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FMUP FACULDADE DE MEDICINA
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POLYPHENOLS IN DIABETIC VASCULAR COMPLICATIONS: FROM MECHANISMS TO IDENTIFICATION OF THERAPEUTIC TARGETS

TESE DE DOUTORAMENTO APRESENTADA

À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO EM

METABOLISMO: CLÍNICA E EXPERIMENTAÇÃO

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List of publications

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I. Costa R, Rodrigues I, Guardão L, Rodrigues S, Magalhães J, Negrão R and Soares R (2017). Dietary polyphenols ameliorate diabetic-related metabolic dysfunctions in mice. J Nutr Biochem 45: 39-47

II. Costa R, Rodrigues I, Guardão L, Lima JQ, Sousa E, Soares R and Negrão R (2017). Polyphenol-induced modulation of VEGF signaling in diabetes: Unveiling the angiogenic paradox and metabolism interplay. Mol Nutr Cell Biol 61(4)
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A REPRODUÇÃO DESTAS PUBLICAÇÕES FOI FEITA COM AUTORIZAÇÃO DAS RESPECTIVAS EDITORAS

Resumo

A diabetes *mellitus* tipo 2 (DM2) é uma doença metabólica crónica que atinge proporções epidémicas a nível mundial. A resistência à insulina e o resultante estado de hiperglicemia crónica são as principais causas da desregulação do metabolismo glicídico e lipídico. As complicações vasculares associadas à diabetes contribuem significativamente para as elevadas taxas de mortalidade e morbidade observadas nesta doença. Diversas evidências científicas sugerem que os polifenóis da dieta, entre outros benefícios, têm a capacidade de modular a angiogénese e de regular certas vias metabólicas. Dado o seu potencial pleiotrópico, os polifenóis têm surgido como candidatos promissores para a prevenção e tratamento de doenças crónicas, nomeadamente a síndrome metabólica e a DM2.

A presente tese tem como objetivo deslindar o efeito do consumo de dois polifenóis derivados da cerveja, o xantohumol (XM) e a 8-prenilnaringenina (8PN), num modelo animal de DM2 induzido pelo consumo de uma dieta hipercalórica, salientando o estudo da desregulação metabólica e angiogénica e nos mecanismos moleculares e vias de sinalização subjacentes.

Os resultados obtidos demonstraram que o XN e a 8PN reduziram o ganho de peso corporal, atenuaram a hiperglicemia, melhoraram a sensibilidade à insulina e a homeostasia da glicose. Ambos os polifenóis melhoraram o perfil lípidico plasmático, reduzindo os triglicerídeos, o colesterol total, conjuntamente com uma melhoria no metabolismo lipídico. Tanto o XN como a 8PN ativaram a proteína cínase ativada pela 5’ adenosina monofosfato, inibindo a expressão da proteína de ligação ao elemento regulador do sterol (SREBP)-1c e das enzimas lipogénicas alvo, carboxílase da acetil-CoA e a síntase dos ácidos gordos. Adicionalmente, o XN e a 8PN reduziram a expressão do translocador de ácidos gordos CD36 e a sinalização despoletada pela ligação do fator de crescimento endotelial vascular-B ao recetor do fator de crescimento endotelial vascular-1, envolvida no transporte e captação de lípideos,

prevenindo assim a acumulação ectópica de gordura. O XN e a 8PN modularam a expressão da cínase 2 da frutose-6-fosfato/fosfátase 2 da frutose-2,6-bisfosfato, uma enzima glicolítica bifuncional cuja expressão está aumentada nas células endoteliais ativas, através da redução da sua expressão no rim e um aumento no ventrículo esquerdo. Este perfil de resultados vai de encontro aos resultados obtidos no chamado paradoxo angiogénico, evidenciado por um aumento da vascularização do rim e uma redução da angiogénese no ventrículo esquerdo. Notavelmente, o XN impediu o aumento da angiogénese renal e a 8PN ativou o processo angiogénico no ventrículo esquerdo, atenuando a desregulação angiogénica.

Os resultados supramencionados ampliam o nosso conhecimento sobre a suplementação nutricional com polifenóis. O XN e a 8PN atenuaram a disfunção metabólica e exerceram efeitos distintos na angiogénese e na ativação da glicólise, de acordo com o microambiente em cada um dos tecidos. Esta relação entre a angiogénese e o metabolismo surge assim como um potencial alvo para os polifenóis, constituindo moléculas promissoras para terapias dirigidas, promovendo uma melhor resolução das complicações associadas à DM2.

Palavras-chave

Diabetes *mellitus*, metabolismo, angiogénese, polifenóis

Abstract

Type 2 diabetes *mellitus* (T2DM) is a lifelong metabolic disorder with epidemic proportions. Insulin resistance and resultant hyperglycemia are the main drive forces for the deregulation of glucose and lipid metabolic pathways. Diabetic vascular complications significantly contribute for the T2DM morbidity and mortality rates. Thus, elucidating how the vascular system interplays with metabolic imbalances is of paramount importance. Growing evidence suggests that dietary polyphenols, among other benefits, are able to modulate angiogenesis and regulate metabolic pathways. As a result of its multifunctional properties, polyphenols have emerged as promising candidates for further development to treat chronic diseases namely metabolic syndrome and T2DM.

