-C (Low-density lipoprotein cholesterol)/HDL-C) and resting blood pressure (Gupta et al., 2018). Hence, prediabetes is a risk factor for developing both diabetes and CVD (American Diabetes Association, 2013).

1.2.2 Pathophysiology of type 2 diabetes

T2D should be viewed and treated as a heterogeneous disorder involving multiple pathophysiological processes, multiple degrees of individuals susceptibilities to complications, and varying responses to therapeutic interventions (DeFronzo et al., 2015).

T2D is primarily caused by insulin resistance and progressively impaired insulin secretion by pancreatic β-cells, which usually occurs in the context of pre-existing insulin resistance in skeletal muscles, liver, and adipose tissue (DeFronzo et al., 2015). At least eight different pathological processes (Figure 3) are known to contribute to the impairment of glucose homeostasis, all of which are well established early in the progression of T2D (DeFronzo, 2009). According to Ralph DeFronzo, these eight “players” (briefly mentioned in the 1.2.1 Prediabetes pathophysiology section) are known as the “ominous octet”: insulin resistance in the muscle and liver, impaired insulin secretion by pancreatic β-cells (β-cell dysfunction), increased glucagon secretion by pancreatic α-cells, accelerated lipolysis in adipose tissue, decreased incretin effect in gastrointestinal tissues, increased glucose reabsorption in the kidney and insulin resistance in the brain (DeFronzo, 2009). In addition, two more players

could be incorporated: activation of the inflammatory pathways and impaired insulin-mediated vasodilatation (DeFronzo et al., 2015).

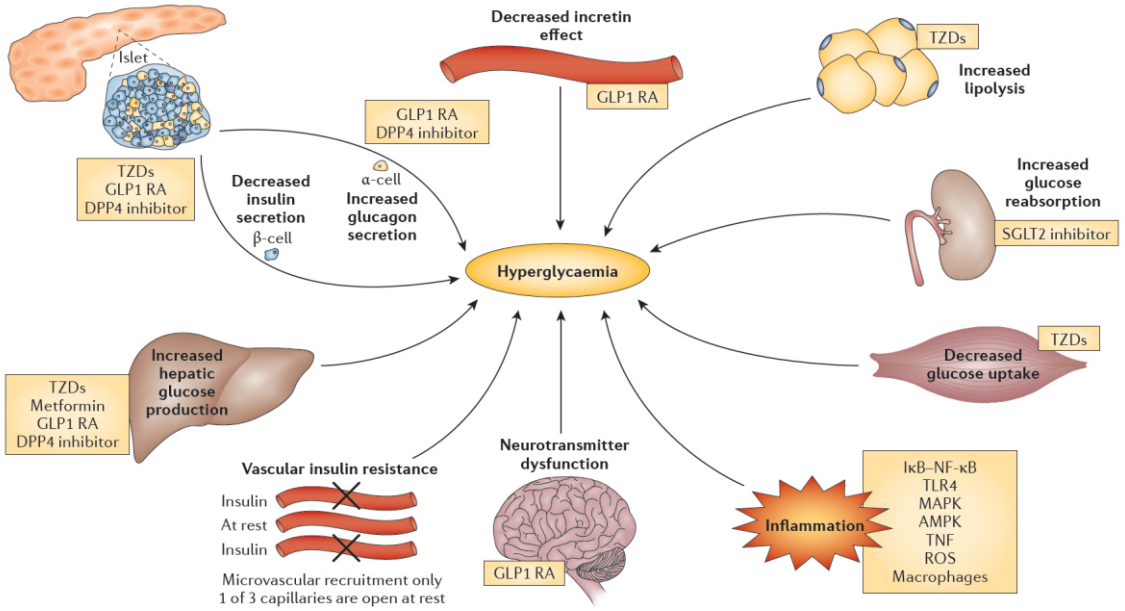

Figure 3: Pathophysiological alterations that contribute to the development of hyperglycaemia in type 2 diabetes. The figure shows the pathophysiological alteration that occurs in the different tissues: liver, pancreas, gastrointestinal tract, adipose tissue, kidneys, muscle, brain, and blood vessels. Each pathological disorder is accompanied by a list of medications designed to treat it. To this list of pathophysiological alterations must be added the activation of proinflammatory pathways. Abbreviations: AMPK: AMP-activated protein kinas, DPP4: dipeptidyl peptidase 4, GLP1 RA: glucagon-like peptide 1 receptor agonist, IκB- NF-κB: inhibitor of nuclear factor-κB, MAPK: mitogen-activated protein kinase, ROS: reactive oxygen species, SGLT2: sodium-glucose cotransporter 2, TLR4: Toll-like receptor 4, TNF: tumour necrosis factor, TZDs: thiazolidinediones. Obtained from (DeFronzo et al., 2015)

β-cell function

Insulin resistance is the earliest disease process found in individuals at risk of developing T2D, and T2D occurs when β-cells are unable to secret necessary amounts of insulin to compensate for insulin resistance (Ferrannini & Mari, 2014). Aging, genetic abnormalities, incretin deficiency/resistance, lipotoxicity, glucotoxicity, insulin resistance leading to β-cell stress, hypersecretion of islet amyloid polypeptide, oxidative stress, and activation of inflammatory pathways are all factors that can affect β-cell function (DeFronzo et al., 2015).

Insulin resistance

Obesity and physical inactivity cause insulin resistance, and combined with a genetic predisposition, stresses β-cell function and results in β-cell failure and a progressive decline in insulin secretion (DeFronzo et al., 2015). Insulin resistance appears years before established T2D (Abdul-Ghani et al., 2006). Insulin resistance is not only present in the muscle and the liver (the two organs in charge of the majority of glucose disposal after carbohydrate ingestion), but

also in adipose, kidney, gastrointestinal tract, vasculature, brain, and pancreatic β-cells (DeFronzo et al., 2015).

1.2.3 Pathophysiology of type 1 diabetes

T1D is characterized by autoimmune destruction of β-cells, and various stages of the disease's progression have been recognized. Classically, T1D is classified as either presymptomatic T1D, which is characterized by a decline in β-cell mass, but individuals present no symptoms; or symptomatic T1D, when individuals have clear symptoms of hyperglycaemia (such us thirst, hunger, polyuria, or weight loss). Alternatively, T1D can be subdivided into three stages: stage 1 is characterized by the presence of autoantibodies and the absence of hyperglycaemia, in stage 2 both autoantibodies and hyperglycaemia are present, and in stage 3 clinical diagnosis of T1D is evident and symptoms of hyperglycaemia appear (American Diabetes Association, 2020; Katsarou et al., 2017). The progression of the disease through these various stages (presymptomatic-symptomatic, stage 1-stage 3) depends on the functional β-cell mass and insulin secretion. Little is known about sequential changes in the insulin secretion and glucose homeostasis that occur as individuals progress through these stages (Bogun et al., 2020). The natural progression of the disease is summarized in Figure 4. Individuals progressing from stage 1 and stage 2 T1D can display IFG or IGT, and can be referred to as "prediabetic"; nonetheless, no formal "pre-type 1" diabetes evidence-based guidelines exist (Jacobsen et al., 2018) and the aforementioned patients should not be confused with prediabetic individuals at risk of developing T2D.

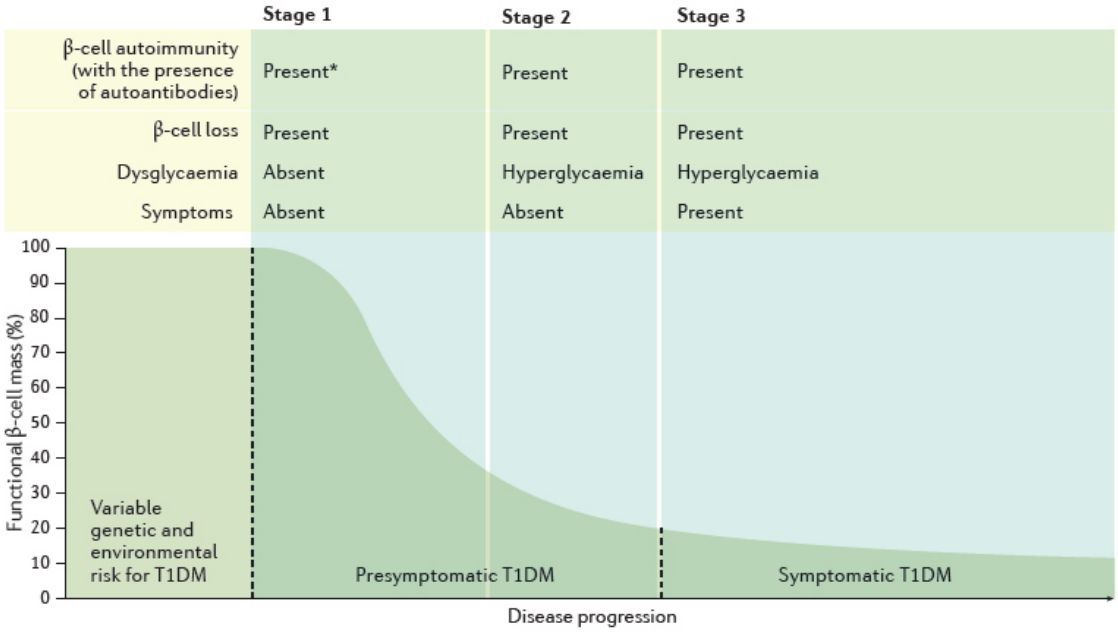

Figure 4: Disease progression of type 1 diabetes.

*Figure shows the classic classification of type 1 diabetes (T1DM) as eighter presymptomatic or symptomatic; or the alternative classification of T1DM subdivided into three stages regarding the presence of autoimmunity, dysglycaemia and symptoms. *β-cell-directed autoimmunity (indicated by the presence of β-cell-targeted autoantibodies) is usually present months to years before the onset of β-cell loss. Obtained from (Katsarou et al., 2017).*

Progression of the disease

T1D is associated with the appearance of autoantibodies months or years before the symptoms appear. These autoantibodies are not thought to be pathogenetic, but rather serve as biomarkers of autoimmunity development. Characteristic autoantibodies associated with T1D are those that target insulin, 65 kDa glutamic acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2) or zinc transporter 8 (ZNT8) (Katsarou et al., 2017). The presence of autoantibodies before the age of 6 months is rare. The first autoantibodies to appear in early childhood are anti-insulin or anti-GAD65, though both antibodies can be present, whereas it is rare to observe anti-IA-2 or ZNT8 first (Katsarou et al., 2017; Krischer et al., 2015). It is not clear what triggers the appearance of β-cell autoantibodies and still under study (Katsarou et al., 2017), but the order of appearance was related to HLA genotypes (Krischer et al., 2015).

It has been speculated that the disease process (continuous) begins with a single autoantibody, followed by intermolecular epitope spreading to multiple autoantibodies, loss of insulin secretory capability due to a combination of β-cell destruction and function inhibition, metabolic changes, and finally diabetes (Krischer et al., 2015). The identification of autoantibodies — particularly anti-insulin or anti-GAD65 — as biomarkers of presymptomatic disease may enable a better understanding of the pathogenesis and epidemiology of T1D (Katsarou et al., 2017). In fact, most people with two or more islet-targeting autoantibodies can develop the symptoms of T1D (Helminen et al., 2015; Ziegler et al., 2013).

1.3 Metabolic alterations in diabetes beyond glucose

Insulin is an anabolic hormone that has metabolic effects all over the body. β-cells regulate insulin production by monitoring glucose levels, amino acids, keto acids, and fatty acids circulating in the plasma. The overall function of insulin is to control energy conservation and utilization during fasting and feeding states. Insulin has a key role in glucose, glycogen, lipid and protein metabolism, as well as a role in inflammation and vasodilatation (Vargas et al., 2022); in this sense, diabetes can impair any of these metabolic pathways.

With the application of comprehensive metabolomic profiling (broadly named metabolomics) it is possible to obtain a detailed snapshot of systemic metabolism and better understand the aetiology and progression of metabolic diseases (Soininen et al., 2015), as well as detecting

early markers of disease and track response to treatment (van Roekel et al., 2019). The metabolome can reflect genetic background, environmental factors, and phenotypes, making it a powerful discovery tool (van Roekel et al., 2019). In humans, several metabolite biomarkers associated with insulin resistance, prediabetes, and diabetes have been identified by using high-throughput metabolomics techniques. An overview is provided below.

1.3.1 Amino acids

The positive association of branch chain amino acids (BCAAs, i.e., leucine, isoleucine, and valine) with diabetes and glycaemic traits has emerged as one of the strongest findings of metabolomics studies (Chen & Gerszten, 2020). Even after adjusting for adiposity, BCAAs were higher in obese people and those with higher insulin resistance (measured by the HOMA-IR) (Newgard et al., 2009). According to a prospective analysis in the Framingham Heart Study (FHS), individuals with BCAA concentrations in the highest quartile had a 2 to 3.5-fold higher odds of developing T2D up to 12 years later compared to individuals in the lowest quartile (T. J. Wang et al., 2011). The association between BCAAs and prediabetes/T2D has been observed in various studies (Guasch-Ferré et al., 2016), although the underlying reason is unknown (Vanweert et al., 2022).

Like BCAAs, several studies have highlighted a positive association between aromatic amino acids (tyrosine and phenylalanine) and the development of diabetes. On the other hand, glutamine (a transamination product of glutamate) has been found to be inversely associated with diabetes, as well as the glutamine:glutamate ratio. Glycine (an amino acid derived from serine) is also inversely related to the development of T2D and impaired glucose tolerance (Chen & Gerszten, 2020; Guasch-Ferré et al., 2016).

1.3.2 Organic acids

α-Hydroxybutyrate—a product of amino acid catabolism that is derived from α-ketobutyrate, a participant in the glutathione production pathway—has been positively associated with incidence of diabetes and is inversely associated with insulin sensitivity in non-diabetic individuals. Acetoacetate—a ketone body generated from fatty acids as an energy source when glucose is low—has also been positively associated with diabetes risk (Chen & Gerszten, 2020).

1.3.3 Lipids and lipoproteins

Lipids play many roles in cellular energy balance, as metabolic substrates, signalling hormones, and cellular membrane building blocks, among others. Increased clinical measurements of

lipids, particularly total triglycerides (also named triacylglycerols), are considered a classic risk factor for T2D. In the 1960s, Randle characterized the competitive interaction between fatty acids and glucose for oxidative cycling and proposed that excessive fatty acid oxidation contributed to impaired glucose homeostasis and insulin resistance. Insulin resistance has also been associated with the intracellular accumulation of fatty acid oxidation products such as diacylglycerols, triacylglycerols, and ceramides (Chen & Gerszten, 2020).

A study on Finnish men found a positive relationship between the ratio of monounsaturated fatty acids (MUFA) to total fatty acids and an elevated risk of developing T2D. The ratios of docosahexaenoic acid (DHA, an omega-3 polyunsaturated fatty acid (PUFA)) and linoleic acid (an omega-6 PUFA) with total fatty acids were found to be inversely associated (Mahendran et al., 2013). In addition, lipoprotein lipid subclass size and composition can be relevant for predicting T2D. Higher levels of large very low-density lipoprotein (VLDL)-particles and small LDL-particles, lower levels of large HDL particles, smaller mean LDL and HDL particle size, and larger mean VLDL particle size are associated with insulin resistance and incidence of diabetes (Mackey et al., 2015). The Lipoprotein Insulin Resistance index (LP-IR; LipoScience) is a new metric that integrates these six lipoprotein characteristics, weighted by the strength of associations with HOMA-IR. In a longitudinal study, higher levels of LP-IR were significantly associated with T2D development (Mackey et al., 2015).

At this point, it is important to highlight that lipid subfractions on lipoproteins are also relevant; it was found that higher cholesterol concentrations in large VLDL particles and non-esterified cholesterol in HDL particles were positively linked with T2D risk (Ahola-Olli et al., 2019).

Regarding apolipoproteins, the Metabolic Syndrome in Men (METSIM) study showed that the strongest predictor of worsening glycaemia was the ratio of apolipoprotein B (ApoB) to LDL-C, and the strongest predictor of future T2D was the ratio of apolipoprotein A (ApoA) to HDL-C (Fizelova et al., 2015).

1.3.4 Inflammation markers

A novel biomarker identified by Nuclear Magnetic Resonance (NMR)-based metabolomics is GlycA, a signal derived from a subset of glycan N-acetylglucosamine residues on enzymatically glycosylated acute-phase proteins (Otvos et al., 2015). GlycA is a composite biomarker that senses the integrated concentrations and glycosylation states of several of the most abundant acute-phase proteins in serum, as opposed to existing biomarkers of inflammation that are discrete molecular species (such as acute-phase proteins or inflammatory cytokines). Thus,

GlycA could provide a more stable measurement of low-grade systemic inflammation (Otvos et al., 2015). Elevated GlycA are stable within individuals for up to a decade and correlate with levels of multiple inflammatory cytokines (such as IL-6, TNF-α, fibrinogen, CRP (C-reactive protein)) in the circulation (Connelly et al., 2017; S. C. Ritchie et al., 2015).

GlycA is associated with incidence of T2D (as shown in the PREVEND study) (Connelly et al., 2016) and also with insulin resistance; both CRP and GlycA demonstrate a statistically independent relation with insulin resistance, suggesting that GlycA reflects an inflammatory pathway distinct from the pathway related to CRP (Lorenzo et al., 2017). Additionally, it has been suggested that GlycA is also a marker of adipose tissue dysfunction, as circulating levels of GlycA were associated with increased lipolysis in nondiabetic adults with obesity (J. A. Levine et al., 2020).

GlycA may be a reliable marker of cardiometabolic risk, as it has been linked to the presence and severity of coronary artery disease (in a secondary prevention cardiovascular cohort CATHGEN), as well as incidence of cardiovascular events in the Women's Health Study (WHS), PREVEND, MESA, and JUPITER trial, independently of traditional CV disease risk factors (Connelly et al., 2017).

1.3.5 Exogenous metabolites

Circulating metabolites are not only produced endogenously through cellular and tissue metabolism, but can also originate from the diet or from xenobiotics, which are foreign chemical substances introduced through food preservatives or containers, environmental exposures (endocrine-disrupting chemicals as pesticides), or the human microbiome (Chen & Gerszten, 2020).

The microbiota in the cecum and colon metabolizes dietary fibre, proteins, and peptides that escape digestion by the host enzymes in the upper gut. Short-chain fatty acids (SCFAs) like acetate, propionate, and butyrate are major products of microbial fermentative activity in the gut and have beneficial effects on multiple organs and host metabolism via G protein-coupled receptors found on enteroendocrine cells, enteric neurons, and enteric leukocytes. SCFAs have also been found to change metabolic profiles and contribute to the improvement of metabolic syndrome by increasing the secretion of peptide hormones such as peptide YY and glucagon-like peptide 1 (GLP1) (Tsutsumi et al., 2021). A study in a sub cohort of the Finnish Diabetes Prevention Study found that serum concentrations of the gut microbiota-produced metabolite indolepropionic acid are inversely associated with T2D and positively associated with total fibre and whole grain diet (de Mello et al., 2017). These discoveries illustrate the essential role

that exogenous sources of metabolites play in metabolic diseases, and they have pushed researchers into rapidly expanding fields of study (Chen & Gerszten, 2020).

1.4 Molecular mechanisms involved in the pathophysiology of diabetes

1.4.1 Inflammation

Inflammation is crucial to pathogenic processes such as diabetes and metabolic syndrome, and innate immunity is particularly implicated in the development of complications in this sense. In T1D, inflammation is a primary event in which infectious (viral) and/or autoimmune processes trigger disease. In T2D, chronic inflammation is typical and thought to be a result of growing insulin resistance and disrupted glucose metabolism (Forrester et al., 2020).

There is a large body of evidence that T2D is an inflammatory disease (Donath & Shoelson, 2011). Elevated circulating levels of acute-phase proteins (such as CRP, haptoglobin, fibrinogen, plasminogen activator inhibitor and serum amyloid A) and sialic acid, as well as cytokines (such as IL-1β, IL-6) and chemokines (Donath & Shoelson, 2011) have been described in T2D. Similarly, in obesity and prediabetes the serum concentration of IL-1 receptor antagonist (IL-1RA) is increased, with an accelerated increase in IL-1RA levels before the onset of T2D (Herder et al., 2009). IL-1RA expression is triggered by IL-1β and is the body's response to counteract increased IL-1β activity (Donath & Shoelson, 2011). Most proinflammatory markers found in high concentrations in the blood of T2D patients are IL-1-dependent, and inhibiting IL-1 activity has been demonstrated to lower their amounts (Larsen et al., 2007).

Increased concentration of IL-1β, IL-6 and acute-phase proteins in T2D may reflect the activation of innate immune cells by increased nutrient concentrations, although levels of these inflammatory markers may not always reflect the degree of inflammation in individual tissues (Donath & Shoelson, 2011). The total volume of pancreatic islets is modest in comparison to that in blood volume. As a result, even severe islet inflammation is unlikely to contribute significantly to circulation levels of these inflammatory factors. In contrast, the mass of adipose tissue (reaching half of the total weight in severe obesity) and the liver (a relatively large organ) may contribute disproportionately to circulating levels of inflammatory markers (Donath & Shoelson, 2011). As shown in Figure 5, excessive levels of nutrients, (like glucose and free fatty acids), will stress the pancreatic islets and insulin sensitive tissues (such as adipose tissue, the liver and muscle) and leukocytes and sustain inflammation, releasing pro-inflammatory cytokines and chemokines and thus promoting inflammation in other tissues (Donath & Shoelson, 2011).

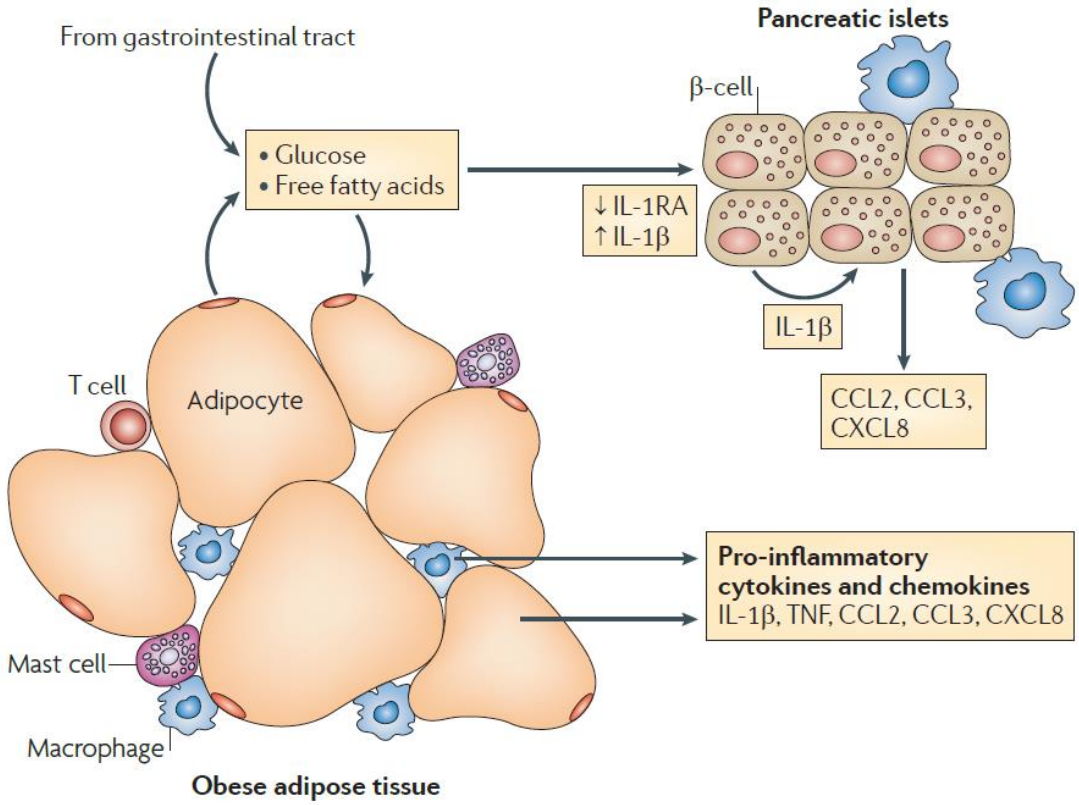

Figure 5: Development of inflammation in type 2 diabetes.
Increased nutrient concentration stresses β-cells and adipose tissue leading to the local production and release of cytokines and chemokines. Moreover, production of IL-1 receptor antagonist (IL-1RA) by β-cells is decreased. Thus, immune cells will be recruited and will contribute to tissue inflammation. The release of cytokines and chemokines from the adipose tissues into the circulation promotes inflammation in other tissues, including the pancreatic islets. CCL2: CC-chemokine ligand 2, CCL3: CC-chemokine ligand 3, CXCL8: CXC-chemokine ligand 8, IL-1RA: IL-1 receptor antagonist, IL-1β: Interleukin-1, TNF: Tumour necrosis factor. Obtained from (Donath & Shoelson, 2011).

Inflammation in response to metabolic stress

The mechanisms behind 'nutrient' (that is, glucose and free fatty acid)-induced activation of IL-1β are complex. Free fatty acids may directly induce the synthesis of pro-inflammatory molecules by activating lipid-sensing TLR2 and TLR4 (Toll-like receptor, which are expressed in immune and non-immune cells) and NF-κB (Nuclear Factor Kappa B) activation (Donath & Shoelson, 2011; van Diepen et al., 2016). The activation of the transcription factor NF-κB increases the expression of NF-κB target genes (such as pro-inflammatory cytokines TNF-α, IL-6 and IL-1β) in the liver and adipose tissue (Arkan et al., 2005). These cytokines can promote insulin resistance in tissues where they are produced, but are also transported through the circulation to affect more distant sites, like vessel walls, skeletal and cardiac muscle, the kidneys, and circulating leukocytes (Arkan et al., 2005; Donath & Shoelson, 2011).

1.4.2 Oxidative stress

Reactive oxygen species

Several forms of 'reactive species' have become the focus of interest in biology and medicine. They are named according to the nature of the reactive atom, that is, oxygen, nitrogen, or sulphur: reactive oxygen species (ROS), reactive nitrogen species and reactive sulphur species. ROS comprise a category of molecules originating from molecular oxygen and created by reduction–oxidation (redox) or electronic excitation. Hydrogen peroxide (H_2O_2), though not exactly a ROS molecule, is considered one of the most important oxidized molecules in redox modulation of biological processes (Sies & Jones, 2020).

The intracellular concentration of H_2O_2 is tightly controlled and maintained in the low nanomolar range (approximately 10^{-9} - 10^{-7} M). Its production is stimulated by metabolic signals or by various stressors, including growth factors, chemokines, and physical stressors, while its removal is accomplished by efficient reducing systems (Sies & Jones, 2020). The cellular concentration of the superoxide anion radical ($O_2^{\bullet -}$) is maintained at around 10^{-11} M and is significantly lower than that of H_2O_2. $O_2^{\bullet -}$ dismutates spontaneously or through the action of superoxide dismutase into H_2O_2 and molecular oxygen (O_2), and is a major source of H_2O_2 (Sies & Jones, 2020). In contrast to physiological levels of H_2O_2, which are essential for signalling, supraphysiological concentrations of H_2O_2 (roughly estimated to be above 10^{-7} M) lead to unspecific oxidation of proteins and altered response patterns, as well as reversible and irreversible damage to biomolecules, resulting in growth arrest and cell death with associated pathological states, a condition known as oxidative distress. All kinds of macromolecules are harmed by oxidative stress, which impairs their function. Furthermore, the products of this damage can function as secondary oxidant signals (Sies & Jones, 2020).

Hyperglycaemia and ROS production

It is currently established that hyperglycaemia causes ROS production in diabetic patients via mitochondrial respiratory chain enzymes, xanthine oxidases, lipoxygenases, cyclooxygenases, nitric oxide synthases, and peroxidases (Volpe et al., 2018). As a major site of ROS production, mitochondria have attracted the attention of researchers because it was discovered that mitochondrial ROS (mtROS) directly stimulates the production of proinflammatory cytokines and pathological conditions such as cancer, autoimmune disease, CVD (X. Li et al., 2013) and diabetes (DeFronzo et al., 2015). In the following sections, we will discuss in more detail the mechanism of hyperglycaemia-induced oxidative stress.

Mechanism of hyperglycaemia-induced ROS production

Electrons from the aerobic breakdown of glucose are primarily stored in NADH (nicotinamide adenine dinucleotide reduced form) for oxygen reduction and ATP (Adenosine triphosphate) generation. As a result, NADH is a reducing molecule, and excess levels can cause reductive stress (Yan, 2014). Overproduction of NADH or a lack of NAD^+ (nicotinamide adenine dinucleotide oxidized form) can cause NADH accumulation, resulting in an imbalance between NADH and NAD^+ and the occurrence of pseudohypoxia. More glucose will flow through the glycolytic pathway under hyperglycaemia, producing higher pyruvate and acetyl-CoA, resulting in more NADH generation (Yan, 2014). Because NADH is an electron carrier, and an excess of it puts stress on the mitochondrial electron transport chain (Yan, 2014). The electron pressure caused by excessive NADH production will exert high stress on mitochondrial complex I, which is the major site for NADH recycling. Complex I will respond within its capacity to oxidize additional NADH to NAD^+ to alleviate the pseudohypoxia state. Because complex I is also involved in proton pumping, more $O_2^{\bullet-}$ will be produced when more NADH is oxidized by complex I, resulting in a corresponding increase in electron leakage that will partially decrease oxygen levels in order to generate $O_2^{\bullet-}$ (Yan, 2014). As a result of the high amount of NADH, an oxidative stress situation will fully develop, completing the transition from reductive stress to oxidative stress (Yan, 2014).

This pathogenic process starts with the release of ferrous ion (Fe^{2+}) from ferritin and iron sulphur cluster-containing proteins in the mitochondria due to increased concentrations of $O_2^{\bullet-}$. The interaction of this released free iron with diffused mitochondrial superoxide-derived H_2O_2 produces hydroxyl radicals, the only ROS species capable of cleaving bonds in macromolecules (Shah & Brownlee, 2016). As a result, ROS-mediated DNA double-strand breaks occur in the nucleus, activating DNA repair mechanisms such as the enzyme poly (ADP-ribose) polymerase 1 (PARP-1). PARP-1 activation inhibits the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by poly ADP-ribosylation and depletes intracellular NAD+ by degrading it to ADP-ribose and nicotinamide (Shah & Brownlee, 2016). Blocking GAPDH causes a bottleneck in glycolysis and Krebs cycle, causing glyceraldehyde 3-phosphate (G3P) accumulation. As a result, all intermediate products above and including G3P must be disposed of via pathogenic pathways that branch off the glycolytic pathways (Katsarou et al., 2017; Yan, 2014).

Activation of glycolytic branching-off pathways

According to Shah and Brownlee, there are four hyperglycaemia-induced pathogenic mechanisms that are activated by overproduction of ROS: activation of 1) Polyol Pathway, 2) Hexosamine Pathway, 3) Protein Kinase C (PKC) pathway and 4) Advanced Glycation End Products (AGEs) (Shah & Brownlee, 2016). Yan proposed a fifth pathway: the Glyceraldehyde Autoxidation (Yan, 2014). These five pathways can diverge from the glycolytic pathway under chronic hyperglycaemia. In conditions of normoglycaemia, these are minor and insignificant to glucose metabolism, but they can become major pathways to dispose excessive glucose levels. All five pathways (Figure 6) have been linked to ROS production, oxidative stress, and the pathogenesis of diabetes and its complications (Yan, 2014).

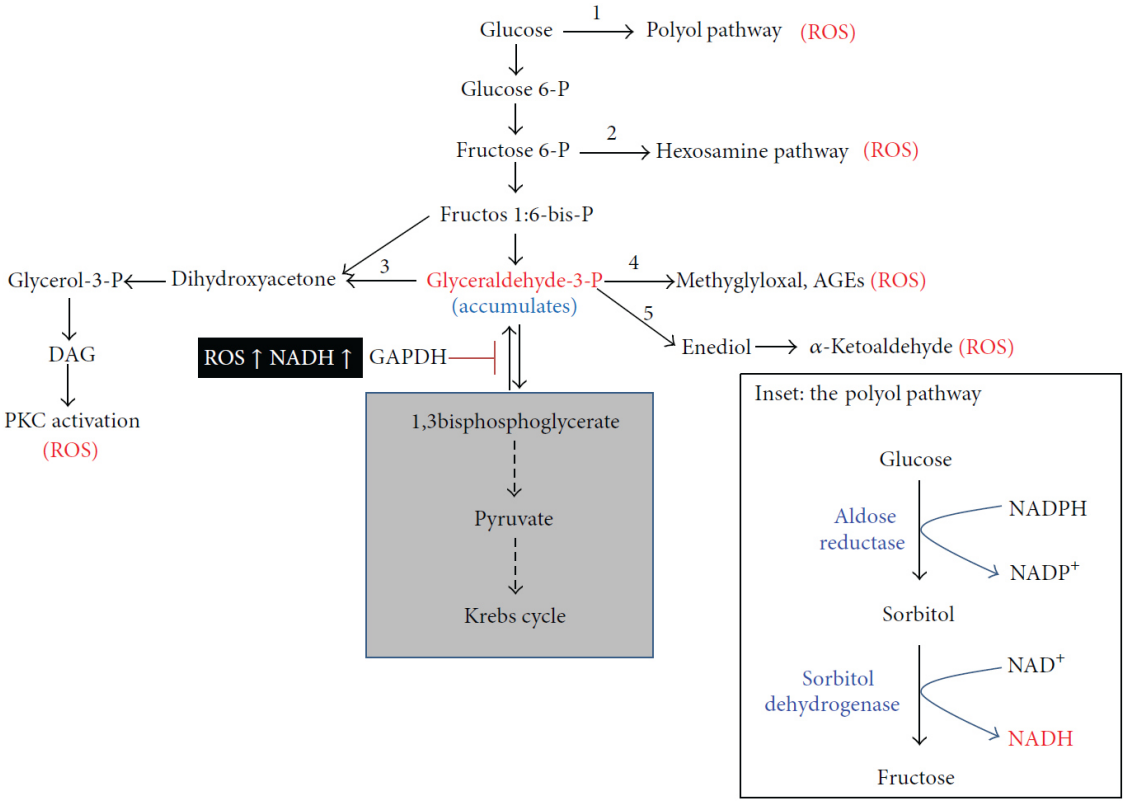

Figure 6: Activation of glycolytic branching-off pathways.
Figure shows the branch-off pathways that are activated to dispose excess glucose when glyceraldehyde-3-dehydrogenase (GAPDH) is inactivated by reactive oxygen species (ROS). These five alternative pathways, in addition to the electron transport chain are linked to ROS production, thus further exacerbating oxidative stress. Inset shows the polyol pathway. Grey area indicates the pathways that are no longer efficient to break down glucose when GAPDH is inactivated. AGEs: Advanced glycation end products, DAG: diacylglycerol, *NAD+/NADH: nicotinamide adenine dinucleotide (oxidized/reduced form), NADP+/NADPH: Nicotinamide adenine dinucleotide phosphate (oxidized/reduced form), -P: phosphate, PKC: Protein Kinase C. Obtained from (Yan, 2014).*

Oxidative stress and diabetes: summary

As summarized in Figure 7, all the above-mentioned pathogenic mechanisms that contribute to diabetes and its complications result from a single hyperglycaemia-induced process: $O_2^{\cdot -}$ overproduction by the mitochondrial electron-transport chain and inhibition of GAPDH (Yan,

2014). Increased ROS levels have been shown to induce insulin resistance, impair insulin synthesis, and impair β-cell insulin secretion. Furthermore, oxidative stress biomarkers have been shown to be elevated in people with insulin resistance and/or impaired insulin secretion. (Yan, 2014). Acute or chronic high glucose levels in diabetes promote ROS generation and induce apoptosis in β-cells. Apoptosis and necroptosis both play key roles during diabetes complications, and they can lead to tissue damage in the heart, retina, kidneys, and nervous system (Volpe et al., 2018).

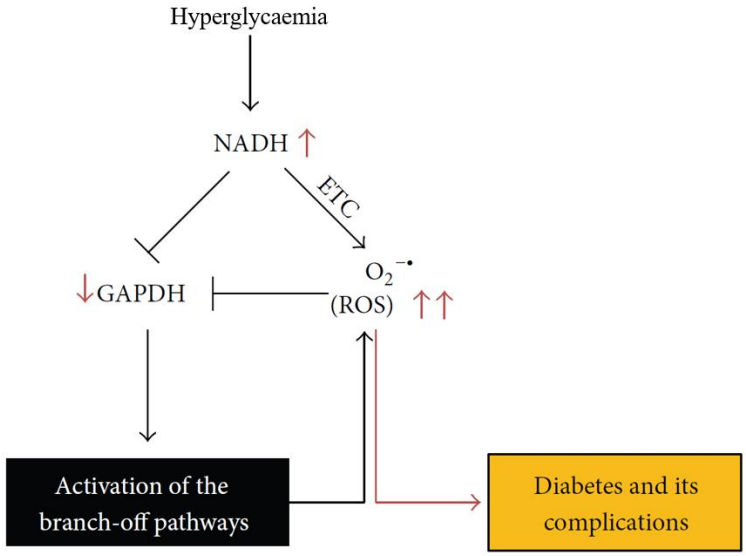

Figure 7: Relationship between hyperglycaemia, oxidative stress, diabetes progression and its complications. Pathogenic mechanisms that contribute to disease progression result from hyperglycaemia-induced superoxide overproduction ($O_2^{-\bullet}$) by the mitochondrial electron transport chain (ETC) and the subsequent inhibition of glyceraldehyde-3-dehydrogenase (GAPDH). NADH: nicotinamide adenine dinucleotide reduced form, ROS: reactive oxygen species. Obtained from (Yan, 2014).

1.4.3 Antioxidant defences

Antioxidants prevent the formation of free radicals, and seek and neutralize or repair the damage caused by them (Clark et al., 1985). There are enzymatic and non-enzymatic antioxidants; additionally, they could be classified as endogenous and exogenous antioxidants from a more informative nutritional perspective. Endogenous antioxidants include all the antioxidants that cells can produce from smaller building blocks. As a result, all enzymatic antioxidants, as well as some non-enzymatic antioxidants, are endogenous (i.e., thiol antioxidants and coenzyme Q10). Exogenous antioxidants, on the other hand, must be ingested, because synthesis is impossible in eukaryotic cells (Sharifi-Rad et al., 2020).

Enzymatic antioxidants

Several enzymes prevent the formation of free radicals; some act directly by scavenging ROS (primary enzymes), whereas "secondary enzymes" play an indirect role by assisting other endogenous antioxidants. Primary enzymes directly — such as Superoxide dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPX) — act on the main ROS produced by incomplete oxygen reduction ($O_2^{\cdot-}$ and H_2O_2) whereas secondary enzymes support this process by regenerating NADPH and reduced glutathione (GSH) and thioredoxin (Trx), as shown in Figure 8 (Amir Aslani & Ghobadi, 2016; Sharifi-Rad et al., 2020).

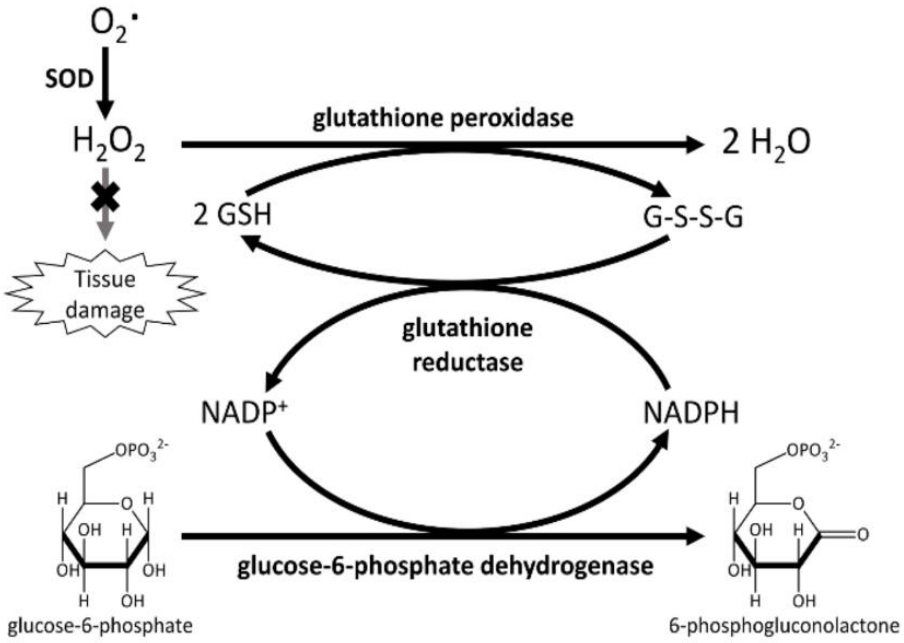

Figure 8: Enzymatic antioxidants system scheme.
Primary enzymes (superoxide dismutase (SOD) or peroxidases) scavenge reactive oxygen species directly. Secondary enzymes like glutathione reductase and glucose-6-phosphate dehydrogenase assist primary enzymes regenerating reduced nicotinamide adenine dinucleotide phosphate (NAPDH) and reduced glutathione (GSH). GSSG: glutathione disulphide, H_2O: water, H_2O_2: hydrogen peroxide, NADP+: nicotinamide adenine dinucleotide phosphate oxidized form, $O_2^{\cdot-}$: superoxide anion. Obtained from (Sharifi-Rad et al., 2020).

Non-enzymatic antioxidants

Some low-weight molecules can also act directly as antioxidants (Sharifi-Rad et al., 2020). In this case, their action is not catalytic, and they always require antioxidant regeneration or dietary supply. Non-enzymatic antioxidants can thus be classified as endogenous (if cells are able to synthesize it) or exogenous (if the antioxidant needs to be supplied through diet) (Amir Aslani & Ghobadi, 2016).

Endogenous non-enzymatic antioxidants

Both GSH and Trx can function as potent ROS scavengers (including OH, H_2O_2, organic peroxides, and $ONOO^-$) without the need for enzymatic assistance. GSH is a tripeptide

composed of γ-glutamyl-cysteinyl-glycine and found primarily in the cytosol, but also in nuclei, peroxisomes, and mitochondria. Despite being ubiquitous, the liver is the primary site of synthesis. During the redox cycling, the thiol (-SH) group on the cysteine amino acid of GSH molecules acid reacts with ROS and dimerizes, forming a disulphide bridge and resulting in a GSSG molecule (glutathione disulphide (oxidized form)) (Amir Aslani & Ghobadi, 2016; Sharifi-Rad et al., 2020).

Other examples of endogenous non-enzymatic antioxidants are Coenzyme Q10, lipoic acid and melatonin (Amir Aslani & Ghobadi, 2016; Sharifi-Rad et al., 2020).

Exogenous non-enzymatic antioxidants

Since their biosynthetic routes are typically only found in microbial and plant cells, exogenous antioxidants must be regularly refilled by the food. Vitamins C and E have notable antioxidant effects (Sharifi-Rad et al., 2020). Carotenoids and polyphenols are also broad classes of plant metabolites and are strong ROS scavengers (Amir Aslani & Ghobadi, 2016).

Antioxidants in diabetes

Several research groups have observed a decrease in antioxidant capacity in the plasma of diabetic patients. A decline in plasma RRR-alpha-tocopherol (a vitamin E stereoisomer) levels was noticed in a prospective study after the onset of T2D. Others have found a decrease in the endogenous antioxidant glutathione in diabetic patients' erythrocytes. The decrease in plasma levels of the antioxidants RRR-alpha-tocopherol or glutathione in diabetic patients could be explained by the increased production of free radicals (Rösen et al., 2001).

1.4.4 Mitochondrial dysfunction

Mitochondria are required for the oxidation of fatty acid, glucose, and amino acid metabolites via the Krebs cycle in the mitochondrial matrix and oxidative phosphorylation in the mitochondrial inner membrane, resulting in ATP and ROS or heat. With dysfunction, this can result in excessive ROS production, causing damage to macromolecules, deficits in energy supply, the production of essential molecules such as haem and nucleotides, and abnormal mitochondrial signalling functions. As a result, in a pathological setting, mitochondrial dysfunction may eventually affect a variety of biological processes (Sorrentino et al., 2018).

Mitochondrial dysfunction has been observed in rodents and humans with obesity, T2D, and metabolic syndrome in the liver, muscle, adipose tissue, and even the brain, including the hypothalamus. The cause is a decrease in mitochondrial density and impaired mitochondrial

function because of aberrant expression of various components of the oxidative phosphorylation system. Disruptions in mitochondrial function contribute to insulin resistance in a variety of ways. Mitochondrial dysfunction has been linked to decreased secretion of adiponectin, a potent insulin-sensitizing adipokine, in adipose tissue. In other tissues, mitochondrial dysfunction has been associated with an increase in ROS, which activates redox sensitive serine kinases, phosphorylates insulin receptor substrate (IRS) proteins, and results in insulin resistance. It is unclear whether mitochondrial dysfunction is the cause or result of insulin resistance. Nevertheless, given the key role of ectopic lipid deposition in causing insulin resistance, mitochondrial dysfunction associated with reduced mitochondrial fatty acid oxidation is at the very least an important exacerbating factor in this process (DeFronzo et al., 2015).

Mitochondrial ROS play a role in the pathogenesis of impaired islet β-cell insulin secretion, which is seen in both T1D and T2D. At the islet β-cell level, acute insulin release is regulated by mitochondrial ATP production, and mitochondrial ROS may contribute to the long-term deterioration of insulin secretory capacity seen in T2D. Furthermore, ROS appears to be involved in the autoimmune destruction of β-cells in T1D, as well as in the pathophysiology of the long-term complications that characterize both types of diabetes (Sivitz & Yorek, 2010).

1.4.5 Autophagy

Autophagy is a catabolic and evolutionary conserved process shared by all eukaryotes. Autophagy has become the central regulatory point in controlling the homeostasis of the body, regulating essential metabolic processes inside cells, diseases such as aging, cancer, and metabolic disorders (Saha et al., 2018). Autophagy breaks down proteins and peptides, and helps cells adapt to a variety of stress circumstances by supplying a pool of amino acids. Moreover, autophagy regulates intracellular conditions at the most fundamental level through cytoplasmic turnover of proteins and organelles. Recent research has defined the concept of selective autophagy, as it has been shown the autophagic machinery is able to detect specific substrates, such as mitochondria, lipid droplets, and peroxisomes, resulting in their turnover. Autophagy has also been shown to play an important role in innate and adaptive immunity; for example, by facilitating the binding of endogenous antigens to major histocompatibility complex-II (MHC-II) molecules recognized by CD4+T cells (Saha et al., 2018).

Current knowledge divides autophagy into three types: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy (herein referred to as autophagy) primarily involves the sequestration of cytoplasmic contents in a double-walled membrane called

autophagosome, followed by fusion with lysosomes. Lysosomal enzymes aid in the breakdown of sequestered products. Microautophagy is defined by the direct engulfment of cytoplasmic cargo by lysosomes. In chaperone-mediated autophagy, proteins are selectively guided to lysosomes by signal peptides and coordinated by chaperones situated on both sides of the targeted membrane. Selective autophagy, in the form of mitophagy, ERphagy, lipophagy, and xenophagy, is used to clear mitochondria, endoplasmic reticulum (ER), lipid droplets, and invading pathogens, respectively (Saha et al., 2018).

The autophagic process

The autophagic process is mediated by multiple autophagy-related (ATGs) proteins and can be divided into at least five sequential steps: (1) initiation, (2) double-membrane nucleation and formation of a pre-autophagosome or phagophore, (3) phagophore elongation and cytoplasmic cargo sequestration, (4) fusion of the autophagosome (the fully enclosed phagophore) to a lysosome, and (5) degradation of sequestered cargo in the autolysosome (Hansen et al., 2018). An illustrative figure of the (macro)autophagy process is provided in Figure 9.

Key upstream regulators of this multistep process include the highly conserved nutrient sensors mammalian Target of Rapamycin (mTOR) and AMPK (AMP-activated protein kinase). mTOR acts as an inhibitor of autophagy by inactivating ATG13 and Unc-51-like kinase 1 (ULK1), while AMPK acts as an activator of autophagy by inactivating mTOR and activating phosphorylation of ULK1 and Beclin1. ULK1 is another conserved kinase that serves as the key upstream initiator of autophagy. A further essential autophagy protein family consists of microtubule-associated protein light chain 3 (LC3) proteins and gamma-aminobutyric acid receptor-associated proteins (GABARAPs). LC3/GABARAP family proteins are proteolytically digested and attached to autophagosomal membranes, where they contribute to cargo detection and recruitment to the phagophore by engaging with different autophagy receptors or cargo receptors associated to proteins or organelles. Autophagy receptors include p62 (also known as sequestosome 1 (SQSTM1)), which identifies ubiquitylated proteins or organelles destined for disposal, and BCL-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), a receptor for mitochondria destined for mitophagy (Hansen et al., 2018).

As shown in Figure 9, ATGs assemble into several complexes: ULK1 initiation complex, the class III PI3K (phosphatidylinositol 3-kinase) nucleation complex and the phosphatidylinositol 3-phosphate (PI3P)-binding complex, which directs the distribution of the machinery that enables autophagosome formation, including the ATG12 and LC3/GABARAPs conjugation

systems (for simplicity, only LC3 is noted in the figure). In the ATG12 conjugation system, ATG12 bonds to ATG5, which then bonds to ATG16L1, followed by dimerization (not shown) and interaction with the PI3P-binding complex (formed by WD repeat domain phosphoinositide-interacting proteins (WIPIs) and zinc-finger FYVE domain-containing protein 1 (DFCP1)). The ATG12–ATG5–ATG16L1 complex then promotes conjugation of LC3 (or GABARAP), whereby LC3 is cleaved by the protease ATG4 to form LC3-I, which is then conjugated with phosphatidylethanolamine (PE) to form LC3-II. This conjugate is incorporated into pre-autophagosomal and autophagosomal membranes, where LC3 can interact with cargo receptors that harbour LC3-interacting motifs (LIRs). Membranes for phagophore expansion are delivered, at least in part, by ATG9-containing vesicles. For simplicity, only the names of vertebrate ATGs are shown. (Hansen et al., 2018).

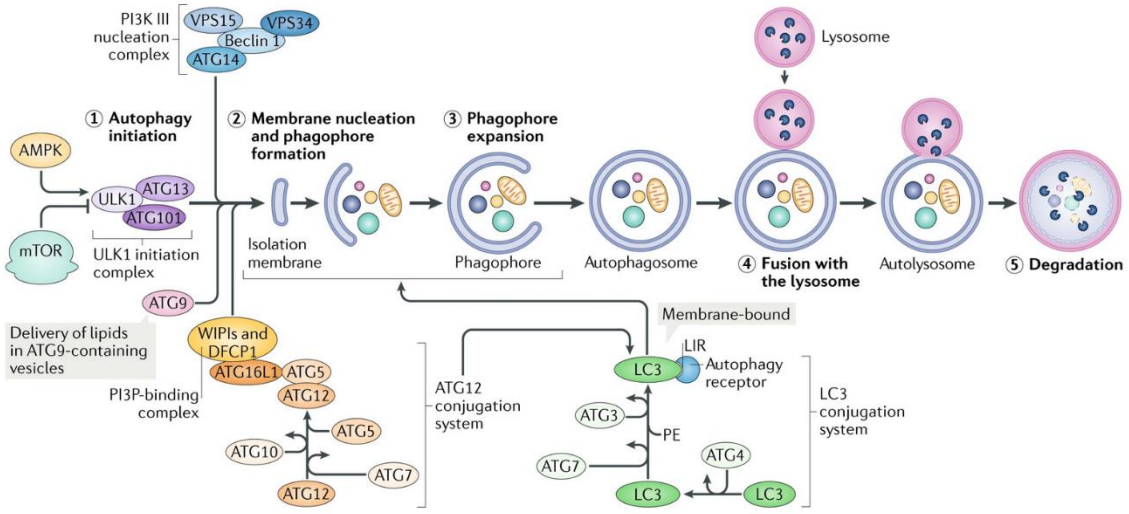

Figure 9: Macroautophagy process.
The schematic depicts the process and main regulatory machinery of macroautophagy. The metabolic sensors AMPK-activated kinase (AMPK) and mammalian target of rapamycin (mTOR) are the main regulators of autophagy. Autophagy consists of five sequential steps, which are regulated by multiple proteins, referred to as autophagy-related proteins (ATGs). ATGs assemble into several complexes: the Unc-51-like kinase 1 (ULK1) initiation complex, the class III PI3K nucleation complex and the phosphatidylinositol 3-phosphate (PI3P)-binding complex. The PI3P-binding complex directs the machinery that enables autophagosome formation, which includes ATG12 and LC3 conjugation systems. DFCP1: zinc- finger FYVE domain- containing protein 1, LC3: light chain 3, LIR: LC3-interacting motifs, PE: phosphatidylethanolamine, VPS15 (also known as Phosphoinositide-3-Kinase Regulatory Subunit 4 (PIK3R4) in humans), VPS34 (also known as phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) in humans), WIPI: WD repeat domain phosphoinositide- interacting proteins. Obtained from (Hansen et al., 2018).

Autophagy and diabetes

Autophagy is controlled by both 'immune-specific' and more general nutrient-response signals (B. Levine et al., 2011), and there are interactions between autophagy and intracellular signalling pathways in diabetes (Gonzalez et al., 2011). There are multiple immune signals that regulate autophagy; for example, autophagy is triggered by several families of pathogen-recognition receptors (such as TLRs, NOD-like receptors, and the double-stranded RNA

dependent protein kinase (PKR)), damage-associated molecular patterns (DAMPs such as ATP, ROS, and misfolded proteins), TNF-α, and NF-κB. On the other hand, it is also inhibited by NF-κB and T helper 2 (TH2) cytokines (B. Levine et al., 2011); and, as we have discussed in previous sections, all the above mentioned pathways are related to diabetes and its complications.

Elevated glucose concentrations result in oxidative stress and damage, a situation that is a common metabolic denominator for both T1D and T2D (Gonzalez et al., 2011). The accumulation of defective organelles within the cell, such as mitochondria, is a likely consequence of impaired autophagy. Mitochondria are the major location of ROS generation, and an imbalance in ROS production relative to autophagy's cytoprotective function may result in the accumulation of ROS (Gonzalez et al., 2011).

Autophagy is essential for cell organelles such as mitochondria and the ER, which play important roles in β-cell survival, insulin production, and insulin action/sensitivity. These findings support the idea that autophagy plays a role in the natural history of diabetes by influencing hormone action and organelle function (Gonzalez et al., 2011).

Diabetic metabolic alterations promote the development of CVD and accelerated atherosclerosis (Giacco & Brownlee, 2010), and autophagy plays a role in these complications. Autophagy most likely has a protective role in atherosclerosis, as it safeguards plaque cells from oxidative stress; it has been shown that autophagy in macrophages result in a more stable plaque, with a lower tendency to rupture (Gonzalez et al., 2011; Martinet & De Meyer, 2009). However, when autophagy is an insufficient response to conditions of pro-atherogenesis (such as very high concentrations of ROS), leakage of mitochondrial proteins, such as cytochrome c, may promote apoptosis (Gonzalez et al., 2011).

Autophagy is normally a protective process; however, under certain extreme conditions, it can be harmful to tissues and induce apoptosis or necrosis. Both autophagy and apoptosis must operate in harmony (Volpe et al., 2018).

1.5 Diabetes and cardiovascular diseases

1.5.1 Impact of diabetes on cardiovascular diseases

Diabetes and the continuum of high blood glucose levels, even below the diabetes diagnostic threshold, are linked to a wide range of cardiovascular conditions, which account for most of the morbidity and mortality in diabetics (International Diabetes Federation, 2019). This is explained by the concept of “glycaemic continuum” across the spectrum of prediabetes, diabetes and cardiovascular risk (Paneni et al., 2013). Diabetes confers roughly a two-fold

excess risk of a wide range of vascular diseases, independently from other conventional risk factors (Emerging Risk Factors Collaboration et al., 2010). Each standard deviation in fasting glucose, HbA1c, or 2-hour OGTT results is associated with a 6-20% increased risk of CVD events. These associations have contributed to a prevalence of coronary artery disease of 21% and CVD of 32% in adults with diabetes living in high- and middle-income countries (International Diabetes Federation, 2019).

Coronary heart disease, cerebrovascular disease, peripheral artery disease, and congestive heart failure are the most common and classic types of CVD associated with diabetes, and they manifest as specific events, hospitalizations, medical procedures, deaths from acute coronary syndromes, myocardial infarction, ischaemic and haemorrhagic stroke, and sudden death. CVD are responsible for between one-third and one-half of all deaths in diabetics (International Diabetes Federation, 2019).

Diabetes, as well as elevated blood glucose levels, increase the risk of CVD through a variety of mechanisms, including insulin resistance (Rask-Madsen et al., 2010), oxidative stress (Giacco & Brownlee, 2010), inflammation and endothelial dysfunction (Gimbrone & García-Cardeña, 2016). Furthermore, elevated blood glucose levels are linked to several other underlying metabolic risk factors, such as hypertension, dyslipidaemia, and central obesity (International Diabetes Federation, 2019; Paneni et al., 2013).

1.5.2 Atherosclerosis

Macrovascular complications are mainly represented by atherosclerotic disease and its associated conditions (Paneni et al., 2013). Diabetic individuals display accelerated atherosclerotic disease, which is associated with several risk factors, including insulin resistance and hyperinsulinemia, activation of inflammatory pathways, and the presence of multiple cardiovascular risk factors (such as hypertriglyceridemia, reduced HDL-C, excessive small dense LDL particles, hypertension, endothelial dysfunction among others (DeFronzo et al., 2015).

Atherosclerosis is the accumulation of fatty and/or fibrous material in the intima, the innermost layer of the arteries. The atherosclerotic plaque can become more fibrous and accumulate calcium mineral over time. Advanced atherosclerotic plaques can obstruct blood flow and cause tissue ischaemia. Atheromatous plaques that do not form a flow-limiting obstruction can disrupt and provoke the formation of a thrombus, which can occlude the lumen and provide a second, usually more acute, route to ischaemia (Libby et al., 2019). The accelerated atherosclerosis process in diabetes mellitus is linked to a higher inflammatory

infiltrate (macrophages and T lymphocytes), a larger necrotic core size, and more diffuse atherosclerosis in the coronary arteries compared to people without diabetes (Shah & Brownlee, 2016).

Atherosclerotic CVD are still the leading cause of vascular disease around the world. When atherosclerosis affects the heart's own circulation, it can result in acute coronary syndromes, such as myocardial infarction, or chronic conditions, such as stable angina pectoris (chest pain or discomfort caused by insufficient perfusion of the heart muscle). Many ischaemic strokes and transient cerebral ischaemic attacks are caused by atherosclerosis. It can result in the formation of aneurysms, including those of the abdominal aorta. When it affects the peripheral arteries, it can cause intermittent claudication, ulceration, and gangrene, which can jeopardize limb viability (Libby et al., 2019).

Mechanisms/pathophysiology

The pathogenesis of atherosclerosis can be considered to have three phases: initiation, progression, and complications (Libby et al., 2019). Figure 10 summarizes the initiation and progression of atherosclerosis.

The normal artery wall is a trilaminar structure. The adventitia, or outermost layer, contains nerve endings, mast cells, and vasa vasorum, which are micro vessels that nourish the outer layer of the media. The tunica media is made up of quiescent smooth muscle cells (SMC) and a well-organized extracellular matrix composed of elastin, collagen, and other macromolecules. The atherosclerotic plaque develops in the intima, the innermost layer. In the early stages of a lesion, LDL particles accumulate in the intima, where they are protected from plasma antioxidants and can undergo oxidative and other modifications that turn them pro-inflammatory and immunogenic (Libby et al., 2019).

Classic monocytes with pro-inflammatory functions circulating in the bloodstream can bind to the adhesion molecules expressed by activated endothelial cells and enter the intima. Chemoattractant cytokines can promote the migration of bound monocytes into the artery wall. Once in the intima, monocytes can mature into macrophages and acquire characteristics associated with the reparative or less pro-inflammatory monocyte/macrophage population. These cells have scavenger receptors, which allow them to bind to lipoprotein particles and transform into foam cells. T lymphocytes, which are less abundant than monocytes, also enter the intima and regulate the functions of innate immune cells, as well as endothelial cells and SMC. SMC in the tunica media can migrate into the intima in response to mediators (such as

Platelet-derived growth factor) produced by accumulating leukocytes (Libby et al., 2019). The role of SMC migration *per se* in atherosclerosis is still unclear (Bennett et al., 2016).

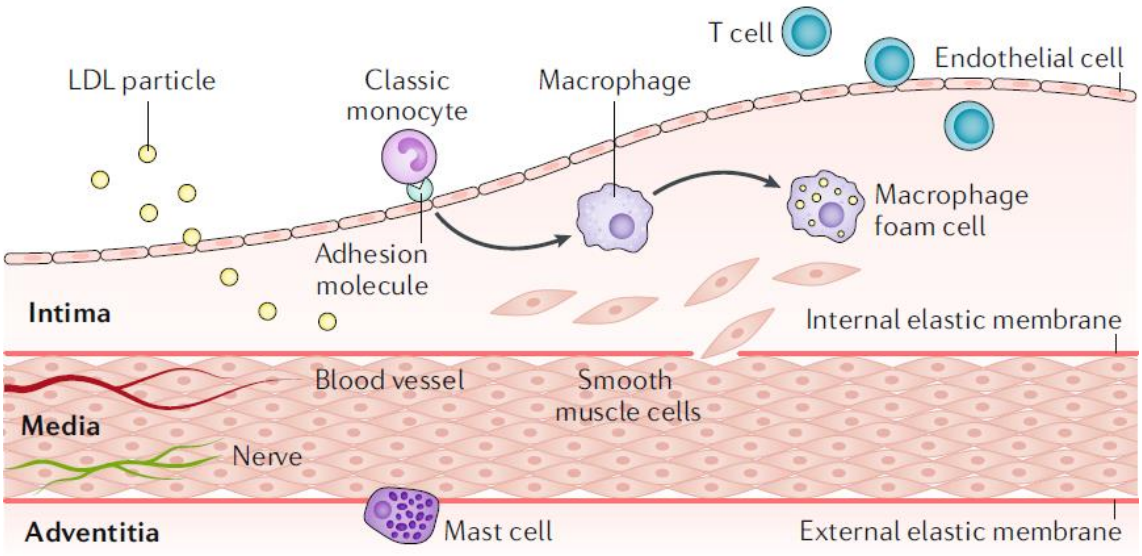

Figure 10: Initiation and progression of atherosclerosis.
Atherosclerosis lesions develop in the intima. In the early stages of lesions, LDL particles accumulate in the intima, where they can undergo oxidative and other modifications and turn proinflammatory. Circulating monocytes can bind to adhesion molecules expressed by the activated endothelium and migrate into the intima. Once in the intima, monocyte mature into macrophages that express scavenger receptors and bind to LDL particles and transform into foam cells. T cells also enters the intima and regulate the functions of innate immune cells, endothelial cells, and smooth muscle cells. Smooth muscle cells from the tunica media can migrate into the intima in response to inflammatory mediators. Obtained from *(Libby et al., 2019)*.

Initiation of atherosclerosis

LDL-C: Atherosclerosis is mainly caused by LDL particle oxidation and will probably not occur in the absence of LDL-C concentrations above physiological needs (about 10-20 mg/dL). Phylogenetic, comparative population studies and pharmacological intervention studies all indicate that LDL-C concentrations in the 20-30 mg/dL range (about 0.5-0.8 mmol/L) are adequate for good health. Despite the recent trends toward lower cholesterol levels, blood cholesterol concentrations in most societies far exceed the biological needs of the organism, thus encouraging atherosclerosis to develop (Libby et al., 2019). The cumulative LDL-C exposure of an artery over time remains a major determinant of disease initiation and progression (Ference et al., 2017).

It is unclear how high LDL-C levels cause atherosclerosis. Decades of research have established that oxidized LDL particles can promote atherogenesis (Miller et al., 2011). Among the sources of LDL particle modification are metal ion catalysis (the Fenton reaction) and the formation of ROS in the intima. The expression of high-capacity scavenger receptors for LDL particles does

not decrease as cellular cholesterol content increases. Thus, these scavenger receptors allow cholesteryl ester overloading of macrophages, resulting in foam cells, a hallmark of the early atherosclerotic lesion. Most models of the onset of atherosclerosis attribute oxidized LDL particles as ligands for scavenger receptors that promote foam cell formation (Libby et al., 2019).

Inflammation: Other risk factors implicated in atherogenesis include hypertension, tobacco, and the components of the metabolic syndrome cluster, such as include elevated blood pressure, visceral adiposity, insulin resistance, and high blood concentrations of triglyceride-rich lipoproteins. As with LDL-C, the mechanisms that link these risk factors to atherogenesis are not completely understood. However, many, if not all, of these risk factors also contribute to the activation of inflammatory pathways (Libby et al., 2019). Inflammation, in turn, can alter the function of artery wall cells, causing atherosclerosis (Gimbrone & García-Cardeña, 2016). As an example, Angiotensin II, which plays a role in the pathogenesis of hypertension, can activate inflammatory pathways regulated by NF-κB in human monocytes (Kranzhöfer et al., 1999). Moreover, visceral adipose tissue, a common concomitant of insulin resistance and T2D, contains inflammatory cells and produces a variety of inflammatory mediators that can affect the distant artery wall and activate cells in the intima (Libby et al., 2019).

Inflammatory biomarkers, such as CRP, predict cardiovascular risk and rise in parallel with many established cardiovascular risk factors (Ridker, 2016). T lymphocytes are also found in human atherosclerotic lesions, as markers of adaptive immune activation. Some T cell subtypes (for example, TH1 cells) promote experimental atherosclerosis, whereas others (for example, regulatory T cells) appear to alleviate atherogenesis. A strong body of evidence from research in mice and humans supports the role of inflammation and immunity in atherosclerosis (Libby et al., 2019; Nus & Mallat, 2016).

The endothelium: Early in atherogenesis, changes occur in the endothelial monolayer, which serves as the interface between the blood and the arterial intima. Under physiological conditions, the arterial endothelium possesses several characteristics that prevent clot formation and promote thrombolysis. Thrombomodulin and heparan sulfate proteoglycans on the endothelial surface, together with endothelial cell production of nitric oxide and prostacyclin, all contribute to the normal endothelial monolayer's anticoagulant and antithrombotic properties. Endothelial dysfunction, which can occur in the presence of atherosclerotic risk factors (such as diabetes, inflammation, high LDL-C), impairs these normal homeostatic properties. A high-cholesterol diet can increase the expression of adhesion

molecules that bind blood leukocytes to the endothelial surface, such as vascular cell adhesion protein 1 (VCAM1), as well as the presence of chemoattractants, which promote the entry of bounded leukocytes into the intima (Libby et al., 2019).

Progression of atherosclerosis

Once formed, atherosclerotic plaques grow due to the continued accumulation of lipid and lipid-engorged cells. For many years, most researchers considered blood monocyte-derived macrophages to be the precursors of lipid-laden foam cells in atheromata. Recent experimental evidence suggests that SMC metaplasia can also give rise to foam cells resembling macrophages (Bennett et al., 2016).

During the progression of an atherosclerotic plaque, resident and recruited SMCs produce extracellular matrix molecules (such as interstitial collagen, elastin, proteoglycans and glycosaminoglycans) that contribute to intimal layer thickening (Bennett et al., 2016). However, T cell mediators such as Interferon-gamma (IFNγ) can impair the SMC's ability to synthesize interstitial collagen, limiting their ability to repair and maintain the fibrous cap that covers the necrotic core. Furthermore, activated macrophages produce more matrix metalloproteinases (MMPs), which degrade the interstitial collagen that gives the fibrous cap strength. The fibrous cap's thinning and structural weakness make the plaque more prone to rupture. In the lesion that evolves as a consequence, SMCs and macrophages can divide/grow but also die through apoptosis. The necrotic, lipid-rich core of the atheroma is formed due to the accumulation of debris from dead and dying cells. Impaired efferocytosis (dead cell clearance) contribute to the formation of the necrotic core (Libby et al., 2019).

Complications

The growing atherosclerotic plaque eventually invades the arterial lumen, resulting in the formation of flow-limiting lesions. Ischaemia and angina pectoris symptoms arise owing to the derived impairment of coronary arterial perfusion, especially when myocardial oxygen demands increase due to physical effort (Libby et al., 2019).

In addition, atherosclerosis plaques can rupture, and this is the most common cause of coronary artery thrombosis, which leads to myocardial infarction. When an atherosclerotic plaque ruptures, the contents of the plaque's interior are exposed to the blood compartment. Thrombogenic material in the plaque core, particularly tissue factor produced by macrophages and SMC, has the potential to cause thrombosis, the ultimate and most feared complication of atherosclerosis. Persistent and occlusive thrombi, in combination with locally impaired luminal

endothelial homeostatic function, can cause ischaemic insults such as acute coronary syndromes and stroke (Libby et al., 2019).

1.5.3 Mechanism of hyperglycaemia-induced cardiovascular damage

Considerable evidence indicates that oxidative stress plays a key role in the pathogenesis of atherosclerosis. Many studies have shown an increase in oxidized lipids and other oxidative stress markers in atherosclerotic lesions since the discovery of lipid hydroperoxides in human atherosclerotic aorta (Forman & Zhang, 2021). For example, 20% of cholesteryl linoleate (Ch18:2) was found to be oxidized in freshly isolated human plaque, whereas it was undetectable in normal arteries (Suarna et al., 1995). Moreover, HNE-modified LDL (HNE stands for 4-hydroxy-2-nonenal, a lipid peroxidation product) was found to be 50% higher in plasma of atherosclerosis patients compared to healthy volunteers (Salomon et al., 2000). Furthermore, isoprostanes (arachidonic acid peroxidation products) were observed to be at least fivefold higher in human atherosclerotic lesions than in human umbilical veins, and oxidized linoleic acid was found only at the sites of lesions (Gniwotta et al., 1997). Oxidative stress is responsible for the conversion of LDL-C into the atherogenic form of oxidized-LDL, which plays an important role in initiating and promoting the inflammatory response and leukocyte recruitment at the lesion site, and contributes to the development of atherosclerosis via SMC activation and reduced nitric oxide bioavailability (Forman & Zhang, 2021).

Furthermore, an increase in inflammatory markers parallels that of oxidative stress, resulting in a state of chronic evolving pathological inflammation. At the organ level, increased accumulation of ROS and inflammation contribute to the cardiovascular functional and structural damage that underlies all major CVDs (Rubattu et al., 2019).

The overproduction of mitochondrial superoxide is the upstream process that activates several hyperglycaemia-induced pathogenic mechanisms (See Figure 11) (Shah & Brownlee, 2016). In section 1.4.2, we summarized how hyperglycaemia induces the overproduction of ROS by saturating the mitochondrial electron transport chain and how this, in turn, activates alternative glucose metabolic pathways that contribute to both diabetes and diabetic complications. Below, we summarize how the activation of these pathways contributes to the development of macrovascular complications, especially atherosclerosis.

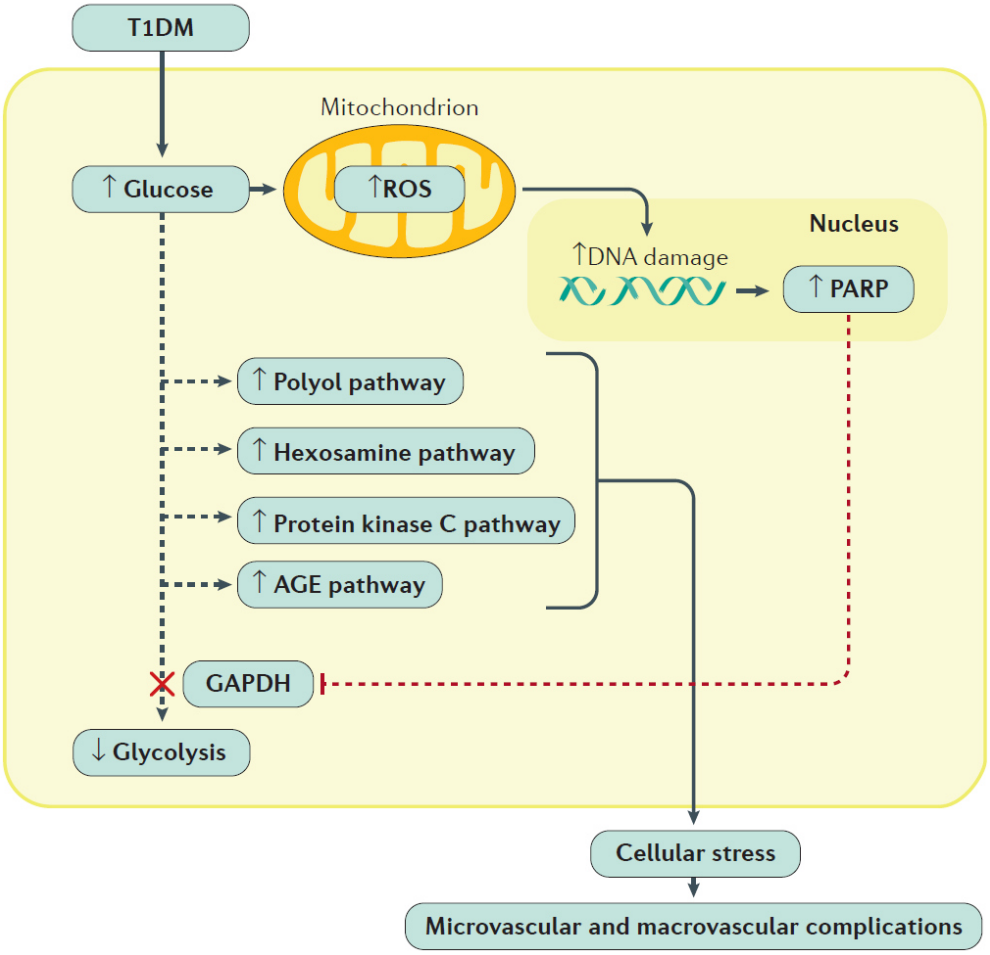

Figure 11: Mechanism of hyperglycaemia-induced cellular damage and cardiovascular complications in diabetes. The activation of these mechanism shares a common element: increased mitochondrial production of reactive oxygen species (ROS) induced by hyperglycaemia. Increased ROS concentrations cause DNA strand breaks, which activates poly (ADP-ribose) polymerase (PARP). PARP produces ADP-ribose polymers that bind to and impact the activity of glyceraldehyde-3-dehydrogenase (GAPDH). GAPDH inhibition causes a bottleneck in glycolysis, causing glycolytic intermediates to be diverted into pathogenetic signalling pathways (dashed arrows). In addition, the accumulation of glycolytic intermediates inactivates two antiatherosclerotic enzymes: endothelial nitric oxide synthase (eNOS) and prostacyclin synthase. AGE: advanced glycation end products, T1DM: type 1 diabetes mellitus. Obtained from *(Katsarou et al., 2017)*.

Mechanisms of Hyperglycaemia-Induced Cardiovascular Damage

Polyol pathway: This pathway is linked to the pathogenesis of several diabetic complications, including diabetic cardiovascular disease (Shah & Brownlee, 2016). Overexpression of human aldose reductase (enzyme that converts glucose into sorbitol, see Figure 6 inset) accelerates atherosclerosis in diabetic apolipoprotein E (ApoE) knockout mice, but pharmacological inhibition of the enzyme prevents this effect (Vedantham et al., 2014).

Hexosamine pathway: The hexosamine pathway causes reversible post-translational modification of intracellular protein serine and threonine residues with N-acetylglucosamine (also called GlcNAc) by the enzyme O-GlcNAc transferase (OGT) (Shah & Brownlee, 2016).

Chronically elevated O-GlcNAc levels have been linked to diabetic cardiovascular complications, as increased O-GlcNAcylation has been associated with decreased mitochondrial function and autophagic signalling and debilitated contractile function in the heart (Shah & Brownlee, 2016). For example, in human arterial endothelial cells, activation of eNOS (endothelial nitric oxide synthase, the enzyme responsible for most of the vascular NO produced for vascular dilatation) by phosphorylation at Serine 1177 is inhibited directly by hyperglycaemia-induced O-GlcNAcylation at this site (Du et al., 2001).

Activation of PKC: PKC activation has been linked to many cellular abnormalities associated with diabetic cardiovascular disease. These include endothelial dysfunction, increased vascular permeability, impaired angiogenesis, and increased apoptosis. PKC-β activation in diabetic ApoE null mice promotes vascular inflammation and accelerates atherosclerosis by increasing the expression of inflammatory mediators (Shah & Brownlee, 2016).

Methylglyoxal formation: AGEs are protein post-translational modifications formed by glucose-derived dicarbonyl reacting with amino groups of proteins. The non-enzymatic fragmentation of the glycolytic intermediate triose-phosphate results in methylglyoxal, which accounts for most of the hyperglycaemia-induced increase in AGEs precursors in diabetic tissues. Methylglyoxal reacts with unprotonated arginine residues in cells to form the major methylglyoxal-derived epitope MG-H1 (methylglyoxal hydroimidazolone 1). Intracellular AGE precursor production damages cells in three ways: 1) it alters proteins and how they function; 2) it alters extracellular matrix components; and 3) it increases the expression of both the pattern recognition receptor for AGEs (RAGEs) and its ligands (Shah & Brownlee, 2016).

Recent research has linked elevated methylglyoxal levels to the pathogenesis of both diabetic atherosclerosis and cardiomyopathy (Shah & Brownlee, 2016). MG-H1 levels in human atherosclerotic plaques were linked to rupture-prone plaques and MG-H1 was found to accumulate predominantly in macrophages near the necrotic core, where it co-localized with cleaved caspase-3 (Hanssen et al., 2014).

1.6 Circulating leukocytes as markers in diabetes translational research

1.6.1 Markers of cardiovascular diseases

Increased oxidative stress is an important mechanism that underlies all major CVDs (Giacco & Brownlee, 2010). In this sense, it is critical to monitor oxidative stress levels in human patients for both diagnostic and therapeutic purposes, as changes in oxidative stress level parallel the progression of the pathological condition, as well as the response to treatment (Yasunari et al.,

2002). A major limitation in this regard is the limited availability of tissue samples from both the heart and blood vessels; in this sense, the use of circulating markers capable of representing the condition within the cardiovascular system is of great benefit (Rubattu et al., 2019).

Several studies conducted over the last 15 years have identified the isolation of circulating leukocytes as a suitable method for representing systemic cardiovascular stress conditions with minimal invasive intervention (Rubattu et al., 2019). Because leukocytes circulate in the bloodstream, they can accurately reflect both systemic and cardiovascular metabolic states. These studies emphasize the importance of measuring oxidative stress levels in circulating leukocytes in various CVDs, with a consistent correlation between degree of oxidative stress and CVD severity and complications (Rubattu et al., 2019). They also point to leukocytes playing a dual role as both a marker of disease condition and a direct contributor to disease progression (Swirski & Nahrendorf, 2013).

Raffa et al. conducted a recent study that highlighted the utility of measuring oxidative stress for predicting and monitoring atherosclerotic diseases. In a cohort of patients with acute coronary syndrome (ACS) or stable chronic angina, peripheral blood mononuclear cells (PBMCs) were used to test the gene expression of a mitochondrial complex I subunit (*NDUFC2,* NADH: Ubiquinone Oxidoreductase Subunit C2) and to determine the level of oxidative stress dependent on mitochondrial dysfunction (Raffa et al., 2019). In ACS patients, *NDUFC2* mRNA was significantly downregulated (which is known to result in altered complex I assembly and activity), along with *UCP2* (Uncoupling Protein 2), *SOD1*, and *SOD2* (antioxidant enzymes) expression. Additionally, ACS patients displayed significant increases in ROS levels, decreased ATP levels, and a higher degree of mitochondrial structural damage and dysfunction. *In vitro*, silencing *NDUFC2* promoted mechanisms associated with atherogenesis and plaque fragility. These observations suggest that oxidative damage of mitochondrial proteins leads to progressive dysfunction and that mitochondrial dysfunction is a mechanism involved in the pathogenesis of several CVDs, including ACS (Raffa et al., 2019).

1.6.2 Markers of metabolic stress

In normal physiology, leukocytes and platelets are programmed for distinct functions, such as regulating the inflammatory response, preventing thrombosis, removing foreign bodies, and sensing and responding to systemic biological signals in the circulation. Dynamic functions of peripheral blood leukocytes and platelets involve an integrated metabolic machinery to meet

the energy demands of normal physiology, which likely involves both glycolysis and mitochondrial oxidative phosphorylation (Kramer et al., 2014).

Many chronic pathological conditions, including metabolic syndrome, cancer, and atherosclerosis, are associated with an inflammatory response characterized by the release of proinflammatory mediators, specifically cytokines. Leukocytes and platelets respond to these proinflammatory mediators in the systemic circulation by an activation process that changes the cellular phenotype and metabolism (Kramer et al., 2014). For example, circulating monocytes search the body for sites of inflammation in response to inflammatory stress signals. They rapidly activate and migrate to injury sites, where they can differentiate into pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes (Zhou et al., 2014). In the M1 state, the activated monocyte–macrophage cell switches from oxidative phosphorylation to glycolysis. During the resolution of inflammation, macrophages transform into the alternatively activated M2 phenotype, which is characterized by increased oxidative phosphorylation. Similarly, lymphocyte activation is associated with a metabolic phenotype change towards an increase in glycolytic function and mitochondrial oxygen consumption (Kramer et al., 2014).

Several studies have been conducted to test the hypothesis that leukocytes and platelets can act as biomarkers of mitochondrial dysfunction in diseases (Kramer et al., 2014). For example, a study in mononuclear cells from T2D patients showed that mitochondria were more polarized and mitochondrial mass was reduced in T2D compared to controls (Widlansky et al., 2010). Moreover, it has been shown that platelet mitochondria from T2D patients displayed decreased oxygen consumption and oxygen-dependent ATP synthesis, along with increased markers of oxidative stress compared to controls (Avila et al., 2012). These observations support the hypothesis that mitochondrial perturbations in T2D extends beyond the classic insulin responsive tissues (Avila et al., 2012).

Another important parameter to evaluate mitochondrial dysfunction is spare/reserve respiratory capacity. Spare respiratory is the difference between maximal oxygen consumption induced by uncoupling and basal consumption and is an estimate of mitochondrial health and the capacity to adapt to pathological stress. Therefore, inadequate spare respiratory capacity is associated with CVD (Marchetti et al., 2020) and the development of diabetic complications (Czajka et al., 2015).

1.7 Clinical management of diabetes

1.7.1 Prevention

TD1 prevention

There is currently no effective and safe intervention to prevent T1D, despite many clinical trials aimed at stopping the on-going autoimmune destruction of pancreatic β cells (Atkinson et al., 2014).

T2D prevention

There is conclusive evidence from randomised controlled trials (RCT) that intensive lifestyle interventions and medication can delay or prevent the onset of T2D in high-risk individuals. The body of evidence supporting these interventions is vast and solid. Four large RCTs demonstrated that a diet and moderate physical activity designed to achieve and maintain a 5–7% reduction in body weight reduced the risk of T2D by 29–58% (DeFronzo et al., 2015). One of them, The Da Qing IGT and Diabetes Study, showed that diet and/or exercise interventions led to a significant decrease in the incidence of diabetes among patients with IGT over a 6-year period. Patients in the diet group were encouraged to consume more vegetables, control alcohol intake, reduce intake of simple sugars and limit total caloric intake (Pan et al., 1997).

1.7.2 Management of type 1 diabetes

T1D management requires the close collaboration of an interdisciplinary team (consisting of physicians, diabetes educators, nurses, dieticians, psychologists, and social workers), the patient, their family and support systems (school or work). To prevent severe hypoglycaemia, hyperglycaemia, and ketoacidosis, it is important to promote healthy lifestyle and glycaemic control (Katsarou et al., 2017).

Medications

In the two and a half decades since the DCCT study, management of T1D has centred on intensive insulin therapy with the aim of maintaining glucose levels as close to normal as possible and preventing hypoglycaemia (Nathan & DCCT/EDIC Research Group, 2014).

1.7.3 Management of type 2 diabetes

Management of T2D is complicated by multiple pathophysiological disturbances (Figure 3) and the profile of the patient (ABCDE - Age, Body mass index, Complications, Duration, Education and Expense, and Etiology) (DeFronzo et al., 2015). Currently available glucose-lowering

therapies target one or more of these pathophysiological disturbances (Chaudhury et al., 2017).

Microvascular complication prevention focuses on glycaemic control, whereas macrovascular complication prevention requires correction of classic cardiovascular risk factors for insulin resistance (metabolic syndrome). HbA1c should ideally be reduced to as close to normal levels as possible, without causing adverse effects, as hypoglycaemia is the greatest concern. Patients with T2D and persistently low HbA1c levels (<6.5%) do not develop retinopathy. Because obesity and inactivity are associated with tissue fat overload (lipotoxicity), lifestyle modification should be a fundamental component of all intervention programmes (DeFronzo et al., 2015).

Lifestyle

Several key lessons have emerged from advances in nutrition science. First, it is evident nowadays that dietary habits influence numerous cardiometabolic risk factors, such as obesity and LDL-C, as well as blood pressure, glucose-insulin homeostasis, lipoprotein concentrations and function, oxidative stress, inflammation, endothelial health, cardiac function, and microbiome composition, among others. A second key lesson is the importance of specific foods and overall diet patterns, rather than single isolated nutrients, for cardiometabolic risk (Mozaffarian, 2016).

Dietary patterns are the combination of foods that are routinely consumed and together produce synergistic health effects. Dietary patterns supported by scientific evidence share several key characteristics; these include more minimally processed foods like fruits, nuts/seeds, vegetables (excluding white potatoes), legumes, whole grains, seafood, yoghurt, and vegetable oils; and fewer red meats, processed (sodium-preserved) meats, refined grains, starches, and added sugars. These diets contain more fibre, vitamins, antioxidants, minerals, phenolics, and unsaturated fats, and less glycaemic index, glycaemic load, salt, and trans-fat (Mozaffarian, 2016).

The most well-studied dietary patterns are traditional Mediterranean and DASH (Dietary Approaches to Stop Hypertension) diets, and both improve a range of risk factors, reduce long-term weight gain, and are consistently associated with a lower risk of clinical events (Mozaffarian, 2016) and diabetes prevention (Salas-Salvadó et al., 2014).

Fish consumption

Many healthy dietary patterns emphasize seafood consumption, as fish contain several healthy components, such as specific proteins, unsaturated fats, vitamin D, selenium, and long-chain omega-3 PUFAs, which include eicosapentaenoic acid (EPA) and DHA. Humans synthesise EPA and DHA from its plant precursor α-linoleic acid (an omega-3). Thus, dietary consumption of EPA and DHA affect their levels in the tissues. Seafood levels of EPA plus DHA vary by > 10-fold. Fatty (oily) fish, such as anchovies, herring, farmed and wild salmon, sardines, trout, and white tuna, tend to have the highest concentrations (Mozaffarian et al., 2011). The association of fish consumption and decreased risk of CVD is well documented and has been largely attributed to the omega-3 PUFA present in fatty fish (Mozaffarian & Rimm, 2006).

Consumption of lean fish has also been related with positive cardiometabolic positive effects; for example in a RCT, dietary cod consumption improved insulin sensitivity in insulin resistant individuals (Ouellet et al., 2007), suggesting that components other than omega-3 PUFA have beneficial cardiometabolic effects, like fish protein. Animal studies suggest that fish protein has beneficial effects on cardiometabolic markers, such as those related to the metabolism of glucose (Lavigne et al., 2001).

Clinical trials with fish protein supplements, primarily derived from lean fish, have demonstrated cardiometabolic benefits, including enhanced glucose metabolism (Vikøren et al., 2013). However, there are few human clinical trials involving protein supplements derived from fatty fish, and the studies carried out to date have been small and have yielded inconsistent results regarding cardiometabolic risk markers. In a recent RCT, Hustad et al. examined the effect of an 8-week intervention with salmon fish protein supplement on cardiometabolic risk markers in individuals at increased risk of T2D, and found no effect of the intervention (Hustad et al., 2021).

Medication

No single medication reverses the multiple abnormalities underlying T2D; hence, combination therapy has gained widespread acceptance and will no doubt continue to grow (DeFronzo et al., 2015). The major classes of oral antidiabetic drugs are biguanides (metformin, the most widely prescribed antidiabetic medication in the world), sulfonylureas, meglitinide, thiazolidinediones, dipeptidyl peptidase 4 (DPP4) inhibitors, GLP1 receptor agonists, sodium-glucose cotransporter 2 inhibitors (iSGLT2), and alpha-glucosidase inhibitors between others. If the HbA1c level increases to 7.5% while taking medication, or if the initial HbA1c level is 9%, combination therapy with two oral agents or insulin may be considered. Though these medications can be used in all patients, regardless of body weight, some medications, such as

liraglutide, may offer distinct advantages in obese patients over lean diabetics (Chaudhury et al., 2017).

Targeting renal glucose absorption

iSGLT2 (dapagliflozin, canagliflozin and empagliflozin between others) prevent glucose absorption in the proximal renal tubule. They reduce the maximum renal glucose reabsorption capacity and the blood glucose threshold at which glucose spills into the urine (to <40 mg per dL) (DeFronzo et al., 2015). Increased glucose removal from the body via glucosuria results in a decrease in plasma glucose, which alleviates glucotoxicity, resulting in improved β-cell function and insulin sensitivity. Their glucose-lowering efficacy is comparable to metformin, and urine calorie loss (4 calories per gram glucose) promotes weight loss. Due to iSGLT2 inhibitory effect on sodium transport, iSGLT2 also enhances natriuresis (excretion of sodium through urine), leading to mild extracellular volume depletion and a reduction in blood pressure (DeFronzo et al., 2015; Ferrannini, 2017). The mechanism of action of iSGLT2 is independent of residual insulin secretion and the presence of insulin resistance, which are two key features in the pathophysiology of T2D. As a result, except in the presence of moderate to severe chronic kidney disease (CKD), these medications can be used as glucose-lowering agents in all stages of T2D (Scheen, 2020).

In addition to their primary anti-hyperglycaemic effects, iSGLT2 exerts numerous metabolic effects that can positively affect cardiovascular and renal prognosis (Scheen, 2020). Evidence from the EMPA-REG OUTCOME study, a double-blind RCT, shows that T2D patients at a high risk of cardiovascular events who received empagliflozin versus a placebo displayed lower rates of death from CVD, nonfatal myocardial infarction, or nonfatal stroke (Zinman et al., 2015). Similar effects were observed with canagliflozin in the CANVAS programme (Neal et al., 2017). Therefore, the evidence from clinical trials suggest that iSGLT2 exerts cardiovascular protection (Scheen, 2020).

1.7.4 Pleiotropic effects of iSGLT2 that can contribute to improving cardiovascular outcomes

There are several potential mechanisms that could contribute to the cardiovascular and renal protection reported with iSGLT2, as shown in Figure 12. These proposed mechanisms include, but are not limited to, endocrine and/or metabolic, haemodynamic, and biochemical mechanisms, which are not mutually exclusive. All are still hypothetical and need to be confirmed by additional studies (Scheen, 2020).

Effects on classic cardiovascular risk factors

Canagliflozin, dapagliflozin, and empagliflozin have demonstrated favourable effects on several classic cardiovascular risk factors (glycaemic control, blood pressure, weight, plasma lipids). The observed protection against CVD, heart failure, and CKD may therefore be partially explained by these combined effects (Scheen, 2020). Nonetheless, a post-hoc exploratory analysis of EMPA-REG OUTCOME suggested that changes in some traditional cardiovascular risk factors make negligible contributions in comparison to markers of plasma volume (haematocrit and haemoglobin) (Inzucchi et al., 2017). Additionally, it was found that an important proportion of the iSGLT2-cardioprotective effect is mediated by a reduction in uric acid (Inzucchi et al., 2017).

Effects on low-grade inflammation

iSGLT2 have been shown to decrease serum leptin, TNF-α, and IL-6 levels, while increasing adiponectin levels (Garvey et al., 2018). While there are numerous animal studies supporting an anti-inflammatory effect of iSGLT2, human data on high-sensitivity C-reactive protein (hs-CRP) and other inflammatory markers are limited (Scheen, 2020).

Multiple mechanisms may be responsible for this anti-inflammatory effect, including weight loss and a reduction in adipose tissue inflammation, a slight increase in ketone bodies and a decrease in uric acid levels, or a reduction in oxidative stress (Bonnet & Scheen, 2018). Through their unique mechanism of action, and by increasing circulating ketone bodies (emerging as potent anti-inflammatory molecules), it has been hypothesized that iSGLT2 can indirectly target the IL-1 pathway, thus consistently reducing low-grade inflammation (Prattichizzo et al., 2018).

While systemic and tissue low-grade inflammation, as well as oxidative stress, can exacerbate vascular complications in T2D patients, the contribution of these effects to the cardiovascular and renal protection observed with iSGLT2 remains unknown. The addition of markers of inflammation and oxidative stress to the list of parameters measured in cardiovascular outcomes trials could provide additional insight into the potential role of these specific effects of iSGLT2 in cardio-renal protection (Scheen, 2020).

Metabolic effects

Increased plasma β-hydroxybutyrate levels associated with iSGLT2 may provide a more energy-efficient fuel than glucose and free fatty acids, which are energy inefficient for the heart and kidneys in a scenario of T2D. A metabolic shift toward an energy-efficient fuel, such as ketone

bodies, may improve myocardial and/or renal efficiency and function, thereby contributing to the cardio-renal protection observed in cardiovascular outcome trials with iSGLT2 (Ferrannini et al., 2016; Scheen, 2020).

Haemodynamic effects

By promoting natriuresis and osmotic diuresis, iSGLT2 may lead, at least transiently, to plasma volume contraction and reduced preload, as well as reductions in blood pressure, arterial stiffness, and afterload in patients at risk of or with heart failure (Scheen, 2020). As previously mentioned, a mediation analysis of the EMPA-REG OUTCOME trial revealed that a change in haematocrit, used as an indirect marker of the change in plasma volume, was the most important mediator of the reduced risk of cardiovascular death associated with empagliflozin compared to a placebo (Inzucchi et al., 2017).

Inhibition of sodium-hydrogen exchange

It has been proposed that the benefits of iSGLT2 in heart failure are mediated by inhibition of sodium-hydrogen exchange in both the kidneys and the myocardium. iSGLT2 may have a direct cardiac effect by decreasing myocardial cytoplasmic sodium and calcium and increasing mitochondrial calcium, effects that are independent of SGLT2 inhibition and are mediated by Sodium/Hydrogen exchanger (NHE) protein activity (Packer et al., 2017).