This thesis aimed to unravel the effect of consumption of two polyphenols, xanthohumol (XN) and 8-prenylnaringenin (8PN), in high-fat diet-induced T2DM mice model, focusing on metabolic and angiogenic deregulation and their underlying molecular mechanisms.

The obtained results demonstrated that XN and 8PN reduced body weight gain, mitigated hyperglycemia and improved insulin sensitivity and glucose homeostasis. Both polyphenols ameliorated plasmatic lipid profile, by reducing triglycerides and cholesterol levels together with an improvement of lipid metabolism. XN and 8PN activated 5' adenosine monophosphate-activated protein kinase, inhibiting the expression of the sterol regulatory binding protein (SREBP)-1c and lipogenic target enzymes, acetyl-CoA carboxylase and fatty acid synthase. Moreover, XN and 8PN reduced the expression of fatty acid translocase/CD36 and vascular endothelial growth factor receptor-1 - vascular endothelial growth factor-B signaling, involved in the lipid transport and uptake, preventing ectopic lipid accumulation. XN and 8PN also modulated the expression of 6-phosphofructo-2-kinase-2/fructose-2,6-bisphosphatase 3, a glycolytic bifunctional enzyme that is up-regulated in active endothelial cells, by reducing its

expression in kidney and increasing in left ventricle. The former results are in agreement with the so-called angiogenic paradox that is evidenced by an increase in kidney vascularization and a reduction of angiogenesis in left ventricle. Remarkably, XN prevented the increase in kidney angiogenesis and 8PN activated angiogenic process in left ventricle.

The aforementioned data increase our knowledge regarding the beneficial effects of nutritional supplementation with polyphenols. XN and 8PN attenuated metabolic dysfunctions and exert distinct effects on angiogenesis and on the glycolysis activation, according to the distinct microenvironment within each tissue. The cross talk between angiogenesis and metabolism arises as a potential target for polyphenols, rendering them putative tissue specific-target agents that may allow a better resolution of T2DM-related complications.

Keywords

Diabetes *mellitus*, metabolism, angiogenesis, polyphenols

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Abbreviations list

3 PO	3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one
6PN	6-prenylnaringenin
8PN	8-prenylnaringenin
ACC	Acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMPK	5’ adenosine monophosphate-activated protein kinase
AUC	Area under the curve
BM-EPC	Bone marrow derived endothelial progenitor cells
CPT	Carnitine palmitoyltransferase
DGAT	Diacylglycerol acyltransferase
DII4	Delta-like 4
DM	Diabetes *mellitus*
DMX	Desmethylxanthohumol
DPP4	Dipeptidyl peptidase
EC	Endothelial cells
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
ET	Endothelin
F-2,6-BP	Fructose-2,6-bisphosphate
FA	Fatty acids
FABP	Fatty acid binding protein
FAS	Fatty acid synthase
FAT	Fatty acid translocase
FATP	Fatty acid transporter proteins

FBS	Fetal bovine serum
FFA	Free fatty acids
FGF	Fibroblast growth factor
GLP	Glucagon-like peptide
GLUT	Glucose transporter
HbA1c	Glycated haemoglobin A1c
HDL-c	High-density lipoprotein cholesterol
HFD	High-fat diet
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HMEC	Human microvascular endothelial growth factor
HOMA	Homeostasis model assessment
ICAM	Intercellular adhesion molecule
IPITT	Intraperitoneal insulin tolerance test
IR	Insulin receptor
IRS	Insulin receptor substrates
IXN	Isoxanthohumol
LCFA	Long-chain fatty acid
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinase
MCP	Mononuclear chemoactrative protein
MMP	Metalloproteinase
MODY	Maturity-onset diabetes of the young
NO	Nitric oxide
NP	Neuropilin
OGTT	Oral glucose tolerance test
ROS	Reactive oxygen species
PAI	Plasminogen activator inhibitor
PDGF	Platelet derived-growth factor

PD-ECGF	Platelet derived-endothelial cell growth factor
PEDF	Pigment epithelium-derived factor
PFK-1	6-phosphofructo-1 kinase
PFKFB3	6-phosphofructo-2-kinase-2/fructose-2,6-bisphosphatase 3
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PlGF	Placental growth factor
PPAR	Peroxisome proliferator-activated receptor
QUICKI	Quantitative insulin sensitivity check index
RNS	Reactive nitrogen species
SD	Standard deviation
SGLT	Sodium-glucose-linked transporter
SREBP	Sterol regulatory element-binding transcription factor
T1DM	Type 1 diabetes *mellitus*
T2DM	Type 2 diabetes *mellitus*
TG	Triglyceride
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TSP	Thrombospondin
TZD	Thiazolidinediones
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VLDL	Very low density lipoprotein
XN	Xanthohumol