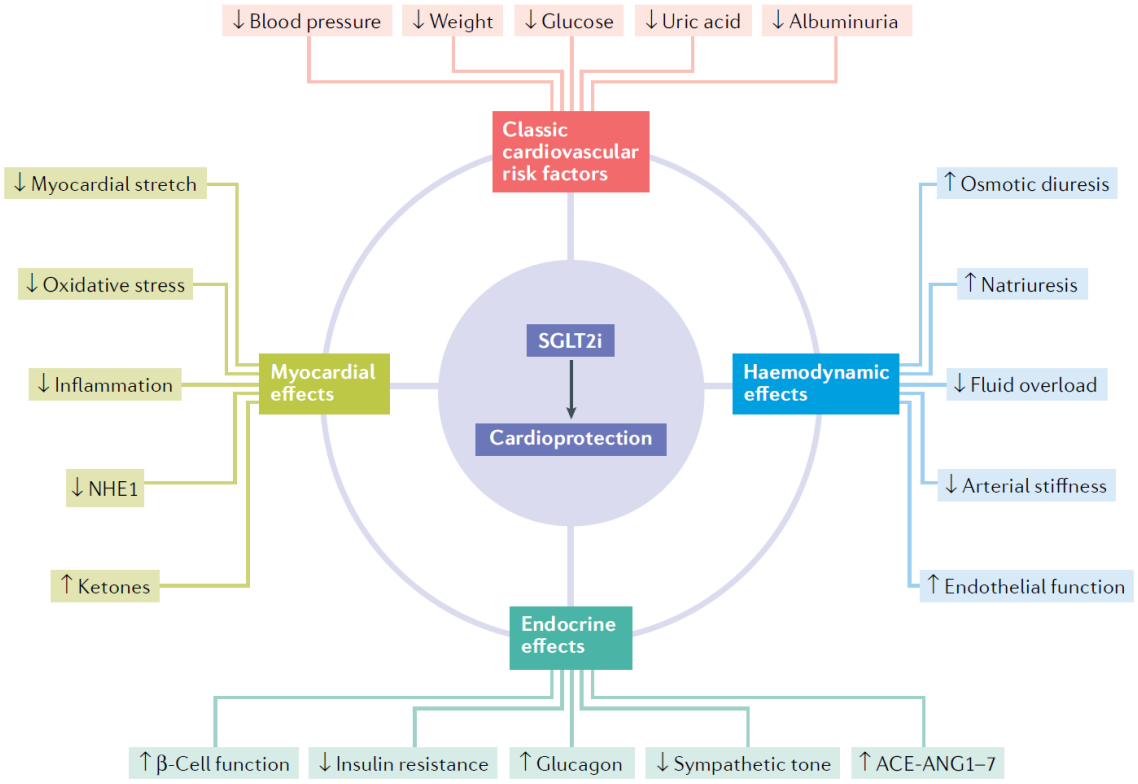

Figure 12: Pleiotropic effects of iSGLT2 that can contribute to improving cardiovascular outcomes.
iSGLT2i can protect the heart through several mechanisms independent of their glucose-lowering effect. It is well accepted that iSGLT2 effects on traditional cardiovascular risk explain only a small portion of their cardiovascular protection. The haemodynamic effects of reduced fluid overload (a marker being an increase in haematocrit) due to osmotic diuresis and enhanced natriuresis, combined with decreased arterial stiffness and improved endothelial function, appear to play a significant role. Direct myocardial effects, such as decreased myocardial stretch, decreased oxidative stress and low-grade inflammation, inhibition of Sodium/Hydrogen exchanger 1 (NHE1) activity (lowering myocardial cytoplasmic sodium and calcium and increasing mitochondrial calcium), and improved energetics due to the use of ketones as super fuel, are also potentially significant effects. Additional positive effects may result from endocrine effects, such as increased glucagon, decreased sympathetic tone, and increased angiotensin-converting enzyme-angiotensin 1-7 pathway (ACE-ANG1-7) activity may have additional beneficial effects, but their roles still hypothetical. Obtained from (Scheen, 2020).

2 OBJECTIVES

Diabetes is a disease characterised by multiple underlying pathophysiological processes in various tissues (insulin resistance in the liver, muscles, and adipose tissue, stressed β-cells, etc.), and the degree of hyperglycaemia reflects the severity of the underlying disease process. In addition to hyperglycaemia, diabetes also affects the metabolism of proteins, lipids, and lipoproteins and drives chronic low-grade inflammation.

Diabetes, even prediabetes, is a risk factor for developing any CVD. This is explained by the concept of the "glycaemic continuum" across the spectrum of prediabetes, diabetes, and cardiovascular risk. Sustained hyperglycaemia induces the overproduction of ROS, leading to oxidative stress. Considerable evidence indicates that oxidative stress plays a key role in the pathogenesis of atherosclerosis.

The overproduction of ROS in general and mitochondrial superoxide in particular are upstream processes that activate several of the hyperglycaemia-induced pathogenic mechanisms that contribute to the progression of diabetes and its cardiovascular complications. In fact, increased mitochondrial ROS production alters mitochondrial function, enhances inflammation, and activates cellular processes, such as autophagy, in order to restore homeostasis.

There are several lifestyle interventions and pharmacological treatments to improve glycaemia in diabetic patients. iSGLT2 are one class of antidiabetic drugs that promote the elimination of glucose through the urine. One such drug is Empagliflozin, which, in addition to resolving glycaemia, also improves cardiovascular outcomes. However, the mechanism of action behind these positive effects is not completely clear. It is hypothesised that empagliflozin exerts its cardioprotective effect through a combination of different mechanisms, such as reducing oxidative stress and inflammation.

Circulating leukocytes can reflect redox state, inflammatory state, and alterations due to treatments in diabetic patients. Moreover, leukocytes are key players in the development of atherosclerosis. In addition, plasma metabolites can reflect systemic metabolism. Hence, leukocytes and plasma metabolomics are useful tools in translational research about diabetes.

In this sense, the aim of this thesis is to evaluate cellular, molecular, and redox-state alterations across the hyperglycaemia continuum—from patients at increased risk of developing diabetes to individuals already diagnosed with T1D or T2D—and to determine if any of these alterations can be modulated by a nutritional or pharmacological intervention.

The three main objectives were:

1. Characterise the plasma metabolomic and PBMCs' transcriptomic profiles of individuals at an increased risk of developing diabetes and identify biomarkers that can predict progression to T2D, and to determine whether any of these can be modulated by a nutritional intervention with salmon protein.
2. Evaluate the bioenergetic and redox state of PBMCs from T1D patients compared to healthy controls and assess autophagy state and leukocyte-endothelium interactions.
3. Evaluate the potential cardioprotective effects of empagliflozin treatment on leukocyte-endothelium interactions, adhesion molecules, mitochondrial ROS production, and inflammatory markers in T2D patients.

3 MATERIAL AND METHODS

3.1 Study population

For the purpose of this thesis, we recruited individuals with increased risk of T2D, T1D and T2D patients, and healthy volunteers. The University of Oslo provided the anonymized clinical, metabolomic, and transcriptomic data for the individuals with increased risk of T2D.

T2D patients on empagliflozin treatment (10mg/daily), T1D patients, and healthy volunteers were recruited from the Endocrinology and Nutrition Service of the Outpatient Clinic of the University Hospital Dr. Peset (Valencia, Spain) between 2017 and 2022. Diagnosis of diabetes (T1D or T2D) was confirmed according to ADA criteria. The exclusion criteria for T1D patients were any documented history of cardiovascular disease (ischaemic cardiopathy, stroke, peripheral arteriopathy, or any CVD associated with cardiovascular risk) or any severe inflammatory, infectious, or autoimmune disease. The exclusion criteria for T2D patients were morbid obesity, significant renal impairment (creatinine >1.5 mg/dL or the estimated glomerular filtration rate < 60 mL/min/1.73 m^2), severe diabetic neuropathy, or chronic diseases other than those directly related to cardiovascular risk. All patients received detailed information about the study procedures and signed a written informed consent. All collected data were anonymized and managed according to the data protection law. The experimental procedures were approved by the hospital's Ethics Committee for Clinical Investigation (ID: 98/19), in line with the ethical principles of the Helsinki Declaration.

Additionally, we analysed the clinical, metabolome, and transcriptome data of individuals with increased risk of developing T2D who had previously participated in the FishMeal study (Hustad et al., 2021). The FishMeal study was a human RCT conducted at the University of Oslo (Norway) between August 2018 and September 2019, and its objective was to investigate the effects of salmon protein intake on cardiometabolic risk markers. The inclusion criteria were non-diabetic men and women ≥20 years old and elevated blood glucose, defined as either fasting serum (s)-glucose ≥5.6 mmol/L (100.9 mg/dL), 2-h OGTT-s-glucose ≥6.5 mmol/L (117.1 mg/dL), or HbA1c ≥40 mmol/mol (≥5.8%). People with diabetes were excluded from the study. A more detailed description of the exclusion criteria is provided in Hustad et al. 2021.

The participants included in the RCT study received capsules containing salmon protein or a placebo for 8 weeks. The daily dose in the experimental group was 5.2 g of salmon protein (corresponding to 7.5 g of fishmeal). The experimental group was given capsules containing salmon protein (250 mg/capsule), microcrystalline cellulose (240 mg/capsule), antioxidants

(tocopherols and rosemary extract), and excipients (magnesium stearate: 5 mg/capsule, tricalcium phosphate: 5 mg/capsule, and silicon dioxide: 2.5 mg/capsule). On the other hand, the placebo group received capsules containing microcrystalline cellulose (250 mg/capsule), along with antioxidants and excipients like the fish protein capsules, but without amino acids.

All the participants gave their written informed consent. The FishMeal study was conducted in accordance with the Helsinki Declaration guidelines and was approved by the Regional Ethics Committee for Medical Research in South-East Norway. The study was registered at ClinicalTrials.gov (ClinicalTrials.gov Identifier: NCT03764423).

3.2 Sample collection and biochemical determinations

Subjects were examined by nurses at the Endocrinology and Nutrition Service of University Hospital Doctor Peset for weight, height, waist circumference, and systolic and diastolic blood pressure. Other relevant information, such as smoker status or pharmacological treatment, was gathered. After 12 hours of fasting, blood samples were extracted from the brachial vein and collected in EDTA tubes and a tube for serum isolation.

Additional blood tubes were collected for routine biochemical measurements, which were conducted by the hospital´s Clinical Analysis Service. The following methods were employed: serum fasting glucose, total cholesterol, and triglycerides were determined by an enzymatic method; insulin levels were calculated by immunochemiluminescence; insulin resistance was measured by homeostasis model assessment (HOMA-IR = [fasting insulin (μU/mL) × fasting glucose (mg/dL)]/405); percentage of glycated haemoglobin % (HbA1c %) was measured with an automated glycohemoglobin analyser (Arkray Inc., Kyoto, Japan); HDL-C levels were assessed by a direct method with a Beckman LX-20 autoanalyzer (Beckman Coulter, La Brea, CA, USA); LDL-C was estimated with Friedewald's formula; and hs-CRP levels were measured by an immunonephelometric assay (Behring Nephelometer II, Newark, DE, USA).

3.3 Plasma metabolomics

These experiments were performed at the University of Oslo. In the population of individuals with increased risk of T2D, circulating metabolites were quantified before and after intervention at a high-throughput and highly automated proton NMR metabolomics facility (Nightingale Health Ltd., Helsinki, Finland). This method gives a comprehensive snapshot of systemic metabolism (Soininen et al., 2015), as it measures 250 metabolites (14 lipoprotein subclasses, their lipid concentrations and composition, apolipoproteins, multiple cholesterol and triglyceride measures, fatty acids, glycolysis-related metabolites, amino acids, ketone

bodies, fluid balance markers, and inflammatory markers) in fasting EDTA plasma. The molar concentrations of these measures are obtained from a single serum sample.

The levels of systemic metabolites in both fasting and non-fasting states result from a broad combination of genetic and lifestyle factors, such as the level of adiposity, diet, and prevalent diseases, as well as conventional cardiovascular risk factors (Soininen et al., 2015). Under normal conditions, the metabolite profile is stable and well-tracked over long time periods (Würtz et al., 2012). These metabolic measures reflecting systemic metabolism can therefore be used as markers of health and disease based on the premise that they extend our knowledge of physiological processes and disease pathogenesis, and potentially permit improved risk prediction beyond conventional risk factors (Kettunen et al., 2012; Soininen et al., 2015).

NMR spectroscopy is one of the most essential techniques in chemistry, especially for the identification and quantification of soluble compounds. In proton NMR spectroscopy, each molecule containing hydrogen atoms emits a characteristic signal whose shape is quantum mechanically distinct and whose area is proportional to the molecule's concentration. Based on these fundamental principles, NMR is the gold standard for the metabolite quantification of low-molecular-weight metabolites and lipid molecules in liquid phase. It is also well-established that NMR is naturally appropriate for detecting and quantifying lipoproteins because the physical particle structure of lipoproteins fundamentally leads to a relationship between particle size and NMR chemical shift (Soininen et al., 2015).

3.4 Serum soluble cytokines and adhesion molecules

Serum samples were employed for measuring levels of pro-inflammatory markers (TNF-α, IL-1β, and IL-6) and cellular adhesion molecules (P-Selectin, Intercellular Adhesion Molecule 1 (ICAM-1), and VCAM-1) using a Luminex ® 200 flow analyser system (Luminex Corp., Austin, TX, USA) and the panels Milliplex® MAP panels Human high sensitivity T Cell and Human Cardiovascular Disease Magnetic Bead Panel (Millipore, Billerica, MA, USA). The intra- and inter-serial coefficients of variation were <5.0% and <15.0%, respectively, for all the measurements.

Luminex® is a bead-based immunoassays that accurately measure multiple analytes in a sample. Color-coded beads are internally dyed with different proportions of red and infrared fluorophores to match a specific spectral signature or bead region. Antibodies specific to a desired analyte are coupled to a unique bead region and are incubated with sample. Then

samples are incubated with a combination of biotinylated detection antibodies and a streptavidin-phycoerythrin reporter. Beads are excited by the laser in a Luminex instrument to determine the bead region and corresponding assigned analyte. The magnitude of the streptavidin-phycoerythrin-derived signal is determined by another laser and is proportional to the amount of analyte bound.

3.5 Blood antioxidant capacity

In healthy controls and T1D patients, blood antioxidant capacity was measured with an electrochemical method using an e-BQC Lab device (Bioquochem, Asturias, Spain) according to the manufacturer's protocol. This device measures the total antioxidant capacity of blood in micro-Coulombs (μC) and distinguishes between fast- and slow-acting antioxidants.

3.6 Functional analysis

3.6.1 Leukocyte isolation

Leukocytes in the T1D and T2D cohorts were extracted using different protocols. In the T1D study, neutrophils and PBMCs were isolated using immunomagnetic depletion of non-target cells from blood collected in EDTA tubes. MACSxpress™ Whole Blood Neutrophil Isolation Kit and MACSprep™ PBMC Isolation Kit human (Miltenyi Biotec, Germany) were used, following the manufacturer's instructions, to isolate neutrophils and PBMCs, respectively. Cells were counted with a fluorescence cell counter and divided into aliquots.

In the T2D study, PBMCs and polymorphonuclear (PMNs) leukocytes were isolated using a Ficoll gradient method. First, citrated blood samples were incubated for 45 min at room temperature with 3% w/v dextran in phosphate-buffered saline solution (PBS; Sigma Aldrich, St. Louis, MO, USA) for erythrocyte sedimentation. Next, the obtained supernatant was laid over a Ficoll-Hypaque medium (GE Healthcare, Barcelona, Spain), and gradient centrifugation was performed (650g for 25 min at room temperature). After centrifugation, the PBMCs were obtained in the buffy coat and the PMNs leukocytes in the sediment. Both samples were treated with erythrocyte lysis buffer (Red Blood Cell Lysis Solution, Miltenyi Biotec, Germany) for 5 min to eliminate the remaining erythrocytes, and were then washed with HBSS (Hank`s Balance Salt Solution; Sigma Aldrich, St. Louis, MO, USA) and finally divided into aliquots.

After extraction, fresh PMNs and PBMCs were immediately used for functional analysis, and the remaining cells were stored at −80°C for subsequent gene and protein determinations.

3.6.2 Static cytometry assay

After extraction, one hundred and fifty thousand PMNs/well were seeded in 48-well plates and incubated for 30 minutes (at 37 °C) with the red mitochondrial superoxide (MitoSOX, 5µM) indicator and the nuclear staining HOECHST 33342 (1µM), both from Thermo Fisher Scientific (Waltham, MA, USA). The wells were then washed with calcium- and magnesium-free HBSS. Visualization and fluorescence measurement were carried out with a fluorescence microscope (IX81; Olympus, Hamburg, Germany) coupled with the static cytometry software ScanR (Olympus, Hamburg, Germany). Patients' cells were seeded in triplicate, and twelve images were recorded per well. Fluorescence was standardized and referred to as a percentage of control.

3.6.3 Flow cytometry assay

Whole blood (500 µL) from patients was incubated with Red Blood Cell lysis solution (Miltenyi Biotec, Germany) and centrifuged to remove the supernatant. The resulting pellet was resuspended in 200 µL of HBSS, labelled with 4 µL of allophycocyanin (APC) anti-human CD45 antibody (BD Biosciences, NJ, USA) to mark the CD45+ cells (which correspond to the leukocyte population), and diluted 1:10 with HBSS. The marked sample was then incubated with 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA, 5 µM, Cat. No. 11500146, Invitrogen, CA, USA) to measure total free radicals or with hydroethidine (HE, 5 µM, Cat. No. 10530533, Invitrogen, CA, USA) to measure $O_2^{\bullet-}$ content. Fluorescence was measured in a C6 Accuri cytometer (BD Biosciences) with a blue laser (488 nm) and FL1 filter (533/30 nm) for DCFH (Ex/Em = 492–495/517–527 nm) or FL2 filter (585/40 nm) for HE (Ex/Em = 518/606 nm). 10,000 cells were analysed in each experiment. The gating of the CD45-positive PMN subpopulation was performed with forward scatter and side scatter parameters.

3.6.4 Leukocyte-endothelium interaction assay

For this study, an *ex vivo* model was used that mimics the interaction between leukocytes and the endothelial cell layer inside the blood vessel and simulates physiological conditions. This assay employs two different cell types: a confluent human umbilical vein endothelial cell (HUVEC) monolayer and PMNs. HUVEC were harvested from fresh umbilical cords obtained from healthy donors by means of collagenase digestion. Primary cultures of HUVEC were grown over fibronectin-coated cell culture dishes (Corning, NY, USA) with complete Endothelial Cell Basal Medium-2 supplemented with Growth Medium-2 Supplement kit (both from PromoCell GmbH, Heidelberg, Germany) until they reached confluence. On the day of the experiment, a 1.2 mL suspension of freshly isolated PMNs (10^6 cells/mL, in RPMI media

supplemented with 10% v/v with fetal bovine serum (Biowest, France) was perfused over the HUVEC monolayer using a parallel-plate flow chamber (Glycotech, Rockville, MD, USA, ref. 31-001) with a 0.5 wide x 0.254 mm high flow rubber gasket at a flow rate of 0.3 mL/min.

Real-time images of the flow-exposed monolayer were recorded for 5 min. During analysis of the video, the following parameters were evaluated: 1) leukocyte rolling flux, which is the number of PMNs that roll over the HUVEC monolayer during 1 minute; 2) rolling velocity, calculated as the time in which 20 PMNs cover 200 µm; and 3) adhesion, calculated as the number of PMNs firmly adhering to the endothelium and resisting flux for at least 30 seconds.

3.6.5 Leukocyte real-time metabolic flux analysis

The mitochondrial function of PBMCs was measured in real-time with a Seahorse XFp analyzer (Agilent, MA, USA) and the XFp Cell Mito Stress Test Kit (Agilent, MA, USA), following the manufacturer's instructions. This method measures the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells. Multiple parameters can be obtained, such as basal respiration, ATP-linked respiration, maximal and reserve capacities, and non-mitochondrial respiration.

Immediately after extraction, an aliquot of 2.0 x 10^6 PBMCs was resuspended on Seahorse XF DMEM medium pH 7.4 (Agilent, MA, USA) supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose (all from Agilent) and seeded on culture miniplates (that had been pre-treated with Poly-d-Lysine 0.1 mg/mL overnight) at a density of 3.5 × 10^5 PBMCs/well in 180 µL of medium. Then, the plate was centrifuged briefly at 250g to ensure PBMCs adhesion, which was confirmed under the microscope. Test compounds were reconstituted in the same medium and loaded into the cartridge injection ports following the manufacturer's volume recommendations, such that the final concentration in each well was Oligomycin A 1.5 µM, FCCP (Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone) 1.0 µM, and Rotenone/Antimycin 1 µM. OCR measurements were normalised with respect to the number of cells.

3.7 Gene and protein expression on leukocytes

3.7.1 Real time PCR and nCounter

Real-Time PCR (qPCR)

We measured gene expression by the qPCR method using frozen PBMCs samples. RNA isolation was performed with the GeneAll Ribospin Total RNA extraction kit (GeneAll

Biotechnology, Hilden, Germany) following the manufacturer's indications. The NanoDrop 200c spectrophotometer (Life Technologies, Thermo Fisher Scientific, MA, USA) was used for RNA quantification, and purity was confirmed with the 260 nm/280 nm and 260 nm/230 nm absorbance ratios, which had to be between 1.8 and 2. cDNA was generated using 1 µg RNA from each sample and the RevertAid first-strand cDNA synthesis kit (Life Technologies, Thermo Fisher Scientific, MA, USA). Working aliquots (1:10 v/v) of the first-strand cDNA were prepared. The reaction mix was prepared with LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany), 2 µL of the working aliquots, and specific primers in a 7500 Fast RT-PCR system (Life Technologies, Carlsbad, CA, USA). The 2−ΔΔCT method was used to analyse the data. β-actin gene expression was employed as an endogenous control, and the average ΔCt of the control group was used to calculate the ΔΔCt values for every sample. The specific sequence, accession number of each primer, and qPCR reaction details are shown in Table 1. qRT-PCR reactions were performed as follows: 10 min at 95 °C, 40 cycles (designed in one step) at 95 °C for 10 s, and one cycle at 60 °C for 30 s, as well as a melting curve stage.

Gene Name	**Primer Name**	**Primer Sequence**	**Sequence Accession Number**
Superoxide dismutase 1	*SOD1* Forward	GGTGTGGCCGATGTGTCTAT	NM_000454
	SOD1 Reverse	TTCCACCTTTGCCCAAGTCA	
Glutathione peroxidase 1	*GPX1* Forward	TTGAGAAGTTCCTGGTGGGC	NM_000581.4
	GPX1 Reverse	CGATGTCAGGCTCGATGTCA	
RELA proto-oncogene, NF-κB subunit	*NFκB p65* Forward	ATCCCATCTTTGACAATCGTGC	NM_021975
	NFκB p65 Reverse	CTGGTCCCGTGAAATACACCTC	
Actin beta	*Actin B* Forward	CCTCGCCTTTGCCGATCC	NM_001101
	Actin B Reverse	CGCGGCGATATCATCATCC	

Table 1: Primer sequences used in qPCR assays.

NanoString nCounter

In addition, we examined the transcriptomic data from the FishMeal study as part of this thesis. The experiments in question were performed by the University of Oslo using the nCounter system (NanoString Technologies, WA, USA). The nCounter is a high throughput, multiplex nucleic acid hybridization technology which uses optical barcodes that hybridise to each target oligonucleotide to enable digital counting of individual RNA molecules without any

enzymatic steps (Goytain & Ng, 2020). This was used with the nCounter Metabolic Pathways Panel (NanoString Technologies, WA, USA, https://nanostring.com/support-documents/metabolic-pathways-panel-gene-list/), which contains code sets covering 768 mRNAs annotated into different metabolic pathways (including 20 reference genes and positive and negative controls). Moreover, the panel was customised with 30 additional code sets covering immune and lipid metabolism-related mRNAs (Table 2). The protocol was performed according to the manufacturer's instructions for nCounter Panel-Plus with an RNA input of 75 ng per sample (PBMCs).

Official Full Name	Official Symbol	Accession
ATP binding cassette subfamily A member 1	ABCA1	NM_005502.3:4048
ATP binding cassette subfamily G member 1	ABCG1	NM_207174.1:943
aryl hydrocarbon receptor	AHR	NM_001621.3:1900
alkB homolog 3, alpha-ketoglutarate dependent dioxygenase	ALKBH3	NM_139178.3:690
apolipoprotein B receptor	APOBR	NM_018690.2:2962
aryl hydrocarbon receptor nuclear translocator	ARNT	NM_178426.1:2235
CAP-Gly domain containing linker protein 1	CLIP1	NM_002956.2:1775
CAP-Gly domain containing linker protein family member 4	CLIP4	NM_024692.4:1708
cytochrome P450 family 2 subfamily B member 6	CYP2B6	NM_000767.4:573
cytochrome P450 family 3 subfamily A member 4	CYP3A4	NM_017460.3:2151
3-hydroxy-3-methylglutaryl-CoA reductase	HMGCR	NM_000859.2:555
insulin like growth factor 1	IGF1	NM_000618.3:491
insulin like growth factor 1 receptor	IGF1R	NM_000875.4:4580
insulin induced gene 1	INSIG1	NM_005542.3:1120
lecitin-cholesterol acyltransferase	LCAT	NM_000229.1:845
low density lipoprotein receptor	LDLR	NM_000527.2:4625
mono-ADP ribosylhydrolase 1	MACROD1	NM_014067.3:1
mono-ADP ribosylhydrolase 2	MACROD2	NM_080676.5:4065
nei like DNA glycosylase 3	NEIL3	NM_018248.2:842
nuclear receptor subfamily 1 group H member 3	NR1H3	NM_005693.2:1575
nuclear receptor subfamily 1 group H member 4	NR1H4	NM_001206977.1:984
poly (ADP-ribose) polymerase 1	PARP1	NM_001618.3:3016
perilipin 2	PLIN2	NM_001122.3:418
Proopiomelanocortin	POMC	NM_000939.2:1092
peroxisome proliferator activated receptor alpha	PPARA	NM_001001928.2:266
peroxisome proliferator activated receptor delta	PPARD	NM_006238.4:895
sirtuin 1	SIRT1	NM_012238.4:840
telomerase associated protein 1	TEP1	NM_007110.3:3115
TCDD inducible poly(ADP-ribose) polymerase	TIPARP	NM_015508.3:835
vitamin D receptor	VDR	NM_000376.2:983

Table 2: Additional code-sets covering immune and inflammation response and lipid metabolism-related mRNAs.

3.7.2 Western blot

The relative protein expression of inflammatory, oxidative stress, and autophagy markers in PBMCs was assessed by Western blot. Protein was extracted from PBMCs previously stored at

-80 °C. First, PBMCs were incubated with lysis buffer (400 mM NaCl, 20 mM HEPES pH 7.5, 0.1 mM EDTA, 20% glycerol, 10 µM Na_2MoO_4, and 0.5% Nonidet P-40) containing protease inhibitors (10 mM β-glycerolphosphate, 10 mM NaF, 10 mM PNP, and 1 mM Na_3VO_4) and 1 mM dithiothreitol or RIPA buffer (Millipore, Billerica, MA, USA) for 15 minutes on ice before being centrifuged at 2100 g for 15 minutes at 4 °C. The supernatant containing the protein was collected in clean tubes, and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, IL, USA).

Protein samples (25 µg) were resolved by polyacrylamide gel electrophoresis (Novex™ WedgeWell™, Invitrogen, CA, USA) and then transferred to nitrocellulose membranes (BioRad, CA, USA) by a wet transfer method. The nitrocellulose membranes were blocked with the corresponding solution (Table 3) for 1 h and then incubated with primary antibodies overnight at 4 °C (Table 3). The next day, membranes were washed three times with Tris-buffered saline with 0.1% Tween® and incubated with the corresponding secondary Horseradish Peroxidase (HRP)-conjugated antibodies for 1 h at room temperature. Chemiluminescent signals were developed by adding SuperSignal West Pico PLUS or SuperSignal West Femto (Thermo Fisher Scientific, Waltham, MA, USA) to the membranes, and signals were detected with a Fusion FX5 acquisition system (Vilbert Lourmat, Marne La Vallée, France). Densitometry analysis was performed with Bio1D software (Vilbert Lourmat, Marne La Vallée, France). In the same sample, protein bands were normalised to β-actin expression.

Primary antibodies						
Targets	**Clonality**	**Brand**	**Reference**	**Dilution**	**Host**	**Blocking buffer**
AMPK (-P)	Monoclonal	Abcam	Ab133448	1:1000	Rabbit	BSA 5%
Beclin 1	Monoclonal	Abcam	Ab207612	1:1000	Rabbit	Milk 5%
LC3	Polyclonal	Cell Signaling	4108S	1:1000	Rabbit	BSA 5%
NF-κB p65 (-P)	Polyclonal	Abcam	Ab28856	1:1000	Rabbit	BSA 5%
SQSTM/P62	Monoclonal	Abnova	H00008878 -M01	1:1000	Mouse	Milk 5%
β-Actin	Polyclonal	Sigma-Aldrich	A5060	1:2000	Rabbit	Milk 5%
Secondary antibodies						
Target	**Clonality**	**Brand**	**Reference**	**Dilution**	**Host**	**Blocking buffer**
Rabbit	Polyclonal	Vector	PI-1000	1:2000	Goat	Same as primary Ab.
Mouse	Polyclonal	Thermo-Fisher	31420	1:2000	Goat	Same as primary Ab.

Table 3: Antibodies and dilutions used in Western Blot.
Tris-buffered saline with 0.1% Tween® solution was used to prepare the blocking buffer. (-P) stands for phosphorylated. Ab: Antibody, BSA: Bovine serum albumin.

3.8 Statistical analysis and bioinformatic tools

All measured variables were evaluated for normality with the Shapiro-Wilk test. Normally distributed variables are reported as mean ± standard deviation and non-normally distributed variables as median and interquartile range.

Comparisons between two groups were evaluated with a t-test for normally distributed variables or with a Mann-Whitney test for non-normal data. Comparisons between the three groups were assessed with a one-way ANOVA followed by a Tukey or Dunnett multiple comparisons test for normal variables, Kruskal-Wallis test followed by a Mann-Whitney U test with multiple comparisons correction for non-normal variables, or the Friedman test followed by Dunn's multiple comparisons test for non-normal variables with repeated measures. Correlations were calculated with Spearman's correlation coefficient. LOESS regression was also applied. The level of significance was set to $\alpha = 0.05$.

R version 4.1.2, the RStudio IDE (www.rstudio.com), the Tidyverse package (Wickham et al., 2019), and GraphPad Prism version 8.02 (GraphPad Software, www.graphpad.com) were used for data management, statistical analysis, and visualization.

The metabolic profile characterization (metabolomics and transcriptomics) of the individuals at increased risk of developing diabetes required additional steps. Subjects were divided into three groups (tertiles) based on their insulin incremental area under the curve (iAUC) levels after a 2-hour OGTT. The insulin iAUC was calculated using two insulin measurements (fasting and 2-hour insulin serum concentration) and the trapezoid method. Individuals in the lowest insulin iAUC tertile (below 33.3%) were assigned to the low insulin iAUC group (n = 24), while those in the highest tertile (above 66.6%) were assigned to the high insulin iAUC group (n = 24).

First, we compared the metabolic profiles of the low vs. high insulin iAUC groups before the intervention (at baseline). Then we analysed changes in the metabolic profile after the intervention, and we compared these changes in the same insulin iAUC groups that received the salmon protein supplementation vs. those that received the placebo.

Transcriptomic and metabolomic data was analysed using the R/Bioconductor software package Limma (M. E. Ritchie et al., 2015), which contains tools for data pre-processing, linear modelling, and gene set testing. Linear models were adjusted for age, sex, and BMI. In addition, transcriptomic analysis was corrected by smoking status.

The data were pre-processed to optimize downstream modelling. Metabolite concentrations were log-transformed (log1p), centred, and scaled. Centring and scaling were done to make metabolites directly comparable in the same downstream analysis and forest plot visualization. For the intervention analysis, metabolite concentrations were centred and scaled independently before and after the intervention, and the difference was then calculated. Gene expression data were log-transformed (log2).

To identify patterns in gene expression that differed between the low and high insulin iAUC groups and were further affected by the salmon protein intervention, we performed gene set tests. A gene set test is a differential expression analysis in which a p-value is assigned to a set of genes as a unit (Wu & Smyth, 2012). We performed gene set enrichment and a competitive gene set test called Camera.

Gene set enrichment consisted of testing if the obtained list of genes overrepresented Gene Ontology (GO) terms (Ashburner et al., 2000; Gene Ontology Consortium, 2021) or KEGG

pathways (Kanehisa & Goto, 2000) more than expected by chance. For this analysis, we used a list of differentially expressed genes with p-values< 0.05 (without false discovery rate correction) obtained from lineal modelling.

The competitive gene set testing allowed us to distinguish the most important biological processes from those that are less important (Wu & Smyth, 2012). For this analysis, the genes present in the nCounter Panel were allocated into gene sets in function of the Hallmark gene sets from the Molecular Signatures Database (MSigDB) (Liberzon et al., 2015). The Hallmark gene sets summarize and represent specific biological states or processes and display coherent expression. Because the Camera method also considers fold changes, we were able to interpret if the biological pathways were up- or downregulated.

4 RESULTS AND DISCUSSION

The ongoing epidemic of diabetes is an important health problem, as it is associated with important rates of mortality and morbidity. It involves several pathophysiological processes occurring in different tissues that, together, contribute to the development of hyperglycaemia. The degree of hyperglycaemia (if present) can change over time and reflects the extent and magnitude of the underlying disease process, rather than the nature of the process (such as β-cell loss or development of insulin resistance). The disease process can be modulated with lifestyle interventions (like exercise, weight reduction or/and diet) and with pharmacological treatment, which, in the case of individuals at-risk of T2D, can effectively delay or prevent the development of the disease.

Diabetes, and even increased glucose levels below the diabetes diagnosis threshold, are risk factors for developing CVD. This is explained by the "hyperglycaemia continuum" between prediabetes, diabetes, and CVD. There is a large body of evidence that supports the idea that hyperglycaemia-induced overproduction of mitochondrial ROS activates cellular pathways that contribute to the development of CVDs.

In the present thesis, we examined molecular and cellular pathways related to the progression of diabetes and the development of CVD across the hyperglycaemia continuum, from individuals at increased risk of diabetes to patients with a diagnosis of T1D or T2D. Furthermore, we explored whether any of these pathways could be modulated by dietary intervention or empagliflozin treatment. During the course of the project, we evaluated three cohorts of patients: a cohort composed of individuals with increased risk of T2D that participated in a recent RCT (the FishMeal study) with salmon protein supplementation (Hustad et al., 2021), conducted by the University of Oslo; a T1D cohort; and a T2D cohort that were initiated with empagliflozin treatment. These three groups were age- and sex-matched with healthy controls, and recruited the T1D and T2D at the Endocrinology and Nutrition Service at University Hospital Dr. Peset, in Valencia, Spain.

The following approaches were applied to these three cohorts: 1) We characterized the plasma metabolomic and PBMC transcriptomic profiles of the population at increased risk of T2D in order to detect biomarkers that could explain the progression to diabetes and further; we explored if these biomarkers were affected by supplementation with salmon protein; 2) We measured the bioenergetic and redox state of PBMCs from T1D versus controls and assessed autophagy state and leukocyte-endothelium interactions; and 3) We evaluated the potential

cardioprotective effects of empagliflozin treatment on leukocyte-endothelium interaction, adhesion molecules, oxidative stress and inflammatory markers in T2D patients.

We analysed the concentrations of circulating biomarkers (in blood, serum, or plasma) and evaluated molecular pathways in circulating leukocytes, as leukocytes can represent systemic and cardiovascular metabolic states and play a key role in the development of atherosclerosis (Rubattu et al., 2019; Swirski & Nahrendorf, 2013).

4.1 Comprehensive characterization of the population at increased risk of diabetes

Characterization of subgroups of patients with different glycaemic regulation

The FishMeal human intervention study analysed data from 74 participants. Due to a lack of data on fasting or 2-hour insulin concentrations, two participants were excluded, resulting in a study population of 72 participants (n = 27 males and 45 females). The study population was further divided into three subgroups based on their insulin iAUC during a 2-h OGTT test (75 g glucose load). Participants in the lowest tertile (below 33.3%) were assigned to the low insulin iAUC group (n = 24), while those in the highest tertile (above 66.6%) were assigned to the high insulin iAUC group (n = 24). The low insulin and high insulin iAUC groups were compared.

There were no significant age differences between the two groups; the median age in the low insulin iAUC group was 53 years and in the high insulin iAUC group it was 61.5 years. In addition, both groups had similar BMIs, with a median BMI of 32.4 kg/m^2 in the low group and 33.0 kg/m^2 in the high insulin iAUC group, respectively. The proportion of women to men was higher in both groups (low insulin iAUC group: 71% and high insulin iAUC group: 63%). The proportion of participants that consumed tobacco daily was higher in the high insulin iAUC group (26.3%) than in the low insulin iAUC group (9.1%). As expected, we found statistical differences in parameters related to glucose metabolism and insulin sensitivity (glycaemic regulation) between these two subgroups: the low insulin iAUC group being the one with the lowest 2-h glucose, fasting insulin, 2-h insulin, and HOMA-IR and with the highest Matsuda index, indicating that the low insulin iAUC group had better glycaemic regulation.

After detecting differences in glucose regulation, we compared the metabolic profile (plasma concentration of 250 metabolites) in these two subgroups. Compared to the low insulin iAUC group, the high insulin iAUC group had higher levels of the inflammatory marker GlycA, lower levels of the amino acid glycine and the SCFA acetate, and a higher ratio of MUFA to total fatty acids (MUFA %).

In line with our results, it has been reported that GlycA is associated with insulin resistance, BMI, metabolic syndrome markers, and the leptin-to-adiponectin ratio (Dullaart et al., 2015; Lorenzo et al., 2017). GlycA is also linked to several inflammatory markers, such as hs-CRP, and is believed to be a biomarker of systemic inflammation and subclinical vascular inflammation (Lawler & Mora, 2016). GlycA has therefore been proposed as a new biomarker of cardiometabolic disease and T2D risk (Connelly et al., 2016; Gruppen et al., 2015). Even though the high insulin iAUC group displayed higher GlycA levels than the low insulin iAUC group, there was no significant difference in hs-CRP between the two groups. The high insulin iAUC group showed a tendency towards a higher hs-CRP concentration, and possibly did not reach statistical significance due to a small sample size, intra-individual variations in hs-CRP, or because hs-CRP did not capture the whole inflammatory process in our participants. Despite GlycA and hs-CRP similarities in disease associations, they probably capture distinct aspects of the inflammatory response. This suggests that GlycA is a more precise risk marker of cardiovascular disease and T2D than hs-CRP (Connelly et al., 2017). Epidemiological studies supporting this hypothesis found that the addition of hs-CRP to the regression model slightly attenuated the association between GlycA and cardiovascular events (Duprez et al., 2016) or T2D (Akinkuolie et al., 2015).

Similar to our findings of reduced plasma glycine levels in the high insulin iAUC, it has been shown in a systematic review that circulating glycine levels are inversely associated with T2D (Guasch-Ferré et al., 2016). A study that measured insulin sensitivity (with the hyperinsulinemic-euglycemic clamp, which is the gold standard) in relationship with circulating amino acids among insulin-sensitive, insulin-resistant, and T2D individuals found that glycine had the strongest positive association with insulin sensitivity, measured as glucose disposition rate (Thalacker-Mercer et al., 2014).

It is not known if lower levels of glycine play an active role in the development of T2D, but interventions that delay or reverse T2D (bariatric surgery or physical activity) increase circulating glycine concentrations (Glynn et al., 2015; Tulipani et al., 2016; Yan-Do & MacDonald, 2017). Moreover, supplementation with glycine has been shown to improve insulin response and glucose tolerance (Gannon et al., 2002; González-Ortiz et al., 2001). There are numerous metabolic effects of circulating glycine, and supplementation with glycine may influence glucose tolerance (Yan-Do & MacDonald, 2017). Current evidence points to a glycine effect in the brain via dorsal vagal complex N-methyl-D-aspartate receptors, systemically reducing oxidative stress and the inflammatory response, and increasing insulin secretion in the islets (Yan-Do & MacDonald, 2017).

Continuing with the plasma amino acids profile, we observed that the high insulin iAUC group tended to have a higher BCAAs plasma concentration than the low insulin iAUC group, despite not reaching statistical significance. Several studies have found a correlation between elevated blood levels of BCAA, prediabetes and T2D (Guasch-Ferré et al., 2016). A study that compared the metabolomic profiles of obese and lean humans revealed a signature of BCAA-related metabolites that is indicative of increased catabolism of BCAA and correlated with insulin resistance (Newgard et al., 2009). Increased levels of BCAA may be a causal factor for developing insulin resistance and T2D, by hampering insulin signalling pathways (Vanweert et al., 2022), and by the accumulation of toxic BCAA metabolites that trigger mitochondrial dysfunction (Lynch & Adams, 2014).

So far, we observed that the high insulin iAUC group had reduced glycine and a tendency towards increased BCAAs. In line with these results, a study in a cohort from the Insulin Resistance Atherosclerosis Study (IRAS) that compared individuals with high and low levels of insulin sensitivity revealed a decrease in glycine and increases in valine and isoleucine (both BCAAs) in the insulin-resistant subjects. This pattern was associated with the development of T2D and remained significant after adjusting for β-cell function, highlighting the association between this metabolic profile and insulin resistance (Palmer et al., 2015).

In terms of fatty acid profile, higher plasma concentrations of saturated fatty acids (SFA) and MUFA were linked to higher HbA1c and fasting glucose levels in the NHANES study (Rhee et al., 2011). This mirrors what we observed in the high insulin iAUC group, where the ratio of MUFA to total fatty acids was higher. Moreover, the FINNRISK study showed that higher levels of MUFA and lower levels of PUFA and linolic acid are linked to a higher risk of heart disease (Würtz et al., 2015). Thus, high circulating MUFA and low PUFA levels are linked to increased cardiovascular risk.

There were no differences in total cholesterol levels, lipid composition of lipoproteins, or LP-IR score between the groups with low and high insulin iAUC. Despite this, we observed a trend that was consistent with a higher LP-IR score in the high insulin iAUC group compared to the low insulin iAUC group: higher levels of large VLDL particles and lower levels of large HDL particles.

Regarding the gene expression analysis, *CPT1A* was the most differentially expressed gene (in terms of p-value) and was upregulated in the high insulin iAUC group compared to the low insulin iAUC group. The CPT1A protein is a key regulatory enzyme of β-oxidation located in the outer membrane of the mitochondria, and it facilitates the translocation of long-chain fatty

acids across the mitochondrial membrane for fatty acid β-oxidation. Similarly, the Framingham Heart Study (FHS) Offspring Cohort found a positive association between mRNA expression of *CPT1A* in whole blood and fasting glucose and triglycerides, as well as BMI (Lai et al., 2020).

In addition, it was reported that fat intake was negatively correlated with CPT1A methylation, which led to a higher level of *CPT1A* gene expression (Lai et al., 2020). In an animal study, mitochondrial dysfunction caused by a high-fat diet was linked to insulin resistance in muscle, implying that excessive CPT1A activity overloads the mitochondria, resulting in incomplete oxidation of long-chain fatty acids (Koves et al., 2008).

In the gene set analysis, we found a down-regulation of the gene set Myc targets V1 and V2 in the high insulin iAUC group and hypothesised that this was indicative of decreased mitochondrial biogenesis, given that Myc induces mitochondrial biogenesis and increases mitochondrial function via multiple pathways (Rosselot et al., 2021). Mitochondrial dysfunction has been observed in multiple tissues in humans with obesity, T2D, and metabolic syndrome (Patti & Corvera, 2010). Altered mitochondrial function contributes to the development of insulin resistance in several ways. For example, in adipose tissue, mitochondrial dysfunction is associated with impaired secretion of adiponectin, a strong insulin-sensitizing adipokine (C.-H. Wang et al., 2013). In other tissues, mitochondrial dysfunction increases ROS production, which contributes to insulin resistance, as it activates redox-sensitive kinases that phosphorylate IRS proteins. It is unclear whether mitochondrial dysfunction is the cause or result of insulin resistance (DeFronzo et al., 2015). In the context of CVD research, it has been demonstrated that PBMCs from T2D patients show signs of mitochondrial dysfunction and increased ROS production (Hernandez-Mijares et al., 2013).

As mentioned at the beginning of this section, our high insulin iAUC group had lower levels of plasma acetate compared to the low insulin iAUC group. Acetate is a SCFA with a similar structure to ketone bodies. It can be generated in the final step of fatty acid β-oxidation in the liver during fasting conditions, but it is also produced in the intestine by the microbiota (Moffett et al., 2020). The microbiome may be a source of biologically active metabolites, which are associated with diet composition (Chen & Gerszten, 2020). Prebiotics have been shown to increase plasma GLP1 and peptide YY concentrations in healthy subjects, while they decrease postprandial plasma glucose response after a standardised meal (Cani et al., 2009). Another RCT found that long-chain MUFA derived from fish oil increased GLP1 secretion in healthy people. The mechanism thought to be responsible is associated with the production of

SCFAs by gut microbiota, which act via G protein-coupled receptors expressed on enteroendocrine cells, enteric neurons, and enteric leukocytes (Tsutsumi et al., 2021).

Effect of the intervention in the patient subgroups

We aimed to evaluate whether the 8-week intervention with salmon protein had influenced glycaemic regulation, metabolomic, and transcriptomic profiles in the low and high insulin iAUC groups. Patients in both groups were assigned the salmon protein or placebo. We measured the same clinical variables, metabolites, and genes in both subgroups after the intervention. We assessed the effect of the intervention regarding changes in metabolite concentration and gene expression before and after the intervention and compared the parameters with those of individuals in the same iAUC group that received a placebo.

We found no effect of the intervention on glycaemic regulation markers such as glucose, insulin, HOMA-IR, and HbA1c after the intervention, as there were no differences between individuals receiving a placebo or fish protein in the low insulin iAUC group or the high insulin iAUC group. However, plasma acetate concentrations increased in the low insulin iAUC group after salmon protein supplementation compared to the placebo group, an effect that was not observed in the high insulin iAUC group. We hypothesised that this could have been due to microbiota composition; indeed, recent research suggests that gut microbial composition can identify individuals who may benefit from dietary interventions (Kovatcheva-Datchary et al., 2015).

With the exception of acetate, the intervention did not change the levels of circulating metabolites. We have previously discussed that glycine supplementation may have an impact on glucose tolerance. For example, it was confirmed in a RCT with healthy individuals that one dose of 5.2 g of salmon protein (equivalent to 7.5 g of fishmeal) was enough to significantly increase the post-prandial concentration of several amino acids, mainly glycine, and resulted in a small but statistically significant reduction in serum glucose and triglycerides after 30–60 min (Hjorth et al., 2022). However, in our 8-week intervention with individuals with an increased risk of T2D, we found no effect of fish protein on glucose metabolism, insulin response, or plasma glycine. Our fishmeal supplement contained 389 mg of glycine per day, which was significantly below the dosages used in other studies; namely, 1 mmol of glycine per kg of lean body mass (approximately 5.6 g for a 75 kg patient) (Gannon et al., 2002) and 5 g of glycine (González-Ortiz et al., 2001).

Most dietary interventions do not cause considerable changes in gene expression, and in our study, we detected only a small number of genes that were expressed differently after the intervention; hence, we did not perform any gene set tests.

Limitations of the study

This study has several strengths and limitations. One strength is the detailed profiling of both metabolites and gene expression in subgroups of people with increased risk of T2D. An important limitation of this study, on the other hand, is the small number of participants in the phenotypic subgroups, especially with respect to the analysis of the intervention data, which increases the risk of both false positive and false negative findings. Indeed, the findings of small studies cannot be extrapolated to the general population. The differences that we observed in metabolites and genes were not statistically significant after adjusting for a false discovery rate, both in the baseline characterization and after the intervention analysis; thus, our results should be interpreted carefully. Furthermore, an inclusion criterion for participation was an increased risk of T2D, in which case there is a lack of a healthy control group. Finally, the current study's short duration does not reflect the possible long-term effects of the intervention in question. Nevertheless, despite this, we were able to identify metabolic biomarkers (GlycA, MUFA %, glycine, and acetate) and candidate gene expression patterns in the group of participants with high insulin iAUC.

4.2 Redox state, mitochondrial function, and autophagy status of T1D leukocytes and their implications in atherosclerosis

Anthropometrical parameters and biochemical determinations

This cohort was composed of forty-four T1D patients and fifty-two age- and sex-matched healthy controls. The mean age was 43.6 years in the T1D group, with a mean duration of diabetes of 15.7 years, while the mean age in the control group was 40.4 years. There was no difference in gender distribution between the two groups, with 55% and 62% of women to men in the T1D and control groups, respectively. There were no differences in weight, waist circumference, BMI, or blood pressure between groups.

The T1D group had higher fasting glucose concentrations and HbA1c than controls, as expected. T1D patients had lower total and LDL-C levels, which may have been attributable to the lipid-lowering medication that 57% of them were taking.

Redox state

Hyperglycaemia promotes the production of ROS by mitochondrial and non-mitochondrial sources, which is linked to the development of cardiovascular complications (Giacco & Brownlee, 2010). We observed that leukocytes from T1D patients produced more total ROS and $O_2^{\cdot-}$ than controls. Furthermore, we assessed blood antioxidant capacity as a measure of systemic oxidative stress and found that T1D patients had lower levels of both fast-acting and total antioxidants. In line with these results, it was previously reported that monocytes from T1D patients without macrovascular disease release higher amounts of $O_2^{\cdot-}$ compared to controls (Devaraj et al., 2006). In addition, Devaraj et al. observed that T1D monocytes produce more IL-1β and IL-6 when activated, in addition to increased concentrations of soluble ICAM1.

The decreased blood antioxidant capacity in T1D patients may reflect an increased utilisation of circulating antioxidants due to a higher production of ROS. Similar to our findings, a study that measured serum antioxidant capacity in T1D patients, their siblings without diabetes, and unrelated controls found that total antioxidant capacity was lower in individuals with T1D than both related and unrelated controls. Moreover, both T1D individuals and their siblings had lower GPX, but increased SOD concentrations compared to unrelated controls. There were no differences in dietary patterns among the three groups, suggesting that the differences in the total antioxidant capacity reflected an enhanced utilisation due to increased production of free radicals rather than lower antioxidant intake (Neyestani et al., 2012).

Mitochondrial function

Chronic hyperglycaemia results in an overloading of the mitochondrial electron transport chain, which increases ROS production and undermines mitochondrial function. Analysis of oxygen consumption rate during the Mito Stress test in T1D vs. control subjects revealed similar basal and ATP-linked respiration in both groups. However, leukocytes from T1D patients displayed a tendency toward a reduced maximal respiration rate (non-significant) and reduced spare respiratory capacity with respect to controls.

Spare capacity is an estimate of mitochondrial health and flexibility, which indicate the cells' ability to change their bioenergetic programme and adapt to pathological stress. Inadequate spare respiratory capacity is associated with cardiovascular and neurological chronic diseases (Marchetti et al., 2020) and with the development of diabetic complications (Czajka et al., 2015; P. Li et al., 2015). In a study to determine if mitochondrial dysfunction plays a role in diabetic nephropathy, Czajka et al. found a similar pattern: while they found comparable basal and ATP-linked respiration in PBMCs from diabetic patients with and without nephropathy,

those from the former group displayed metabolic inflexibility, which was evident in their reduced maximal and spare respiratory capacities (Czajka et al., 2015).

Even though patients in our T1D group did not have nephropathy, we observed a similar bioenergetic maladaptation among them. Regarding this discrepancy with the Czajka et al. study, their nephropathy group included a large proportion of T2D patients, and so additional factors could explain the mitochondrial impairment they observed, such as insulin resistance.

Autophagy status

Together with mitochondrial dysfunction and excessive ROS, we have observed a classic pattern of autophagy activation in T1D leukocytes involving increased levels of P-AMPK and Beclin-1, both markers of autophagy induction; decreased amounts of the cargo proteins P62 and NBR1; and enhanced conversion of LC3-I to LC3-II (that is, an increased LC3-II/I ratio), indicative of autophagosome maturation.

AMPK has been pointed to as a protein marker of autophagy induction upon mitochondrial ROS production (Filomeni et al., 2015), as it becomes activated upon exposure to H_2O_2, a process that occurs through oxidative modifications such as S-glutathionylation of the AMPKα and AMPKβ subunits (Zmijewski et al., 2010). Atg4 can also be modified by H_2O_2, inactivating its hydrolysing activity towards LC3, allowing accumulation of the pro-autophagic LC3-II isoform (Scherz-Shouval et al., 2007).

Given that autophagy is an important mechanism by which cellular homeostasis is restored, we analysed the expression of autophagy markers in function of glycaemic control. We observed a positive correlation between HbA1c (%) and the LC3-II/I ratio and a tendency for this ratio to increase when HbA1c exceeded 5.7% (the threshold for normal values), thereby supporting an association between increased autophagy and poor glycaemic control and suggesting that it is an indication of excessive cellular stress due to chronic hyperglycaemia.

Inflammation

Adhesion of leukocytes to the endothelium is a hallmark of the inflammatory process at the onset of atherosclerosis. We simulated interactions between leukocytes and the endothelium using an *ex vivo* model based on a parallel flow chamber. Leukocytes from T1D patients interacted more with the endothelium than those from control subjects, since T1D leukocytes had lower rolling velocities, higher rolling flux (the number of leukocytes that interact with the endothelium), and higher leukocyte adhesion (the number of leukocytes that attach to the endothelium and remain stationary).

This finding is consistent with a prior study carried out by our group in a different T1D cohort, which showed increased leukocyte-endothelium interactions in diabetics (Iannantuoni et al., 2020). At the same time, Iannantuoni et al. and Devaraj et al. reported that T1D patients displayed higher levels of circulating cellular adhesion molecules (such as ICAM1) and inflammatory markers than controls. This could be the reason why leukocytes are attracted to the endothelial walls in diabetes.

Providing further evidence of this proinflammatory state, Bradshaw et al. demonstrated that monocytes isolated directly from the blood of T1D patients spontaneously secrete more pro-inflammatory cytokines, such as IL-1β and IL-6, than healthy controls (Bradshaw et al., 2009). Serum levels of monocyte-derived cytokines are known to be elevated in diabetic patients. At the onset of clinical disease and in long-standing diabetic subjects, levels of serum TNF-α, IL-1α, IL-1β and IL-6 are higher in T1D patients than in controls (Bradshaw et al., 2009).

Cellular adhesion molecules play an important role in tethering circulating leukocytes to the vascular endothelium at sites of inflammation and mediating leukocyte transmigration from blood vessels into adjacent inflamed tissues. In the absence of signals that stimulate the expression of adhesion molecules, the adhesive forces between leukocytes and the vascular endothelium are below the level necessary to tether leukocytes. Several cytokines, including TNF-α and IL-1β, considerably boost the expression of many adhesion molecules, increasing the adhesiveness of leukocytes to the endothelium (Meager, 1999).

Crosstalk between oxidative stress, mitochondrial function, inflammation, and autophagy

There is a crosstalk between increased ROS production, mitochondrial dysfunction, inflammation, and autophagy. The activation of the JNK (c-JUN N-terminal kinase) pathway and inhibition of the Akt pathway by TNF-α are the mechanisms by which ROS production induces upregulation of autophagy (Jia et al., 2006; Menikdiwela et al., 2020). Furthermore, animal studies have shown that the proinflammatory transcription factor NF-κB is involved in the induction of autophagy in response to ROS (Menikdiwela et al., 2020; Zeng et al., 2013).

In the context of atherosclerosis, there are several factors that can stimulate autophagy in endothelial cells, SMC, and leukocytes, such as oxidative stress, inflammation, and metabolic stress (hypoxia combined with nutrient deprivation). For example, infiltrated T lymphocytes and monocytes in the arterial intima at an early stage of plaque formation release proinflammatory cytokines such as IFNγ and TNF-α, which can activate autophagy in macrophages and non-immune cells (Martinet & De Meyer, 2009). In fact, TNFα stimulates the

expression of LC3 mRNA and Beclin 1 protein in SMC isolated from human atherosclerotic plaques (Jia et al., 2006).

4.3 Potential molecular mechanisms underlying empagliflozin`s cardioprotective effect.

Anthropometrical parameters and biochemical determinations

This research began with eighteen T2D patients who initiated treatment with 10 mg empagliflozin per day, according to normal clinical practice (Schernthaner et al., 2016). Before being recruited for the study, all patients had been on stable glucose-lowering therapy for at least 12 months, and they continued this therapy in combination with empagliflozin for 24 weeks. Subjects were asked to follow a scheduled visit programme that included follow-up at 12 and 24 weeks after the first visit. Two subjects were excluded from the study due to a lack of adherence to the empagliflozin treatment, resulting in a final study sample of sixteen participants. With respect to concomitant medication taken by the study participants; 81.3% were on lipid-lowering medication, 50% were on antihypertensive drugs, 31.3% were on antithrombotic drugs, and 37.5% were on diuretics. In terms of clinical history, four patients were affected by retinopathy and five by nephropathy.

The mean BMI of the participants at the start of the study was 31.4 kg/m^2, the mean age was 59.7 years, and the proportion of women to men was 31.3%. We observed a progressive reduction in body weight, BMI, and waist circumference at 12 and 24 weeks of treatment. Empagliflozin treatment improved glycaemic control, as fasting glucose and HbA1c levels decreased from 12 weeks onward. Patients receiving insulin as part of their treatment were excluded from HOMA-IR and insulin assessments, but empagliflozin treatment did not affect these parameters. There were no differences in triglycerides and hs-CRP concentrations after 24 weeks.

Total cholesterol levels increased at 12 weeks and remained higher than baseline until the end of the study; however, there were no changes in LDL-C or HDL-C. A previous RCT demonstrated that 24 weeks of empagliflozin treatment produced small increases with respect to baseline in total cholesterol, HDL-C, and LDL-C compared to the placebo (Häring et al., 2014). It is possible that we did not observe these changes in HDL-C and LDL-C due to our smaller study population. The mechanism by which iSGLT2 raises cholesterol levels remains unknown. It has been proposed that the rises in HDL-C and LDL-C are due in part to haemoconcentration, because iSGLT2 causes volume contraction in response to increased urinary volume (Kohler

et al., 2017; Pieber et al., 2015). The clinical significance of minimal changes in lipids (that is, minor increases in LDL-C, HDL-C, and non HDL-C levels and inconsistent changes in plasma triglyceride levels) brought on by iSGLT2 treatment is unclear (Scheen, 2020).

Leukocyte-endothelium interactions

We evaluated the interactions between patients' leukocytes and endothelial cells at baseline and 12 and 24 weeks after initiation of empagliflozin treatment, using the same *ex vivo* model as in the T1D cohort. At week 24, empagliflozin treatment reduced leukocyte-endothelial interactions, as the patients' leukocytes exhibited increased rolling velocity, decreased rolling flux, and adhesion compared to baseline. These findings suggest that empagliflozin exerts a beneficial effect by protecting against the early stages of the atherosclerotic process.

These interactions are mediated by the levels of adhesion molecules resulting from vascular inflammation and dysfunction, and are involved in the recruitment of immune cells and platelets to the endothelium. In consonance, we observed lower levels of the adhesion molecules P-selectin and ICAM1 after 24 weeks of treatment.

Pleiotropic effects of iSGLT2 that may improve cardiovascular outcomes includes positive effects on endothelial function and inflammation (Scheen, 2020). In diabetic murine models in vivo, empagliflozin treatment has been shown to reduce glucotoxicity and thus prevent the development of endothelial dysfunction, as well as reducing oxidative stress and exerting anti-inflammatory effects. In the same study, P-selectin and ICAM1 mRNA expression was upregulated in the aorta of diabetic rats compared to controls, and it was observed that empagliflozin treatment reduced and normalized P-selectin and ICAM1 expression in diabetic rats (Steven et al., 2017). It has also been demonstrated that empagliflozin improves endothelial and cardiomyocyte function in human heart failure with preserved ejection fraction, effects that were related to reduced pro-inflammatory (ICAM-1, VCAM-1, TNF-α, and IL-6) and oxidative pathways (Kolijn et al., 2021).

Oxidative stress

Hyperglycaemia is a key factor in the atherosclerosis process and is related to mitochondrial impairment and oxidative stress (Shah & Brownlee, 2016) and enhanced leukocyte–endothelium interactions (de Marañón et al., 2021). Our data confirms the positive effect of empagliflozin treatment on glycaemia; in consonance, we observed that mitochondrial $O_2^{\cdot-}$ production in leukocytes was reduced at 24 weeks of treatment. In addition, we found that *SOD1* mRNA expression increased at 24 weeks, while there were no changes in *GPX* mRNA.

These results are in accordance with a previous study by our group that showed how empagliflozin treatment promotes an antioxidant response in leukocytes, as patients' leukocytes displayed an increased GSH content and higher *GSR* and Catalase mRNA expression after treatment (Iannantuoni et al., 2019).

A recent *in vitro* study with human endothelial cells demonstrated that empagliflozin treatment reduced mitochondrial Ca^{2+} overload and ROS production triggered by high glucose levels, as well as improving cell viability in response to oxidative stress (Mone et al., 2022). Moreover, a study in a diabetic mouse model reported that 8 weeks of empagliflozin treatment improved diabetic myocardial structure and function, decreased myocardial oxidative stress, and ameliorated myocardial fibrosis through inhibition of the TGF-β (transforming growth factor β)/Smad pathway and activation of the Nrf2/ARE (nuclear erythroid 2-related factor 2/antioxidant response element) signalling pathways (C. Li et al., 2019). Li and co-workers found that levels of the antioxidant enzymes SOD and GPX were increased in the cardiac tissue of mice treated with empagliflozin. Overall, these results suggest that empagliflozin's antioxidant effect is modulated by other cellular mechanisms in addition to its positive effect on glycaemia.

Inflammation

Increased total fat mass and visceral adiposity are linked to both insulin resistance and chronic inflammation. This chronic low-grade inflammation is characterized by a reduction in adiponectin; at the same time, adipocytes, macrophages, and immune cells secrete many adipokines, cytokines, and chemokines, including TNF-α and IL-6, in a way that promotes oxidative stress and endothelial dysfunction. In fact, TNF-α and IL-6 can stimulate the hepatic production of CRP, exacerbating inflammation and promoting endothelial dysfunction through VCAM1 (Bonnet & Scheen, 2018).

In consonance with a reduction of BMI and waist circumference at 24 weeks of empagliflozin treatment, we observed a decrease in serum concentrations of IL-6, but without changes in TNF-α and IL-1β. Our group has previously shown that empagliflozin treatment can have an anti-inflammatory effect, as it reduced serum levels of the inflammatory enzyme myeloperoxidase and increased the anti-inflammatory interleukin-10 (IL-10) (Iannantuoni et al., 2019).

A study that compared the effect of canagliflozin versus glimepiride (a sulfonylurea) on low-grade inflammation markers found that, following 52 weeks of treatment, canagliflozin reduced serum leptin by 25% and increased adiponectin by 17%, as well as reducing serum IL-6

concentrations by 22% compared to glimepiride (Garvey et al., 2018). It is important to note that the increase in adiponectin and decrease in IL-6 with canagliflozin occurred independently of changes in HbA1c, weight, or lipids (Garvey et al., 2018).

To understand the potential molecular mechanism underlying empagliflozin's anti-inflammatory effect, we measured the protein and gene expression of NF-κB in patients' leukocytes and found that both were reduced at 24 weeks of treatment compared to baseline. In accordance with our results, it was demonstrated that empagliflozin reduced the expression of inflammatory cytokines and NF-κB in the heart, liver, and kidneys in a non-diabetic mouse model treated with doxorubicin. The mice that received empagliflozin displayed improved cardiac function and reduced inflammation compared to those that only received doxorubicin (Quagliariello et al., 2021).

Overall, the present study suggests that empagliflozin treatment reduces leukocyte-endothelium interactions, oxidative stress, and inflammation, and that these mechanisms underlie the cardioprotective effect of empagliflozin.

The study has some limitations; for example, the reported effects of empagliflozin are based on observational and prospective studies with a limited number of patients. However, in its defence, the primary goal of this pilot study was to estimate average values and variability in order to plan future studies in larger populations.

5 CONCLUSIONS

1) The metabolic characterization of individuals at increased risk of T2D suggests that several plasma metabolites (GlycA, MUFA %, glycine, and acetate) serve as biomarkers to predict the progression of T2D; however, their usefulness requires further testing and validation in future prospective studies. Whether these new biomarkers are more sensitive to dietary interventions in people with an increased risk of T2D is still to be determined.

2) Inflammation and oxidative stress are key mechanisms contributing to atherosclerosis, and leukocytes play a pivotal role in this process. Our characterization of leukocytes from T1D patients reveals augmented ROS, impaired mitochondrial respiration rate, and activated autophagy. These alterations occur in parallel to an overall reduction in antioxidant defences and increased interaction between leukocytes and endothelial cells. These findings support the idea that T1D impairs mitochondrial function and promotes oxidative stress and autophagy in leukocytes, and that these mechanisms contribute to an increased risk of atherosclerosis by augmenting leukocyte-endothelial interactions.

3) Empagliflozin decreases leukocyte–endothelium interactions, adhesion molecules, mitochondrial ROS, IL-6, and NF-κB expression in T2D and enhances antioxidant activity. This highlights the value of this drug for preventing the atherosclerotic process, inflammation, and, consequently, possible cardiovascular events in T2D patients.

6 CITED LITERATURE

Abdul-Ghani, M. A., Sabbah, M., Kher, J., Minuchin, O., Vardi, P., & Raz, I. (2006). Different contributions of insulin resistance and beta-cell dysfunction in overweight Israeli Arabs with IFG and IGT. *Diabetes/Metabolism Research and Reviews*, *22*(2), 126-130. https://doi.org/10.1002/dmrr.595

Ahola-Olli, A. V., Mustelin, L., Kalimeri, M., Kettunen, J., Jokelainen, J., Auvinen, J., Puukka, K., Havulinna, A. S., Lehtimäki, T., Kähönen, M., Juonala, M., Keinänen-Kiukaanniemi, S., Salomaa, V., Perola, M., Järvelin, M.-R., Ala-Korpela, M., Raitakari, O., & Würtz, P. (2019). Circulating metabolites and the risk of type 2 diabetes: A prospective study of 11,896 young adults from four Finnish cohorts. *Diabetologia*, *62*(12), 2298-2309. https://doi.org/10.1007/s00125-019-05001-w

Akinkuolie, A. O., Pradhan, A. D., Buring, J. E., Ridker, P. M., & Mora, S. (2015). Novel Protein Glycan Side-Chain Biomarker and Risk of Incident Type 2 Diabetes Mellitus. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *35*(6), 1544-1550. https://doi.org/10.1161/ATVBAHA.115.305635

American Diabetes Association. (2013). Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*, *37*(Supplement_1), S81-S90. https://doi.org/10.2337/dc14-S081

American Diabetes Association. (2020). 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2021. *Diabetes Care*, *44*(Supplement_1), S15-S33. https://doi.org/10.2337/dc21-S002

Amir Aslani, B., & Ghobadi, S. (2016). Studies on oxidants and antioxidants with a brief glance at their relevance to the immune system. *Life Sciences*, *146*, 163-173. https://doi.org/10.1016/j.lfs.2016.01.014

Arkan, M. C., Hevener, A. L., Greten, F. R., Maeda, S., Li, Z.-W., Long, J. M., Wynshaw-Boris, A., Poli, G., Olefsky, J., & Karin, M. (2005). IKK-beta links inflammation to obesity-induced insulin resistance. *Nature Medicine*, *11*(2), 191-198. https://doi.org/10.1038/nm1185

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., & Sherlock, G. (2000). Gene Ontology: Tool for the unification of biology. *Nature genetics*, *25*(1), 25-29. https://doi.org/10.1038/75556

Atkinson, M. A., Eisenbarth, G. S., & Michels, A. W. (2014). Type 1 diabetes. *Lancet (London, England)*, *383*(9911), 69-82. https://doi.org/10.1016/S0140-6736(13)60591-7

Avila, C., Huang, R. J., Stevens, M. V., Aponte, A. M., Tripodi, D., Kim, K. Y., & Sack, M. N. (2012). Platelet Mitochondrial Dysfunction is Evident in Type 2 Diabetes in Association with Modifications of Mitochondrial Anti-Oxidant Stress Proteins. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association*, *120*(4), 248-251. https://doi.org/10.1055/s-0031-1285833

Bennett, M. R., Sinha, S., & Owens, G. K. (2016). Vascular Smooth Muscle Cells in Atherosclerosis. *Circulation Research*, *118*(4), 692-702. https://doi.org/10.1161/CIRCRESAHA.115.306361

Bogun, M. M., Bundy, B. N., Goland, R. S., & Greenbaum, C. J. (2020). C-Peptide Levels in Subjects Followed Longitudinally Before and After Type 1 Diabetes Diagnosis in TrialNet. *Diabetes Care*, *43*(8), 1836-1842. https://doi.org/10.2337/dc19-2288

Bonnet, F., & Scheen, A. J. (2018). Effects of SGLT2 inhibitors on systemic and tissue low-grade inflammation: The potential contribution to diabetes complications and cardiovascular

disease. *Diabetes & Metabolism*, *44*(6), 457-464. https://doi.org/10.1016/j.diabet.2018.09.005

Bradshaw, E. M., Raddassi, K., Elyaman, W., Orban, T., Gottlieb, P. A., Kent, S. C., & Hafler, D. A. (2009). Monocytes from patients with type 1 diabetes spontaneously secrete proinflammatory cytokines inducing Th17 cells. *Journal of Immunology (Baltimore, Md.: 1950)*, *183*(7), 4432-4439. https://doi.org/10.4049/jimmunol.0900576

Cani, P. D., Lecourt, E., Dewulf, E. M., Sohet, F. M., Pachikian, B. D., Naslain, D., De Backer, F., Neyrinck, A. M., & Delzenne, N. M. (2009). Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. *The American Journal of Clinical Nutrition*, *90*(5), 1236-1243. https://doi.org/10.3945/ajcn.2009.28095

Chaudhury, A., Duvoor, C., Reddy Dendi, V. S., Kraleti, S., Chada, A., Ravilla, R., Marco, A., Shekhawat, N. S., Montales, M. T., Kuriakose, K., Sasapu, A., Beebe, A., Patil, N., Musham, C. K., Lohani, G. P., & Mirza, W. (2017). Clinical Review of Antidiabetic Drugs: Implications for Type 2 Diabetes Mellitus Management. *Frontiers in Endocrinology*, *8*. https://www.frontiersin.org/articles/10.3389/fendo.2017.00006

Chen, Z.-Z., & Gerszten, R. E. (2020). Metabolomics and Proteomics in Type 2 Diabetes. *Circulation Research*, *126*(11), 1613-1627. https://doi.org/10.1161/CIRCRESAHA.120.315898

Clark, I. A., Cowden, W. B., & Hunt, N. H. (1985). Free radical-induced pathology. *Medicinal Research Reviews*, *5*(3), 297-332. https://doi.org/10.1002/med.2610050303

Connelly, M. A., Gruppen, E. G., Wolak-Dinsmore, J., Matyus, S. P., Riphagen, I. J., Shalaurova, I., Bakker, S. J. L., Otvos, J. D., & Dullaart, R. P. F. (2016). GlycA, a marker of acute phase glycoproteins, and the risk of incident type 2 diabetes mellitus: PREVEND study. *Clinica Chimica Acta*, *452*, 10-17. https://doi.org/10.1016/j.cca.2015.11.001

Connelly, M. A., Otvos, J. D., Shalaurova, I., Playford, M. P., & Mehta, N. N. (2017). GlycA, a novel biomarker of systemic inflammation and cardiovascular disease risk. *Journal of Translational Medicine*, *15*(1), 219. https://doi.org/10.1186/s12967-017-1321-6

Czajka, A., Ajaz, S., Gnudi, L., Parsade, C. K., Jones, P., Reid, F., & Malik, A. N. (2015). Altered Mitochondrial Function, Mitochondrial DNA and Reduced Metabolic Flexibility in Patients With Diabetic Nephropathy. *EBioMedicine*, *2*(6), 499-512. https://doi.org/10.1016/j.ebiom.2015.04.002

Danaei, G., Finucane, M. M., Lu, Y., Singh, G. M., Cowan, M. J., Paciorek, C. J., Lin, J. K., Farzadfar, F., Khang, Y.-H., Stevens, G. A., Rao, M., Ali, M. K., Riley, L. M., Robinson, C. A., Ezzati, M., & Global Burden of Metabolic Risk Factors of Chronic Diseases Collaborating Group (Blood Glucose). (2011). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: Systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. *Lancet (London, England)*, *378*(9785), 31-40. https://doi.org/10.1016/S0140-6736(11)60679-X

de Marañón, A. M., Canet, F., Abad-Jiménez, Z., Jover, A., Morillas, C., Rocha, M., & Victor, V. M. (2021). Does Metformin Modulate Mitochondrial Dynamics and Function in Type 2 Diabetic Patients? *Antioxidants & Redox Signaling*, *35*(5), 377-385. https://doi.org/10.1089/ars.2021.0019

de Mello, V. D., Paananen, J., Lindström, J., Lankinen, M. A., Shi, L., Kuusisto, J., Pihlajamäki, J., Auriola, S., Lehtonen, M., Rolandsson, O., Bergdahl, I. A., Nordin, E., Ilanne-Parikka, P., Keinänen-Kiukaanniemi, S., Landberg, R., Eriksson, J. G., Tuomilehto, J., Hanhineva, K., & Uusitupa, M. (2017). Indolepropionic acid and novel lipid metabolites are associated with a lower risk of type 2 diabetes in the Finnish Diabetes Prevention Study. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/srep46337

DeFronzo, R. A. (2009). From the Triumvirate to the Ominous Octet: A New Paradigm for the Treatment of Type 2 Diabetes Mellitus. *Diabetes*, *58*(4), 773-795. https://doi.org/10.2337/db09-9028

DeFronzo, R. A., Ferrannini, E., Groop, L., Henry, R. R., Herman, W. H., Holst, J. J., Hu, F. B., Kahn, C. R., Raz, I., Shulman, G. I., Simonson, D. C., Testa, M. A., & Weiss, R. (2015). Type 2 diabetes mellitus. *Nature Reviews Disease Primers*, *1*(1), Article 1. https://doi.org/10.1038/nrdp.2015.19

Devaraj, S., Glaser, N., Griffen, S., Wang-Polagruto, J., Miguelino, E., & Jialal, I. (2006). Increased monocytic activity and biomarkers of inflammation in patients with type 1 diabetes. *Diabetes*, *55*(3), 774-779. https://doi.org/10.2337/diabetes.55.03.06.db05-1417

Donath, M. Y., & Shoelson, S. E. (2011). Type 2 diabetes as an inflammatory disease. *Nature Reviews. Immunology*, *11*(2), 98-107. https://doi.org/10.1038/nri2925

Du, X. L., Edelstein, D., Dimmeler, S., Ju, Q., Sui, C., & Brownlee, M. (2001). Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *The Journal of Clinical Investigation*, *108*(9), 1341-1348. https://doi.org/10.1172/JCI11235

Dullaart, R. P. F., Gruppen, E. G., Connelly, M. A., Otvos, J. D., & Lefrandt, J. D. (2015). GlycA, a biomarker of inflammatory glycoproteins, is more closely related to the leptin/adiponectin ratio than to glucose tolerance status. *Clinical Biochemistry*, *48*(12), 811-814. https://doi.org/10.1016/j.clinbiochem.2015.05.001

Duprez, D. A., Otvos, J., Sanchez, O. A., Mackey, R. H., Tracy, R., & Jacobs, D. R. (2016). Comparison of the Predictive Value of GlycA and Other Biomarkers of Inflammation for Total Death, Incident Cardiovascular Events, Noncardiovascular and Noncancer

Inflammatory-Related Events, and Total Cancer Events. *Clinical Chemistry*, *62*(7), 1020-1031. https://doi.org/10.1373/clinchem.2016.255828

Emerging Risk Factors Collaboration, Sarwar, N., Gao, P., Seshasai, S. R. K., Gobin, R., Kaptoge, S., Di Angelantonio, E., Ingelsson, E., Lawlor, D. A., Selvin, E., Stampfer, M., Stehouwer, C. D. A., Lewington, S., Pennells, L., Thompson, A., Sattar, N., White, I. R., Ray, K. K., & Danesh, J. (2010). Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: A collaborative meta-analysis of 102 prospective studies. *Lancet (London, England)*, *375*(9733), 2215-2222. https://doi.org/10.1016/S0140-6736(10)60484-9

Ference, B. A., Ginsberg, H. N., Graham, I., Ray, K. K., Packard, C. J., Bruckert, E., Hegele, R. A., Krauss, R. M., Raal, F. J., Schunkert, H., Watts, G. F., Borén, J., Fazio, S., Horton, J. D., Masana, L., Nicholls, S. J., Nordestgaard, B. G., van de Sluis, B., Taskinen, M.-R., … Catapano, A. L. (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *European Heart Journal*, *38*(32), 2459-2472. https://doi.org/10.1093/eurheartj/ehx144

Ferrannini, E. (2017). Sodium-Glucose Co-transporters and Their Inhibition: Clinical Physiology. *Cell Metabolism*, *26*(1), 27-38. https://doi.org/10.1016/j.cmet.2017.04.011

Ferrannini, E., & Mari, A. (2014). β-Cell function in type 2 diabetes. *Metabolism: Clinical and Experimental*, *63*(10), 1217-1227. https://doi.org/10.1016/j.metabol.2014.05.012

Ferrannini, E., Mark, M., & Mayoux, E. (2016). CV Protection in the EMPA-REG OUTCOME Trial: A «Thrifty Substrate» Hypothesis. *Diabetes Care*, *39*(7), 1108-1114. https://doi.org/10.2337/dc16-0330

Filomeni, G., De Zio, D., & Cecconi, F. (2015). Oxidative stress and autophagy: The clash between damage and metabolic needs. *Cell Death and Differentiation*, *22*(3), 377-388. https://doi.org/10.1038/cdd.2014.150

Fizelova, M., Miilunpohja, M., Kangas, A. J., Soininen, P., Kuusisto, J., Ala-Korpela, M., Laakso, M., & Stančáková, A. (2015). Associations of multiple lipoprotein and apolipoprotein measures with worsening of glycemia and incident type 2 diabetes in 6607 non-diabetic Finnish men. *Atherosclerosis*, *240*(1), 272-277. https://doi.org/10.1016/j.atherosclerosis.2015.03.034

Forman, H. J., & Zhang, H. (2021). Targeting oxidative stress in disease: Promise and limitations of antioxidant therapy. *Nature Reviews Drug Discovery*, *20*(9), Article 9. https://doi.org/10.1038/s41573-021-00233-1

Forrester, J. V., Kuffova, L., & Delibegovic, M. (2020). The Role of Inflammation in Diabetic Retinopathy. *Frontiers in Immunology*, *11*. https://www.frontiersin.org/articles/10.3389/fimmu.2020.583687

Gannon, M. C., Nuttall, J. A., & Nuttall, F. Q. (2002). The metabolic response to ingested glycine. *The American Journal of Clinical Nutrition*, *76*(6), 1302-1307. https://doi.org/10.1093/ajcn/76.6.1302

Garvey, W. T., Van Gaal, L., Leiter, L. A., Vijapurkar, U., List, J., Cuddihy, R., Ren, J., & Davies, M. J. (2018). Effects of canagliflozin versus glimepiride on adipokines and inflammatory biomarkers in type 2 diabetes. *Metabolism: Clinical and Experimental*, *85*, 32-37. https://doi.org/10.1016/j.metabol.2018.02.002

Gene Ontology Consortium. (2021). The Gene Ontology resource: Enriching a GOld mine. *Nucleic Acids Research*, *49*(D1), D325-D334. https://doi.org/10.1093/nar/gkaa1113

Giacco, F., & Brownlee, M. (2010). Oxidative Stress and Diabetic Complications. *Circulation Research*, *107*(9), 1058-1070. https://doi.org/10.1161/CIRCRESAHA.110.223545

Gimbrone, M. A., & García-Cardeña, G. (2016). Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. *Circulation Research*, *118*(4), 620-636. https://doi.org/10.1161/CIRCRESAHA.115.306301

Glynn, E. L., Piner, L. W., Huffman, K. M., Slentz, C. A., Elliot-Penry, L., AbouAssi, H., White, P. J., Bain, J. R., Muehlbauer, M. J., Ilkayeva, O. R., Stevens, R. D., Porter Starr, K. N., Bales, C. W., Volpi, E., Brosnan, M. J., Trimmer, J. K., Rolph, T. P., Newgard, C. B., & Kraus, W. E. (2015). Impact of combined resistance and aerobic exercise training on branched-chain amino acid turnover, glycine metabolism and insulin sensitivity in overweight humans. *Diabetologia*, *58*(10), 2324-2335. https://doi.org/10.1007/s00125-015-3705-6

Gniwotta, C., Morrow, J. D., Roberts, L. J., & Kühn, H. (1997). Prostaglandin F2-Like Compounds, F2-Isoprostanes, Are Present in Increased Amounts in Human Atherosclerotic Lesions. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *17*(11), 3236-3241. https://doi.org/10.1161/01.ATV.17.11.3236

Gonzalez, C. D., Lee, M.-S., Marchetti, P., Pietropaolo, M., Towns, R., Vaccaro, M. I., Watada, H., & Wiley, J. W. (2011). The emerging role of autophagy in the pathophysiology of diabetes mellitus. *Autophagy*, *7*(1), 2-11. https://doi.org/10.4161/auto.7.1.13044

González-Ortiz, M., Medina-Santillán, R., Martínez-Abundis, E., & von Drateln, C. R. (2001). Effect of glycine on insulin secretion and action in healthy first-degree relatives of type 2 diabetes mellitus patients. *Hormone and Metabolic Research = Hormon- Und Stoffwechselforschung = Hormones Et Metabolisme*, *33*(6), 358-360. https://doi.org/10.1055/s-2001-15421

Goytain, A., & Ng, T. (2020). NanoString nCounter Technology: High-Throughput RNA Validation. En H. Li & J. Elfman (Eds.), *Chimeric RNA: Methods and Protocols* (pp. 125-139). Springer US. https://doi.org/10.1007/978-1-4939-9904-0_10

Gruppen, E. G., Riphagen, I. J., Connelly, M. A., Otvos, J. D., Bakker, S. J. L., & Dullaart, R. P. F. (2015). GlycA, a Pro-Inflammatory Glycoprotein Biomarker, and Incident Cardiovascular Disease: Relationship with C-Reactive Protein and Renal Function. *PLoS ONE*, *10*(9), e0139057. https://doi.org/10.1371/journal.pone.0139057

Guasch-Ferré, M., Hruby, A., Toledo, E., Clish, C. B., Martínez-González, M. A., Salas-Salvadó, J., & Hu, F. B. (2016). Metabolomics in Prediabetes and Diabetes: A Systematic Review and Meta-analysis. *Diabetes Care*, *39*(5), 833-846. https://doi.org/10.2337/dc15-2251

Gupta, A. K., Menon, A., Brashear, M., & Johnson, W. D. (2018). Chapter 2 - Prediabetes: Prevalence, Pathogenesis, and Recognition of Enhanced Risk. En D. Bagchi & S. Nair (Eds.), *Nutritional and Therapeutic Interventions for Diabetes and Metabolic Syndrome (Second Edition)* (pp. 15-32). Academic Press. https://doi.org/10.1016/B978-0-12-812019-4.00002-7

Hansen, M., Rubinsztein, D. C., & Walker, D. W. (2018). Autophagy as a promoter of longevity: Insights from model organisms. *Nature Reviews Molecular Cell Biology*, *19*(9), Article 9. https://doi.org/10.1038/s41580-018-0033-y

Hanssen, N. M. J., Wouters, K., Huijberts, M. S., Gijbels, M. J., Sluimer, J. C., Scheijen, J. L. J. M., Heeneman, S., Biessen, E. A. L., Daemen, M. J. A. P., Brownlee, M., de Kleijn, D. P., Stehouwer, C. D. A., Pasterkamp, G., & Schalkwijk, C. G. (2014). Higher levels of advanced glycation endproducts in human carotid atherosclerotic plaques are associated with a rupture-prone phenotype. *European Heart Journal*, *35*(17), 1137-1146. https://doi.org/10.1093/eurheartj/eht402

Häring, H.-U., Merker, L., Seewaldt-Becker, E., Weimer, M., Meinicke, T., Broedl, U. C., Woerle, H. J., & on behalf of the EMPA-REG MET Trial Investigators. (2014). Empagliflozin as Add-On to Metformin in Patients With Type 2 Diabetes: A 24-Week, Randomized,

Double-Blind, Placebo-Controlled Trial. *Diabetes Care*, *37*(6), 1650-1659. https://doi.org/10.2337/dc13-2105

Helminen, O., Aspholm, S., Pokka, T., Hautakangas, M.-R., Haatanen, N., Lempainen, J., Ilonen, J., Simell, O., Knip, M., & Veijola, R. (2015). HbA1c Predicts Time to Diagnosis of Type 1 Diabetes in Children at Risk. *Diabetes*, *64*(5), 1719-1727. https://doi.org/10.2337/db14-0497

Herder, C., Brunner, E. J., Rathmann, W., Strassburger, K., Tabák, A. G., Schloot, N. C., & Witte, D. R. (2009). Elevated levels of the anti-inflammatory interleukin-1 receptor antagonist precede the onset of type 2 diabetes: The Whitehall II study. *Diabetes Care*, *32*(3), 421-423. https://doi.org/10.2337/dc08-1161

Hernandez-Mijares, A., Rocha, M., Rovira-Llopis, S., Bañuls, C., Bellod, L., de Pablo, C., Alvarez, A., Roldan-Torres, I., Sola-Izquierdo, E., & Victor, V. M. (2013). Human Leukocyte/Endothelial Cell Interactions and Mitochondrial Dysfunction in Type 2 Diabetic Patients and Their Association With Silent Myocardial Ischemia. *Diabetes Care*, *36*(6), 1695-1702. https://doi.org/10.2337/dc12-1224

Hjorth, M., Galigniana, N. M., Ween, O., Ulven, S. M., Holven, K. B., Dalen, K. T., & Sæther, T. (2022). Postprandial Effects of Salmon Fishmeal and Whey on Metabolic Markers in Serum and Gene Expression in Liver Cells. *Nutrients*, *14*(8), Article 8. https://doi.org/10.3390/nu14081593

Hustad, K. S., Ottestad, I., Hjorth, M., Dalen, K. T., Sæther, T., Sheikh, N. A., Strømnes, M., Ulven, S. M., & Holven, K. B. (2021). No effect of salmon fish protein on 2-h glucose in adults with increased risk of type 2 diabetes: A randomised controlled trial. *British Journal of Nutrition*, *126*(9), 1304-1313. https://doi.org/10.1017/S0007114521000040

Iannantuoni, F., M. de Marañon, A., Abad-Jiménez, Z., Canet, F., Díaz-Pozo, P., López-Domènech, S., Morillas, C., Rocha, M., & Víctor, V. M. (2020). Mitochondrial

Alterations and Enhanced Human Leukocyte/Endothelial Cell Interactions in Type 1 Diabetes. *Journal of Clinical Medicine*, *9*(7), Article 7. https://doi.org/10.3390/jcm9072155

Iannantuoni, F., M. de Marañon, A., Diaz-Morales, N., Falcon, R., Bañuls, C., Abad-Jimenez, Z., Victor, V. M., Hernandez-Mijares, A., & Rovira-Llopis, S. (2019). The SGLT2 Inhibitor Empagliflozin Ameliorates the Inflammatory Profile in Type 2 Diabetic Patients and Promotes an Antioxidant Response in Leukocytes. *Journal of Clinical Medicine*, *8*(11), Article 11. https://doi.org/10.3390/jcm8111814

International Diabetes Federation. (2019). *IDF Diabetes Atlas 9th edition* (9th ed.). https://diabetesatlas.org/atlas/ninth-edition/

International Diabetes Federation. (2021). *IDF Diabetes Atlas 10th edition* (10th ed.). https://www.diabetesatlas.org/

Inzucchi, S. E., Zinman, B., Fitchett, D., Wanner, C., Ferrannini, E., Schumacher, M., Schmoor, C., Ohneberg, K., Johansen, O. E., George, J. T., Hantel, S., Bluhmki, E., & Lachin, J. M. (2017). How Does Empagliflozin Reduce Cardiovascular Mortality? Insights From a Mediation Analysis of the EMPA-REG OUTCOME Trial. *Diabetes Care*, *41*(2), 356-363. https://doi.org/10.2337/dc17-1096

Jacobsen, L. M., Haller, M. J., & Schatz, D. A. (2018). Understanding Pre-Type 1 Diabetes: The Key to Prevention. *Frontiers in Endocrinology*, *9*, 70. https://doi.org/10.3389/fendo.2018.00070

Jia, G., Cheng, G., Gangahar, D. M., & Agrawal, D. K. (2006). Insulin-like growth factor-1 and TNF-α regulate autophagy through c-jun N-terminal kinase and Akt pathways in human atherosclerotic vascular smooth cells. *Immunology & Cell Biology*, *84*(5), 448-454. https://doi.org/10.1111/j.1440-1711.2006.01454.x

Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, *28*(1), 27-30. https://doi.org/10.1093/nar/28.1.27

Katsarou, A., Gudbjörnsdottir, S., Rawshani, A., Dabelea, D., Bonifacio, E., Anderson, B. J., Jacobsen, L. M., Schatz, D. A., & Lernmark, Å. (2017). Type 1 diabetes mellitus. *Nature Reviews. Disease Primers*, *3*, 17016. https://doi.org/10.1038/nrdp.2017.16

Kettunen, J., Tukiainen, T., Sarin, A.-P., Ortega-Alonso, A., Tikkanen, E., Lyytikäinen, L.-P., Kangas, A. J., Soininen, P., Würtz, P., Silander, K., Dick, D. M., Rose, R. J., Savolainen, M. J., Viikari, J., Kähönen, M., Lehtimäki, T., Pietiläinen, K. H., Inouye, M., McCarthy, M. I., … Ripatti, S. (2012). Genome-wide association study identifies multiple loci influencing human serum metabolite levels. *Nature Genetics*, *44*(3), Article 3. https://doi.org/10.1038/ng.1073

Kohler, S., Zeller, C., Iliev, H., & Kaspers, S. (2017). Safety and Tolerability of Empagliflozin in Patients with Type 2 Diabetes: Pooled Analysis of Phase I–III Clinical Trials. *Advances in Therapy*, *34*(7), 1707-1726. https://doi.org/10.1007/s12325-017-0573-0

Kolijn, D., Pabel, S., Tian, Y., Lódi, M., Herwig, M., Carrizzo, A., Zhazykbayeva, S., Kovács, Á., Fülöp, G. Á., Falcão-Pires, I., Reusch, P. H., Linthout, S. V., Papp, Z., van Heerebeek, L., Vecchione, C., Maier, L. S., Ciccarelli, M., Tschöpe, C., Mügge, A., … Hamdani, N. (2021). Empagliflozin improves endothelial and cardiomyocyte function in human heart failure with preserved ejection fraction via reduced pro-inflammatory-oxidative pathways and protein kinase Gα oxidation. *Cardiovascular Research*, *117*(2), 495-507. https://doi.org/10.1093/cvr/cvaa123

Kovatcheva-Datchary, P., Nilsson, A., Akrami, R., Lee, Y. S., De Vadder, F., Arora, T., Hallen, A., Martens, E., Björck, I., & Bäckhed, F. (2015). Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of Prevotella. *Cell Metabolism*, *22*(6), 971-982. https://doi.org/10.1016/j.cmet.2015.10.001

Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R. B., Newgard, C. B., Lopaschuk, G. D., & Muoio, D. M. (2008). Mitochondrial Overload and Incomplete Fatty Acid Oxidation Contribute to Skeletal Muscle Insulin Resistance. *Cell Metabolism*, *7*(1), 45-56. https://doi.org/10.1016/j.cmet.2007.10.013

Kramer, P. A., Ravi, S., Chacko, B., Johnson, M. S., & Darley-Usmar, V. M. (2014). A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: Implications for their use as bioenergetic biomarkers. *Redox Biology*, *2*, 206-210. https://doi.org/10.1016/j.redox.2013.12.026

Kranzhöfer, R., Browatzki, M., Schmidt, J., & Kübler, W. (1999). Angiotensin II activates the proinflammatory transcription factor nuclear factor-kappaB in human monocytes. *Biochemical and Biophysical Research Communications*, *257*(3), 826-828. https://doi.org/10.1006/bbrc.1999.0543

Krischer, J. P., Lynch, K. F., Schatz, D. A., Ilonen, J., Lernmark, Å., Hagopian, W. A., Rewers, M. J., She, J.-X., Simell, O. G., Toppari, J., Ziegler, A.-G., Akolkar, B., Bonifacio, E., & the TEDDY Study Group. (2015). The 6 year incidence of diabetes-associated autoantibodies in genetically at-risk children: The TEDDY study. *Diabetologia*, *58*(5), 980-987. https://doi.org/10.1007/s00125-015-3514-y

Lai, C.-Q., Parnell, L. D., Smith, C. E., Guo, T., Sayols-Baixeras, S., Aslibekyan, S., Tiwari, H. K., Irvin, M. R., Bender, C., Fei, D., Hidalgo, B., Hopkins, P. N., Absher, D. M., Province, M. A., Elosua, R., Arnett, D. K., & Ordovas, J. M. (2020). Carbohydrate and fat intake associated with risk of metabolic diseases through epigenetics of CPT1A. *The American Journal of Clinical Nutrition*, *112*(5), 1200-1211. https://doi.org/10.1093/ajcn/nqaa233

Larsen, C. M., Faulenbach, M., Vaag, A., Vølund, A., Ehses, J. A., Seifert, B., Mandrup-Poulsen, T., & Donath, M. Y. (2007). Interleukin-1-receptor antagonist in type 2 diabetes

mellitus. *The New England Journal of Medicine*, *356*(15), 1517-1526. https://doi.org/10.1056/NEJMoa065213

Lavigne, C., Tremblay, F., Asselin, G., Jacques, H., & Marette, A. (2001). Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats. *American Journal of Physiology-Endocrinology and Metabolism*, *281*(1), E62-E71. https://doi.org/10.1152/ajpendo.2001.281.1.E62

Lawler, P. R., & Mora, S. (2016). Glycosylation Signatures of Inflammation Identify Cardiovascular Risk: Some Glyc It Hot. *Circulation Research*, *119*(11), 1154-1156. https://doi.org/10.1161/CIRCRESAHA.116.310005

Levine, B., Mizushima, N., & Virgin, H. W. (2011). Autophagy in immunity and inflammation. *Nature*, *469*(7330), Article 7330. https://doi.org/10.1038/nature09782

Levine, J. A., Han, J. M., Wolska, A., Wilson, S. R., Patel, T. P., Remaley, A. T., Periwal, V., Yanovski, J. A., & Demidowich, A. P. (2020). Associations of GlycA and high-sensitivity C-reactive protein with measures of lipolysis in adults with obesity. *Journal of Clinical Lipidology*, *14*(5), 667-674. https://doi.org/10.1016/j.jacl.2020.07.012

Li, C., Zhang, J., Xue, M., Li, X., Han, F., Liu, X., Xu, L., Lu, Y., Cheng, Y., Li, T., Yu, X., Sun, B., & Chen, L. (2019). SGLT2 inhibition with empagliflozin attenuates myocardial oxidative stress and fibrosis in diabetic mice heart. *Cardiovascular Diabetology*, *18*(1), 15. https://doi.org/10.1186/s12933-019-0816-2

Li, P., Wang, B., Sun, F., Li, Y., Li, Q., Lang, H., Zhao, Z., Gao, P., Zhao, Y., Shang, Q., Liu, D., & Zhu, Z. (2015). Mitochondrial respiratory dysfunctions of blood mononuclear cells link with cardiac disturbance in patients with early-stage heart failure. *Scientific Reports*, *5*, 10229. https://doi.org/10.1038/srep10229

Li, X., Fang, P., Mai, J., Choi, E. T., Wang, H., & Yang, X. (2013). Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. *Journal of Hematology & Oncology*, *6*(1), 19. https://doi.org/10.1186/1756-8722-6-19

Libby, P., Buring, J. E., Badimon, L., Hansson, G. K., Deanfield, J., Bittencourt, M. S., Tokgözoğlu, L., & Lewis, E. F. (2019). Atherosclerosis. *Nature Reviews Disease Primers*, *5*(1), Article 1. https://doi.org/10.1038/s41572-019-0106-z

Liberzon, A., Birger, C., Thorvaldsdóttir, H., Ghandi, M., Mesirov, J. P., & Tamayo, P. (2015). The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell systems*, *1*(6), 417-425. https://doi.org/10.1016/j.cels.2015.12.004

Lorenzo, C., Festa, A., Hanley, A. J., Rewers, M. J., Escalante, A., & Haffner, S. M. (2017). Novel Protein Glycan-Derived Markers of Systemic Inflammation and C-Reactive Protein in Relation to Glycemia, Insulin Resistance, and Insulin Secretion. *Diabetes Care*, *40*(3), 375-382. https://doi.org/10.2337/dc16-1569

Lynch, C. J., & Adams, S. H. (2014). Branched-chain amino acids in metabolic signalling and insulin resistance. *Nature Reviews Endocrinology*, *10*(12), Article 12. https://doi.org/10.1038/nrendo.2014.171

Mackey, R. H., Mora, S., Bertoni, A. G., Wassel, C. L., Carnethon, M. R., Sibley, C. T., & Goff, D. C., Jr. (2015). Lipoprotein Particles and Incident Type 2 Diabetes in the Multi-Ethnic Study of Atherosclerosis. *Diabetes Care*, *38*(4), 628-636. https://doi.org/10.2337/dc14-0645

Mahendran, Y., Cederberg, H., Vangipurapu, J., Kangas, A. J., Soininen, P., Kuusisto, J., Uusitupa, M., Ala-Korpela, M., & Laakso, M. (2013). Glycerol and fatty acids in serum predict the development of hyperglycemia and type 2 diabetes in Finnish men. *Diabetes Care*, *36*(11), 3732-3738. https://doi.org/10.2337/dc13-0800

Marchetti, P., Fovez, Q., Germain, N., Khamari, R., & Kluza, J. (2020). Mitochondrial spare respiratory capacity: Mechanisms, regulation, and significance in non-transformed and cancer cells. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *34*(10), 13106-13124. https://doi.org/10.1096/fj.202000767R

Martinet, W., & De Meyer, G. R. Y. (2009). Autophagy in Atherosclerosis. *Circulation Research*, *104*(3), 304-317. https://doi.org/10.1161/CIRCRESAHA.108.188318

Meager, A. (1999). Cytokine regulation of cellular adhesion molecule expression in inflammation. *Cytokine & Growth Factor Reviews*, *10*(1), 27-39. https://doi.org/10.1016/s1359-6101(98)00024-0

Menikdiwela, K. R., Ramalingam, L., Rasha, F., Wang, S., Dufour, J. M., Kalupahana, N. S., Sunahara, K. K. S., Martins, J. O., & Moustaid-Moussa, N. (2020). Autophagy in metabolic syndrome: Breaking the wheel by targeting the renin–angiotensin system. *Cell Death & Disease*, *11*(2), Article 2. https://doi.org/10.1038/s41419-020-2275-9

Miller, Y. I., Choi, S.-H., Wiesner, P., Fang, L., Harkewicz, R., Hartvigsen, K., Boullier, A., Gonen, A., Diehl, C. J., Que, X., Montano, E., Shaw, P. X., Tsimikas, S., Binder, C. J., & Witztum, J. L. (2011). Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circulation Research*, *108*(2), 235-248. https://doi.org/10.1161/CIRCRESAHA.110.223875

Moffett, J. R., Puthillathu, N., Vengilote, R., Jaworski, D. M., & Namboodiri, A. M. (2020). Acetate Revisited: A Key Biomolecule at the Nexus of Metabolism, Epigenetics and Oncogenesis—Part 1: Acetyl-CoA, Acetogenesis and Acyl-CoA Short-Chain Synthetases. *Frontiers in Physiology*, *11*. https://www.frontiersin.org/articles/10.3389/fphys.2020.580167

Mone, P., Varzideh, F., Jankauskas, S. S., Pansini, A., Lombardi, A., Frullone, S., & Santulli, G. (2022). SGLT2 Inhibition via Empagliflozin Improves Endothelial Function and Reduces Mitochondrial Oxidative Stress: Insights From Frail Hypertensive and Diabetic Patients. *Hypertension*, *79*(8), 1633-1643. https://doi.org/10.1161/HYPERTENSIONAHA.122.19586

Moreno-Aliaga, M., Campion, J., Milagro, F., Berjón, A., & Alfredo, M. (2005). Adiposity and proinflammatory state: The chicken or the egg. *Adipocytes*, *1*, 1-16.