I

Introduction

1. Diabetes *mellitus*

1.1. Epidemiology and background of diabetes *mellitus*

Diabetes *mellitus* (DM) is a world epidemic and growing health problem, affecting 415 million people presently and predicted to affect more than 600 million by 2040 (Ogurtsova *et al.*, 2017). According to the latest diabetes atlas from the International Diabetes Federation, DM was responsible for 4.6 million annually deaths worldwide, corresponding to one death every seven seconds. With its increasing prevalence, DM is becoming a leading cause of morbidity and mortality, placing a huge demand on the economic burden costs to health care systems and lifetime medical resources (Ogurtsova *et al.*, 2017).

There are four main forms of DM: gestational, maturity-onset diabetes of the young (MODY-1), type 1 DM (T1DM) and type 2 DM (T2DM). T2DM is the most prevalent one and accounts for almost 90% to 95% of diabetes cases (Gardner *et al.*, 2012; DeFronzo *et al.*, 2015; Al-Aissa *et al.*, 2017).

T2DM arises as a result of insulin resistance that initially is compensated by increased pancreatic β-cells mass and insulin secretion (Gerich, 2003; Bergman, 2013). With disease development, pancreatic β-cells mass and function progressively decline, being no longer able to release sufficient insulin in order to promote glucose uptake in peripheral tissues, such as skeletal muscle and adipose tissue. This condition is termed glucotoxicity and takes place when insulin-dependent tissues become desensitized to insulin, resulting in β-cell dysfunction and chronic hyperglycemia (Ye, 2013).

T2DM is complex and multifactorial disease. Several risk factors including obesity, physical inactivity, older age, genetic predisposition, high blood pressure and unfavorable lipid profile, namely increased cholesterol and triglycerides (TG) levels, play a causative role in the progression of T2DM (DeFronzo *et al.*, 2015). Endothelial dysfunction (Sena *et al.*, 2013), oxidative stress (Brownlee, 2001; Ceriello *et al.*, 2004) and chronic inflammation (Pfutzner *et*

al., 2006) are interrelated conditions in the etiology of T2DM. T2DM associated comorbidities result from micro and macrovascular complications together with defects in whole body glucose and lipid metabolism.

1.2. Diagnosis and available therapeutic approaches to type 2 diabetes *mellitus*

According to American Diabetes Association, T2DM may be diagnosed through plasma glucose criteria, either the fasting plasma glucose (≥126 mg/dL; 7.0 mmol/L), or the 2-hour oral glucose tolerance test (OGTT) after a 75 g glucose load (≥200 mg/dL; 11.1 mmol/L) or glycated hemoglobin A1c (HbA1c) criteria (HbA1c ≥6.5%; 48 mmol/mol) (American Diabetes, 2017). An early diagnosis is extremely important to delay or prevent its comorbidities (Tuomilehto *et al.*, 2001; Knowler *et al.*, 2002) and early screening is crucial since almost 30% of T2DM patients are undiagnosed (DeFronzo *et al.*, 2015).

Nutritional and physical activity approaches are still the cornerstone of treatment. When a good glycemic control is not attained with these lifestyle modifications, glucose-lowering drugs are required (Tuomilehto *et al.*, 2001; Knowler *et al.*, 2002; Tiwari, 2015). Despite recent therapeutic breakthroughs, the available treatments for diabetes management are not completely efficient, as highlighted by the elevated mortality and morbidity rates. Presently, the most well established pharmacological approach in T2DM management is the use of glucose-lowering drugs that maintain blood glucose concentration within the normal range. Metformin is one of these agents, which can be used alone or in combination with other agents with complementary mechanisms of action, performing a specific treatment strategy for each diabetic patient. These therapeutic agents include sulfonylureas, to promote insulin secretion, thiazolidinediones (TZD) insulin-sensitizing molecules (Del Prato, 2009), as well as more recent therapeutic agents namely incretin-based therapies including the glucagon-like

peptide (GLP)-1 receptor agonist, an insulinotropic molecule poorly secreted by diabetic individuals, and dipeptidyl peptidase (DPP)-4 inhibitors, which prolong GLP-1 half-life (Del Prato, 2009; DeFronzo *et al.*, 2015; Meece, 2017). Moreover, there is a novel therapeutic strategy, the sodium-glucose-linked transporter (SGLT)-2 inhibitors, which have been clinically used to target renal glucose absorption (Ahuja *et al.*, 2016), promoting urinary glucose elimination. Management of T2DM frequently requires the combination of two or three of the aforementioned agents to accomplish a durable glycemic control with a specific management of the therapeutic strategy for each patient (Raz *et al.*, 2013). When those pharmacological agents fail to normalize glycemic levels, an additional therapy with insulin is often required (Yacoub, 2014). Despite of the benefits, some of the current available treatments have limited efficacy, safety, cost-effectiveness or present unwanted side-effects (Tahrani *et al.*, 2016). Therefore, it is essential to investigate the molecular signaling pathways involved in the pathogenesis of T2DM to develop new therapies, ideally with increased efficacy and reduced adverse effects.