Mozaffarian, D. (2016). Dietary and Policy Priorities for Cardiovascular Disease, Diabetes, and Obesity – A Comprehensive Review. *Circulation*, *133*(2), 187-225. https://doi.org/10.1161/CIRCULATIONAHA.115.018585

Mozaffarian, D., Appel, L. J., & Van Horn, L. (2011). Components of a Cardioprotective Diet. *Circulation*, *123*(24), 2870-2891. https://doi.org/10.1161/CIRCULATIONAHA.110.968735

Mozaffarian, D., & Rimm, E. B. (2006). Fish intake, contaminants, and human health: Evaluating the risks and the benefits. *JAMA*, *296*(15), 1885-1899. https://doi.org/10.1001/jama.296.15.1885

Nathan, D. M. & DCCT/EDIC Research Group. (2014). The diabetes control and complications trial/epidemiology of diabetes interventions and complications study at 30 years: Overview. *Diabetes Care*, *37*(1), 9-16. https://doi.org/10.2337/dc13-2112

Neal, B., Perkovic, V., Mahaffey, K. W., de Zeeuw, D., Fulcher, G., Erondu, N., Shaw, W., Law, G., Desai, M., & Matthews, D. R. (2017). Canagliflozin and Cardiovascular and Renal Events in Type 2 Diabetes. *New England Journal of Medicine*, *377*(7), 644-657. https://doi.org/10.1056/NEJMoa1611925

Newgard, C. B., An, J., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Lien, L. F., Haqq, A. M., Shah, S. H., Arlotto, M., Slentz, C. A., Rochon, J., Gallup, D., Ilkayeva, O., Wenner, B. R.,

Yancy, W. S., Eisenson, H., Musante, G., Surwit, R. S., Millington, D. S., … Svetkey, L. P. (2009). A Branched-Chain Amino Acid-Related Metabolic Signature that Differentiates Obese and Lean Humans and Contributes to Insulin Resistance. *Cell Metabolism*, *9*(4), 311-326. https://doi.org/10.1016/j.cmet.2009.02.002

Neyestani, T. R., Ghandchi, Z., Eshraghian, M.-R., Kalayi, A., Shariatzadeh, N., & Houshiarrad, A. (2012). Evidence for augmented oxidative stress in the subjects with type 1 diabetes and their siblings: A possible preventive role for antioxidants. *European Journal of Clinical Nutrition*, *66*(9), Article 9. https://doi.org/10.1038/ejcn.2012.81

Nus, M., & Mallat, Z. (2016). Immune-mediated mechanisms of atherosclerosis and implications for the clinic. *Expert Review of Clinical Immunology*, *12*(11), 1217-1237. https://doi.org/10.1080/1744666X.2016.1195686

Otvos, J. D., Shalaurova, I., Wolak-Dinsmore, J., Connelly, M. A., Mackey, R. H., Stein, J. H., & Tracy, R. P. (2015). GlycA: A Composite Nuclear Magnetic Resonance Biomarker of Systemic Inflammation. *Clinical Chemistry*, *61*(5), 714-723. https://doi.org/10.1373/clinchem.2014.232918

Ouellet, V., Marois, J., Weisnagel, S. J., & Jacques, H. (2007). Dietary Cod Protein Improves Insulin Sensitivity in Insulin-Resistant Men and Women: A randomized controlled trial. *Diabetes Care*, *30*(11), 2816-2821. https://doi.org/10.2337/dc07-0273

Packer, M., Anker, S. D., Butler, J., Filippatos, G., & Zannad, F. (2017). Effects of Sodium-Glucose Cotransporter 2 Inhibitors for the Treatment of Patients With Heart Failure: Proposal of a Novel Mechanism of Action. *JAMA Cardiology*, *2*(9), 1025-1029. https://doi.org/10.1001/jamacardio.2017.2275

Palmer, N. D., Stevens, R. D., Antinozzi, P. A., Anderson, A., Bergman, R. N., Wagenknecht, L. E., Newgard, C. B., & Bowden, D. W. (2015). Metabolomic Profile Associated With Insulin Resistance and Conversion to Diabetes in the Insulin Resistance Atherosclerosis Study.

The Journal of Clinical Endocrinology & Metabolism, *100*(3), E463-E468. https://doi.org/10.1210/jc.2014-2357

Pan, X.-R., Li, G.-W., Hu, Y.-H., Wang, J.-X., Yang, W.-Y., An, Z.-X., Hu, Z.-X., Juan-Lin, Xiao, J.-Z., Cao, H.-B., Liu, P.-A., Jiang, X.-G., Jiang, Y.-Y., Wang, J.-P., Zheng, H., Zhang, H., Bennett, P. H., & Howard, B. V. (1997). Effects of Diet and Exercise in Preventing NIDDM in People With Impaired Glucose Tolerance: The Da Qing IGT and Diabetes Study. *Diabetes Care*, *20*(4), 537-544. https://doi.org/10.2337/diacare.20.4.537

Paneni, F., Beckman, J. A., Creager, M. A., & Cosentino, F. (2013). Diabetes and vascular disease: Pathophysiology, clinical consequences, and medical therapy: part I. *European Heart Journal*, *34*(31), 2436-2443. https://doi.org/10.1093/eurheartj/eht149

Patti, M.-E., & Corvera, S. (2010). The Role of Mitochondria in the Pathogenesis of Type 2 Diabetes. *Endocrine Reviews*, *31*(3), 364-395. https://doi.org/10.1210/er.2009-0027

Pieber, T. R., Famulla, S., Eilbracht, J., Cescutti, J., Soleymanlou, N., Johansen, O. E., Woerle, H. J., Broedl, U. C., & Kaspers, S. (2015). Empagliflozin as adjunct to insulin in patients with type 1 diabetes: A 4-week, randomized, placebo-controlled trial (EASE-1). *Diabetes, Obesity and Metabolism*, *17*(10), 928-935. https://doi.org/10.1111/dom.12494

Prattichizzo, F., De Nigris, V., Micheloni, S., La Sala, L., & Ceriello, A. (2018). Increases in circulating levels of ketone bodies and cardiovascular protection with SGLT2 inhibitors: Is low-grade inflammation the neglected component? *Diabetes, Obesity and Metabolism*, *20*(11), 2515-2522. https://doi.org/10.1111/dom.13488

Quagliariello, V., De Laurentiis, M., Rea, D., Barbieri, A., Monti, M. G., Carbone, A., Paccone, A., Altucci, L., Conte, M., Canale, M. L., Botti, G., & Maurea, N. (2021). The SGLT-2 inhibitor empagliflozin improves myocardial strain, reduces cardiac fibrosis and pro-

inflammatory cytokines in non-diabetic mice treated with doxorubicin. *Cardiovascular Diabetology*, *20*(1), 150. https://doi.org/10.1186/s12933-021-01346-y

Raffa, S., Chin, X. L. D., Stanzione, R., Forte, M., Bianchi, F., Cotugno, M., Marchitti, S., Micaloni, A., Gallo, G., Schirone, L., Tocci, G., Violini, R., Torrisi, M. R., Volpe, M., & Rubattu, S. (2019). The reduction of NDUFC2 expression is associated with mitochondrial impairment in circulating mononuclear cells of patients with acute coronary syndrome. *International Journal of Cardiology*, *286*, 127-133. https://doi.org/10.1016/j.ijcard.2019.02.027

Rajala, M. W., & Scherer, P. E. (2003). Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology*, *144*(9), 3765-3773. https://doi.org/10.1210/en.2003-0580

Rask-Madsen, C., Li, Q., Freund, B., Feather, D., Abramov, R., Wu, I.-H., Chen, K., Yamamoto-Hiraoka, J., Goldenbogen, J., Sotiropoulos, K. B., Clermont, A., Geraldes, P., Dall'Osso, C., Wagers, A. J., Huang, P. L., Rekhter, M., Scalia, R., Kahn, C. R., & King, G. L. (2010). Loss of insulin signaling in vascular endothelial cells accelerates atherosclerosis in apolipoprotein E null mice. *Cell Metabolism*, *11*(5), 379-389. https://doi.org/10.1016/j.cmet.2010.03.013

Rhee, E. P., Cheng, S., Larson, M. G., Walford, G. A., Lewis, G. D., McCabe, E., Yang, E., Farrell, L., Fox, C. S., O'Donnell, C. J., Carr, S. A., Vasan, R. S., Florez, J. C., Clish, C. B., Wang, T. J., & Gerszten, R. E. (2011). Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. *The Journal of Clinical Investigation*, *121*(4), 1402-1411. https://doi.org/10.1172/JCI44442

Richter, B., Hemmingsen, B., Metzendorf, M.-I., & Takwoingi, Y. (2018). Development of type 2 diabetes mellitus in people with intermediate hyperglycaemia. *The Cochrane Database*

of Systematic Reviews, *10*, CD012661. https://doi.org/10.1002/14651858.CD012661.pub2

Ridker, P. M. (2016). A Test in Context: High-Sensitivity C-Reactive Protein. *Journal of the American College of Cardiology*, *67*(6), 712-723. https://doi.org/10.1016/j.jacc.2015.11.037

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research*, *43*(7), e47. https://doi.org/10.1093/nar/gkv007

Ritchie, S. C., Würtz, P., Nath, A. P., Abraham, G., Havulinna, A. S., Fearnley, L. G., Sarin, A.-P., Kangas, A. J., Soininen, P., Aalto, K., Seppälä, I., Raitoharju, E., Salmi, M., Maksimow, M., Männistö, S., Kähönen, M., Juonala, M., Ripatti, S., Lehtimäki, T., … Inouye, M. (2015). The Biomarker GlycA Is Associated with Chronic Inflammation and Predicts Long-Term Risk of Severe Infection. *Cell Systems*, *1*(4), 293-301. https://doi.org/10.1016/j.cels.2015.09.007

Rösen, P., Nawroth, P. P., King, G., Möller, W., Tritschler, H.-J., & Packer, L. (2001). The role of oxidative stress in the onset and progression of diabetes and its complications: Asummary of a Congress Series sponsored byUNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes/Metabolism Research and Reviews*, *17*(3), 189-212. https://doi.org/10.1002/dmrr.196

Rosselot, C., Baumel-Alterzon, S., Li, Y., Brill, G., Lambertini, L., Katz, L. S., Lu, G., Garcia-Ocaña, A., & Scott, D. K. (2021). The many lives of Myc in the pancreatic β-cell. *Journal of Biological Chemistry*, *296*, 100122. https://doi.org/10.1074/jbc.REV120.011149

Rubattu, S., Forte, M., & Raffa, S. (2019). Circulating Leukocytes and Oxidative Stress in Cardiovascular Diseases: A State of the Art. *Oxidative Medicine and Cellular Longevity*, *2019*, e2650429. https://doi.org/10.1155/2019/2650429

Saha, S., Panigrahi, D. P., Patil, S., & Bhutia, S. K. (2018). Autophagy in health and disease: A comprehensive review. *Biomedicine & Pharmacotherapy*, *104*, 485-495. https://doi.org/10.1016/j.biopha.2018.05.007

Salas-Salvadó, J., Bulló, M., Estruch, R., Ros, E., Covas, M.-I., Ibarrola-Jurado, N., Corella, D., Arós, F., Gómez-Gracia, E., Ruiz-Gutiérrez, V., Romaguera, D., Lapetra, J., Lamuela-Raventós, R. M., Serra-Majem, L., Pintó, X., Basora, J., Muñoz, M. A., Sorlí, J. V., & Martínez-González, M. A. (2014). Prevention of diabetes with Mediterranean diets: A subgroup analysis of a randomized trial. *Annals of Internal Medicine*, *160*(1), 1-10. https://doi.org/10.7326/M13-1725

Salomon, R. G., Kaur, K., Podrez, E., Hoff, H. F., Krushinsky, A. V., & Sayre, L. M. (2000). HNE-derived 2-pentylpyrroles are generated during oxidation of LDL, are more prevalent in blood plasma from patients with renal disease or atherosclerosis, and are present in atherosclerotic plaques. *Chemical Research in Toxicology*, *13*(7), 557-564. https://doi.org/10.1021/tx000007u

Scheen, A. J. (2020). Sodium–glucose cotransporter type 2 inhibitors for the treatment of type 2 diabetes mellitus. *Nature Reviews Endocrinology*, *16*(10), Article 10. https://doi.org/10.1038/s41574-020-0392-2

Schernthaner, G., Schernthaner-Reiter, M. H., & Schernthaner, G.-H. (2016). EMPA-REG and Other Cardiovascular Outcome Trials of Glucose-lowering Agents: Implications for Future Treatment Strategies in Type 2 Diabetes Mellitus. *Clinical Therapeutics*, *38*(6), 1288-1298. https://doi.org/10.1016/j.clinthera.2016.04.037

Scherz-Shouval, R., Shvets, E., & Elazar, Z. (2007). Oxidation as a Post-Translational Modification that Regulates Autophagy. *Autophagy*, *3*(4), 371-373. https://doi.org/10.4161/auto.4214

Serrano-Rìos, M., Goday, A., & Martìnez Larrad, T. (1999). Migrant populations and the incidence of type 1 diabetes mellitus: An overview of the literature with a focus on the Spanish-heritage countries in Latin America. *Diabetes/Metabolism Research and Reviews*, *15*(2), 113-132. https://doi.org/10.1002/(sici)1520-7560(199903/04)15:2<113::aid-dmrr25>3.0.co;2-i

Shah, M. S., & Brownlee, M. (2016). Molecular and Cellular Mechanisms of Cardiovascular Disorders in Diabetes. *Circulation Research*, *118*(11), 1808-1829. https://doi.org/10.1161/CIRCRESAHA.116.306923

Sharifi-Rad, M., Anil Kumar, N. V., Zucca, P., Varoni, E. M., Dini, L., Panzarini, E., Rajkovic, J., Tsouh Fokou, P. V., Azzini, E., Peluso, I., Prakash Mishra, A., Nigam, M., El Rayess, Y., Beyrouthy, M. E., Polito, L., Iriti, M., Martins, N., Martorell, M., Docea, A. O., … Sharifi-Rad, J. (2020). Lifestyle, Oxidative Stress, and Antioxidants: Back and Forth in the Pathophysiology of Chronic Diseases. *Frontiers in Physiology*, *11*. https://www.frontiersin.org/articles/10.3389/fphys.2020.00694

Sies, H., & Jones, D. P. (2020). Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. *Nature Reviews. Molecular Cell Biology*, *21*(7), 363-383. https://doi.org/10.1038/s41580-020-0230-3

Sivitz, W. I., & Yorek, M. A. (2010). Mitochondrial Dysfunction in Diabetes: From Molecular Mechanisms to Functional Significance and Therapeutic Opportunities. *Antioxidants & Redox Signaling*, *12*(4), 537-577. https://doi.org/10.1089/ars.2009.2531

Soininen, P., Kangas, A. J., Würtz, P., Suna, T., & Ala-Korpela, M. (2015). Quantitative Serum Nuclear Magnetic Resonance Metabolomics in Cardiovascular Epidemiology and Genetics. *Circulation: Cardiovascular Genetics*, *8*(1), 192-206. https://doi.org/10.1161/CIRCGENETICS.114.000216

Sorrentino, V., Menzies, K. J., & Auwerx, J. (2018). Repairing Mitochondrial Dysfunction in Disease. *Annual Review of Pharmacology and Toxicology*, *58*, 353-389. https://doi.org/10.1146/annurev-pharmtox-010716-104908

Steven, S., Oelze, M., Hanf, A., Kröller-Schön, S., Kashani, F., Roohani, S., Welschof, P., Kopp, M., Gödtel-Armbrust, U., Xia, N., Li, H., Schulz, E., Lackner, K. J., Wojnowski, L., Bottari, S. P., Wenzel, P., Mayoux, E., Münzel, T., & Daiber, A. (2017). The SGLT2 inhibitor empagliflozin improves the primary diabetic complications in ZDF rats. *Redox Biology*, *13*, 370-385. https://doi.org/10.1016/j.redox.2017.06.009

Suarna, C., Dean, R. T., May, J., & Stocker, R. (1995). Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of alpha-tocopherol and ascorbate. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *15*(10), 1616-1624. https://doi.org/10.1161/01.atv.15.10.1616

Swirski, F. K., & Nahrendorf, M. (2013). Leukocyte behavior in atherosclerosis, myocardial infarction, and heart failure. *Science (New York, N.Y.)*, *339*(6116), 161-166. https://doi.org/10.1126/science.1230719

Thalacker-Mercer, A. E., Ingram, K. H., Guo, F., Ilkayeva, O., Newgard, C. B., & Garvey, W. T. (2014). BMI, RQ, diabetes, and sex affect the relationships between amino acids and clamp measures of insulin action in humans. *Diabetes*, *63*(2), 791-800. https://doi.org/10.2337/db13-0396

Tsutsumi, R., Yamasaki, Y., Takeo, J., Miyahara, H., Sebe, M., Bando, M., Tanba, Y., Mishima, Y., Takeji, K., Ueshima, N., Kuroda, M., Masumoto, S., Harada, N., Fukuda, D., Yoshimoto, R., Tsutsumi, Y. M., Aihara, K., Sata, M., & Sakaue, H. (2021). Long-chain monounsaturated fatty acids improve endothelial function with altering microbial flora. *Translational Research*, *237*, 16-30. https://doi.org/10.1016/j.trsl.2021.03.016

Tulipani, S., Griffin, J., Palau-Rodriguez, M., Mora-Cubillos, X., Bernal-Lopez, R. M., Tinahones, F. J., Corkey, B. E., & Andres-Lacueva, C. (2016). Metabolomics-guided insights on bariatric surgery versus behavioral interventions for weight loss. *Obesity*, *24*(12), 2451-2466. https://doi.org/10.1002/oby.21686

van Diepen, J. A., Thiem, K., Stienstra, R., Riksen, N. P., Tack, C. J., & Netea, M. G. (2016). Diabetes propels the risk for cardiovascular disease: Sweet monocytes becoming aggressive? *Cellular and Molecular Life Sciences*, *73*(24), 4675-4684. https://doi.org/10.1007/s00018-016-2316-9

van Roekel, E. H., Loftfield, E., Kelly, R. S., Zeleznik, O. A., & Zanetti, K. A. (2019). Metabolomics in epidemiologic research: Challenges and opportunities for early-career epidemiologists. *Metabolomics : Official journal of the Metabolomic Society*, *15*(1), 9. https://doi.org/10.1007/s11306-018-1468-z

Vanweert, F., Schrauwen, P., & Phielix, E. (2022). Role of branched-chain amino acid metabolism in the pathogenesis of obesity and type 2 diabetes-related metabolic disturbances BCAA metabolism in type 2 diabetes. *Nutrition & Diabetes*, *12*(1), Article 1. https://doi.org/10.1038/s41387-022-00213-3

Vargas, E., Joy, N. V., & Carrillo Sepulveda, M. A. (2022). Biochemistry, Insulin Metabolic Effects. En *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK525983/

Vedantham, S., Thiagarajan, D., Ananthakrishnan, R., Wang, L., Rosario, R., Zou, Y. S., Goldberg, I., Yan, S. F., Schmidt, A. M., & Ramasamy, R. (2014). Aldose reductase drives hyperacetylation of Egr-1 in hyperglycemia and consequent upregulation of proinflammatory and prothrombotic signals. *Diabetes*, *63*(2), 761-774. https://doi.org/10.2337/db13-0032

Vikøren, L. A., Nygård, O. K., Lied, E., Rostrup, E., & Gudbrandsen, O. A. (2013). A randomised study on the effects of fish protein supplement on glucose tolerance, lipids and body composition in overweight adults. *The British Journal of Nutrition*, *109*(4), 648-657. https://doi.org/10.1017/S0007114512001717

Volpe, C. M. O., Villar-Delfino, P. H., dos Anjos, P. M. F., & Nogueira-Machado, J. A. (2018). Cellular death, reactive oxygen species (ROS) and diabetic complications. *Cell Death & Disease*, *9*(2), Article 2. https://doi.org/10.1038/s41419-017-0135-z

Wang, C.-H., Wang, C.-C., Huang, H.-C., & Wei, Y.-H. (2013). Mitochondrial dysfunction leads to impairment of insulin sensitivity and adiponectin secretion in adipocytes. *The FEBS Journal*, *280*(4), 1039-1050. https://doi.org/10.1111/febs.12096

Wang, T. J., Larson, M. G., Vasan, R. S., Cheng, S., Rhee, E. P., McCabe, E., Lewis, G. D., Fox, C. S., Jacques, P. F., Fernandez, C., O'Donnell, C. J., Carr, S. A., Mootha, V. K., Florez, J. C., Souza, A., Melander, O., Clish, C. B., & Gerszten, R. E. (2011). Metabolite profiles and the risk of developing diabetes. *Nature Medicine*, *17*(4), 448-453. https://doi.org/10.1038/nm.2307

Widlansky, M. E., Wang, J., Shenouda, S. M., Hagen, T. M., Smith, A. R., Kizhakekuttu, T. J., Kluge, M. A., Weihrauch, D., Gutterman, D. D., & Vita, J. A. (2010). Altered mitochondrial membrane potential, mass, and morphology in the mononuclear cells of humans with type 2 diabetes. *Translational Research*, *156*(1), 15-25. https://doi.org/10.1016/j.trsl.2010.04.001

World Health Organization. (2016). *Global report on diabetes*. World Health Organization. https://www.who.int/publications/i/item/9789241565257

World Health Organization. (2019). *Classification of diabetes mellitus*. World Health Organization.

Wu, D., & Smyth, G. K. (2012). Camera: A competitive gene set test accounting for inter-gene correlation. *Nucleic Acids Research*, *40*(17), e133. https://doi.org/10.1093/nar/gks461

Würtz, P., Havulinna, A. S., Soininen, P., Tynkkynen, T., Prieto-Merino, D., Tillin, T., Ghorbani, A., Artati, A., Wang, Q., Tiainen, M., Kangas, A. J., Kettunen, J., Kaikkonen, J., Mikkilä, V., Jula, A., Kähönen, M., Lehtimäki, T., Lawlor, D. A., Gaunt, T. R., … Salomaa, V. (2015). Metabolite profiling and cardiovascular event risk: A prospective study of 3 population-based cohorts. *Circulation*, *131*(9), 774-785. https://doi.org/10.1161/CIRCULATIONAHA.114.013116

Würtz, P., Raiko, J. R., Magnussen, C. G., Soininen, P., Kangas, A. J., Tynkkynen, T., Thomson, R., Laatikainen, R., Savolainen, M. J., Laurikka, J., Kuukasjärvi, P., Tarkka, M., Karhunen, P. J., Jula, A., Viikari, J. S., Kähönen, M., Lehtimäki, T., Juonala, M., Ala-Korpela, M., & Raitakari, O. T. (2012). High-throughput quantification of circulating metabolites improves prediction of subclinical atherosclerosis. *European Heart Journal*, *33*(18), 2307-2316. https://doi.org/10.1093/eurheartj/ehs020

Yan, L.-J. (2014). Pathogenesis of chronic hyperglycemia: From reductive stress to oxidative stress. *Journal of Diabetes Research*, *2014*, 137919. https://doi.org/10.1155/2014/137919

Yan-Do, R., & MacDonald, P. E. (2017). Impaired "Glycine"-mia in Type 2 Diabetes and Potential Mechanisms Contributing to Glucose Homeostasis. *Endocrinology*, *158*(5), 1064-1073. https://doi.org/10.1210/en.2017-00148

Yasunari, K., Maeda, K., Nakamura, M., & Yoshikawa, J. (2002). Oxidative stress in leukocytes is a possible link between blood pressure, blood glucose, and C-reacting protein. *Hypertension (Dallas, Tex.: 1979)*, *39*(3), 777-780. https://doi.org/10.1161/hy0302.104670

Zeng, M., Wei, X., Wu, Z., Li, W., Li, B., Zhen, Y., Chen, J., Wang, P., & Fei, Y. (2013). NF-κB-mediated induction of autophagy in cardiac ischemia/reperfusion injury. *Biochemical and Biophysical Research Communications*, *436*(2), 180-185. https://doi.org/10.1016/j.bbrc.2013.05.070

Zhou, D., Huang, C., Lin, Z., Zhan, S., Kong, L., Fang, C., & Li, J. (2014). Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways. *Cellular Signalling*, *26*(2), 192-197. https://doi.org/10.1016/j.cellsig.2013.11.004

Ziegler, A. G., Rewers, M., Simell, O., Simell, T., Lempainen, J., Steck, A., Winkler, C., Ilonen, J., Veijola, R., Knip, M., Bonifacio, E., & Eisenbarth, G. S. (2013). Seroconversion to Multiple Islet Autoantibodies and Risk of Progression to Diabetes in Children. *JAMA*, *309*(23), 2473-2479. https://doi.org/10.1001/jama.2013.6285

Zinman, B., Wanner, C., Lachin, J. M., Fitchett, D., Bluhmki, E., Hantel, S., Mattheus, M., Devins, T., Johansen, O. E., Woerle, H. J., Broedl, U. C., & Inzucchi, S. E. (2015). Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes. *New England Journal of Medicine*, *373*(22), 2117-2128. https://doi.org/10.1056/NEJMoa1504720

Zmijewski, J. W., Banerjee, S., Bae, H., Friggeri, A., Lazarowski, E. R., & Abraham, E. (2010). Exposure to hydrogen peroxide induces oxidation and activation of AMP-activated protein kinase. *The Journal of Biological Chemistry*, *285*(43), 33154-33164. https://doi.org/10.1074/jbc.M110.143685

7 ANNEX I: PUBLICATIONS

Title: Glycated Proteins, Glycine, Acetate, and Monounsaturated Fatty Acids May Act as New Biomarkers to Predict the Progression of Type 2 Diabetes: Secondary Analyses of a Randomized Controlled Trial

Authors: Canet F, Christensen JJ, Victor VM, Hustad KS, Ottestad I, Rundblad A, Sæther T, Dalen KT, Ulven SM, Holven KB, Telle-Hansen VH.

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Glycated Proteins, Glycine, Acetate, and Monounsaturated Fatty Acids May Act as New Biomarkers to Predict the Progression of Type 2 Diabetes: Secondary Analyses of a Randomized Controlled Trial

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Abstract: Food protein or food-derived peptides may regulate blood glucose levels; however, studies have shown inconsistent results. The aim of the present study was to characterize subgroups of individuals with increased risk of type 2 diabetes (T2D) and to investigate the cardiometabolic effects of fish protein in the same subgroups. We first divided participants into high insulin$_{iAUC}$ and low insulin$_{iAUC}$ subjects based on their insulin incremental area under the curve (iAUC) levels after a 2 h oral glucose tolerance test (OGTT), and secondly based on whether they had received 5.2 g salmon fish protein or placebo for 8 weeks, in a previously conducted randomized controlled trial (RCT). We then profiled these groups by analyzing plasma metabolomics and peripheral blood mononuclear cell (PBMC) gene expression. Compared to the low insulin$_{iAUC}$ group, the high insulin$_{iAUC}$ group had higher plasma concentrations of monounsaturated fatty acids (MUFAs) and glycated proteins (GlycA) and lower concentrations of glycine and acetate. After intervention with fish protein compared to placebo, however, only acetate was significantly increased in the low insulin$_{iAUC}$ group. In conclusion, we identified metabolic biomarkers known to be associated with T2D; also, intervention with fish protein did not affect cardiometabolic risk markers in subgroups with increased risk of T2D.

Keywords: diabetes; salmon fish protein; fishmeal; metabolome; transcriptome; metabolic profile

1. Introduction

Type 2 diabetes (T2D) is estimated to affect more than 425 million individuals globally [1]. Impaired insulin secretion due to dysfunctional pancreatic beta-cells and/or peripheral insulin resistance is established characteristics of T2D, giving dysregulated metabolism of carbohydrates, fatty acids, and protein [2]. Prediabetes is an early stage of this continuum and is a high-risk condition for developing T2D [2]. The term prediabetes is increasingly being used to refer to individuals who are at increased risk of developing T2D [3]. Individuals with prediabetes have elevated levels of fasting serum insulin and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), indicating a direct

relationship between prediabetes and insulin resistance [4]. However, individuals with prediabetes are a diverse population in terms of pathophysiology and clinical presentation [2] and may display variations in fasting or postprandial blood glucose concentrations [4]. Excess adipose tissue increases insulin resistance [5], and the prevalence of prediabetes gradually increases with increasing body mass index (BMI) and waist circumference [4]. Moreover, prediabetes alters serum lipid profiles (increased triglycerides and decreased high-density lipoprotein (HDL)) and increases cardiac risk ratios (total-cholesterol/HDL-C and low-density lipoprotein cholesterol (LDL-C)/HDL-C) and resting blood pressure. Thus, prediabetes is also an important predictor of metabolic syndrome [4]. Individuals with prediabetes and/or metabolic syndrome are at high risk of developing T2D and cardiovascular diseases [4].

The ability to predict progression from impaired glucose tolerance to T2D is, therefore, highly valuable for improved prevention. However, identifying those at the highest risk remains challenging. Some new biomarkers for cardiovascular and T2D risk have been suggested, including lipoprotein insulin resistance (LP-IR) score [6–8], and circulating glycoprotein acetyls (GlycA) [6,9] branched-chain amino acids (BCAAs) [6] and glycine [6]. Furthermore, fatty acids are important signaling molecules [10,11], and circulating levels of several fatty acids are increased with insulin resistance [12]. Increased plasma levels of monounsaturated fatty acids (MUFAs) are associated with cardiovascular risk [13] and with increased HbA1c and fasting glucose levels [14]. The effect of fatty acids on diabetic risk is suggested to be through inflammatory mechanisms [15], oxidative stress [16], and hepatic de novo lipogenesis [17]. However, prediabetes is still sub-optimally characterized with respect to a broad risk assessment, and whether these newly suggested biomarkers are more sensitive to dietary intervention in people with increased risk of T2D is still to be elucidated.

There is conclusive evidence from randomized controlled trials (RCT) that intensive lifestyle interventions (diet and/or exercise) [2,18] and medications (such as metformin, alpha-glucosidase inhibitors, and thiazolidinediones) can delay or prevent the onset of T2D in high-risk individuals [2]. Observational follow-up of RCT participants has shown that the beneficial effects of lifestyle interventions may persist over time, improve quality of life, and are both safe and cost-effective [2,19]. A healthy diet is important for both the prevention and management of T2D [20]. Food protein or food-derived peptides have been found to regulate blood glucose levels [21–24]. Although interventions with fish proteins have shown promising effects on cardiometabolic risk markers [25–29] and glycemic regulation in human studies [28,30], these observations are inconsistent [26,31–33]. In a recent RCT, Hustad et al. investigated the effect of an 8-week intervention with salmon fish protein on cardiometabolic risk markers in people with increased risk of T2D [34]. They found that intervention with salmon fish protein had no effect on glycemic regulation in individuals with an increased risk of T2D [34]. However, the participants in the study had large variability in plasma glucose and insulin values. Hence, the lack of a group effect on glycemic regulation could be due to the heterogeneous nature of prediabetes in the studied individuals.

The aim of the present study was to characterize subgroups of individuals with increased risk of T2D who participated in the previous intervention study by Hustad et al. [34]. Participants were grouped based on their insulin incremental area under the curve (iAUC) levels after a 2 h oral glucose tolerance test (OGTT). Metabolome and transcriptome analyses were applied to measure alterations in cardiometabolic risk markers after intake of salmon fish protein in subgroups with low insulin$_{iAUC}$ and high insulin$_{iAUC}$.

2. Materials and Methods

2.1. Subjects

In the present study, we used clinical, metabolome, and transcriptome data from the FishMeal study [34]. The FishMeal study was a human RCT investigating the effects of salmon fish protein intake on cardiometabolic risk markers. The inclusion criteria were non-diabetic men and women ≥20 years and elevated blood glucose defined as either fasting serum (s)-glucose ≥5.6 mmol/L, 2 h OGTT-s-glucose ≥6.5 mmol/L or HbA1c ≥40 mmol/mol (≥5.8%). People with diabetes were excluded from the study. Further description of the exclusion criteria is available in Hustad et al. [34]. The participants were included in an 8-week double-blind, randomized controlled parallel study with 5.2 g of salmon fish protein (corresponding to 7.5 g of fishmeal) compared with a placebo, as previously described. The participants were instructed to limit their fish and seafood intake to a maximum of 150 g per week and otherwise maintain their usual lifestyle habits throughout the study. The salmon fishmeal contained 69.7 g of protein and 13.2 g of fat/100 g. A more detailed description of the content can be found in [34].

The FishMeal human intervention study analyzed data from 74 participants. For the present study, two participants were excluded due to a lack of data on fasting or 2 h insulin concentrations, resulting in a study sample of 72 participants.

2.2. Blood Sampling and Biochemical Routine Measures

Venous blood samples were drawn after an overnight fast (≥10 h). Serum was obtained from silica gel tubes (Becton, Dickinson, and Company, San Jose, CA, USA) and kept at room temperature for 30–60 min until centrifugation (1500× *g*, 15 min). Plasma was obtained from K2EDTA tubes (Becton, Dickinson, and Company), immediately placed on ice, and centrifuged within 10 min (2000× *g*, 4 °C, 15 min).

Serum concentrations of fasting glucose, insulin, HbA1c, triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, high-sensitive C-reactive protein (hsCRP), and glucose and insulin after a 2 h OGTT (75 g glucose load) were measured by standard methods at an accredited routine laboratory (Fürst Medical Laboratory, Oslo, Norway).

2.3. NMR Spectroscopy

Circulating metabolites, before and after the intervention, were quantified at a high throughput proton NMR metabolomics facility (Nightingale Health Ltd., Helsinki, Finland), giving a snapshot of systemic metabolism [35]. The method measures 250 metabolites (lipoprotein subclasses, fatty acids, glycolysis-related metabolites, amino acids, ketone bodies, fluid balance markers, and inflammatory markers) in fasting EDTA plasma.

2.4. Isolation of Peripheral Blood Mononuclear Cells and RNA

Peripheral blood mononuclear cells (PBMCs) from blood samples drawn at fasting, before and after the intervention, were isolated with BD Vacutainer Cell Preparation tubes (Becton, Dickinson). The method is well-documented for PBMCs isolation with more than 90% purity. PBMCs were isolated according to the manufacturer's instructions and stored at −80 °C until RNA isolation. Total RNA was isolated using the RNeasy kit with QIAshredder homogenization of the cell lysates and DNase treatment using the automated protocol for the QIAcube according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The quantity and quality of the isolated RNA were analyzed with the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Gothenburg, Sweden) and the Agilent RNA 6000 Nano kit using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Six participants were excluded due to missing PBMCs and hence RNA, leaving 66 participants for the gene expression analyses.

2.5. Nanostring Gene Expression Assay

RNA expression analysis was performed using the NanoString nCounter system and the nCounter Metabolic Pathways Panel (Nanostring Technologies, https://nanostring.com/support-documents/metabolic-pathways-panel-gene-list/, accessed on 14 October 2022). This panel contains code sets that cover 768 mRNAs annotated to different metabolic pathways, including 20 reference genes, as well as positive and negative controls. All gene name abbreviations are listed in Supplementary Table S1. In addition, the panel was customized by adding 30 additional code sets covering immune and inflammation response and lipid metabolism-related mRNAs (Supplementary Table S2). The procedure was performed according to the manufacturer's instructions for nCounter Panel-Plus with an RNA input of 75 ng per sample.

Samples with an imaging quality control >75%, binding density between 0.1 and 1.8, positive control linearity >0.95, and the lowest positive control (0.5 fm) count higher than 2 SD above the negative controls were included. Samples were normalized to remove both technical and biological variation. The background threshold was set to be the geometric mean of the negative controls. Each sample was normalized to the mean of its positive controls relative to the geometric mean of positive controls in all samples. Finally, all samples were normalized to all 20 reference genes included in the panel (ABCF1, FCF1, SAP130, MRPS5, COG7, TBP, USP39, EDC3, DHX16, UBB, NRDE2, TLK2, DNAJC14, POLR2A, SDHA, G6PD, OAZ1, TBC1D10B, STK11IP, and AGK). The quality control and the technical and biological normalization were performed in the nSolver analysis software version 4.0 (NanoString Technologies). The stability of the 20 reference genes was assessed using NormFinder.

2.6. Statistics and Bioinformatics Analyses

2.6.1. Tools

All analyses were performed in R version 4.1.2 and RStudio IDE (https://www.rstudio.com/, accessed on 2 December 2021). In the following sections, we indicate the packages and specific functions that were relevant in the following format: *package*::*function (setting)*. We used tidyverse tools for data management and visualization [36].

2.6.2. Linear Regression Models

We used the R/Bioconductor software package *limma* [37] for running the linear models and assessing differential metabolite concentrations and gene expressions. We adjusted for age, sex, and BMI in the linear models for the metabolomics data and the gene expression data. We also included smoking as a covariate in the gene expression analyses.

2.6.3. Pre-Processing

We pre-processed the data to optimize downstream modeling. Metabolite concentrations were log-transformed (*base::log1p*), centered, and scaled (*base::scale*). Centering and scaling were conducted to make metabolites directly comparable in the same downstream analysis and forest plot visualization. For the intervention data, we centered and scaled independently the before and after intervention values and then calculated the difference in metabolites concentration. Gene expression data were log transformed (*base::log2*).

2.6.4. Stratification of the Participants and Metabolome and Transcriptome Characterization before Intervention

Before the intervention, the participants in the FishMeal study were divided into three groups based on their insulin incremental area under the curve (iAUC) levels after a 2 h OGTT. We calculated the insulin iAUC using two insulin measurements (fasting and 2 h insulin serum concentration) and the trapezoid method (*DescTools::AUC* [38]). Participants in the lowest tertile (below 33.3%) were classified into the low insulin$_{iAUC}$ group (n = 24), whereas those in the highest tertile (above 66.6%) to the high insulin$_{iAUC}$ group (n = 24). The two groups were characterized and compared for metabolic profiling (includ-

ing metabolites and PBMC gene expression profiles). We included all three groups in the linear modeling and focused on the comparison between the low and high $insulin_{iAUC}$ groups. The linear model of the group characterizations before intervention (*limma::lmFit* followed by *limma::eBayes*) was defined as: metabolite or gene = $insulin_{iAUC}$ group + covariates. We considered that metabolites concentrations and gene transcripts expression with $p < 0.05$ (no FDR correction) were different between the low and high $insulin_{iAUC}$ groups. Supplementary Figure S1 illustrates the outline of the analysis pipeline for the characterization of the subgroups before intervention.

2.6.5. Analyses of Metabolic and Gene Expression Profiles in the Subgroups after Intervention

The same metabolites and gene transcripts were measured after intervention with fish protein or placebo in the low $insulin_{iAUC}$ groups ($n = 16$ and $n = 8$, respectively) and the high $insulin_{iAUC}$ groups ($n = 12$ and $n = 12$, respectively). We assessed changes in plasma metabolite concentrations and changes in PBMC gene expression levels before and after intervention in the low and high $insulin_{iAUC}$ groups.

We defined the linear model (*limma::lmFit* followed by *limma::eBayes*) for the effects of the fish protein intervention as changes in metabolite or gene =$insulin_{iAUC}$ group + treatment group + covariates. Then, we computed the estimated coefficient for the contrasts between fish protein-treated low $insulin_{iAUC}$ vs. placebo-treated low $insulin_{iAUC}$ and fish protein-treated high $insulin_{iAUC}$ vs. placebo-treated high $insulin_{iAUC}$. Metabolite concentrations and gene transcript expressions with $p < 0.05$ (no FDR correction) were considered altered when compared to placebo. Supplementary Figure S2 illustrates the outline of the analysis pipeline of the fish protein intervention.

2.6.6. Gene Set Enrichment Analysis and Competitive Gene Set Testing

We used the list of genes (obtained with *limma::lmFit* followed by *limma::eBayes*) (p values < 0.05, no FDR correction) and searched for "enriched pathways" in order to understand patterns in gene expression that could be different between the low $insulin_{iAUC}$ and high $insulin_{iAUC}$ groups and affected by the fish protein intervention. This consisted of testing if the obtained list of genes over-represented Gene Ontology (GO) terms [39,40] or KEGG pathways [41] more than expected by chance. The lists of genes were tested with the *limma::kegga* function for KEGG pathways and the *limma::goana* function for GO terms. We restricted the universe of genes only to genes found in our nCounter panel (Supplementary Tables S1 and S2). The p-values returned by *limma::goana* and *limma::kegga* functions are unadjusted for multiple testing because GO terms and KEGG pathways are often overlapping, and standard methods of p-value adjustment may be very conservative [42].

To broaden our search, we used the Hallmark gene sets from the Molecular Signatures Database (MSigDB) [43]. The Hallmarks gene sets summarize and represent specific biological states or processes and display coherent expression [43]. With the Hallmark gene sets, we performed competitive gene set testing, which allowed us to distinguish the most important biological process from those that are less important. For this analysis, we used the Hallmark gene set and the Camera method (*limma::camera*) [44]. Because the Camera method also considers fold changes, we could interpret if the biological pathways were up- or downregulated.

3. Results

3.1. Subgroup Characterization before Intervention

For this exploratory study, 72 individuals with a high risk of T2D ($n = 27$ males/45 females) were divided into three groups (tertiles) based on their insulin iAUC values before intervention. The groups with the lowest and the highest $insulin_{iAUC}$ were compared.

Table 1 shows the anthropometric and biochemical characteristics of the study population before intervention. The median age was 53.0 years in the low $insulin_{iAUC}$ group and 61.5 years in the high $insulin_{iAUC}$ group. The two groups had similar BMI (median

BMI in the low insulin$_{iAUC}$ group: 32.4 kg/m^2 and high insulin$_{iAUC}$ group: 33.0 kg/m^2). The proportion of women to men was higher in both groups (low insulin$_{iAUC}$ group: 71% and high insulin$_{iAUC}$ group: 63%). The proportion of participants that used tobacco daily was higher in the high insulin$_{iAUC}$ group (26.3%) compared to the low insulin$_{iAUC}$ group (9.1%). We found differences in parameters related to glucose metabolism and insulin sensitivity (glycemic regulation) between the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ groups as expected, in which the low insulin$_{iAUC}$ group being the one with the lowest glucose 2 h, fasting insulin, insulin 2 h, and HOMA-IR and with the highest Matsuda index (Table 1).

Table 1. Anthropometric and biochemical characteristics of subgroups before intervention.

Variable	Low Insulin$_{iauc}$	High Insulin$_{iauc}$	*p* Value
	n = 24	*n* = 24	
Sex (*n* female, %)	17 (71%)	15 (63%)	
Tobacco use daily (*n*, %)	2 (9.1%)	5 (26.3%)	
Age (y)	53.0 (46.3–64.0)	61.5 (49.5–65.0)	n.s
Weight (kg)	94.9 ± 16.8	100.3 ± 19.0	n.s
BMI (kg m^{-2})	32.4 (28.9–35.1)	33.0 (31.0–36.4)	n.s
f. Glucose (mmol L^{-1})	5.4 (5.0–5.8)	5.2 (5.1–5.7)	n.s
Glucose 2 h (mmol L^{-1})	4.7 ± 1.1	6.9 ± 1.1	***
HbA1c (%)	5.8 ± 0.3	5.9 ± 0.3	n.s
f. Insulin (pmol L^{-1})	56 (44–92)	124 (102–196)	***
Insulin 2 h (pmol L^{-1})	154 (120–225)	804 (661–1152)	***
Insulin iAUC (pmol h L^{-1})	113 (60–163)	766 (647–1058)	***
HOMA-IR	2.4 (1.7–3.7)	4.9 (3.7–5.8)	***
Matsuda index	6.9 (4.9–10.7)	1.8 (1.1–2.1)	***
Triglycerides (mmol L^{-1})	1.4 ± 0.7	1.6 ± 0.6	n.s
Total cholesterol (mmol L^{-1})	5.3 ± 1.3	5.0 ± 0.8	n.s
HDL-C (mmol L^{-1})	1.4 (1.2–1.8)	1.2 (1.1–1.4)	n.s
LDL-C (mmol L^{-1})	3.5 (2.6–4.3)	3.3 (2.9–4.2)	n.s
ApoA1 (g L^{-1})	1.7 (1.5–1.8)	1.5 (1.4–1.7)	n.s
Apo B (g L^{-1})	1.1 ± 0.3	1.0 ± 0.2	n.s
hsCRP (mg L^{-1})	3.4 (2.1–4.6)	4.2 (2.6–7.3)	n.s
Systolic BP (mm Hg)	117 ± 13	123 ± 17	n.s
Diastolic BP (mm Hg)	69 (58–78)	71 (66–78)	n.s

*** $p < 0.001$ low insulin$_{iAUC}$ vs. high insulin$_{iAUC}$. Parametric data are expressed as mean ± SD, and non-parametric data as median and interquartile range. Differences between tertiles were assessed using an ANOVA with Tukey posthoc test for parametric variables or Kruskal-Wallis rank sum test with Pairwise Wilcoxon Rank Sum Tests for non-parametric variables. Abbreviations: ApoA1: apolipoprotein A-I, Apo B: apolipoprotein B, BMI: body mass index, BP: blood pressure, f.: fasting, HbA1c: hemoglobin A1c, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostatic model assessment for insulin resistance, hsCRP: high sensitivity C-reactive protein, iAUC: incremental Area Under the Curve, LDL-C: low-density lipoprotein cholesterol, n.s: not significant.