1.3. Metabolic disturbances in the pathogenesis of type 2 diabetes *mellitus*

Liver and skeletal muscle are crucial tissues in the regulation of whole-body homeostasis and energy metabolism. The sustained elevations in glucose and FA blood levels observed in T2DM, lead to impairment of several cellular pathways and contribute to the pathogenesis of the disease. It is of paramount importance to fully elucidate the underlying mechanisms of this disease to provide guidance for T2DM prevention and treatment.

1.3.1. Glucose metabolism imbalance in type 2 diabetes *mellitus*

The liver is the main producer of the endogenous glucose that is released into the circulation to be uptaken by peripheral organs. The liver is therefore a central organ in glycemic control, since it regulates the balance between anabolic and catabolic pathways (Meyer *et al.*, 2002). In the fed state, the organism responds to the short-term disturbance in blood glucose levels following the intake of nutrients with a variety of systemic neuroendocrine responses, including the secretion of insulin by the pancreas that together with other hormones plays a central role in the regulation of glucose and lipid metabolism in the liver. In response to enhanced glucose concentration, pancreatic β-cells release insulin, which binds to insulin receptor (IR) in the plasma membrane of insulin-sensitive cells, subsequently inducing the phosphorylation of selective adaptor proteins, the insulin receptor substrate (IRS)-1. The entire process is tightly regulated in distinct steps, both positively and negatively in a time-controlled manner to attain a proper signal duration and intensity (Moore *et al.*, 2003; Boucher *et al.*, 2014). After a meal, insulin elicits signaling cascades that result in suppression of endogenous glucose production through the inhibition of gluconeogenesis and glycogenolysis. Insulin also stimulates glucose utilization through increased glycolysis and glycogenesis. Conversely, in the fasting state, the glucose disposal is ascribed by insulin-independent tissues (Moore *et al.*, 2003). These effects are facilitated by an increase of gluconeogenic substrates including certain aminoacids, lactate, and glycerol and mediated by the activation of gluconeogenic enzymes, such as pyruvate carboxylase, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. Together with the activation of glycogenolysis, these processes account for approximately 90 % of glucose disposal (Bergman, 2013).

In T2DM, however, the dysfunction in insulin signaling impairs the regulation of the liver glucose metabolism. For example, the insulin-induced suppression of glucose production through gluconeogenesis and glycogenolysis is promptly prevented. As a consequence,

sustained hyperglycemia and hyperinsulinemia arise and exert deleterious effects in the entire organism(Boden, 2011; Boucher *et al.*, 2014).

Blood glucose originates either from the intestine after dietary intake or from endogenous production in the liver and kidney and is uptaken by peripheral tissues, most notably by the skeletal muscle. Being the key site of glucose uptake, the skeletal muscle is therefore one of the main targets for the management of insulin resistance-related diseases, such as T2DM (Bouzakri *et al.*, 2005).

The glucose transporter (GLUT)-4 controls the majority of glucose transport mediated by insulin, while GLUT-1 mediates basal glucose transport (Saltiel *et al.*, 2001). Together, GLUT-4 and GLUT-1 maintain glucose blood levels within a tight physiological range.

In the skeletal muscle, insulin controls glucose uptake by increasing the translocation of glucose GLUT-4 into the plasma membrane (Bouzakri *et al.*, 2005; Thong *et al.*, 2005). Insulin binds to its receptor inducing tyrosine phosphorylation of IRS-1. Therefore, IRS-1 activates phosphatidylinositol 3-kinase (PI3K), which in turn phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) in the membrane and generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Thong *et al.*, 2005). The cascade of events involving PIP3-dependent kinases, activates AKT protein and phosphorylates its substrate, AS160. Once activated, AS160 promotes GLUT-4 translocation from the intracellular vesicles to cell membrane increasing the glucose uptake in skeletal muscle (Kramer *et al.*, 2006). In T2DM, the protein content of GLUT-4 at the cell surface is markedly reduced and the insulin-stimulated glucose transport is compromised (Ryder *et al.*, 2000; Bouzakri *et al.*, 2005). This can also be attributed to decreased expression of AS160 in T2DM (Karlsson *et al.*, 2005; Karlsson *et al.*, 2006).

Notwithstanding, the recruitment of GLUT-4 to the cell membrane to mediate the glucose transporter can also be promoted by insulin-independent mechanisms in response to muscular fibers contraction during exercise and by a nucleoside analogue, 5-aminoimidazole-4-

carboxamide ribonucleotide (AICAR). Muscle contraction and AICAR are both activators of the 5 adenosine monophosphate-activated protein kinase (AMPK) pathway (Kramer *et al.*, 2006; Treebak *et al.*, 2006). Once activated, AMPK facilitates the glucose transport by increasing AS160 and consequently GLUT-4 trafficking in skeletal muscle (Kramer *et al.*, 2006; Treebak *et al.*, 2006). Importantly, AMPK also constitutes a cellular energy sensor that acts as a major regulator of the whole-body energy balance by modulating tissue-specific metabolic pathways, rendering this protein an important therapeutic target.