3.2. Subgroup Differences in Metabolic Profile before Intervention

To characterize individuals in the two subgroups (low insulin$_{iAUC}$ and high insulin$_{iAUC}$), we measured the plasma concentration of 250 metabolites. Compared to the low insulin$_{iAUC}$ group, the high insulin$_{iAUC}$ group had a higher ratio of MUFAs to total fatty acids (MUFA%) (Figure 1), lower levels of glycine and acetate, and a higher level of the inflammatory marker GlycA (Figure 2). Although there were no significant differences between the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ groups regarding BCAAs, we observed a tendency to a higher individual ($p = 0.09$ for valine) and total BCAAs ($p = 0.10$) in the high insulin$_{iAUC}$ group compared to the low insulin$_{iAUC}$ group (Figure 2).

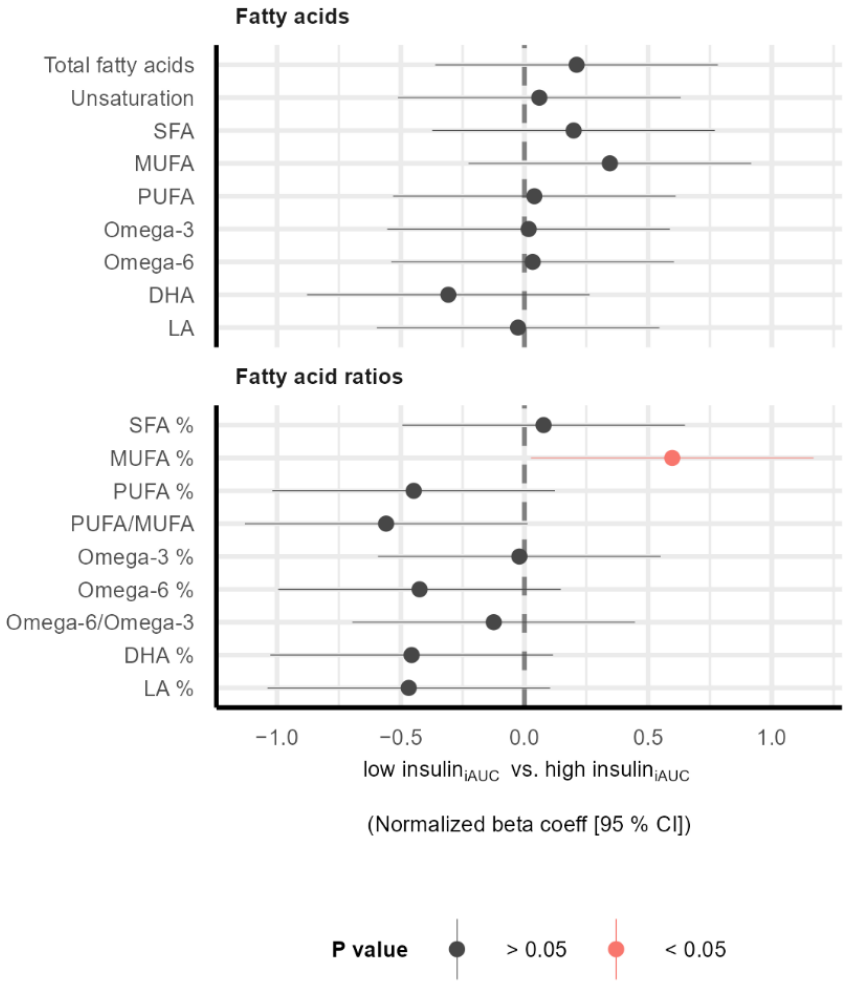

Figure 1. Fatty acids profile in plasma before intervention. The forest plot displays the normalized beta coefficients (mean difference) and 95% confidence interval for the difference between the low insulin$_{iAUC}$ and high insulin$_{iAUC}$groups. Estimates on the left and right side of the zero-line translate to lower and higher in the high insulin$_{iAUC}$ group compared to the low insulin$_{iAUC}$ group, respectively. Color denotes the *p* value. Abbreviations: DHA: Docosahexaenoic acid, DHA%: Ratio of docosahexaenoic acid to total fatty acids, iAUC: incremental Area Under the Curve, LA: Linoleic acid, LA%: Ratio of linoleic acid to total fatty acids, MUFA: Monounsaturated fatty acids, MUFA%: Ratio of monounsaturated fatty acids to total fatty acids, Omega-3%: Ratio of omega-3 fatty acids to total fatty acids, Omega-6%: Ratio of omega-6 fatty acids to total fatty acids, PUFA: Polyunsaturated fatty acids, PUFA%: Ratio of polyunsaturated fatty acids to total fatty acids, SFA: Saturated fatty acids, SFA%: Ratio of saturated fatty acids to total fatty acids, Unsaturation: Degree of unsaturation.

There were no differences in total cholesterol levels or lipid composition of lipoproteins between the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ groups (Supplementary Figure S3) neither on the LP-IR score (data not shown). Despite this, we observed a tendency coherent with higher LP-IR score in the high insulin$_{iAUC}$ compared to the low insulin$_{iAUC}$ group: higher levels of large very low-density lipoproteins (VLDL) particles and lower levels of large HDL particles (Supplementary Figure S4). See Supplementary Table S3 for more information about all the NMR metabolites, fold-changes, *p* values, and adjusted *p* values.

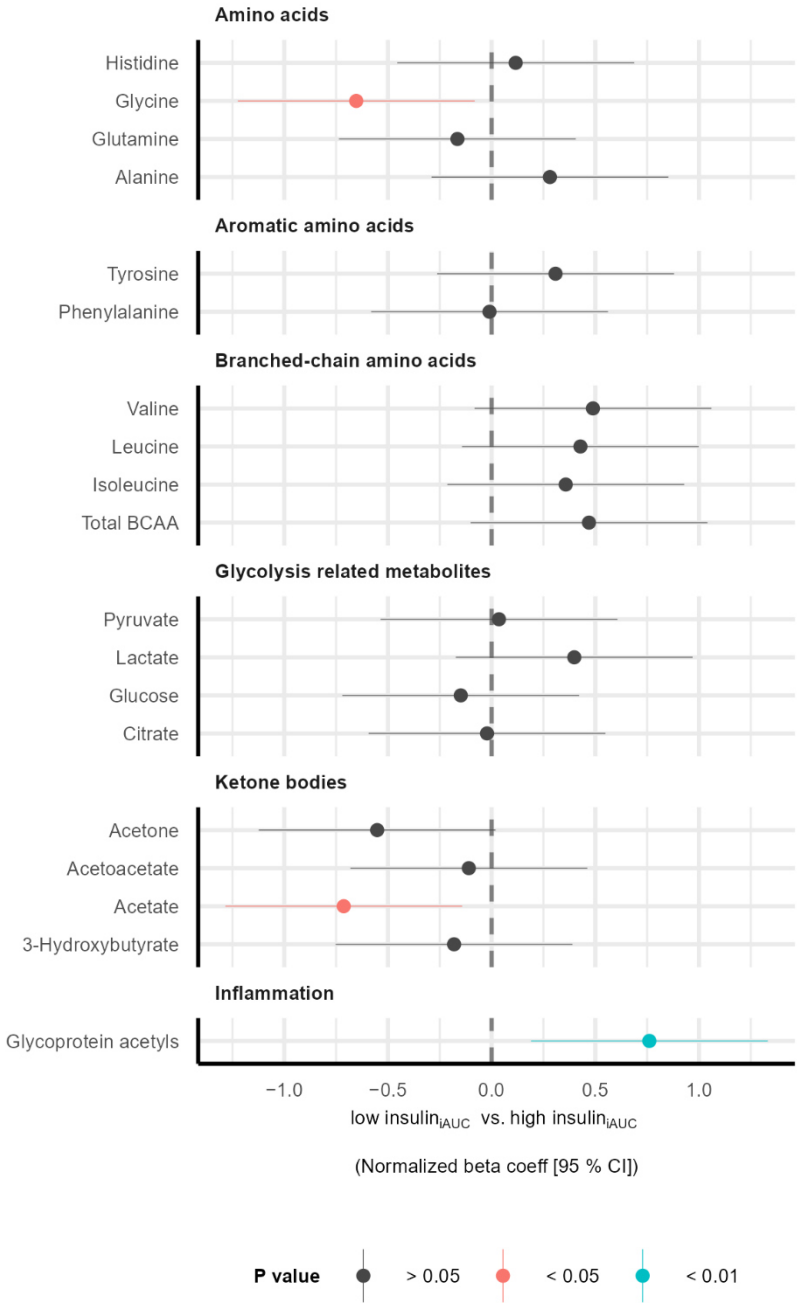

Figure 2. Amino acids, glycolysis-related metabolites, ketone bodies, and inflammation markers concentration in plasma before intervention. The forest plot displays the normalized beta coefficients (mean difference) and 95% confidence interval for the difference between the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ groups. Estimates on the left and right side of the zero-line translate to lower and higher in the high insulin$_{iAUC}$ group compared to the low insulin$_{iAUC}$ group, respectively. Color denotes the *p* value. Abbreviations: BCAA: Branched-chain amino acids, iAUC: incremental Area Under the Curve.

3.3. Subgroup Differences in Gene Expression before Intervention

We measured the gene expression of 778 gene transcripts involved in immunometabolism in PBMCs to investigate if the observed differences in insulin sensitivity and metabolites between the groups could be associated with differences in gene expression. We found 39 genes with a different expressions between the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ groups at a nominal significance level of 0.05 (Figure 3). *CPT1A* (carnitine palmitoyl transferase 1A) was the most differentially expressed gene in terms of *p* value ($p = 0.0004$) and fold change

(log2 fold-change = 0.24) and was higher expressed in the high insulin$_{iAUC}$ group compared to the low insulin$_{iAUC}$ group (Figure 3). However, none of the genes were significantly different between the groups after adjustment for false discovery rate (FDR < 0.1). See Supplementary Table S4 for more information about all the tested gene transcripts, fold-changes, *p* values, and adjusted *p* values.

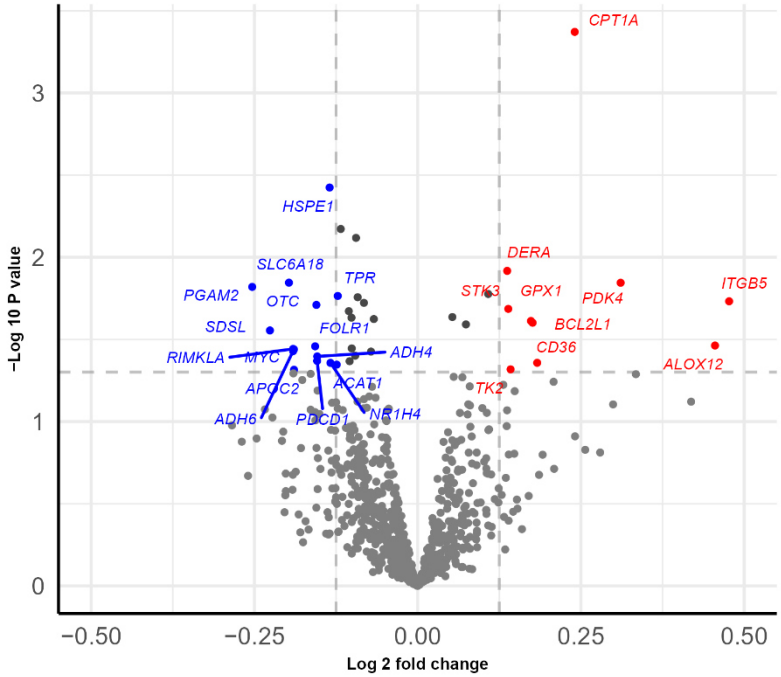

Figure 3. Volcano plot of differentially expressed genes before intervention. The linear model was adjusted by age, sex, BMI, and daily use of tobacco. Genes with *p* value < 0.05 (no FDR correction) and with an absolute fold change above 1.10 were highlighted. Genes on the left side of the volcano plot are down-regulated, and those on the right are up-regulated in the high insulin$_{iAUC}$ group compared to the low insulin$_{iAUC}$ group. Abbreviations: *ACAT1*: acetyl-CoA acetyltransferase 1, *ADH4*: alcohol dehydrogenase 4 (class II) pi polypeptide, *ADH6*: Alcohol dehydrogenase 6 (class V), *ALOX12*: arachidonate 12-lipoxygenase 12S type, *APOC2*: apolipoprotein C2, *BCL2L1*: BCL2 such as 1, *CD36*: CD36 molecule (alternatively *FAT*: Fatty acid traslocase, or *SCARB3*: Scavenger receptor class B member 3), *CPT1A*: carnitine palmitoyltransferase 1A, *DERA*: deoxyribose-phosphate aldolase, *FOLR1*: folate receptor alpha, *GPX1*: glutathione peroxidase 1, *HSPE1*: heat shock protein family E (Hsp10) member 1, iAUC: incremental Area Under the Curve, *ITGB5*: integrin subunit beta 5, *MYC*: MYC proto-oncogene, bHLH transcription factor, *NR1H4*: nuclear receptor subfamily 1 group H member 4 (alternatively *FXR*: Farnesol Receptor HRR-1), *OTC*: ornithine transcarbamylase, *PDCD1*: programmed cell death 1, *PDK4*: pyruvate dehydrogenase kinase 4, *PGAM2*: phosphoglycerate mutase 2, *RIMKLA*: ribosomal modification protein rimK such as family member A, *SDSL*: serine dehydratase like, *SLC6A18*: solute carrier family 6 member 18, *STK3*: serine/threonine kinase 3, *TK2*: thymidine kinase 2, *TPR*: translocated promoter region, nuclear basket protein.

3.4. Gene Set Enrichment Analysis and Competitive Gene Set Testing before Intervention

To understand patterns in gene expression between the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ groups, we performed a gene enrichment analysis including the 39 genes with different expression ($p < 0.05$, without FDR correction) and using KEGG pathways and Gene Ontology (GO) terms. The top three KEGG pathways that showed enrichment in the high insulin$_{iAUC}$ compared with the low insulin$_{iAUC}$ group were thyroid cancer, ribosome, and fatty acid degradation (Table 2). In concordance, the top three GO terms (molecular function) were rRNA binding, large ribosomal subunit rRNA binding, and core promoter sequence-specific DNA binding. Furthermore, we performed a competitive gene set test (CAMERA) using the hallmark gene sets from MSigDB. Comparing the low insulin$_{iAUC}$

and high insulin$_{iAUC,}$ we found that the two most important altered gene sets were those related to MYC targets V1 (FDR = 0.0005) and MYC targets V2 (FDR = 0.02), which were both downregulated in the high insulin$_{iAUC}$ group (Table 3).

Table 2. Enriched KEGG pathways and top 5 GO terms before intervention.

	Pathway Name	Ratio	*p* Value	Genes Symbol
KEGG Pathway ID				
hsa05216	Thyroid cancer	4/12	2.00×10^{-3}	MYC, MAPK1, TP53, TPR
hsa03010	Ribosome	2/2	2.45×10^{-3}	RPLP0, RPL23
hsa00071	Fatty acid degradation	4/17	8.02×10^{-3}	ACAT1, ADH4, ADH6, CPT1A
hsa05219	Bladder cancer	3/14	2.89×10^{-2}	MYC, MAPK1, TP53
GO term ID				
GO:0019843	rRNA binding	3/3	1.17×10^{-4}	NPM1, RPLP0, RPL23
GO:0070180	large ribosomal subunit rRNA binding	2/2	2.45×10^{-3}	RPLP0, RPL23
GO:0001046	core promoter sequence-specific DNA binding	3/7	3.55×10^{-3}	MYC, NPM1, TP53
GO:0003735	structural constituent of ribosome	2/3	7.12×10^{-3}	RPLP0, RPL23
GO:0003723	RNA binding	7/50	9.25×10^{-3}	HSPE1, IMPDH2, NPM1, RPLP0, TP53, TPR, RPL23

For KEGG pathways and GO terms (molecular function), we performed a gene enrichment analysis using the list of 39 differentially expressed genes (p value < 0.05, no FDR correction) obtained with the adjusted linear model as input. All the analyses were performed using the genes in the nCounter Human Metabolic Pathways Panel plus 30 additional genes from the custom panel (Supplementary Tables S1 and S2). The displayed pathways are differentially expressed in the high insulin$_{iAUC}$ group compared to the low insulin$_{iAUC}$ group. Pathways are sorted by p value. The ratio column represents the number of genes with differential expression in that set and the total number of genes in that pathway. The symbols of the differentially expressed genes in each pathway are shown.

Table 3. Top 10 differentially expressed hallmark gene sets (MSigDB) before intervention.

Hallmark (MSigDB) Gene Sets	Number of Genes in the SET	Direction	*p* Value	FDR
MYC targets V1	22	Down	1.18×10^{-5}	5.92×10^{-4}
MYC targets V2	6	Down	9.73×10^{-4}	2.43×10^{-2}
IL6 JAK STAT3 signaling	19	Up	3.15×10^{-3}	5.05×10^{-2}
Epithelial mesenchymal transition	11	Up	5.12×10^{-3}	5.05×10^{-2}
TGF beta signaling	4	Up	5.63×10^{-3}	5.05×10^{-2}
Wnt beta catenin signaling	4	Down	6.05×10^{-3}	5.05×10^{-2}
TNFA signaling via NFKB	31	Up	1.55×10^{-2}	1.11×10^{-1}
Apoptosis	21	Up	2.63×10^{-2}	1.64×10^{-1}
Protein secretion	6	Up	3.26×10^{-2}	1.69×10^{-1}
Interferon gamma response	28	Up	3.37×10^{-2}	1.69×10^{-1}
Angiogenesis	2	Up	4.46×10^{-2}	2.03×10^{-1}

For hallmark gene sets, we performed a competitive gene set test named CAMERA. The analysis was performed using the genes in the nCounter Human Metabolic Pathways Panel plus 30 additional genes from the custom panel (Supplementary Tables S1 and S2). The displayed gene sets are differentially expressed in the high insulin$_{iAUC}$ group compared to the low insulin$_{iAUC}$ group. Gene sets are sorted by p value. The Direction column indicates if the gene set is down- or up-regulated in the high insulin$_{iAUC}$ group compared to the low insulin$_{iAUC}$ group. Abbreviations: FDR: False Discovery Rate, IL6: Interleukin-6, JAK: Janus Kinase, MSigDB: Molecular Signature Database, MYC: Myc proto-oncogene bHLH transcription factor, NFKB: Nuclear factor-kappa B, STAT3: Signal Transducer and Activator of Transcription 3, TGF beta: Transforming Growth Factor Beta, TNFA: Tumor Necrosis Factor-Alpha.

3.5. Characterization of the Study Population in the Different Intervention Groups

We further investigated the effect of intervention with salmon fish protein on cardiometabolic risk markers in the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ subgroups. Table 4 shows the anthropometric and biochemical characteristics of the study population allocated into distinct groups (low insulin$_{iAUC}$ and high insulin$_{iAUC}$) and treatments (placebo or fish protein) before the intervention. We observed the same pattern in glucose metabolism and insulin sensitivity as before subdividing into intervention groups: individuals in both low

insulin$_{iAUC}$ groups had lower glucose 2 h, fasting insulin, insulin 2 h, and HOMA-IR and higher Matsuda index, compared to individuals in the high insulin$_{iAUC}$ groups (Table 4). We did not find significant differences in glycemic regulation (glucose, insulin, HOMA-IR, and HbA1c) after the intervention between individuals receiving a placebo or fish protein for the low insulin$_{iAUC}$ or the high insulin$_{iAUC}$ groups (data not shown).

Table 4. Anthropometric and biochemical characteristics of the study population in the different intervention groups.

	Low Insulin$_{iauc}$ Placebo	**Low Insulin$_{iauc}$ Fish Protein**	**High Insulin$_{iauc}$ Placebo**	**High Insulin$_{iAUC}$ Fish Protein**	***p* Value**
	n = 8	*n* = 16	*n* = 12	*n* = 12	
Sex (*n* female, %)	5 (62%)	12 (75%)	9 (75%)	6 (50%)	
Tobacco use daily (*n*, %)	2 (25%)	0 (0%)	2 (10%)	3 (25%)	
Age (y)	56.9 ± 13.9	52.1 ± 10.6	59.0 ± 9.2	56.1 ± 10.6	n.s
Weight (kg)	92.9 ± 19.6	95.9 ± 15.8	97.0 ± 13.9	104.0 ± 23.3	n.s
BMI (kg/m^2)	30.2 (28.5–34.1)	32.8 (29.4–35.7)	34.1 (31.2–36.0)	32.9 (30.8–37.6)	n.s
f. Glucose (mmol L^{-1})	5.8 (5.0–6.2)	5.2 (5.0–5.6)	5.3 (5.0–5.7)	5.2 (5.1–5.8)	n.s
Glucose 2 h (mmol L^{-1})	4.9 ± 1.1	4.7 ± 0.4	6.8 ± 1.4	6.9 ± 0.8	**, ###
HbA1c (%)	5.9 (5.7–6.1)	5.8 (5.7–5.9)	5.7 (5.6–5.8)	5.9 (5.7–6.3)	n.s
f. Insulin (pmol L^{-1})	73 ± 65	66 ± 61	139 ± 70	147 ± 62	**, ###
Insulin 2 h (pmol L^{-1})	148 (133–228)	164 (115–218)	764 (685–1152)	861 (661–1171)	***, ###
Insulin iAUC (pmol h L^{-1})	116 (73–163)	113 (53–159)	766 (647–1058)	830 (638–1078)	***, ###
HOMA-IR	2.2 (1.6–3.2)	2.4 (1.8–3.7)	4.3 (3.4–8.6)	5.1 (4.4–7.6)	###
Matsuda index	6.2 (4.6–11.2)	6.8 (5.1–8.6)	1.8 (1.2–2.2)	1.7 (1.1–2.0)	***, ###
Triglycerides (mmol L^{-1})	1.2 ± 0.4	1.5 ± 0.4	1.6 ± 0.7	1.6 ± 0.4	n.s
Total cholesterol (mmol L^{-1})	5.0 ± 0.8	5.4 ± 1.6	4.9 ± 0.8	5.2 ± 0.8	n.s
HDL-C (mmol L^{-1})	1.5 (1.2–1.8)	1.4 (1.2–1.8)	1.3 (1.1–1.5)	1.2 (1.0–1.3)	n.s
LDL-C (mmol L^{-1})	3.2 ± 0.6	3.7 ± 1.0	3.1 ± 0.2	3.8 ± 1.0	n.s
ApoA1 (gL^{-1})	1.8 (1.5–1.8)	1.7 (1.5–1.8)	1.6 (1.4–1.7)	1.5 (1.4–1.6)	n.s
ApoB (gL^{-1})	0.9 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	n.s
hsCRP (mg L^{-1})	4.0 ± 2.4	3.5 ± 3.1	4.6 ± 3.0	5.4 ± 3.1	n.s
Systolic BP (mm Hg)	119 ± 16	116 ± 13	124 ± 20	122 ± 13	n.s
Diastolic BP (mm Hg)	68 ± 13	70 ± 11	70 ± 8	71 ± 11	n.s

** $p < 0.01$ and *** $p < 0.001$ low insulin$_{iAUC}$ placebo vs. high insulin$_{iAUC}$ placebo. ### $p < 0.001$ low insulin$_{iAUC}$ fish protein vs. high insulin$_{iAUC}$ fish protein. Parametric data are expressed as mean ± SD, and non-parametric data as median and interquartile range. Differences between groups were assessed using an ANOVA with Tukey posthoc test for parametric variables or Kruskal-Wallis rank sum test with Pairwise Wilcoxon Rank Sum Tests for non-parametric variables. Abbreviations: ApoA1: apolipoprotein A-I, Apo B: apolipoprotein B, BMI: body mass index, BP: blood pressure, f: fasting, HbA1c: hemoglobin A1c, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostatic model assessment for insulin resistance, hsCRP: high sensitivity C-reactive protein, iAUC: incremental Area Under the Curve, LDL-C: low-density lipoprotein cholesterol, n.s: not significant.

3.6. Effect on Metabolic Profile after Fish Protein Intervention

We investigated the effect on plasma metabolites after 8-weeks of salmon fish protein intake compared to placebo in the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ subgroups. We observed that fish protein-treated low insulin$_{iAUC}$ participants had higher acetate levels than placebo-treated low insulin$_{iAUC}$ participants (Figure 4). We did not find any other significant changes in plasma metabolites in the low insulin$_{iAUC}$ or high insulin$_{iAUC}$ groups after the intervention (Supplementary Figures S5–S7). See Supplementary Tables S5 and S6 for more information about all the NMR metabolites, fold-changes, *p* values, and adjusted *p* values.

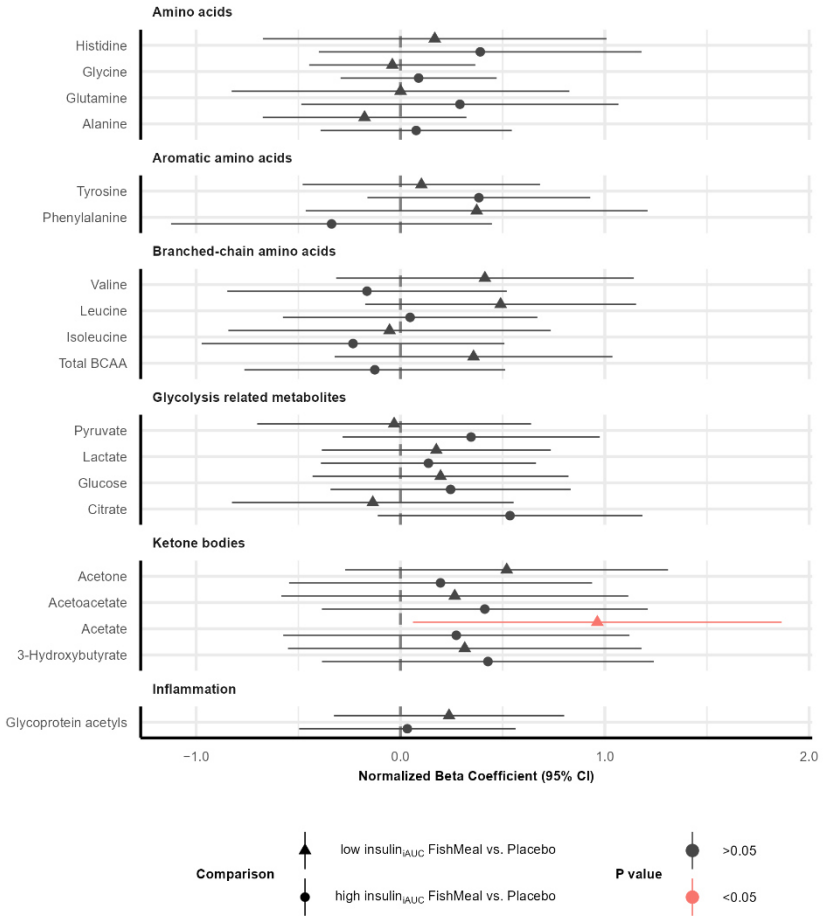

Figure 4. Changes in amino acids, glycolysis-related metabolites, ketone bodies, and inflammation markers concentration in plasma after the intervention. The forest plot displays the normalized beta coefficients (mean difference) and 95% confidence interval for the differences between the fish protein-treated low insulin$_{iAUC}$ group and placebo-treated low insulin$_{iAUC}$ group (triangles) and the differences between the fish protein-treated high insulin$_{iAUC}$ group and placebo-treated high insulin$_{iAUC}$ group (circles). Estimates on the left and right side of the zero-line translate to lower and higher in individuals that received the fish protein than those that received the placebo, respectively. Color denotes the *p* value. Abbreviations: BCAA: Branched-chain amino acids, iAUC: incremental Area Under the Curve.

3.7. Effect on Gene Expression after Fish Protein Intervention

Fold changes in gene expression were evaluated from before to after intervention with fish protein compared to placebo in the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ groups. We found nine genes with altered expression in the fish protein-treated low insulin$_{iAUC}$ group compared to the placebo-treated low insulin$_{iAUC}$ group and 14 genes with altered expression in the fish protein-treated high insulin$_{iAUC}$ group compared to placebo-treated high insulin$_{iAUC}$ group, with a significance level below 0.05. After adjusting for FDR, none of the genes showed significant changes. Fold changes in gene expression during dietary interventions are typically not large, and in our study, the most altered genes in terms of fold change were *AMDHD1* (Amidohydrolase Domain-Containing Protein 1, log2 fold-change = 0.43) and *CD274* (CD274 molecule, log2 fold-change = −0.43) in the fish protein-treated low insulin$_{iAUC}$ compared to placebo-treated low insulin$_{iAUC}$ group. At the same time, *ABCA1* (ATP Binding Cassette Subfamily A Member 1, log2 fold-change = 0.32) and *IDO1* (Indoleamine 2,3-Dioxygenase 1, log2 fold-change = −0.51) were the most altered genes in the fish protein-treated high insulin$_{iAUC}$ group compared to placebo-treated high insulin$_{iAUC}$ group (Figure 5). We did not conduct gene enrichment analysis due to the small number of differentially expressed genes after the intervention. See Supplementary

Tables S7 and S8 for more information about all the genes, fold changes, *p* values, and adjusted *p* values.

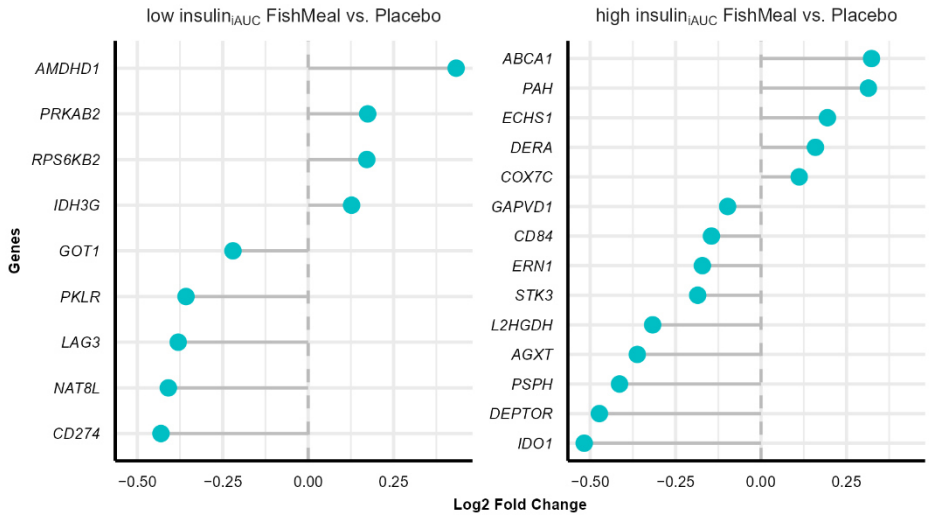

Figure 5. Differentially expressed genes in the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ groups after the intervention. The figure shows the differentially expressed genes (*p* value < 0.05, no FDR correction) after the intervention in the low insulin$_{iAUC}$ and high insulin$_{iAUC}$ groups, sorted by fold change (blue dots). The genes with altered expression were obtained using a linear model adjusted for covariates (age, sex, BMI, and daily use of tobacco). Fold change represent the difference in PBMCs gene expression between fish protein-treated low insulin$_{iAUC}$ and placebo-treated low insulin$_{iAUC}$ group (**left panel**) and the difference between fish protein-treated high insulin$_{iAUC}$ and placebo-treated high insulin$_{iAUC}$ (**right panel**). Abbreviations: *ABCA1*: ATP binding cassette subfamily A member 1, *AGXT*: alanine-glyoxylate and serine-pyruvate aminotransferase, *AMDHD1*: amidohydrolase domain containing 1, *CD274*: CD274 molecule, *CD84*: CD84 molecule, *COX7C*: cytochrome c oxidase subunit 7C, *DEPTOR*: DEP domain containing MTOR interacting protein, *DERA*: deoxyribose-phosphate aldolase, *ECHS1*: enoyl-CoA hydratase, short chain 1, *ERN1*: endoplasmic reticulum to nucleus signaling 1, *GAPVD1*: GTPase activating protein and VPS9 domains 1, *GOT1*: glutamic-oxaloacetic transaminase 1, IDH3G: Isocitrate Dehydrogenase (NAD(+)) 3 Non-Catalytic Subunit Gamma, *IDO1*: indoleamine 2,3-dioxygenase 1, iAUC: incremental Area Under the Curve, *L2HGDH*: L-2-hydroxyglutarate dehydrogenase, *LAG3*: lymphocyte activating 3, *NAT8L*: N-acetyltransferase 8 like, *PAH*: phenylalanine hydroxylase, *PKLR*: pyruvate kinase L/R, *PRKAB2*: protein kinase AMP-activated non-catalytic subunit beta 2, *PSPH*: phosphoserine phosphatase, *RPS6KB2*: ribosomal protein S6 kinase B2, *STK3*: serine/threonine kinase 3.

4. Discussion

In the present study, we investigated the metabolic and gene expression profile of subgroups of participants with different insulin responses to identify biomarkers of cardiometabolic risk and further determine whether any of these subgroups respond to an 8-week intervention with fish protein. Before the intervention, we found that the plasma level of GlycA and MUFAs, and the gene expression of *CPT1A* were higher, and glycine and acetate levels were lower in individuals in the high insulin$_{iAUC}$ group compared to individuals in the low insulin$_{iAUC}$ group. After the intervention, only acetate was increased in the low insulin$_{iAUC}$ group treated with fish protein compared to the placebo-treated low insulin$_{iAUC}$ group. However, none of the metabolites and gene transcripts before and after the intervention were significantly altered after adjustment for FDR, and hence the results should be carefully interpreted.

We found that GlycA levels were higher in the high insulin$_{iAUC}$ group, that is, the group with less insulin response (increased 2 h glucose, fasting insulin, 2 h insulin, and reduced Matsuda index), compared to the low insulin$_{iAUC}$ group before intervention. GlycA is shown to be positively correlated with insulin resistance, BMI, markers of metabolic syn-

drome, and the ratio of leptin to adiponectin [45,46]. Furthermore, GlycA is associated with different inflammatory markers and is considered a biomarker of systemic inflammation and subclinical vascular inflammation [47]. Hence, GlycA is suggested as a new biomarker of cardiometabolic disease and T2D risk [19,22]. Although GlycA levels were higher in the high $insulin_{iAUC}$ group compared to the low $insulin_{iAUC}$ group, we did not observe any significant difference in hsCRP between the groups (Tables S1 and S4). The high $insulin_{iAUC}$ group showed a tendency to a higher hsCRP concentration, and possibly we did not reach statistical significance due to a small sample size, intra-individual variations in hsCRP, or hsCRP did not capture the whole inflammatory process in our participants. This suggests that GlycA may act as a more precise risk marker of CVD and T2D compared to hsCRP [48]. Epidemiological studies supporting this hypothesis found that the association between GlycA and cardiovascular events, or T2D, was slightly attenuated by the addition of hsCRP to the regression model [48].

Before the intervention, we found that the high $insulin_{iAUC}$ group had a higher level of MUFAs to total fatty acids ratio compared to the low $insulin_{iAUC}$ group. In the NHANES study, the higher plasma concentrations of SFAs and MUFAs were associated with elevated HbA1c and fasting glucose levels [14], which is in line with our results. Furthermore, previous research has also shown that higher MUFA levels and lower PUFA and linolic acid levels are associated with increased cardiovascular risk in the FINNRISK study [13]. Hence, high circulating MUFA and low PUFA levels are linked to increased cardiovascular risk.

CPT1A was upregulated in the high $insulin_{iAUC}$ group compared to the low $insulin_{iAUC}$ group before intervention and was one of the most differentially expressed genes in terms of the *p* value. The CPT1A protein, a key regulatory enzyme of β-oxidation, is in the outer membrane of the mitochondria. CPT1A facilitates the translocation of long-chain fatty acids across the mitochondrial membrane for fatty acid β-oxidation. Hence, our results may reflect a shift in metabolic sources from carbohydrates to fatty acids in the high $insulin_{iAUC}$ group. In accordance with our observations, the Framingham Heart Study (FHS) Offspring Cohort found a positive association between mRNA expression of *CPT1A* in whole blood with fasting glucose and triglycerides, as well as BMI. Moreover, it was described that fat intake was negatively associated with *CPT1A* methylation, therefore, increasing *CPT1A* gene expression [49]. In an animal study, mitochondrial dysfunction caused by a high-fat diet was linked to insulin resistance in muscle, implying that excessive CPT1A activity overloads the mitochondria resulting from incomplete oxidation of long-chain fatty acids [50].

In our population, we found a tendency for a higher BCAAs plasma concentration in the high $insulin_{iAUC}$ group compared to the low $insulin_{iAUC}$ group before the intervention (Figure 2). We reached significance for valine and total BCAAs without age, sex, and BMI correction (data not shown). Both BCAAs and aromatic amino acids have been positively correlated with insulin resistance in T2D [51,52], and BCAAs are suggested as a metabolic signature associated with insulin resistance [53,54]. Increased levels of BCAAs may be a causal factor for developing insulin resistance and T2D by hampering insulin signaling pathways [55], and the accumulation of toxic BCAA metabolites triggers mitochondrial dysfunction [56].

Before the intervention, the individuals in the high $insulin_{iAUC}$ group had lower levels of plasma glycine compared to the low $insulin_{iAUC}$ group. In line with our results, most studies suggest an inverse association between glycine and prediabetes and T2D [51]. People with nondiabetic insulin resistance or impaired glucose tolerance have reduced circulating glycine [52]. It is unclear if reduced levels of glycine have an active role in the development of T2D, but it has been observed that interventions with delayed or reversed T2D (bariatric surgery or physical activity) are associated with an increase in circulating glycine concentrations [57–59]. Furthermore, glycine supplementation has been shown to give enhanced insulin response and glucose tolerance [60,61]. The consumption of 5.2 g of salmon protein (corresponding to 7.5 g fishmeal) was enough to significantly increase the post-prandial concentration of several amino acids, especially glycine [62]. However, in

the present intervention study lasting for 8 weeks, we did not find an effect of fish protein on glucose metabolism, insulin response, or plasma glycine concentration in subgroups of individuals with increased T2D risk. Our fishmeal supplement provided 389 mg of glycine per day, which is considerably lower than the dosages used by others, including 1 mmol glycine/kg lean body mass (~5.63 g for a 75 kg patient) [60] and 5 g of glycine [61]. There are many metabolic effects of circulating glycine, and glycine supplementation may impact glucose tolerance [59]. Current evidence point to a glycine effect in the brain via dorsal vagal complex N-methyl-D-aspartate (NMDA) receptors, systemically reducing oxidative stress and inflammatory response and increasing insulin secretion in the islets via glycine receptors (GlyRs) [59].

Increased glucose levels promote reactive oxygen species (ROS) overproduction [63], resulting in morphological changes in mitochondria [64]. Excessive ROS could damage the proteins, lipids, and DNA in the mitochondria, leading to mitochondrial dysfunction and reduced mitochondria biogenesis [64]. Several studies have found a link between mitochondrial dysfunction and insulin resistance in various tissues [64]. We found a down-regulation in the Myc targets V1 and V2 pathways in the high insulin$_{iAUC}$ group (Table 3), which could be a sign of reduced mitochondrial biogenesis, as Myc induces mitochondrial biogenesis and increases mitochondrial function through many pathways [65]. Moreover, alterations in the oxidizing environment of the endoplasmic reticulum (ER) can induce ER stress. There is evidence that beta-cell ER stress in patients with T2D can cause beta-cell dysfunction by decreasing insulin synthesis and secretion [66]. This could be a reason for the ribosome-enriched pathway found in the high insulin$_{iAUC}$ group before intervention (Table 2).

We observed lower concentrations of acetate in the high insulin$_{iAUC}$ compared to the low insulin$_{iAUC}$ group before intervention (Figure 2). In contrast, acetate was associated with prediabetes and T2D in other studies [51]. Acetate is a short-chain fatty acid produced by microbiota. The microbiome is a potential source of biologically active metabolites, frequently linked with diet composition [67]. It has been shown in healthy subjects that prebiotics increased plasma glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) concentrations, whereas postprandial plasma glucose response decreased after a standardized meal [68]. In another RCT, healthy individuals treated with long-chain MUFAs derived from fish oil increased GLP-1 secretion. The proposed mechanism is linked to gut microbiota producing short-chain fatty acids that act through the G protein-coupled receptors expressed on enteroendocrine cells, enteric neurons, and enteric leukocytes [69]. In our study, acetate was increased in the low insulin$_{iAUC}$ group after fish protein intervention compared to placebo treatment, while no change was observed in the high insulin$_{iAUC}$ group. Recent studies show that gut microbial composition may help to identify individuals who may benefit from dietary interventions [70].

This study has several strengths and limitations. One strength is the detailed profiling of both metabolites and gene expressions in subgroups of people with increased T2D risk. The small number of participants in the phenotypic subgroups in the intervention is a limitation of the study, giving a higher risk of both false positive and false negative findings. The results from small studies cannot be generalized to the population as a whole. Furthermore, the inclusion criteria for participation were increased risk of T2D, and hence the lack of a healthy control group is a limitation of the present study. Lastly, the short-term duration of the present study does not reflect the long-term effects. Despite this, we were able to identify metabolic biomarkers (GlycA, MUFA %, glycine, and acetate), as well as candidate gene expression patterns in the group of participants that displayed high insulin$_{iAUC}$.

5. Conclusions

In conclusion, our results support that several plasma metabolites (GlycA, MUFA %, glycine, and acetate) may serve as biomarkers to predict the progression of T2D; however, the usefulness of these needs further testing and validation in prospective studies. Whether these newly suggested biomarkers are more sensitive to dietary interventions in people with increased risk of T2D is still to be elucidated.

Results from this and similar intervention studies are important for hypothesis generation in terms of pathophysiology and clinical manifestation in subgroups of individuals in heterogeneous populations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14235165/s1, Figure S1: Methods flow chart before intervention; Figure S2: Methods flow chart after the intervention; Figure S3: Cholesterol concentration, lipoprotein sizes, and lipid composition and apolipoprotein concentration in plasma before intervention; Figure S4: Lipoprotein subclasses concentration and composition in plasma before intervention; Figure S5: Changes in fatty acid profile in plasma after the intervention; Figure S6: Changes in cholesterol concentration, lipoprotein size and lipid composition and apolipoprotein concentration in plasma after the intervention; Figure S7: Changes in lipoprotein subclass concentration and composition in plasma after the intervention. Table S1: Nanostring nCounter Metabolic Pathway Panel; Table S2: Additional code-sets covering immune and inflammation response and lipid metabolism-related mRNAs; Table S3: Metabolomic analysis before the intervention. Low $insulin_{iAUC}$ vs. high $insulin_{iAUC}$ group; Table S4: Gene expression analysis before the intervention. Low $insulin_{iAUC}$ vs. high $insulin_{iAUC}$ group; Table S5: Metabolomic analysis after the intervention. Fish protein-treated low $insulin_{iAUC}$ vs. placebo-treated low $insulin_{iAUC}$; Table S6: Metabolomic analysis after the intervention. Fish-protein-treated high $insulin_{iAUC}$ vs. placebo-treated high $insulin_{iAUC}$; Table S7: Gene expression after the intervention. Fish-protein-treated low $insulin_{iAUC}$ vs. placebo-treated low $insulin_{iAUC}$; Table S8: Gene expression analysis after the intervention. Fish-protein-treated high $insulin_{iAUC}$ vs. placebo-treated high $insulin_{iAUC}$.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets used are available from the corresponding author upon reasonable request.

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References

1. Zheng, Y.; Ley, S.H.; Hu, F.B. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat. Rev. Endocrinol.* **2018**, *14*, 88–98. [CrossRef]
2. DeFronzo, R.A.; Ferrannini, E.; Groop, L.; Henry, R.R.; Herman, W.H.; Holst, J.J.; Hu, F.B.; Kahn, C.R.; Raz, I.; Shulman, G.I.; et al. Type 2 diabetes mellitus. *Nat. Rev. Dis. Primers* **2015**, *1*, 15019. [CrossRef]
3. American Diabetes, A. Diagnosis and classification of diabetes mellitus. *Diabetes Care* **2013**, *36* (Suppl. 1), S67–S74. [CrossRef] [PubMed]
4. Alok, K.; Gupta, A.M.; Brashear, M.; William, D.J. Chapter 2—Prediabetes: Prevalence, Pathogenesis, and Recognition of Enhanced Risk. In *Nutritional and Therapeutic Interventions for Diabetes and Metabolic Syndrome*, 2nd ed.; Academic Press: Cambridge, MA, USA, 2018; p. 18. [CrossRef]
5. Rajala, M.W.; Scherer, P.E. Minireview: The adipocyte–at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* **2003**, *144*, 3765–3773. [CrossRef]
6. Chung, S.T.; Matta, S.T.; Meyers, A.G.; Cravalho, C.K.; Villalobos-Perez, A.; Dawson, J.M.; Sharma, V.R.; Sampson, M.L.; Otvos, J.D.; Magge, S.N. Nuclear Magnetic Resonance Derived Biomarkers for Evaluating Cardiometabolic Risk in Youth and Young Adults Across the Spectrum of Glucose Tolerance. *Front. Endocrinol.* **2021**, *12*, 665292. [CrossRef] [PubMed]
7. Shalaurova, I.; Connelly, M.A.; Garvey, W.T.; Otvos, J.D. Lipoprotein insulin resistance index: A lipoprotein particle-derived measure of insulin resistance. *Metab. Syndr. Relat. Disord.* **2014**, *12*, 422–429. [CrossRef] [PubMed]
8. Harada, P.H.N.; Demler, O.V.; Dugani, S.B.; Akinkuolie, A.O.; Moorthy, M.V.; Ridker, P.M.; Cook, N.R.; Pradhan, A.D.; Mora, S. Lipoprotein insulin resistance score and risk of incident diabetes during extended follow-up of 20 years: The Women's Health Study. *J. Clin. Lipidol.* **2017**, *11*, 1257–1267.e1252. [CrossRef]
9. Otvos, J.D.; Shalaurova, I.; Wolak-Dinsmore, J.; Connelly, M.A.; Mackey, R.H.; Stein, J.H.; Tracy, R.P. GlycA: A Composite Nuclear Magnetic Resonance Biomarker of Systemic Inflammation. *Clin. Chem.* **2015**, *61*, 714–723. [CrossRef]
10. Jump, D.B. Fatty acid regulation of gene transcription. *Crit. Rev. Clin. Lab. Sci.* **2004**, *41*, 41–78. [CrossRef]
11. Gonzalez-Becerra, K.; Ramos-Lopez, O.; Barron-Cabrera, E.; Riezu-Boj, J.I.; Milagro, F.I.; Martinez-Lopez, E.; Martinez, J.A. Fatty acids, epigenetic mechanisms and chronic diseases: A systematic review. *Lipids Health Dis.* **2019**, *18*, 178. [CrossRef]
12. Saltiel, A.R.; Kahn, C.R. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **2001**, *414*, 799–806. [CrossRef] [PubMed]
13. Wurtz, P.; Havulinna, A.S.; Soininen, P.; Tynkkynen, T.; Prieto-Merino, D.; Tillin, T.; Ghorbani, A.; Artati, A.; Wang, Q.; Tiainen, M.; et al. Metabolite profiling and cardiovascular event risk: A prospective study of 3 population-based cohorts. *Circulation* **2015**, *131*, 774–785. [CrossRef] [PubMed]
14. Yu, E.A.; Hu, P.J.; Mehta, S. Plasma fatty acids in de novo lipogenesis pathway are associated with diabetogenic indicators among adults: NHANES 2003-2004. *Am. J. Clin. Nutr.* **2018**, *108*, 622–632. [CrossRef] [PubMed]
15. Odegaard, J.I.; Chawla, A. Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis. *Science* **2013**, *339*, 172–177. [CrossRef] [PubMed]
16. Delarue, J.; Magnan, C. Free fatty acids and insulin resistance. *Curr. Opin. Clin. Nutr. Metab. Care* **2007**, *10*, 142–148. [CrossRef]
17. Sanders, F.W.; Griffin, J.L. De novo lipogenesis in the liver in health and disease: More than just a shunting yard for glucose. *Biol. Rev. Camb. Philos. Soc.* **2016**, *91*, 452–468. [CrossRef]
18. Pan, X.R.; Li, G.W.; Hu, Y.H.; Wang, J.X.; Yang, W.Y.; An, Z.X.; Hu, Z.X.; Lin, J.; Xiao, J.Z.; Cao, H.B.; et al. Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance. The Da Qing IGT and Diabetes Study. *Diabetes Care* **1997**, *20*, 537–544. [CrossRef]
19. Li, G.; Zhang, P.; Wang, J.; Gregg, E.W.; Yang, W.; Gong, Q.; Li, H.; Li, H.; Jiang, Y.; An, Y.; et al. The long-term effect of lifestyle interventions to prevent diabetes in the China Da Qing Diabetes Prevention Study: A 20-year follow-up study. *Lancet* **2008**, *371*, 1783–1789. [CrossRef]
20. Micha, R.; Shulkin, M.L.; Penalvo, J.L.; Khatibzadeh, S.; Singh, G.M.; Rao, M.; Fahimi, S.; Powles, J.; Mozaffarian, D. Etiologic effects and optimal intakes of foods and nutrients for risk of cardiovascular diseases and diabetes: Systematic reviews and meta-analyses from the Nutrition and Chronic Diseases Expert Group (NutriCoDE). *PLoS ONE* **2017**, *12*, e0175149. [CrossRef]
21. Nong, N.T.P.; Hsu, J.L. Characteristics of Food Protein-Derived Antidiabetic Bioactive Peptides: A Literature Update. *Int. J. Mol. Sci.* **2021**, *22*, 9508. [CrossRef]
22. Jin, R.; Teng, X.; Shang, J.; Wang, D.; Liu, N. Identification of novel DPP-IV inhibitory peptides from Atlantic salmon (Salmo salar) skin. *Food Res. Int.* **2020**, *133*, 109161. [CrossRef] [PubMed]
23. Gao, J.; Gong, H.; Mao, X. Dipeptidyl Peptidase-IV Inhibitory Activity and Related Molecular Mechanism of Bovine alpha-Lactalbumin-Derived Peptides. *Molecules* **2020**, *25*, 3009. [CrossRef] [PubMed]
24. Fosgerau, K.; Hoffmann, T. Peptide therapeutics: Current status and future directions. *Drug Discov. Today* **2015**, *20*, 122–128. [CrossRef] [PubMed]

25. Dale, H.F.; Jensen, C.; Hausken, T.; Lied, E.; Hatlebakk, J.G.; Bronstad, I.; Lihaug Hoff, D.A.; Lied, G.A. Effect of a cod protein hydrolysate on postprandial glucose metabolism in healthy subjects: A double-blind cross-over trial. *J. Nutr. Sci.* **2018**, *7*, e33. [CrossRef] [PubMed]
26. Kawasaki, T.; Seki, E.; Osajima, K.; Yoshida, M.; Asada, K.; Matsui, T.; Osajima, Y. Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects. *J. Hum. Hypertens* **2000**, *14*, 519–523. [CrossRef] [PubMed]
27. Nobile, V.; Duclos, E.; Michelotti, A.; Bizzaro, G.; Negro, M.; Soisson, F. Supplementation with a fish protein hydrolysate (Micromesistius poutassou): Effects on body weight, body composition, and CCK/GLP-1 secretion. *Food Nutr. Res.* **2016**, *60*, 29857. [CrossRef] [PubMed]
28. Vikoren, L.A.; Nygard, O.K.; Lied, E.; Rostrup, E.; Gudbrandsen, O.A. A randomised study on the effects of fish protein supplement on glucose tolerance, lipids and body composition in overweight adults. *Br. J. Nutr.* **2013**, *109*, 648–657. [CrossRef]
29. Zhu, C.F.; Li, G.Z.; Peng, H.B.; Zhang, F.; Chen, Y.; Li, Y. Treatment with marine collagen peptides modulates glucose and lipid metabolism in Chinese patients with type 2 diabetes mellitus. *Appl. Physiol. Nutr. Metab.* **2010**, *35*, 797–804. [CrossRef]
30. Ouellet, V.; Marois, J.; Weisnagel, S.J.; Jacques, H. Dietary cod protein improves insulin sensitivity in insulin-resistant men and women: A randomized controlled trial. *Diabetes Care* **2007**, *30*, 2816–2821. [CrossRef]
31. Hovland, I.H.; Leikanger, I.S.; Stokkeland, O.; Waage, K.H.; Mjos, S.A.; Brokstad, K.A.; McCann, A.; Ueland, P.M.; Slizyte, R.; Carvajal, A.; et al. Effects of low doses of fish and milk proteins on glucose regulation and markers of insulin sensitivity in overweight adults: A randomised, double blind study. *Eur. J. Nutr.* **2020**, *59*, 1013–1029. [CrossRef]
32. Nenseter, M.S.; Osterud, B.; Larsen, T.; Strom, E.; Bergei, C.; Hewitt, S.; Holven, K.B.; Hagve, T.A.; Mjos, S.A.; Solvang, M.; et al. Effect of Norwegian fish powder on risk factors for coronary heart disease among hypercholesterolemic individuals. *Nutr. Metab. Cardiovasc. Dis.* **2000**, *10*, 323–330. [PubMed]
33. Jensen, C.; Fjeldheim Dale, H.; Hausken, T.; Hatlebakk, J.G.; Bronstad, I.; Lied, G.A.; Hoff, D.A.L. Supplementation with Low Doses of a Cod Protein Hydrolysate on Glucose Regulation and Lipid Metabolism in Adults with Metabolic Syndrome: A Randomized, Double-Blind Study. *Nutrients* **2020**, *12*, 1991. [CrossRef] [PubMed]
34. Hustad, K.S.; Ottestad, I.; Hjorth, M.; Dalen, K.T.; Saether, T.; Sheikh, N.A.; Stromnes, M.; Ulven, S.M.; Holven, K.B. No effect of salmon fish protein on 2-h glucose in adults with increased risk of type 2 diabetes: A randomised controlled trial. *Br. J. Nutr.* **2021**, *126*, 1304–1313. [CrossRef] [PubMed]
35. Soininen, P.; Kangas, A.J.; Wurtz, P.; Suna, T.; Ala-Korpela, M. Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. *Circ. Cardiovasc. Genet.* **2015**, *8*, 192–206. [CrossRef] [PubMed]
36. Wickham, H.; Averick, M.; Bryan, J.; Chang, W.; McGowan, L.D.A.; François, R.; Grolemund, G.; Hayes, A.; Henry, L.; Hester, J.; et al. Welcome to the Tidyverse. *J. Open Source Softw.* **2019**, *4*, 1686. [CrossRef]
37. Ritchie, M.E.; Phipson, B.; Wu, D.; Hu, Y.; Law, C.W.; Shi, W.; Smyth, G.K. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **2015**, *43*, 13. [CrossRef]
38. Signorell, A.; Aho, K.; Alfons, A.; Anderegg, N.; Aragon, T.; Arachchige, C.; Arppe, A.; Baddeley, A.; Barton, K.; Bolker, B.; et al. DescTools: Tools for Descriptive Statistics. 2022. Available online: https://cran.r-project.org/package=DescTools (accessed on 22 October 2022).
39. Ashburner, M.; Ball, C.A.; Blake, J.A.; Botstein, D.; Butler, H.; Cherry, J.M.; Davis, A.P.; Dolinski, K.; Dwight, S.S.; Eppig, J.T.; et al. Gene ontology: Tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* **2000**, *25*, 25–29. [CrossRef]
40. Gene Ontology, C. The Gene Ontology resource: Enriching a GOld mine. *Nucleic Acids Res.* **2021**, *49*, D325–D334. [CrossRef]
41. Kanehisa, M.; Goto, S. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **2000**, *28*, 27–30. [CrossRef]
42. Ritchie, S.C.; Wurtz, P.; Nath, A.P.; Abraham, G.; Havulinna, A.S.; Fearnley, L.G.; Sarin, A.P.; Kangas, A.J.; Soininen, P.; Aalto, K.; et al. The Biomarker GlycA Is Associated with Chronic Inflammation and Predicts Long-Term Risk of Severe Infection. *Cell Syst.* **2015**, *1*, 293–301. [CrossRef]
43. Liberzon, A.; Birger, C.; Thorvaldsdottir, H.; Ghandi, M.; Mesirov, J.P.; Tamayo, P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst.* **2015**, *1*, 417–425. [CrossRef] [PubMed]
44. Wu, D.; Smyth, G.K. Camera: A competitive gene set test accounting for inter-gene correlation. *Nucleic Acids Res.* **2012**, *40*, e133. [CrossRef] [PubMed]
45. Dullaart, R.P.; Gruppen, E.G.; Connelly, M.A.; Otvos, J.D.; Lefrandt, J.D. GlycA, a biomarker of inflammatory glycoproteins, is more closely related to the leptin/adiponectin ratio than to glucose tolerance status. *Clin. Biochem.* **2015**, *48*, 811–814. [CrossRef]
46. Lorenzo, C.; Festa, A.; Hanley, A.J.; Rewers, M.J.; Escalante, A.; Haffner, S.M. Novel Protein Glycan-Derived Markers of Systemic Inflammation and C-Reactive Protein in Relation to Glycemia, Insulin Resistance, and Insulin Secretion. *Diabetes Care* **2017**, *40*, 375–382. [CrossRef] [PubMed]
47. Lawler, P.R.; Mora, S. Glycosylation Signatures of Inflammation Identify Cardiovascular Risk: Some Glyc It Hot. *Circ. Res.* **2016**, *119*, 1154–1156. [CrossRef] [PubMed]
48. Connelly, M.A.; Otvos, J.D.; Shalaurova, I.; Playford, M.P.; Mehta, N.N. GlycA, a novel biomarker of systemic inflammation and cardiovascular disease risk. *J. Transl. Med.* **2017**, *15*, 219. [CrossRef] [PubMed]
49. Lai, C.Q.; Parnell, L.D.; Smith, C.E.; Guo, T.; Sayols-Baixeras, S.; Aslibekyan, S.; Tiwari, H.K.; Irvin, M.R.; Bender, C.; Fei, D.; et al. Carbohydrate and fat intake associated with risk of metabolic diseases through epigenetics of CPT1A. *Am. J. Clin. Nutr.* **2020**, *112*, 1200–1211. [CrossRef] [PubMed]

50. Koves, T.R.; Ussher, J.R.; Noland, R.C.; Slentz, D.; Mosedale, M.; Ilkayeva, O.; Bain, J.; Stevens, R.; Dyck, J.R.; Newgard, C.B.; et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab.* **2008**, *7*, 45–56. [CrossRef] [PubMed]
51. Guasch-Ferre, M.; Hruby, A.; Toledo, E.; Clish, C.B.; Martinez-Gonzalez, M.A.; Salas-Salvado, J.; Hu, F.B. Metabolomics in Prediabetes and Diabetes: A Systematic Review and Meta-analysis. *Diabetes Care* **2016**, *39*, 833–846. [CrossRef]
52. Thalacker-Mercer, A.E.; Ingram, K.H.; Guo, F.; Ilkayeva, O.; Newgard, C.B.; Garvey, W.T. BMI, RQ, diabetes, and sex affect the relationships between amino acids and clamp measures of insulin action in humans. *Diabetes* **2014**, *63*, 791–800. [CrossRef]
53. Newgard, C.B.; An, J.; Bain, J.R.; Muehlbauer, M.J.; Stevens, R.D.; Lien, L.F.; Haqq, A.M.; Shah, S.H.; Arlotto, M.; Slentz, C.A.; et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab.* **2009**, *9*, 311–326. [CrossRef] [PubMed]
54. Palmer, N.D.; Stevens, R.D.; Antinozzi, P.A.; Anderson, A.; Bergman, R.N.; Wagenknecht, L.E.; Newgard, C.B.; Bowden, D.W. Metabolomic profile associated with insulin resistance and conversion to diabetes in the Insulin Resistance Atherosclerosis Study. *J. Clin. Endocrinol. Metab.* **2015**, *100*, E463–E468. [CrossRef] [PubMed]
55. Vanweert, F.; Schrauwen, P.; Phielix, E. Role of branched-chain amino acid metabolism in the pathogenesis of obesity and type 2 diabetes-related metabolic disturbances BCAA metabolism in type 2 diabetes. *Nutr Diabetes* **2022**, *12*, 35. [CrossRef] [PubMed]
56. Lynch, C.J.; Adams, S.H. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat. Rev. Endocrinol.* **2014**, *10*, 723–736. [CrossRef]
57. Tulipani, S.; Griffin, J.; Palau-Rodriguez, M.; Mora-Cubillos, X.; Bernal-Lopez, R.M.; Tinahones, F.J.; Corkey, B.E.; Andres-Lacueva, C. Metabolomics-guided insights on bariatric surgery versus behavioral interventions for weight loss. *Obesity* **2016**, *24*, 2451–2466. [CrossRef]
58. Glynn, E.L.; Piner, L.W.; Huffman, K.M.; Slentz, C.A.; Elliot-Penry, L.; AbouAssi, H.; White, P.J.; Bain, J.R.; Muehlbauer, M.J.; Ilkayeva, O.R.; et al. Impact of combined resistance and aerobic exercise training on branched-chain amino acid turnover, glycine metabolism and insulin sensitivity in overweight humans. *Diabetologia* **2015**, *58*, 2324–2335. [CrossRef]
59. Yan-Do, R.; MacDonald, P.E. Impaired "Glycine"-mia in Type 2 Diabetes and Potential Mechanisms Contributing to Glucose Homeostasis. *Endocrinology* **2017**, *158*, 1064–1073. [CrossRef]
60. Gannon, M.C.; Nuttall, J.A.; Nuttall, F.Q. The metabolic response to ingested glycine. *Am. J. Clin. Nutr.* **2002**, *76*, 1302–1307. [CrossRef]
61. Gonzalez-Ortiz, M.; Medina-Santillan, R.; Martinez-Abundis, E.; von Drateln, C.R. Effect of glycine on insulin secretion and action in healthy first-degree relatives of type 2 diabetes mellitus patients. *Horm. Metab. Res.* **2001**, *33*, 358–360. [CrossRef]
62. Hjorth, M.; Galigniana, N.M.; Ween, O.; Ulven, S.M.; Holven, K.B.; Dalen, K.T.; Saether, T. Postprandial Effects of Salmon Fishmeal and Whey on Metabolic Markers in Serum and Gene Expression in Liver Cells. *Nutrients* **2022**, *14*, 1593. [CrossRef]
63. Yan, L.J. Pathogenesis of chronic hyperglycemia: From reductive stress to oxidative stress. *J. Diabetes Res.* **2014**, *2014*, 137919. [CrossRef] [PubMed]
64. Bhatti, J.S.; Bhatti, G.K.; Reddy, P.H. Mitochondrial dysfunction and oxidative stress in metabolic disorders—A step towards mitochondria based therapeutic strategies. *Biochim. Biophys. Acta Mol. Basis Dis.* **2017**, *1863*, 1066–1077. [CrossRef] [PubMed]
65. Rosselot, C.; Baumel-Alterzon, S.; Li, Y.; Brill, G.; Lambertini, L.; Katz, L.S.; Lu, G.; Garcia-Ocana, A.; Scott, D.K. The many lives of Myc in the pancreatic beta-cell. *J. Biol. Chem.* **2021**, *296*, 100122. [CrossRef] [PubMed]
66. Eizirik, D.L.; Pasquali, L.; Cnop, M. Pancreatic beta-cells in type 1 and type 2 diabetes mellitus: Different pathways to failure. *Nat. Rev. Endocrinol.* **2020**, *16*, 349–362. [CrossRef] [PubMed]
67. Chen, Z.Z.; Gerszten, R.E. Metabolomics and Proteomics in Type 2 Diabetes. *Circ Res* **2020**, *126*, 1613–1627. [CrossRef]
68. Cani, P.D.; Lecourt, E.; Dewulf, E.M.; Sohet, F.M.; Pachikian, B.D.; Naslain, D.; De Backer, F.; Neyrinck, A.M.; Delzenne, N.M. Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. *Am. J. Clin. Nutr.* **2009**, *90*, 1236–1243. [CrossRef]
69. Tsutsumi, R.; Yamasaki, Y.; Takeo, J.; Miyahara, H.; Sebe, M.; Bando, M.; Tanba, Y.; Mishima, Y.; Takeji, K.; Ueshima, N.; et al. Long-chain monounsaturated fatty acids improve endothelial function with altering microbial flora. *Transl. Res.* **2021**, *237*, 16–30. [CrossRef]
70. Kovatcheva-Datchary, P.; Nilsson, A.; Akrami, R.; Lee, Y.S.; De Vadder, F.; Arora, T.; Hallen, A.; Martens, E.; Bjorck, I.; Backhed, F. Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of Prevotella. *Cell Metab.* **2015**, *22*, 971–982. [CrossRef]

8 ANNEX II: PUBLICATIONS

Title: Mitochondrial redox impairment and enhanced autophagy in peripheral blood mononuclear cells from type 1 diabetic patients

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Mitochondrial redox impairment and enhanced autophagy in peripheral blood mononuclear cells from type 1 diabetic patients

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ABSTRACT

Type 1 diabetes (T1D) involves critical metabolic disturbances that contribute to an increased cardiovascular risk. Leukocytes are key players in the onset of atherosclerosis due to their interaction with the endothelium. However, whether mitochondrial redox impairment, altered bioenergetics and abnormal autophagy in leukocytes contribute to T1D physiopathology is unclear.

In this study we aimed to evaluate the bioenergetic and redox state of peripheral blood mononuclear cells (PBMCs) from T1D patients in comparison to those from healthy subjects, and to assess autophagy induction and leukocyte-endothelial interactions.

T1D patients presented lower levels of fast-acting and total antioxidants in their blood, and their leukocytes produced higher amounts of total reactive oxygen species (ROS) and superoxide radical with respect to controls. Basal and ATP-linked respiration were similar in PBMCs from T1D and controls, but T1D PBMCs exhibited reduced spare respiratory capacity and a tendency toward decreased maximal respiration and reduced non-mitochondrial respiration, compared to controls. The autophagy markers P-AMPK, Beclin-1 and LC3-II/LC3-I were increased, while P62 and NBR1 were decreased in T1D PBMCs versus those from controls. Leukocytes from T1D patients displayed lower rolling velocity, higher rolling flux and more adhesion to the endothelium versus controls.

Our findings show that T1D impairs mitochondrial function and promotes oxidative stress and autophagy in leukocytes, and suggest that these mechanisms contribute to an increased risk of atherosclerosis by augmenting leukocyte-endothelial interactions.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by insulin deficiency due to pancreatic beta cell destruction, which leads to hyperglycaemia [1].

T cells, classically described as the main mediators of beta cell destruction in T1D [2], are the most abundant cell type in peripheral blood mononuclear cells (PBMCs), making up approximately 45–70% [3]. The study of PBMCs provides important insight into the immunological status of patients with T1D [4], and circulating leukocytes are

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sensors of metabolic stress in patients and act as bioenergetic biomarkers [5]. Importantly, mitochondrial dysfunction in PBMCs is known to reflect the systemic inflammation and oxidative stress that underlie cardiovascular diseases [6].

The risk of cardiovascular disease among T1D patients is well established, and cardiovascular disease is the co-morbidity responsible for the greatest proportion of deaths in T1D patients [7]. The major mechanisms underlying hyperglycaemia-induced vascular damage are activated by mitochondrial overproduction of reactive oxygen species (ROS) [8]. Superoxide is the initial ROS molecule formed by the mitochondrial electron-transport chain and is then converted to other reactive species that can damage cells in several ways [8]. ROS are one of the major inducers of autophagy [9]. Physiological ROS levels maintain cellular homeostasis and autophagy supports this balance. However, under oxidative stress conditions autophagy is triggered and acts as a critical mediator of pathological responses to excessive ROS production [10,11].

Given the critical role of peripheral circulating cells as sensors of metabolic stress and their potential involvement in the vascular complications of T1D, we aimed to determine the bioenergetic and redox state of leukocytes, their interaction with the endothelium, and autophagy induction in T1D patients and to compare these parameters with those in healthy subjects.

2. Material and methods

2.1. Study population

Fifty-two healthy volunteers and forty-four patients with T1D were recruited from the Endocrinology and Nutrition Service of University Hospital Dr. Peset, in Valencia, Spain and were matched according to age and sex. Participants were informed of the details of the study and gave their written consent. The Ethics Committee of Clinical Investigation of the University Hospital Dr. Peset reviewed and approved the study protocol (ID: 98/19). The study was conducted under the ethical principles of the Helsinki Declaration.

Exclusion criteria were any documented history of cardiovascular disease (ischaemic cardiopathy, stroke, peripheral arteriopathy or any cardiovascular diseases associated with cardiovascular risk); and any severe inflammatory, infectious, or autoimmune disease.

2.2. Physical and biochemical determinations

Patients underwent a physical examination to determine weight, height, waist circumference, systolic (SBP) and diastolic (DBP) blood pressure. After 12 h of fasting, blood samples were collected and biochemical determinations were carried out in our hospital's Clinical Analysis Service, as previously described [12].

2.3. Leukocyte extraction

Neutrophils and PBMCs were isolated from blood collected in EDTA tubes as described previously [13]. For neutrophils, 1.0×10^6 cells were resuspended in RPMI media supplemented with 10% v/v FBS (Biowest) and used for leukocyte-endothelium interaction assays. In the case of PBMCs, an aliquot of 2.0×10^6 cells was used for real-time metabolic flux analysis and the remaining cells were stored at −80 °C for subsequent western blotting.

2.4. Leukocyte-endothelium interaction assays

To mimic the interaction between leukocytes and the endothelial cell layer inside the blood vessel and thus simulate physiological conditions, we used an *ex vivo* model based on a parallel plate flow chamber as described before [14]. In short, a suspension of leukocytes (1.0×10^6 cells/mL) was perfused over a monolayer of primary human umbilical vein endothelial cells (HUVECs) at a flow rate of 0.36 ml/min. Real time images of the flow-exposed monolayer were recorded for 5 min and analysed. Leukocyte rolling flux, rolling velocity and adhesion were determined as described elsewhere [15].

2.5. Measurement of blood antioxidant capacity

Blood antioxidant capacity was measured using e-BQC Lab device (Bioquochem) according to the manufacturer's protocol. This electrochemical method measures the total antioxidant capacity of blood in micro-Coulombs (μC) and distinguishes between fast- and slow-acting antioxidants.

2.6. Flow cytometry assay

The whole blood of patients (500 μl) was processed as previously described [13]. The fluorochromes (Invitrogen) 2′,7′-Dichlorodihydrofluorescein diacetate (5 μM, DCFH) and Hydroethidine (5 μM, HE) were employed to measure total free radicals and superoxide content, respectively. 10,000 cells were analysed in each experiment.

2.7. Real-time metabolic flux analysis

The mitochondrial function of PBMCs was measured in real-time with a Seahorse XFp analyzer (Agilent) using the XFp Cell Mito Stress Test Kit (Agilent) and following the manufacturer's instructions.

Immediately after extraction of PBMCs, the pellets were resuspended with Seahorse XF DMEM medium pH 7.4 (Agilent) containing 1 mM pyruvate, 2 mM glutamine and 10 mM glucose and seeded on culture miniplates that had been pretreated with Poly-D-Lysine (0.1 mg/ml) at a density of 3.5×10^5 cells/well in 180 μL. Compounds were loaded into the cartridge injection ports following the manufacturer's volume recommendations, such that the final concentration in each well was Oligomycin A 1.5 μM, FCCP 1.0 μM and Rotenone/Antimycin 1 μM.

2.8. Protein expression analysis of autophagy markers

PBMC protein extraction, quantification and Western blot were carried out as described previously [12,16]. The details of primary antibodies for AMPK-P, Beclin 1, P62, NBR1, LC3A/B and β-Actin are in Supplementary Table 1.

2.9. Statistical analysis

R version 4.1.2, RStudio IDE (www.rstudio.com) and the Tidyverse package [17] were employed for data management and visualization. GraphPad Prism version 8.02 (GraphPad Software, www.graphpad.com) was used for statistical analysis and data presentation. Statistical significance (P-value <0.05) between two groups was assessed by an unpaired T test for normally distributed variables and Mann–Whitney *U* test for non-normally distributed variables. For correlation analysis we calculated Spearman's correlation coefficient and applied LOESS regression.

3. Results

3.1. Anthropometrical parameters and biochemical determinations

Table 1 shows the anthropometric and biochemical data of the fifty-two healthy controls and forty-four T1D patients. There was no difference in gender distribution between the groups, with 62% and 55% women in the control and T1D groups, respectively. The mean age was 40.4 years among controls and 43.6 years in the T1D group, and the mean duration of diabetes in the latter group was 15.7 years. There were no significant differences in weight, waist circumference, body mass index (BMI) or blood pressure between groups.

Table 1
Anthropometric characteristics, biochemical determinations, and pharmacological treatment of the study population.

Characteristics	Control	Type 1 diabetes	p-value
n	52	44	
Sex (% women)	62%	55%	ns
Age (years)	40.4 ± 11.5	43.6 ± 12.3	ns
Duration of diabetes (years)	–	15.7 ± 8.7	
Weight (kg)	75.4 ± 16.3	73.9 ± 13.3	ns
Waist circumference (cm)	85.1 ± 13.0	87.8 ± 13.8	ns
BMI (kg/m^2)	25.9 ± 4.8	25.7 ± 4.4	ns
SBP (mm Hg)	118 ± 13	125 ± 20	ns
DBP (mm Hg)	73 ± 9	74 ± 10	ns
Glucose (mg/dL)	88.0 (83.75–96.0)	133.0 (112.5–232.0)	<0.001
HbA1c-DCCT (%)	5.2 ± 0.3	7.6 ± 1.0	<0.001
Total cholesterol (mg/dL)	185.6 ± 31.1	160.4 ± 26.7	<0.001
HDL-c (mg/dL)	57.1 ± 11.9	57.7 ± 14.6	ns
LDL-c (mg/dL)	112.1 ± 25.7	86.2 ± 23.0	<0.001
Triglycerides (mg/dL)	71.5 (53.8–104.3)	71.0 (55.5–92.5)	ns
hs-CRP (mg/L)	0.95 (0.4–2.25)	1.5 (0.8–3.6)	ns
Treatment			
Non-insulin antidiabetic drugs			13.6%
Metformin		–	2.3%
Metformin + SGLT2 inhibitors		–	4.5%
Metformin + DPP4 inhibitors		–	2.3%
SGLT2 inhibitors			4.5%
Lipid-lowering medication			56.8%
Statins		–	34.1%
Statins + Fibrates		–	4.5%
Statins + Ezetimibe		–	18.2%
Antihypertensive medication		–	13.6%

Data are expressed as mean ± SD for normally distributed variables or median and interquartile range for non-normally distributed data. A Chi-square test was performed to determine differences in the proportion of sexes between groups. An unpaired T test or Mann Whitney *U* Test was performed to determine differences between groups. Abbreviations: BMI: body mass index, DBP: diastolic blood pressure, HbA1c: glycated hemoglobin, HDL-c: high-density lipoprotein cholesterol, hs-CRP: high-sensitive C-reactive protein, LDL-c: low-density lipoprotein cholesterol; ns: not significant, SBP: systolic blood pressure.

As expected, the T1D group displayed higher fasting glucose concentrations and HbA1c than controls. T1D patients presented lower levels of total cholesterol and LDL cholesterol, which may have been due to the lipid-lowering medication that 57% of them were taking.

3.2. ROS production and antioxidant capacity

Hyperglycaemia contributes to the production of large amounts of ROS by mitochondrial and non-mitochondrial sources, which are associated with the development of cardiovascular complications [18,19]. Leukocytes from T1D patients produced higher amounts of total ROS (Fig. 1A, $p < 0.05$) and the superoxide radical (Fig. 1B, $p < 0.01$) than those from controls. In addition, we measured blood antioxidant capacity as a reflection of systemic oxidative stress and found that T1D patients had lower levels of fast-acting and total antioxidants (Fig. 1C–D, both $p < 0.05$).

3.3. Measurement of mitochondrial function

Analysis of oxygen consumption rate (OCR) during the Mito Stress test in T1D vs control subjects (Fig. 2A) revealed similar basal and ATP-linked respiration in both groups (Supplementary Figs. 1A–B). Interestingly, leukocytes from T1D patients displayed reduced maximal respiration rate ($p = 0.06$), spare capacity ($p < 0.05$) and non-mitochondrial respiration ($p = 0.05$), compared to controls (Fig. 2B–D). There were no differences in mitochondrial coupling efficiency (Supplementary Fig. 1C).

3.4. Autophagy marker expression

Given that autophagy is a major defence against oxidative stress, we next determined whether autophagic flux was activated in the leukocytes of our T1D patients. We observed increased protein expression of P-AMPK and Beclin-1 (Fig. 3A–B, both $p < 0.05$), both markers of autophagy induction. Moreover, leukocytes from T1D displayed increased conversion of LC3-I to LC3-II (Fig. 3C, $p < 0.05$) and decreased amounts of the cargo proteins P62 (Fig. 3D, $p < 0.05$) and NBR1 (Fig. 3E, $p < 0.01$), all indicative of autophagosome maturation.

Interestingly, we found a positive correlation between HbA1c (%) and the LC3-II/I ratio ($r = 0.40$, $p < 0.01$, Supplementary Fig. 2A), and a tendency for said ratio to increase when HbA1c exceeded 5.7% (Supplementary Fig. 2B).

3.5. Leukocyte-endothelium interactions

The adhesion of leukocytes to the endothelium is a hallmark of the inflammatory process at the onset of atherosclerosis. In the present study we found that T1D patients presented increased leukocyte-endothelium interactions, as their leukocytes displayed reduced rolling velocity (Fig. 4A, $p < 0.01$) and higher numbers of their leukocytes were observed to roll (Fig. 4B, $p < 0.05$) or attach to the endothelium and remain stationary (Fig. 4C, $p < 0.01$) in comparison to those of control subjects.

4. Discussion

T1D is a serious chronic illness usually diagnosed at a young age. T1D patients depend on exogenous insulin throughout life, and their wellbeing is typically compromised by both hyperglycaemia (a consequence of poor control of the disease) and hypoglycaemia, due to improper insulin treatment [1].

Chronic hyperglycaemia results in the overloading of the mitochondrial electron transport chain, which increases ROS production and undermines mitochondrial function. In T1D subjects, we have observed decreased blood antioxidant capacity with respect to controls, as well as higher leukocyte total ROS and superoxide production. In line with our findings, Devaraj et al. observed higher superoxide anion levels in monocytes from T1D patients compared with controls [20]. In addition, we observed that mitochondria in leukocytes from T1D patients displayed a reduced spare respiratory capacity. Spare capacity estimates mitochondrial health and flexibility, indicating the cell's bioenergetic adaptation to pathological stress. Inadequate spare respiratory capacity is associated with cardiovascular and neurological chronic diseases [21], and with the development of diabetic complications [22,23]. In this context, Czajka et al. compared PBMCs from diabetic patients with and without nephropathy; while they found similar basal and ATP-linked respiration in both groups, they detected metabolic inflexibility in the former, manifested as reduced maximal and spare respiratory capacity, [22]. In our T1D cohort, we observed similar bioenergetic maladaptation, even in the absence of nephropathy. With regard to this discrepancy, the nephropathy group in the Czajka et al. study included a large proportion of type 2 diabetic patients, and so additional factors, such as insulin resistance, could have been driving the mitochondrial impairment observed.

Together with mitochondrial dysfunction and excessive ROS, we have witnessed a classic pattern of autophagy activation, with low p62 and NBR1 protein levels and increased levels of Beclin1 and the LC3-II isoform. Interestingly, P-AMPK levels were also increased in PBMCs of T1D patients. In this sense, AMPK may be a protein marker of autophagy induction upon mitochondrial ROS production [9], as it becomes activated upon exposure to H_2O_2, a process that occurs through oxidative modifications (including *S*-glutathionylation of the AMPKα and AMPKβ subunits) [9,24]. Moreover, modifications of Atg4 by H_2O_2 have been found to inactivate its hydrolysing activity towards LC3, which allows

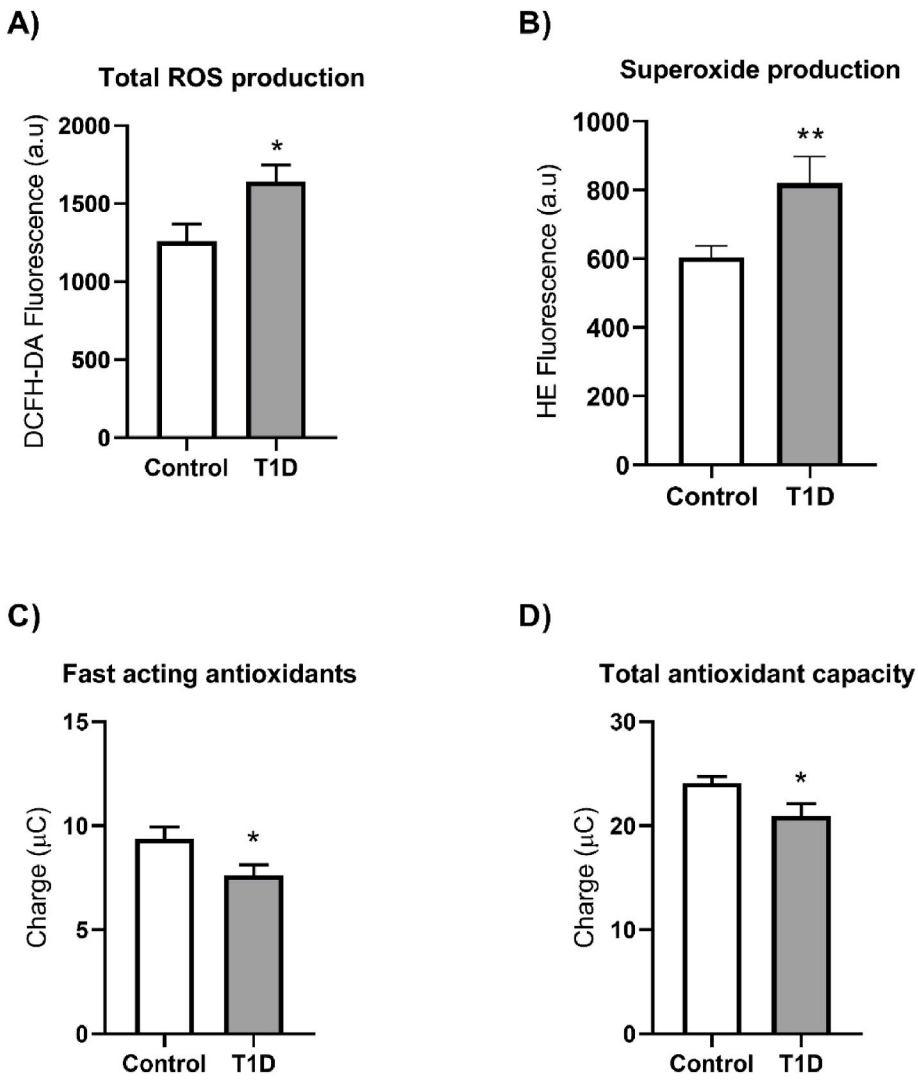

Fig. 1. Leukocyte reactive oxygen species production and blood antioxidant capacity in control and type 1 diabetic patients. A) Total ROS production measured by flow cytometry as DCFH-DA fluorescence intensity (a.u), B) Superoxide production measured by flow cytometry as HE fluorescence intensity (a.u), C) Fast acting antioxidant measurement in blood (μC) and D) Blood total antioxidant capacity (μC), measured by an electrochemistry method. Values in the bar charts represent mean ± SEM. At least 11 samples for each group were included. Comparisons were made using a Mann-Whitney test for Total ROS production and using a *t*-test for Superoxide production, Fast acting antioxidants, and Total antioxidant capacity. *$p < 0.05$, **$p < 0.01$. Abbreviations: a.u.: arbitrary units, DCFH-DA: 2′,7′-Dichlorofluorescin diacetate, HE: Hydroethidine, ROS: reactive oxygen species, T1D: type 1 diabetes.

accumulation of the pro-autophagic LC3-II isoform [9,25].

Furthermore, our data show a positive correlation between HbA1c and LC3-II, supporting an association between increased autophagy and poor glycaemic control, and might be an indication of excessive cellular stress due to chronic hyperglycaemia.

There is a crosstalk between increased ROS production, mitochondrial dysfunction, inflammation, and autophagy. In obesity, it has been observed that autophagy is upregulated due to over activated pro-inflammatory signals [26]. Upregulation of autophagy by TNFα is induced by ROS production and is mediated by activation of the Jun kinase pathway and inhibition of the Akt pathway [26–28]. Accordingly, we have previously observed increased levels of serum TNFα in T1D patients [29]. Moreover, it has been shown in animal studies that the proinflammatory transcription factor NF-κB is involved in the induction of autophagy in response to ROS [26,30].

Lastly, and in accordance with a previous analysis performed by our group in a different cohort of T1D patients [29], we demonstrate that leukocyte-endothelial interactions are exacerbated in the diabetic population. In support of this proinflammatory state, other authors have shown that monocytes isolated directly from the blood of T1D patients secrete pro-inflammatory cytokines such as IL-1β and IL-6 [31]. In relation to this, we and other groups have previously demonstrated an increase in circulating adhesion molecules in T1D, which could be the reason why leukocytes are attracted to the endothelial walls [20,29].

5. Conclusions

Inflammation and oxidative stress are key mechanisms contributing to atherosclerosis, and leukocytes play a pivotal role in this process. In this study, we have characterized the phenotype of leukocytes from T1D patients, and this phenotype includes augmented ROS, impaired mitochondrial respiration rate and activated autophagy. These alterations occur in parallel to an overall reduction in antioxidant defences and increased interaction between leukocytes and endothelial cells. These findings support the idea that T1D impairs mitochondrial function and promotes oxidative stress and autophagy in leukocytes, and that these mechanisms contribute to an increased risk of atherosclerosis by augmenting leukocyte-endothelial interactions. Further studies are needed to confirm the directionality of these processes.

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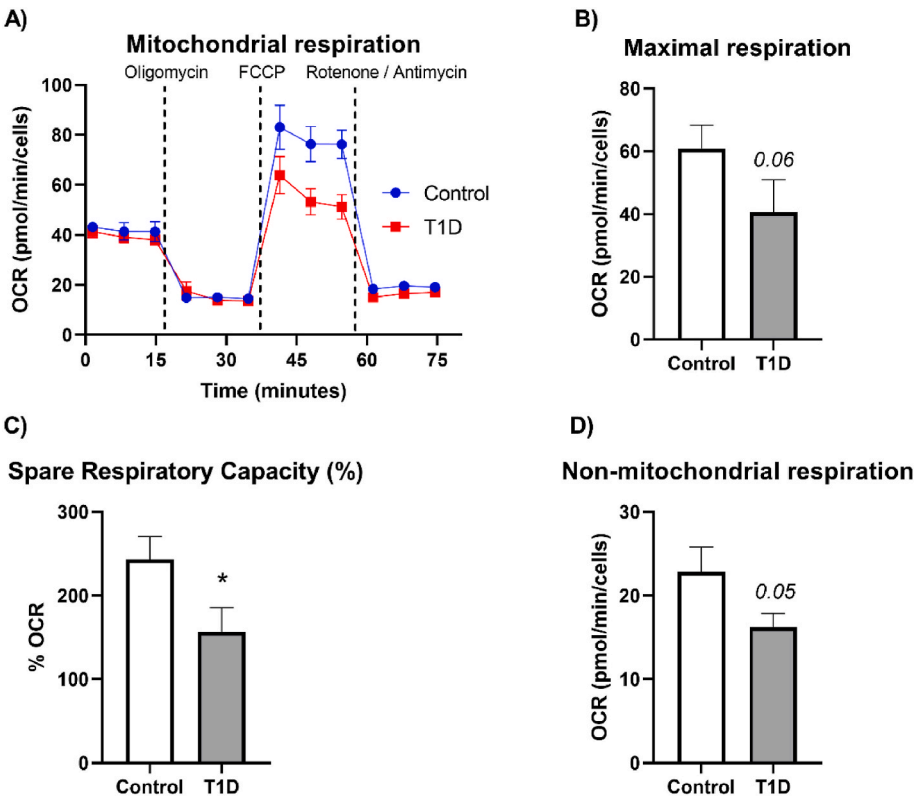

Fig. 2. Mitochondrial function on leukocytes from control and type 1 diabetic patients. A) Representative OCR during Mito Stress Test, B) Maximal respiration is the maximum rate of respiration that the cell can achieve, C) Spare respiratory capacity (%) indicates the capability of the cells to adapt to an energy demand, expressed as percentage of basal respiration and D) Non-mitochondrial respiration, indicating how much oxygen is still consumed by a subset of cellular enzymes after the injection of rotenone and antimycin A. Values in the bar charts represent mean ± SEM of at least 7 independent experiments. Comparisons were made using a *t*-test. *p < 0.05. Abbreviations: OCR: Oxygen consumption rate, T1D: type 1 diabetes.

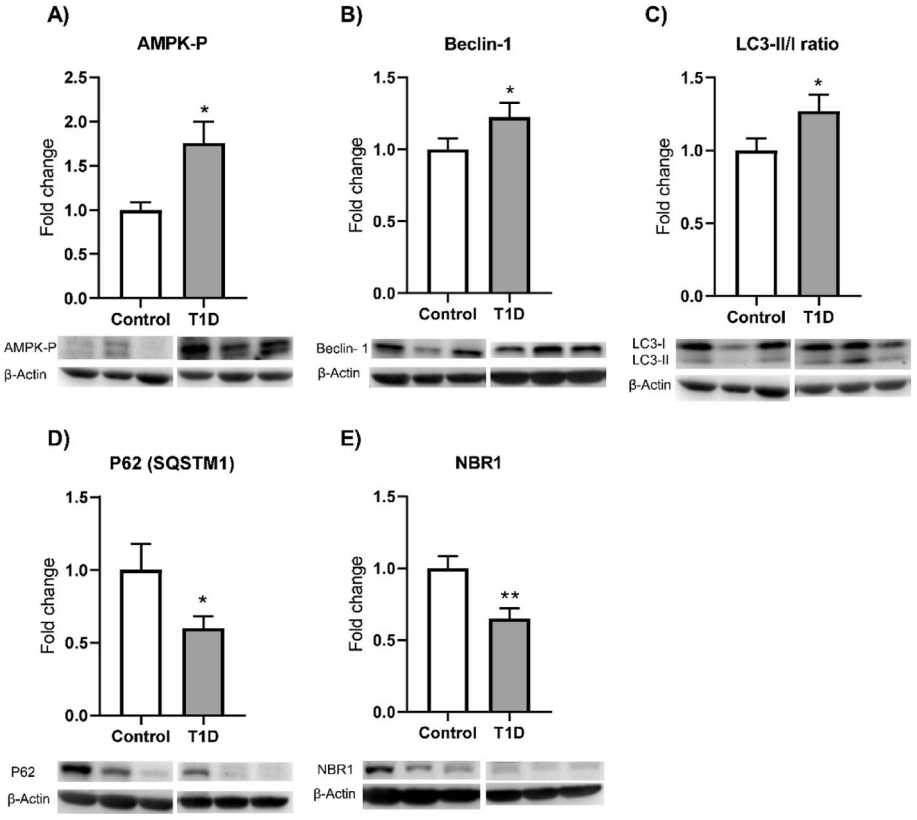

Fig. 3. Expression of autophagy markers on leukocytes from control and type 1 diabetic patients. Protein levels of autophagy markers relative to β-Actin signal A) AMPK-P, B) Beclin-1, C) LC3-II/I ratio. D) P62 (SQSTM1) and E) NBR1; expressed as fold-change with respect to the control group. Representative images of the Western blot experiments are shown for each assessed protein. Values in the bar charts represent mean ± SEM. Quantification of Beclin-1, P62, NBR1 and LC3-II/I ratio was performed using at least 15 samples for each group, and at least 8 samples for each group were used for quantification of AMPK-P. Images show representative western blots including 3 biological replicates. Comparisons were made using a *t*-test. *p < 0.05, **p < 0.01. Abbreviations: T1D: type 1 diabetes.

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Declaration of competing interest

The authors have no competing interest to declare.

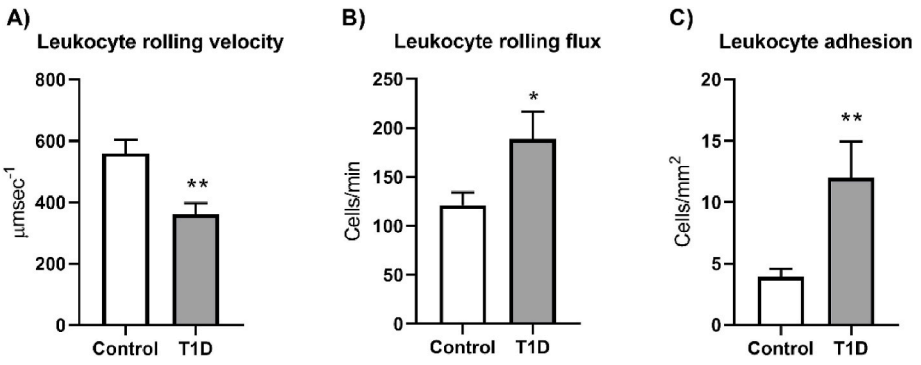

Fig. 4. Leukocyte-endothelium interaction parameters in control and type 1 diabetic patients. A) Mean velocity of rolling leukocytes across the endothelial cell layer (µm/sec), B) Number of leukocytes rolling across the endothelial cell layer per unit of time (cells/min) and C) Number of leukocytes attached to 1 mm^2 of endothelial cell layer (cell/mm^2). Values in the bar charts represent mean ± SEM. Parameters were measured using an *ex vivo* model based on a parallel plate flow chamber and using at least 12 samples for each group. Comparisons were made using a Mann-Whitney test for rolling velocity and a *t*-test for rolling flux and adhesion. *$p < 0.05$, **$p < 0.01$.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102551.

References

[1] A. Katsarou, S. Gudbjörnsdottir, A. Rawshani, D. Dabelea, E. Bonifacio, B. J. Anderson, L.M. Jacobsen, D.A. Schatz, Å. Lernmark, Type 1 diabetes mellitus, Nat. Rev. Dis. Prim. 3 (2017), 17016, https://doi.org/10.1038/nrdp.2017.16.

[2] Q. Ling, L. Shen, W. Zhang, D. Qu, H. Wang, B. Wang, Y. Liu, J. Lu, D. Zhu, Y. Bi, Increased plasmablasts enhance T cell-mediated beta cell destruction and promote the development of type 1 diabetes, Mol. Med. 28 (2022) 18, https://doi.org/10.1186/s10020-022-00447-y.