1.3.2. Lipid metabolism impairment in type 2 diabetes *mellitus*

Impairment in insulin signaling and in glucose homeostasis also promotes and amplifies lipid metabolic dysfunction. Plasma free fatty acids (FFA) are elevated during fasting, and decreased in postprandial state, being those levels tightly controlled by insulin signaling (Ye, 2013). FA can be obtained not only from lipid intake, but also from *de novo* lipogenesis. Dietary lipids, being mostly TG, are hydrolyzed into monoacylglycerides and FFA by the pancreatic lipase. Once in the intestinal epithelial cells, monoacylglycerides are re-esterified into TG, which are incorporated into lipoprotein particles, chylomicrons. Then, chylomicrons enter the bloodstream through the lymphatic system, carrying dietary TG, which are hydrolyzed in FFA and glycerol by the lipoprotein lipase of endothelial cell (EC), allowing their delivery to tissues. Next, FFA are either re-synthesized in TG that can be stored in adipose tissue intracellular lipid droplets, or oxidized by β-oxidation to supply energy. Insulin regulates many of the abovementioned processes. For instance, insulin stimulates lipoprotein lipase and TG synthesis in both liver and adipose tissue and inhibits hormone-sensitive lipase in adipose tissue, reducing lipolysis (Moore *et al.*, 2003).

When insulin resistance emerges, even in the presence of nutrient abundance, insulin is not able to suppress peripheral tissues lipolysis resulting in increased and sustained levels of

FFA in the bloodstream. This contributes to a metabolic impairment leading to an early T2DM development (Boden, 2011; Bergman, 2013; Sears *et al.*, 2015). Moreover, low-grade chronic inflammation that accompanies T2DM, negatively affects insulin signaling due to adipokines and inflammatory mediators released by the adipose tissue (Hotamisligil, 2006). T2DM is also characterized by hyperlipidemia, including hypercholesterolemia and hypertriglyceridemia. This results from a deregulation in TG-rich lipoproteins. Increased levels of plasma TG, decreased high-density lipoprotein cholesterol (HDL-c), and increased levels of small dense low density lipoproteins (LDL) particles are commonly found in T2DM. These are accompanied by an elevated hepatic production and lower clearance of very low density lipoprotein (VLDL) and intestinal-derived chylomicrons (Howard *et al.*, 2003; Bergman, 2013). Altogether, these features contribute to the lipotoxicity associated with T2DM (Bergman, 2013). FFA released by the adipose tissue in insulin resistance conditions, are mainly stored in the liver and skeletal muscle, and contribute to lipid toxicity (Ye, 2013). Furthermore, an imbalance between the rate of FA uptake and mitochondrial β-oxidation, *de novo* FA synthesis and secretion of TG may lead to the intracellular accumulation of TG and several intermediate metabolites of the FFA re-esterification pathway, such as diacylglycerol, ceramides and long-chain fatty Acyl-CoA (Boden, 2011). These lipid metabolites are intracellular signaling molecules that alter the insulin signaling and, consequently, its action (Ye, 2013; Holland *et al.*, 2007). The increment in FFA uptake and β-oxidation impairs not only lipid, but also glucose metabolism. This is due to the inhibition of both glucose transporters and glycolysis together with the stimulation of gluconeogenesis as a result of the increased availability of plasma gluconeogenic substrates.

Another important pathway increasingly investigated is the above-mentioned AMPK, a fundamental cornerstone of the metabolic regulation. Besides its role in the glycolytic process regulation, this important kinase is also a master regulator of lipid metabolism in the liver, skeletal muscle and adipose tissue, being an important target for metabolic diseases

management (Carling, 2017). Accordingly, AMPK signaling transduction pathway may underlie new mechanisms to control T2DM. Once activated, AMPK phosphorylates and inactivates the sterol regulatory element-binding transcription factor (SREBP)-1c, an important transcription factor involved in the synthesis and uptake of FA, acting as a key regulator of lipogenesis (Coughlan *et al.*, 2014; Kim, Yang*, et al.*, 2016).