[3] D. Lozano-Ojalvo, R. López-Fandiño, I. López-Expósito, PBMC-derived T cells, in: K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, H. Wichers (Eds.), The Impact of Food Bioactives on Health: in Vitro and Ex Vivo Models, Springer, Cham, 2015 (CH), http://www.ncbi.nlm.nih.gov/books/NBK500146/. (Accessed 27 July 2022).

[4] A.S. Santos, E. Cunha-Neto, N.V. Gonfinetti, F.B. Bertonha, P. Brochet, A. Bergon, C.A. Moreira-Filho, C. Chevillard, M.E.R. da Silva, Prevalence of inflammatory pathways over immuno-tolerance in peripheral blood mononuclear cells of recent-onset type 1 diabetes, Front. Immunol. 12 (2021), 765264, https://doi.org/10.3389/fimmu.2021.765264.

[5] P.A. Kramer, S. Ravi, B. Chacko, M.S. Johnson, V.M. Darley-Usmar, A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: implications for their use as bioenergetic biomarkers, Redox Biol. 2 (2014) 206–210, https://doi.org/10.1016/j.redox.2013.12.026.

[6] A. Alfatni, M. Riou, A.-L. Charles, A. Meyer, C. Barnig, E. Andres, A. Lejay, S. Talha, B. Geny, Peripheral blood mononuclear cells and platelets mitochondrial dysfunction, oxidative stress, and circulating mtDNA in cardiovascular diseases, J. Clin. Med. 9 (2020) E311, https://doi.org/10.3390/jcm9020311.

[7] S.P. Laing, A.J. Swerdlow, S.D. Slater, A.C. Burden, A. Morris, N.R. Waugh, W. Gatling, P.J. Bingley, C.C. Patterson, Mortality from heart disease in a cohort of 23,000 patients with insulin-treated diabetes, Diabetologia 46 (2003) 760–765, https://doi.org/10.1007/s00125-003-1116-6.

[8] F. Giacco, M. Brownlee, Oxidative stress and diabetic complications, Circ. Res. 107 (2010) 1058–1070, https://doi.org/10.1161/CIRCRESAHA.110.223545.

[9] G. Filomeni, D. De Zio, F. Cecconi, Oxidative stress and autophagy: the clash between damage and metabolic needs, Cell Death Differ. 22 (2015) 377–388, https://doi.org/10.1038/cdd.2014.150.

[10] G. Kroemer, G. Mariño, B. Levine, Autophagy and the integrated stress response, Mol. Cell 40 (2010) 280–293, https://doi.org/10.1016/j.molcel.2010.09.023.

[11] I. Szumiel, Autophagy, reactive oxygen species and the fate of mammalian cells, Free Radic. Res. 45 (2011) 253–265, https://doi.org/10.3109/10715762.2010.525233.

[12] F. Canet, F. Iannantuoni, A.M. de Marañon, P. Díaz-Pozo, S. López-Domènech, T. Vezza, B. Navarro, E. Solá, R. Falcón, C. Bañuls, C. Morillas, M. Rocha, V. M. Víctor, Does empagliflozin modulate leukocyte–endothelium interactions, oxidative stress, and inflammation in type 2 diabetes? Antioxidants 10 (2021) 1228, https://doi.org/10.3390/antiox10081228.

[13] A.M. de Marañón, P. Díaz-Pozo, F. Canet, N. Díaz-Morales, Z. Abad-Jiménez, S. López-Domènech, T. Vezza, N. Apostolova, C. Morillas, M. Rocha, V.M. Víctor, Metformin modulates mitochondrial function and mitophagy in peripheral blood mononuclear cells from type 2 diabetic patients, Redox Biol. 53 (2022), 102342, https://doi.org/10.1016/j.redox.2022.102342.

[14] C. Bañuls, A.M. de Marañon, S. Veses, I. Castro-Vega, S. López-Domènech, C. Salom-Vendrell, S. Orden, Á. Álvarez, M. Rocha, V.M. Víctor, A. Hernández-Mijares, Malnutrition impairs mitochondrial function and leukocyte activation, Nutr. J. 18 (2019) 89, https://doi.org/10.1186/s12937-019-0514-7.

[15] S. Rovira-Llopis, M. Rocha, R. Falcon, C. de Pablo, A. Alvarez, A. Jover, A. Hernandez-Mijares, V.M. Victor, Is myeloperoxidase a key component in the ROS-induced vascular damage related to nephropathy in type 2 diabetes? Antioxidants Redox Signal. 19 (2013) 1452–1458, https://doi.org/10.1089/ars.2013.5307.

[16] A.M. de Marañón, F. Canet, Z. Abad-Jiménez, A. Jover, C. Morillas, M. Rocha, V. M. Victor, Does metformin modulate mitochondrial dynamics and function in type 2 diabetic patients? Antioxidants Redox Signal. 35 (2021) 377–385, https://doi.org/10.1089/ars.2021.0019.

[17] H. Wickham, M. Averick, J. Bryan, W. Chang, L.D. McGowan, R. François, G. Grolemund, A. Hayes, L. Henry, J. Hester, M. Kuhn, T.L. Pedersen, E. Miller, S. M. Bache, K. Müller, J. Ooms, D. Robinson, D.P. Seidel, V. Spinu, K. Takahashi, D. Vaughan, C. Wilke, K. Woo, H. Yutani, Welcome to the Tidyverse, J. Open Source Softw. 4 (2019) 1686, https://doi.org/10.21105/joss.01686.

[18] Y. Taniyama, K.K. Griendling, Reactive oxygen species in the vasculature: molecular and cellular mechanisms, Hypertension 42 (2003) 1075–1081, https://doi.org/10.1161/01.HYP.0000100443.09293.4F.

[19] T. Yuan, T. Yang, H. Chen, D. Fu, Y. Hu, J. Wang, Q. Yuan, H. Yu, W. Xu, X. Xie, New insights into oxidative stress and inflammation during diabetes mellitus-accelerated atherosclerosis, Redox Biol. 20 (2019) 247–260, https://doi.org/10.1016/j.redox.2018.09.025.

[20] S. Devaraj, N. Glaser, S. Griffen, J. Wang-Polagruto, E. Miguelino, I. Jialal, Increased monocytic activity and biomarkers of inflammation in patients with type 1 diabetes, Diabetes 55 (2006) 774–779, https://doi.org/10.2337/diabetes.55.03.06.db05-1417.

[21] P. Marchetti, Q. Fovez, N. Germain, R. Khamari, J. Kluza, Mitochondrial spare respiratory capacity: mechanisms, regulation, and significance in non-transformed and cancer cells, Faseb. J. 34 (2020) 13106–13124, https://doi.org/10.1096/fj.202000767R.

[22] A. Czajka, S. Ajaz, L. Gnudi, C.K. Parsade, P. Jones, F. Reid, A.N. Malik, Altered mitochondrial function, mitochondrial DNA and reduced metabolic flexibility in patients with diabetic nephropathy, EBioMedicine 2 (2015) 499–512, https://doi.org/10.1016/j.ebiom.2015.04.002.

[23] P. Li, B. Wang, F. Sun, Y. Li, Q. Li, H. Lang, Z. Zhao, P. Gao, Y. Zhao, Q. Shang, D. Liu, Z. Zhu, Mitochondrial respiratory dysfunctions of blood mononuclear cells link with cardiac disturbance in patients with early-stage heart failure, Sci. Rep. 5 (2015), 10229, https://doi.org/10.1038/srep10229.

[24] J.W. Zmijewski, S. Banerjee, H. Bae, A. Friggeri, E.R. Lazarowski, E. Abraham, Exposure to hydrogen peroxide induces oxidation and activation of AMP-activated protein kinase, J. Biol. Chem. 285 (2010) 33154–33164, https://doi.org/10.1074/jbc.M110.143685.

[25] R. Scherz-Shouval, E. Shvets, Z. Elazar, Oxidation as a post-translational modification that regulates autophagy, Autophagy 3 (2007) 371–373, https://doi.org/10.4161/auto.4214.

[26] K.R. Menikdiwela, L. Ramalingam, F. Rasha, S. Wang, J.M. Dufour, N. S. Kalupahana, K.K.S. Sunahara, J.O. Martins, N. Moustaid-Moussa, Autophagy in metabolic syndrome: breaking the wheel by targeting the renin–angiotensin system, Cell Death Dis. 11 (2020) 1–17, https://doi.org/10.1038/s41419-020-2275-9.

[27] D. Chen, J. Liu, L. Lu, Y. Huang, Y. Wang, M. Wang, Y. Liu, D. Xie, J. Chen, J. Diao, L. Wei, M. Wang, Emodin attenuates TNF-α-induced apoptosis and autophagy in mouse C2C12 myoblasts though the phosphorylation of Akt, Int. Immunopharm. 34 (2016) 107–113, https://doi.org/10.1016/j.intimp.2016.02.023.

[28] J. Harris, Autophagy and cytokines, Cytokine 56 (2011) 140–144, https://doi.org/10.1016/j.cyto.2011.08.022.

[29] F. Iannantuoni, A.M. de Marañon, Z. Abad-Jiménez, F. Canet, P. Díaz-Pozo, S. López-Domènech, C. Morillas, M. Rocha, V.M. Víctor, Mitochondrial alterations

and enhanced human leukocyte/endothelial cell interactions in type 1 diabetes, J. Clin. Med. 9 (2020) 2155, https://doi.org/10.3390/jcm9072155.

[30] M. Zeng, X. Wei, Z. Wu, W. Li, B. Li, Y. Zhen, J. Chen, P. Wang, Y. Fei, NF-κB-mediated induction of autophagy in cardiac ischemia/reperfusion injury, Biochem. Biophys. Res. Commun. 436 (2013) 180–185, https://doi.org/10.1016/j.bbrc.2013.05.070.

[31] E.M. Bradshaw, K. Raddassi, W. Elyaman, T. Orban, P.A. Gottlieb, S.C. Kent, D. A. Hafler, Monocytes from patients with type 1 diabetes spontaneously secrete proinflammatory cytokines inducing Th17 cells, J. Immunol. 183 (2009) 4432–4439, https://doi.org/10.4049/jimmunol.0900576.

9 ANNEX III: PUBLICATIONS

Title: Does Empagliflozin Modulate Leukocyte–Endothelium Interactions, Oxidative Stress, and Inflammation in Type 2 Diabetes?

Authors: Canet F, Iannantuoni F, Marañon AM, Díaz-Pozo P, López-Domènech S, Vezza T, Navarro B, Solá E, Falcón R, Bañuls C, Morillas C, Rocha M, Víctor VM.

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Does Empagliflozin Modulate Leukocyte–Endothelium Interactions, Oxidative Stress, and Inflammation in Type 2 Diabetes?

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Abstract: Sodium-glucose co-transporter 2 inhibitors (iSGLT2) have been linked to cardiovascular risk reduction in patients with type 2 diabetes (T2D). However, their underlying molecular mechanisms remain unclear. This study aimed to evaluate the effects of empagliflozin, a novel potent and selective iSGLT-2, on anthropometric and endocrine parameters, leukocyte–endothelium interactions, adhesion molecules, ROS production, and NFkB-p65 transcription factor expression. According to standard clinical protocols, sixteen T2D patients receiving 10 mg/day of empagliflozin were followed-up for 24 weeks. Anthropometric and analytical measurements were performed at baseline, 12 weeks, and 24 weeks. Interactions between polymorphonuclear leukocytes and human umbilical vein endothelial cells (HUVECs), serum levels of adhesion molecules (P-Selectin, VCAM-1 and ICAM-1) and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), mitochondrial ROS levels, antioxidant enzymes (*SOD1* and *GPX1*), and NFkB-p65 were measured. We observed a decrease in body weight, BMI, and HbA1C levels from 12 weeks of treatment, which became more pronounced at 24 weeks and was accompanied by a significant reduction in waist circumference and glucose. Leukocyte–endothelium interactions were reduced due to an enhancement in the leukocyte rolling velocity from 12 weeks onwards, together with a significant decrease in leukocyte rolling flux and adhesion at 24 weeks. Accordingly, a significant decrease in ICAM-1 levels, mitochondrial ROS levels, and IL-6 and NFkB-p65 expression was observed, as well as an increase in *SOD1*. This pilot study provides evidence of the anti-inflammatory and antioxidant properties of empagliflozin treatment in humans, properties which may underlie its beneficial cardiovascular effects.

Keywords: cardiovascular risk; empagliflozin; inflammation; oxidative stress; type 2 diabetes

1. Introduction

Cardiovascular diseases (CVDs) are the most common cause of mortality in type 2 diabetic (T2D) patients, and hyperglycemia, hypertension, dyslipidemia, and obesity are important risk factors for CVDs. In particular, under chronic hyperglycemic conditions, elevated levels of circulating advanced glycation end products (AGEs) play a central role in the pathogenesis of the micro- and macrovascular complications related to T2D [1], promoting cellular dysfunction and regulating endothelial cell permeability, monocyte migration, and expression of adhesion molecules [2]. Another important aspect in the development of CVDs is the atherosclerotic process, which is mediated by peripheral

polymorphonuclear leukocytes (PMNs). PMNs are activated under chronic hyperglycemia and play a crucial role in CVDs by promoting cellular and endothelial impairment due to vessel recruitment and leukocyte aggregation [3] or through reactive oxygen species (ROS) production, which reduces antioxidant defense systems such as superoxide dismutase 1 (SOD1) and glutathione peroxidase 1 (GPX1), thus leading to oxidative stress. Consequently, these events promote NF-κB activation, altering, in turn, pro-inflammatory gene expression and eventually inducing cardiovascular impairment [4].

In addition, the relationship between glycated hemoglobin levels (HbA1C), inflammation, and CVDs points to modulation of HbA1C levels as a potentially interesting therapeutic goal. In this sense, inhibitors of sodium and glucose co-transporter 2 (iSGLT2) are one of many classes of anti-diabetic agents and could represent an effective therapeutic strategy given their safety and potential both as a monotherapy and in combination with other anti-diabetic drugs [5]. Empagliflozin is an iSGLT2 approved for the treatment of adults with T2D and, as demonstrated by the EMPA-REG OUTCOME study, exerts both cardioprotective and renoprotective effects [6].

In the present pilot study, we investigated the potential therapeutic benefits of empagliflozin treatment (12 and 24 weeks) on leukocyte–endothelial interactions, adhesion molecules, mitochondrial ROS production, and NFkB-p65 expression, all of which are implicated in the development of atherosclerosis and CVDs.

2. Materials and Methods

2.1. Patients and Sample Collection

This is an observational and prospective follow-up study of a cohort of eighteen patients diagnosed with T2D according to the American Diabetes Association's criteria and attending the Endocrinology Department of the University Hospital Doctor Peset (Valencia, Spain). Patients were recruited when physicians added empagliflozin to their usual treatment according to the hospital's standard clinical protocols. Two of the eighteen patients were excluded from the study due to a lack of treatment adherence. Subjects were asked to follow a scheduled visit program that included follow-up at 12 and 24 weeks after the first visit.

The inclusion criteria were as follows: age between 40 and 70 years and evolution of diabetes greater than 10 years. The exclusion criteria were as follows: severe diabetic neuropathy, significant renal impairment (creatinine > 1.5 mg/dL or eGFR < 60 mL/min/1.73 m^2), morbid obesity (BMI > 40 kg/m^2), smoking habit or frequent alcohol intake, and chronic diseases other than those directly related to cardiovascular risk. Empagliflozin was administered orally at doses of 10 mg/day according to the normal clinical practice [6]. Measurements were assessed at baseline and at 12 and 24 weeks of empagliflozin treatment. The most common side effects for iSGLT2 treatment described in the literature are genital and urinary tract infections, but none of our subjects developed any of these conditions during the study. All subjects were informed about the study procedures and gave their informed written consent. The study was performed in compliance with the statement of ethical principles for medical research of the Declaration of Helsinki and obtained approval from the hospital's ethics committee (CEIC 98/19).

2.2. Anthropometric and Biochemical Analysis

During the first and follow-up appointments, patients underwent a physical examination to determine the following anthropometrical parameters: weight (kg), height (m), waist circumference (cm), and systolic (SBP) and diastolic blood pressures (DBP, mmHg). After 12 h of overnight fasting, blood samples were taken from 8:00 to 10:00 a.m. and centrifuged (1.500 *g*, 10 min, 4 °C) to separate serum or plasma prior to determining biochemical and molecular parameters. Biochemical determinations were carried out by our hospital´s Clinical Analysis Service and evaluated as usual: glucose, triglycerides, and total cholesterol levels in serum were measured by means of an enzymatic method; insulin levels were calculated by immunochemiluminescence and insulin resistance was

measured by homeostasis model assessment (HOMA-IR = [fasting insulin (μU/mL) × fasting glucose (mg/dL)]/405); percentage of HbA1C was measured with an automated glycohemoglobin analyzer (Arkray Inc., Kyoto, Japan); levels of high-density lipoprotein cholesterol (HDL-c) were assessed with a Beckman LX-20 autoanalyzer (Beckman Coulter, La Brea, CA, USA); low-density lipoprotein cholesterol (LDL-c) was estimated with Friedewald's formula; and high-sensitive C-reactive protein (hs-CRP) levels were determined by an immunonephelometric assay (Behring Nephelometer II, Newark, DE, USA).

2.3. Leukocyte Isolation

Citrated blood samples were incubated for 45 min with 3% *w*/*v* dextran in phosphate-buffered saline solution (PBS; Sigma Aldrich, St. Louis, MO, USA). To isolate PMNs, supernatants were placed on Ficoll-Hypaque (GE Healthcare, Barcelona, Spain), and gradient centrifugation was performed (650g for 25 min at RT). The supernatant was discarded, and the bottom phase containing the PMN pellet was incubated for 5 min at RT with lysis buffer to eliminate the remaining erythrocytes. The sample was then centrifuged (1.200 rpm, 5 min) and washed twice with Hank´s Balance Salt Solution (HBSS; Sigma Aldrich, St. Louis, MO, USA). Finally, the pellet was resuspended in complete RPMI medium (Biowest-bw, Nuaillé, France) supplemented with 10% FBS. Aliquots of 1.0×10^6 cell/mL were employed in the subsequent experiments.

2.4. Leukocyte–Endothelium Interactions, Pro-Inflammatory Cytokines, and Cellular Adhesion Molecule Evaluation

For adhesion assays, we used an ex vivo model based on a parallel plate flow chamber, as described before [7]. In brief, human umbilical vein endothelial cells (HUVECs) were harvested from fresh umbilical cords obtained from healthy donors. Primary cultures of HUVECs were grown over fibronectin-coated cell culture dishes (Corning, NY, USA) and incubated with complete Endothelial Cell Basal Medium-2 supplemented with Growth Medium-2 Supplement kit (both from PromoCell GmbH, Heidelberg, Germany) until HUVECs reached confluence. A portion of 5 × 25 mm of the HUVEC monolayer was exposed to the PMN flux and recorded using an inverted microscope (Nikon Eclipse TE 2000-S, Amstelveen, The Netherlands) coupled to a video camera (Sony Exware HAD, Koeln, Germany). Along the HUVEC monolayer, suspensions of PMNs were perfused at a flow rate of 0.36 mL/min (human blood flow rate in physiological condition). Real-time images of the flow-exposed monolayer were recorded for 5 min and further analyzed to extrapolate leukocyte rolling flux, rolling velocity, and adhesion [8].

Levels of pro-inflammatory markers (TNF-α, IL-1β, and IL-6) and cellular adhesion molecules in serum samples (P-Selectin, ICAM-1, and VCAM-1) were evaluated using a Luminex 200 flow analyzer system (Luminex Corp., Austin, TX, USA). Milliplex® MAP human high sensitivity T Cell and Human Cardiovascular Disease Magnetic Bead Panel were purchased from Millipore Corporation (Billerica, MA, USA). The intra-serial CV was <5.0%, and the inter-serial CV was <15.0%, for all determinations.

2.5. Evaluation of Mitochondrial ROS Production in Leukocytes

Mitochondrial ROS production was evaluated by static cytometry using a fluorescence microscope (IX81; Olympus, Hamburg, Germany) coupled with the static cytometry software ScanR (Olympus, Hamburg, Germany). After extraction, fresh PMNs were seeded in 48-well plates (at 1.5 × 105 PMNs per well) and incubated for 30 min with red mitochondrial superoxide indicator (MitoSOX, 5 μM). Cells from each patient were seeded in triplicate, and 12 images per well were recorded. To visualize nuclei, we coupled both fluorochromes with Hoechst 33342 (4 μM, Sigma Aldrich, St. Louis, MO, USA). Fluorescence was standardized and referred to as a percentage of control.

2.6. Western Blot Analysis

Leukocytes were incubated for 15 min on ice with a lysis buffer (400 mM NaCl, 20 mM HEPES pH 7.5, 0.1 mM EDTA, 20% glycerol, 10 μM Na2MoO4, and 0.5% Nonidet P-40)

containing protease inhibitors (10 mM β-glycerolphosphate, 10 mM NaF, 10 mM PNP, and 1 mM Na3VO4) and 1 mM dithiothreitol and were then centrifuged at 4 °C for 15 min. Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific, Chicago, IL, USA). Protein samples (25 µg) were resolved by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After blocking, they were incubated with primary antibodies overnight at 4 °C. We used the following primary antibodies: anti-NFκB-p65 (phospho S536) rabbit polyclonal antibody (Abcam, Cambridge, MA, USA) and anti-β actin rabbit polyclonal antibody (Sigma Aldrich, MO, USA). Blots were incubated with goat anti-rabbit HRP secondary antibody (Millipore Iberica, Madrid, Spain) and developed for 2 min with supersignal west femto (Thermo Fisher Scientific, IL, USA). Chemiluminescence signals were detected with a Fusion FX5 acquisition system (Vilbert Lourmat, Marne La Vallée, France) and analyzed by densitometry using Bio1D software (Vilbert Lourmat, Marne La Vallée, France). Protein bands were normalized to the expression of β-actin in the same sample.

2.7. Gene Expression Analysis

RNA was isolated from leukocytes using a GeneAll Ribospin Total RNA extraction kit (GeneAll Biotechnology, Hilden, Germany) following the manufacturer's indications. RNA quantification was obtained using a NanoDrop 200c spectrophotometer (Life Technologies, Thermo Fisher Scientific), and purity was confirmed with the 260 nm/280 nm and 260 nm/230 nm absorbance ratios. cDNA was generated with the RevertAid first-strand cDNA synthesis kit (Life Technologies, Thermo Fisher Scientific). Working aliquots (1:10 v/v) of the first-strand cDNA were prepared, and 2 µL of these aliquots was used in further steps. We assessed NFkB-p65(RelA) relative gene expression using quantitative RT-PCR in a 7500 Fast RT-PCR system (Life Technologies, Carlsbad, CA, USA) and the $2-\Delta\Delta CT$ method. β-actin gene expression was employed as an endogenous control, and we calculated the average ΔCt of the basal group to calculate the ΔΔCt values for every sample. qRT-PCR reactions were carried out as follows: 10 min at 95 °C, 40 cycles (designed in one step) at 95 °C for 10 s, and one cycle at 60 °C for 30 s, as well as a melting curve stage. For the reaction mix, we used LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany). Data were analyzed with Expression Suite software (Life Technologies, Thermo Fisher Scientific) and Microsoft Excel. The specific sequence, accession number, and annealing temperature of each primer are shown in Table 1.

Table 1. Primer sequences used in qRT-PCR assays.

Gene Name	Primer Name	Primer Sequence	Sequence Accession Number
Superoxide dismutase 1	*SOD1* Forward	GGTGTGGCCGATGTGTCTAT	NM_000454
	SOD1 Reverse	TTCCACCTTTGCCCAAGTCA	
Glutathione peroxidase 1	*GPX1* Forward	TTGAGAAGTTCCTGGTGGGC	NM_000581.4
	GPX1 Reverse	CGATGTCAGGCTCGATGTCA	
RELA proto-oncogene, NF-kB subunit	*NFkB p65* Forward	ATCCCATCTTTGACAATCGTGC	NM_021975
	NFkB p65 Reverse	CTGGTCCCGTGAAATACACCTC	
Actin beta	*Actin B* Forward	CCTCGCCTTTGCCGATCC	NM_001101
	Actin B Reverse	CGCGGCGATATCATCATCC	

2.8. Statistical Analysis

Statistical analysis was carried out using GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com, accessed 15 January 2021). Normality was confirmed using the Shapiro–Wilk test. Parametric data were expressed as mean ± standard deviation (SD), and non-parametric data as a median with 25th and 75th percentiles. Statistical significance between groups was assessed by one-way ANOVA followed by a Tukey or Dunnett multiple comparisons test for parametric data, or the Friedman test followed by Dunn's multiple comparisons test for non-parametric data. A

paired or unpaired *t*-test was employed when two groups were compared. Differences of $p < 0.05$ were considered statistically significant. Bar graphs show mean ± standard error of the mean (SEM).

3. Results

3.1. Anthropometric and Biochemical Analysis

This study initially involved eighteen T2D patients who initiated treatment with empagliflozin. All patients had received stable glucose-lowering therapy for at least 12 months before being recruited for the study, and they continued with this therapy in combination with empagliflozin during the entire study period. The information compiled regarding the concomitant medications taken by the participants in our study is summarized in Table 2.

Table 2. List of concomitant medications.

Patient	Lipid-Lowering Medication	Antihypertensive Medication	Antithrombotic Medication	Diuretic Medication
1	-	-	-	-
2	Atorvastatin	-	-	-
3	Excluded from the study			
4	Atorvastatin	-	ASA	-
5	Atorvastatin	Enalapril + bimatoprost + timolol	-	HCTZ
6	Simvastatin	Valsartan + Amlodipine	-	HCTZ
7	-	-	-	-
8	Atorvastatin	Ramipril	ASA	-
9	Atorvastatin	Nebivolol + Valsartan	Clopidogrel + ASA	-
10	Pravastatin + Fenofibrate	-	-	-
11	Atorvastatin	-	ASA	-
12	-	-	-	-
13	Simvastatin	Manidipine + Olmesartan	-	HCTZ
14	Atorvastatin	Amlodipine + Irbesartan	-	HCTZ
15	Atorvastatin	Eprosartan		HCTZ
16	Simvastatin	Telmisartan	ASA	HCTZ
17	Atorvastatin	-	-	-
18	Excluded from the study			

Abbreviations: ASA, acetylsalicylic acid; HCTZ, hydrochlorothiazide.

In terms of the medical history of microvascular complications, four and five diabetic patients were affected by retinopathy and nephropathy, respectively.

Table 3 shows the anthropometric and biochemical data for our study population. We observed that empagliflozin significantly reduced the body weight of the participants at 12 weeks of treatment ($p < 0.01$) and that this reduction was maintained at 24 weeks ($p < 0.01$ vs. baseline and $p < 0.05$ vs. 12 weeks). In parallel, a significant waist circumference reduction was observed at 24 weeks ($p < 0.01$ vs. baseline and $p < 0.05$ vs. 12 weeks). These data were confirmed by a progressive reduction in BMI ($p < 0.05$ at 12 weeks and $p < 0.01$ at 24 weeks, both vs. baseline). The data show a decrease in glucose levels after 24 weeks ($p < 0.05$) and in HbA1C levels from 12 weeks onwards ($p < 0.05$).

Table 3. Anthropometric characteristics of the study population at baseline, and at 12- and 24-week follow-up.

	Baseline	12-Week Empagliflozin	24-Week Empagliflozin
N	16	16	16
Age (years)	59.7 ± 10.8	-	-
Sex (female)	5		
Weight (kg)	85.7 ± 20.1	82.9 ± 20.3 **	81.6 ± 20.3 ** #
Waist circumference (cm)	102.7 ± 12.3	99.8 ± 13.5	97.1 ± 13.7 ** #
BMI	31.4 ± 5.3	30.3 ± 5.4 *	29.9 ± 5.6 **
SBP (mmHg)	139.5 ± 26.9	139.6 ± 24.6	133.9 ± 21.7
DBP (mmHg)	76.4 ± 14.1	81.0 ± 16.3	73.9 ± 12.7
Glucose (mg/dL)	149.1 ± 35.9	134.1 ± 32.6	125.2 ± 19.9 *
HbA_{1C} (%)	7.6 ± 1.3	7.2 ± 1.3 *	6.8 ± 0.9 *
Insulin (μUI/mL)	9.6 ± 5.4	9.6 ± 5.5	9.5 ± 5.9
HOMA-IR	3.88 ± 2.16	3.32 ± 1.54	3.10 ± 2.10
Total cholesterol (mg/dL)	141.0 ± 25.4	154.7 ± 27.6 *	149.5 ± 27.1 *
LDL-c (mg/dL)	82.3 ± 16.9	87.2 ± 16.6	89.1 ± 19.8
HDL-c (mg/dL)	46.1 ± 6.1	43.2 ± 7.1	47.5 ± 3.7
Triglycerides (mg/dL)	92 (83–131)	113 (100–168)	104 (82–122)
hs-RCP	2.42 (1.2–11.5)	4.28 (1.5–7.8)	1.9 (1.3–5.8)

Data are expressed as mean ± SD for parametric variables and median (interquartile range) for non-parametric data. The following statistical analyses were performed: for parametric variables, a repeated measures one-way ANOVA followed by Tukey's multiple comparisons test; for non-parametric variables, a Friedman test followed by Dunn's multiple comparisons test. * $p < 0.05$ vs. baseline, ** $p < 0.01$ vs. baseline, # $p < 0.05$ vs. 12-week empagliflozin treatment. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HbA1C, glycated hemoglobin A1C; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; hs-CRP, high-sensitivity C-reactive protein; LDL-c, low-density lipoprotein cholesterol; SBP, systolic blood pressure.

Total cholesterol levels were increased at 12 weeks ($p < 0.05$) and were maintained at 24 weeks ($p < 0.05$). No significant changes in LDL-c and HDL-c were observed after treatment with empagliflozin. Patients receiving insulin as part of their treatment were excluded from HOMA-IR and insulin assessments. Lastly, we did not observe differences in triglyceride and hs-CRP levels.

3.2. Leukocyte–Endothelium Interactions and Adhesion Molecule Expression

The leukocyte rolling velocity (Figure 1A) was enhanced at 12 and 24 weeks of treatment with empagliflozin ($p < 0.05$ and $p < 0.01$, respectively) compared to baseline. Regarding the PMN rolling flux and adhesion (Figure 1B,C), data for both show a tendency to decrease, which became significant at 24 weeks of treatment ($p < 0.05$ both). To explore, in more depth, the results obtained during the leukocyte–endothelium interaction assays, we studied the expression of adhesion molecules in the serum at 24 weeks of treatment. We observed a significant reduction in P-Selectin and ICAM-1 expression levels at 24 weeks (Figure 1D,F; $p < 0.05$), but not in the expression of VCAM-1 (Figure 1D,E).

3.3. Mitochondrial Superoxide Production

To evaluate whether empagliflozin had an effect on oxidative stress parameters, we measured mitochondrial superoxide production. The fluorescence of MitoSOX decreased significantly at 24 weeks of treatment (Figure 2A; $p < 0.05$), indicating a reduction in oxidative stress in leukocytes from T2D patients. Interestingly, this reduction was associated with a significant increase in the mRNA expression of *SOD1* (Figure 2B; $p < 0.05$), and a tendency for *GPX1* to rise after 24 weeks of treatment (Figure 2C).

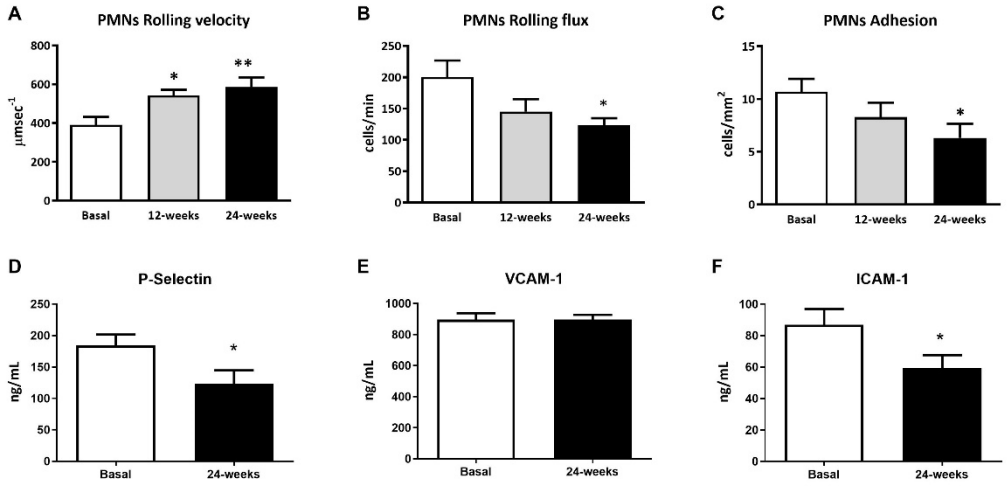

Figure 1. Effects of empagliflozin treatment on leukocyte–endothelium interactions and serum soluble cell adhesion molecules in type 2 diabetic patients at baseline and at 12 and 24 weeks of treatment. (**A**) Leukocyte rolling velocity ($\mu m \cdot sec^{-1}$), (**B**) rolling flux (cells/min), (**C**) leukocyte adhesion (cells/mm^2), (**D**) P-Selectin levels (ng/mL), (**E**) VCAM-1 levels (ng/mL), (**F**) ICAM-1 levels (ng/mL). For leukocyte–endothelium interactions, an ANOVA followed by Dunnett's multiple comparisons test was performed. For serum soluble cell adhesion molecules, Student's t-test was performed. * $p < 0.05$ and ** $p < 0.01$ vs. baseline. Data are expressed as mean ± SEM. Abbreviations: PMN, polymorphonuclear leukocytes; ICAM-1, intercellular adhesion molecule-1; P-Selectin, platelet selectin; VCAM-1, vascular cell adhesion molecule-1.

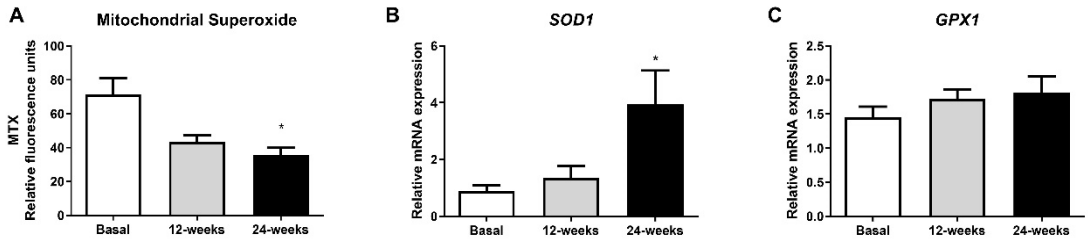

Figure 2. Effects of empagliflozin treatment on mitochondrial superoxide production and expression of *SOD1* and *GPX1* in leukocytes from type 2 diabetic patients at baseline and at 12 and 24 weeks of treatment. (**A**) Mitochondrial superoxide production, (**B**) mRNA expression of *SOD1*, and (**C**) mRNA expression of *GPX1* in human leukocytes. Data are expressed as mean ± SEM. Mitochondrial superoxide was measured as relative MitoSOX fluorescence by static cytometry, and data were normalized with respect to fluorescence at baseline. The values of *SOD1* and *GPX1* gene expression were normalized to mean baseline mRNA expression levels and calculated using the $2-\Delta\Delta CT$ method. For mitochondrial superoxide production, an ANOVA followed by Dunnett's multiple comparisons test was performed. For mRNA expression of *SOD1* and *GPX1*, a paired Student's t-test was performed. * $p < 0.05$ vs baseline. Abbreviations: MTX, MitoSOX Red mitochondrial superoxide indicator; *GPX1*, glutathione peroxidase 1; *SOD1*, superoxide dismutase 1.

3.4. Inflammatory Parameters

Changes in diabetes-related inflammatory status were measured in terms of IL-6, TNF-α, and IL-1β levels in the serum and phospho NFκB-p65 protein expression. Empagliflozin treatment was able to markedly reduce IL-6 serum levels, although no changes in TNF-α and IL-1β were detected (Figure 3A; $p < 0.05$, Figure 3B,C). Moreover, a decrease in phospho NFκB-p65 levels in leukocytes from T2D patients at 24 weeks of empagliflozin treatment (Figure 3E; $p < 0.05$) was observed. In parallel, we obtained similar results after evaluating gene expression (Figure 3D; $p < 0.05$).

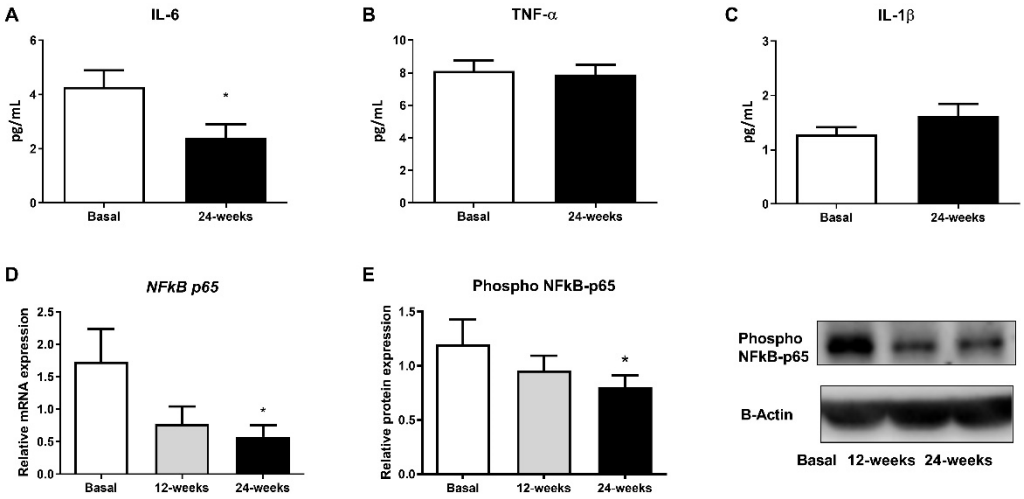

Figure 3. Effects of empagliflozin treatment on serum levels of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β, as well as production and expression of NFκB-p65, in leukocytes from T2D patients at baseline and at 12 and 24 weeks of treatment. Serum levels of (**A**) IL-6, (**B**) TNF-α, and (**C**) IL-1β, (**D**) mRNA expression of NFkB-p65, and (**E**) protein levels of phospho NFκB-p65, representative WB images in human leukocytes. Data are expressed as mean ± SEM. The values of NFkB-p65 gene expression were normalized to mean baseline mRNA expression levels and calculated using the $2-\Delta\Delta CT$ method. For mRNA expression of NFkB-p65, a paired Student's t-test was performed. * $p < 0.05$ vs. baseline. Abbreviations: IL, interleukin; NFκB-p65, nuclear factor kappa-light-chain-enhancer of activated B cells; TNF-α, tumor necrosis factor alpha.

4. Discussion

In this observational, prospective follow-up study, we analyzed the effects of the iSGLT2 empagliflozin on cardiovascular parameters, the atherosclerotic process, and inflammation, including leukocyte–endothelium interactions, adhesion molecules, mitochondrial ROS, and the serum and gene expression profile of different pro-inflammatory markers after 12 and 24 weeks of treatment. Furthermore, we explored some of the beneficial effects of empagliflozin, including weight reduction, decreased BMI and waist circumference, and improved glucose and HbA1C levels [5].

We evaluated the effects of empagliflozin on leukocyte–endothelial cell interactions by using an ex vivo parallel-flow chamber assay that mimics the physiological blood flow. As a result, we observed that empagliflozin increases the PMN rolling velocity in consonance with a decrease in the PMN rolling flux and adhesion at 24 weeks of treatment. These actions suggest that this drug exerts a beneficial effect by protecting against the early stages of the atherosclerotic process. Hyperglycemia and increased levels of HbA1C are key factors in the atherosclerosis process and are related to enhanced leukocyte–endothelium interactions, mitochondrial impairment, and oxidative stress [8]. Enhanced leukocyte–endothelium interactions have also been linked to insulin resistance [8]. Our data confirm that empagliflozin treatment reduces hyperglycemia in general, which would improve insulin resistance. In line with the beneficial effect of empagliflozin, the drug has been reported to protect the heart from inflammation and energy depletion via AMPK activation, as well as preventing doxorubicin-induced myocardial dysfunction [9].

Of note, an increase in total cholesterol, LDL-c, and HDL-c plasma levels has also been shown in T2D patients treated with empagliflozin, as previously reported [10,11]. The mechanism by which SGLT2 inhibition raises these levels is yet to be determined. It has been suggested that the increase in cholesterol is partly attributable to hemoconcentration, as SGLT2 inhibitors induce volume contraction as a response to the increased urinary volume [12,13]. It is important to note that, although our study participants tended to have higher levels of cholesterol following treatment with empagliflozin, the mean of these concentrations was lower than that of healthy individuals, since the former group had been

receiving statins, which are known to block the rate-limiting step of cholesterol synthesis and reduce the relative risk of cardiovascular disease associated with diabetes [14].

In addition, endothelial–leukocyte interactions depend on the levels of adhesion molecules resulting from vascular inflammation and dysfunction and involved in the recruitment of immune cells and platelets to the endothelium. In this sense, the present results show a reduction in the expression of the adhesion molecules P-selectin and ICAM-1 after 24 weeks of empagliflozin treatment. These results are in accordance with those previously reported by our group showing that empagliflozin reduces the levels of the inflammatory enzyme myeloperoxidase—which is actively involved in the development of microvascular alterations and increased release of the anti-inflammatory interleukin-10 (IL-10). Considered as a whole, these data suggest that empagliflozin ameliorates the inflammatory state and reduces the risk of CVDs. In support of this, empagliflozin has been shown to reduce inflammation and boost the antioxidant response of leukocytes from T2D patients [15].

It is well known that T2D is related to oxidative stress and that this leads to pro-inflammatory responses. In fact, enhanced ROS levels activate the pro-inflammatory nuclear factor NFκB, thus contributing to insulin resistance. Considering this, we decided to explore whether empagliflozin can modulate mitochondrial ROS levels as well as NFκB-p65 protein and gene expression in leukocytes from T2D patients. Interestingly, we showed that empagliflozin decreases mitochondrial ROS production. This reduction was associated with a significant increase in the mRNA expression of *SOD1*, and a tendency towards an increased mRNA expression of *GPX1* expression, two critical markers of the mitochondrial antioxidant capacity. Moreover, the expression of p65 (phospho S563) was reduced by the treatment, highlighting empagliflozin as a molecule with anti-inflammatory properties that, by decreasing IL-6 levels, modulates not only oxidative stress and leukocyte–endothelium interactions but also the inflammatory response.

5. Conclusions

In conclusion, this preliminary study provides evidence that treatment with empagliflozin decreases leukocyte–endothelium interactions, adhesion molecules, mitochondrial ROS, and IL-6 and NFkB expression in T2D and enhances antioxidant activity. This highlights the value of this drug for preventing the atherosclerotic process, inflammation, and, consequently, possible cardiovascular events in T2D patients.

6. Study's Limitations

This study has some limitations. The potential effects of empagliflozin are based on observational and prospective studies with a limited number of patients. However, we performed this pilot study with the primary aim of estimating average values and variability in order to plan future studies in larger populations. In this sense, although the sample size is quite limited, this study provides valuable preliminary evidence of the anti-inflammatory and antioxidant properties of empagliflozin treatment in humans, properties which may underlie its beneficial cardiovascular effects.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Doctor Peset (CEIC 30/17).

Informed Consent Statement: Informed written consent was obtained from all the participants involved in this study.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The data are not publicly available due to patient's privacy.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Strain, W.D.; Paldanius, P.M. Diabetes, cardiovascular disease and the microcirculation. *Cardiovasc. Diabetol.* **2018**, *17*, 57. [CrossRef] [PubMed]
2. Bevilacqua, M.P.; Stengelin, S.; Gimbrone, M.A., Jr.; Seed, B. Endothelial leukocyte adhesion molecule 1: An inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science* **1989**, *243*, 1160–1165. [CrossRef] [PubMed]
3. Jay, D.; Hitomi, H.; Griendling, K.K. Oxidative stress and diabetic cardiovascular complications. *Free Radic. Biol. Med.* **2006**, *40*, 183–192. [CrossRef] [PubMed]
4. Brownlee, M. Biochemistry and molecular cell biology of diabetic complications. *Nature* **2001**, *414*, 813–820. [CrossRef] [PubMed]
5. Frampton, J.E. Empagliflozin: A Review in Type 2 Diabetes. *Drugs* **2018**, *78*, 1037–1048. [CrossRef] [PubMed]
6. Schernthaner, G.; Schernthaner-Reiter, M.H.; Schernthaner, G.H. EMPA-REG and Other Cardiovascular Outcome Trials of Glucose-lowering Agents: Implications for Future Treatment Strategies in Type 2 Diabetes Mellitus. *Clin. Ther.* **2016**, *38*, 1288–1298. [CrossRef] [PubMed]
7. Banuls, C.; de Maranon, A.M.; Veses, S.; Castro-Vega, I.; Lopez-Domenech, S.; Salom-Vendrell, C.; Orden, S.; Alvarez, A.; Rocha, M.; Victor, V.M.; et al. Malnutrition impairs mitochondrial function and leukocyte activation. *Nutr. J.* **2019**, *18*, 89. [CrossRef] [PubMed]
8. de Maranon, A.M.; Canet, F.; Abad-Jimenez, Z.; Jover, A.; Morillas, C.; Rocha, M.; Victor, V.M. Does Metformin Modulate Mitochondrial Dynamics and Function in Type 2 Diabetic Patients? *Antioxid. Redox Signal.* **2021**. [CrossRef] [PubMed]
9. Koyani, C.N.; Plastira, I.; Sourij, H.; Hallstrom, S.; Schmidt, A.; Rainer, P.P.; Bugger, H.; Frank, S.; Malle, E.; von Lewinski, D. Empagliflozin protects heart from inflammation and energy depletion via AMPK activation. *Pharmacol. Res.* **2020**, *158*, 104870. [CrossRef] [PubMed]
10. Nauck, M.A. Update on developments with SGLT2 inhibitors in the management of type 2 diabetes. *Drug Des. Dev. Ther.* **2014**, *8*, 1335–1380. [CrossRef] [PubMed]
11. Haring, H.U.; Merker, L.; Seewaldt-Becker, E.; Weimer, M.; Meinicke, T.; Broedl, U.C.; Woerle, H.J.; Investigators, E.-R.M.T. Empagliflozin as add-on to metformin in patients with type 2 diabetes: A 24-week, randomized, double-blind, placebo-controlled trial. *Diabetes Care* **2014**, *37*, 1650–1659. [CrossRef] [PubMed]
12. Pieber, T.R.; Famulla, S.; Eilbracht, J.; Cescutti, J.; Soleymanlou, N.; Johansen, O.E.; Woerle, H.J.; Broedl, U.C.; Kaspers, S. Empagliflozin as adjunct to insulin in patients with type 1 diabetes: A 4-week, randomized, placebo-controlled trial (EASE-1). *Diabetes Obes. Metab.* **2015**, *17*, 928–935. [CrossRef] [PubMed]
13. Kohler, S.; Zeller, C.; Iliev, H.; Kaspers, S. Safety and Tolerability of Empagliflozin in Patients with Type 2 Diabetes: Pooled Analysis of Phase I-III Clinical Trials. *Adv. Ther.* **2017**, *34*, 1707–1726. [CrossRef] [PubMed]
14. Stroes, E. Statins and LDL-cholesterol lowering: An overview. *Curr. Med. Res. Opin.* **2005**, *21* (Suppl. S6), S9–S16. [CrossRef] [PubMed]
15. Iannantuoni, F.; de Marañon, A.M.; Diaz-Morales, N.; Falcon, R.; Banuls, C.; Abad-Jimenez, Z.; Victor, V.M.; Hernandez-Mijares, A.; Rovira-Llopis, S. The SGLT2 Inhibitor Empagliflozin Ameliorates the Inflammatory Profile in Type 2 Diabetic Patients and Promotes an Antioxidant Response in Leukocytes. *J. Clin. Med.* **2019**, *8*, 1814. [CrossRef] [PubMed]