SREBP-1c is a key regulator of lipogenesis controlling the expression of lipogenic enzymes, namely acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), major biosynthetic enzymes for FA synthesis (Hardie *et al.*, 1997; Zhou *et al.*, 2001; Li *et al.*, 2011). Lipogenesis encompasses the process of FA synthesis through the action of ACC and FAS and their subsequent esterification into TG. This process takes place mainly in the liver, but it also occurs in adipose tissue (Boden, 2011). The inhibition of ACC activity, through phosphorylation by AMPK or by downregulation of SREBP-1c, reduces the carboxylation of acetyl-CoA into malonyl-CoA (Figure 1.1). This molecule, resultant from the rate-limiting step of lipogenesis, is itself an allosteric inhibitor of carnitine palmitoyltransferase (CPT)-1, a major enzymatic regulator of FA oxidation. CPT-1 catalyzes the covalent binding of carnitine and long-chain fatty acids (LCFA) to enable LCFA to cross the mitochondrial membrane. Consequently, there is a stimulation of FA uptake by the mitochondria, where they undergo β-oxidation. Therefore, SREBP-1c expression downregulated by AMPK reduces lipogenesis and increases FA oxidation.

The activation of AMPK has been recently proposed as an attractive pharmacological target to treat insulin resistance-associated diseases as T2DM (Coughlan *et al.*, 2014; Carling, 2017; Ramesh *et al.*, 2016). In fact, the mechanisms of action of several pharmacological agents used in the treatment of T2DM are powerful inducers of AMPK. For instance, the insulin-sensitizing drugs from TZD family, which are activators of peroxisome proliferator-activated receptor (PPAR)-γ, the hypoglycemic drug metformin, GLP-1 agonists, and DPP4 inhibitors exert their effects through regulation of AMPK activity, demonstrating its ability on the prevention of

overt T2DM (Dutta *et al.*, 2016). Therefore, the identification of more specific and potent AMPK activators appears to be appellative in the treatment of T2DM.

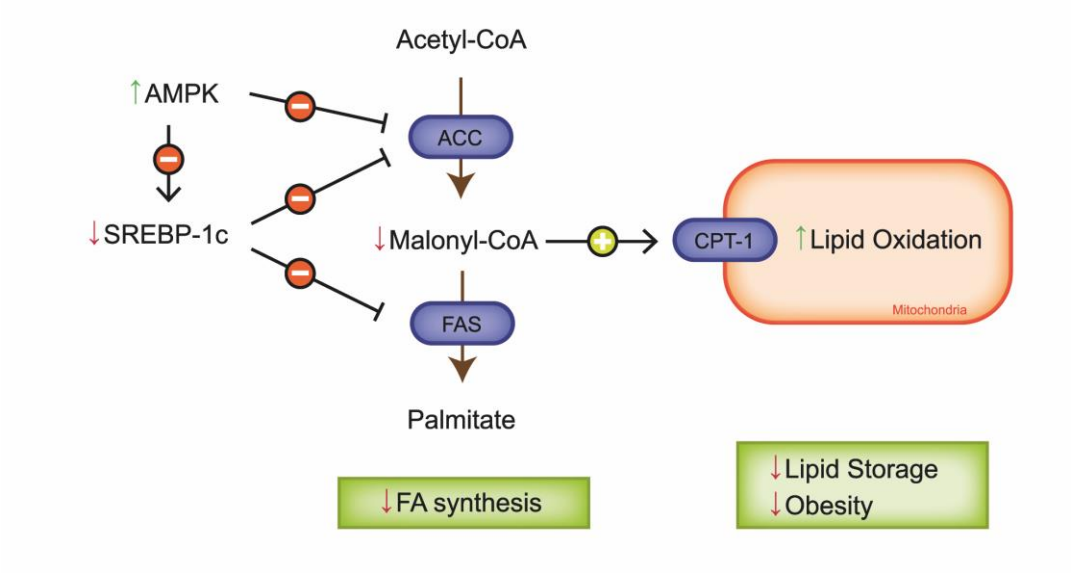

Figure 1.1 - Schematic representation of the inhibition of lipogenesis and activation of lipid oxidation mediated by 5' adenosine monophosphate-activated protein kinase (AMPK). Abbreviations: acetyl-CoA carboxylase (ACC); carnitine palmitoyl transferase (CPT)-1; fatty acid (FA); fatty acid synthase (FAS); sterol regulatory element binding protein (SREBP)-1c.

1.3.2.1. Lipid uptake by peripheral tissues

One of the main regulatory mechanisms to modulate the plasma lipid levels consists in the control of FFA entry and metabolism in peripheral tissues, being this process mediated by fatty acid transporter proteins (FATP). These transporters facilitate FA uptake into cells and subcellular organelles to rapidly provide substrate for metabolic demands or storage (Glatz *et al.*, 2010). The hydrolysis of TG-containing lipoproteins through the lipoprotein lipases action releases FFA into the circulation that together with the plasma FFA bound to albumin are internalized into different tissues across cell surface by specific transporters (Goldberg *et al.*, 2009). The fatty acid translocase (FAT), also recognized as the scavenger receptor CD36, is one of the regulators of lipid metabolism, since it controls FA entry into the cell, as well as oxidized

LDL, oxidized phospholipids and LCFA, by mediating their movement across the cell membrane in several tissues (Goldberg *et al.*, 2009). In fact, some studies performed with FAT/CD36 knockout mice have established the requirement of this translocase to assist FA uptake, since this transport is blunted in the skeletal muscle, cardiac muscle and adipose tissue of these animals. Additionally, increased CD36 levels in liver and skeletal muscle contributes to T2DM-related dyslipidemia (Koonen *et al.*, 2007). Another group of lipid transporters responsible for the increased rate of FA uptake are the FATP. FATP are expressed in EC and promote lipid transport and uptake to peripheral tissues. In particular, FATP-3 and FATP-4 have recently been shown to be upregulated in response to vascular endothelial growth factor (VEGF)-B (Li, 2010). Although belonging to the family of angiogenic factors, the signaling transduction pathway triggered by the binding between VEGF-B and its receptor, the vascular endothelial growth factor receptor (VEGFR)-1, and co-receptor neuropilin (NP)-1 has recently been associated with lipid metabolism, as represented in Figure 1.2 (Karpanen *et al.*, 2008; Li, 2010; Hagberg *et al.*, 2010).

The inhibition of this signaling pathway has attracted considerable attention, since it may prevent ectopic lipid accumulation and restore insulin sensitivity in obesity and T2DM animal models (Carmeliet *et al.*, 2012; Hagberg *et al.*, 2013; Sun *et al.*, 2014; Muhl *et al.*, 2016). As a matter of fact, several studies, in both humans and animal models, have implicated the membrane FA transporters in the pathogenesis of diseases such as insulin resistance and T2DM, being considered as a promising therapeutic target to control lipid fluxes in the body in a tissue-specific manner (Glatz *et al.*, 2010; Hagberg *et al.*, 2012).

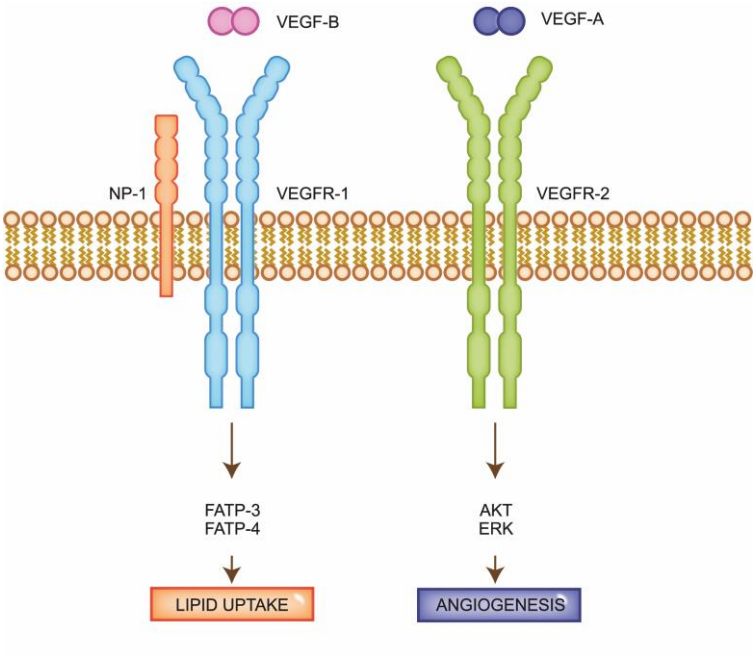

Figure 1.2 - Schematic representation of vascular endothelial growth factor (VEGF) function. Vascular endothelial growth factor (VEGFR)-1 and VEGFR-2, the receptors for VEGF-B and VEGF-A, respectively, and neuropilin-1 (NP-1), the co-receptor of VEGFR-1 are expressed on the surface of endothelial cells. The binding of VEGF-A to VEGFR-2 is the major mediator of angiogenesis through the activation of AKT and ERK. VEGF-B signaling mediated by its binding to VEGFR-1 and NP-1 has recently associated to endothelial targeting of lipids to peripheral tissues, mainly to the upregulation of fatty acid transporter protein (FATP)-3 and FATP-4.

1.4. Vascular complications in type 2 diabetes *mellitus*

Experimental and clinical evidence indicate that persistent hyperglycemia and insulin resistance, implicated in the development of T2DM, are major risk factors in the pathogenesis of vascular complications in diabetes (Ginsberg, 2000).

The functional and structural changes in blood vessels under hyperglycemia are induced by endothelial dysfunction. These alterations are also attributable to increased oxidative stress and chronic low-grade inflammation. These are important angiogenic inducers implicated in T2DM pathophysiology and responsible for the development and progression of T2DM-related vascular complications (Giacco *et al.*, 2010).

Early in the course of T2DM, EC are subjected to pathological alterations in response to hyperglycemia. Increased inflammatory factors, including C-reactive protein, interleukins and

tumor necrosis factor alpha (TNF-α), contribute to the chronic inflammation state. Additionally, high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) lead to oxidative stress, inducing uncoupled endothelial nitric oxide synthase (eNOS), which is responsible for the reduced production and bioavailability of nitric oxide (NO), an important vasodilator. Remarkably, overexpression of adhesion molecules including intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, mononuclear chemoactrative protein (MCP)-1, protein kinase C (PKC), leading to increased monocyte adhesion, as well as alterations in platelet functions and some markers of fibrinolysis, namely increased levels of plasminogen activator inhibitor (PAI)-1 are also associated with vascular modifications occurring in T2DM (Hempel *et al.*, 1997; Festa *et al.*, 2002; Inoguchi *et al.*, 2000; Kolluru *et al.*, 2012).

Beyond its important role in the regulation of glucose and FA metabolism and homeostasis, insulin also affects the vascular endothelium, through the regulation of blood flow and vascular tone (Baron, 1994; Steinberg *et al.*, 2002). The hemodynamic impact of insulin in blood vessels is mediated through binding to EC receptors and activation of two distinct pathways. The activation of PI3K pathway increases eNOS production and the subsequent release of NO. The resultant effect is the increase in the relaxation of surrounding smooth muscle cells and endothelial vasodilation, allowing a proper blood flow rate to target tissues (Steinberg *et al.*, 2002). The activation of the mitogen-activated protein kinase (MAPK) pathway leads to endothelin (ET)-1 release, causing vasoconstriction due to binding to its receptor ET_A on EC. The insulin-mediated balance between these effects leads to increased insulin diffusion, improving insulin sensitivity (Kolka *et al.*, 2013; De Nigris *et al.*, 2015). Impairment in insulin endothelial signaling affects blood flow and insulin delivery to tissues, independently of insulin direct effects on each tissue. Several lines of evidence described that the exposure to high glucose levels impairs insulin signaling in EC *in vitro*. EC derived from

obese animal models have diminished eNOS phosphorylation and reduced NO production, which prevents insulin-mediated vasodilation (Kubota *et al.*, 2011; De Nigris *et al.*, 2015).

Additionally, raised levels of circulating FA also impair insulin-mediated vasodilation by reducing the activity of PI3K and AKT in vascular cells, contributing to endothelial dysfunction and insulin resistance in the microvasculature (Steinberg *et al.*, 2002).

T2DM induces a widespread damage of the endothelium leading to micro and macrovascular complications that affect many organs and tissues (Chawla *et al.*, 2016). However, the underlying molecular mechanisms are still largely unknown. It is crucial to better understand the causes of T2DM-related vascular complications and unravel potential targets to develop therapeutic approaches aiming for their reduction or prevention.

1.4.1. Endothelial cell physiology and function

The vascular endothelium constitutes not only a selective, permeable barrier between the blood and tissues, but plays also, an important role in cellular metabolism, vasomotor balance and vascular-tissue homeostasis (Haller, 1997; Sharma *et al.*, 2012; Bierhansl *et al.*, 2017). The endothelium is composed by a monolayer of EC that line the entire inner surface of blood vessels, sitting on a basement membrane and surrounded by mural cells namely pericytes or smooth muscle cell layers, according to the vessel's caliber (Carmeliet, 2003). In the last decades, endothelium is being recognized as an active metabolic tissue with autocrine, paracrine and endocrine actions, mediating important physiological processes. In response to diverse mechanical and chemical stimuli, EC synthesize and release a large number of vasoactive molecules, growth factors, and mediate the transport of oxygen and nutrients, cytokines and hormones between the bloodstream and tissues, while removing toxic metabolic products. Endothelium has the capacity to ensure the maintenance of vascular tone by producing vasodilator or vasoconstrictor molecules to maintain normal blood flow; regulate

vascular smooth muscle cells and fibroblast proliferation, as well as transendothelial leucokyte adhesion and migration; control the production of thrombotic and fibrinolytic and platelet aggregation components; generate new blood vessels, among other functions (Quyyumi, 1998; Esper *et al.*, 2006).

1.4.2. Angiogenic process

In physiological conditions, EC remain quiescent in the adulthood, except under specific situations, namely wound-healing, pregnancy, menstrual cycle and hair growth, conditions where neovascularization is promoted (Carmeliet *et al.*, 2011). When EC are activated in response to angiogenic stimuli, they undergo vascular sprouting inducing the formation of new blood vessels from pre-existing ones, a process termed angiogenesis. Angiogenesis is a multistep process, involving a large variety of growth factors, receptors, and diverse cellular types that orchestrate the entire process of vessel sprouting in a coordinated and synergistic manner (Folkman, 1971; Duh *et al.*, 1999; Yancopoulos *et al.*, 2000; Ribatti *et al.*, 2000; Elayappan *et al.*, 2009; Carmeliet *et al.*, 2011; Tykhomyrov *et al.*, 2015). Additionally, the formation of blood vessels could also occur through vasculogenesis, a process that allows the formation of *de novo* blood vessels involving the recruitment, incorporation and differentiation of bone marrow-derived endothelial progenitor cells (BM-EPC) (Conway *et al.*, 2001; Carmeliet, 2003). Vasculogenesis is of major importance during embryonic development, but plays also a role in adulthood in different pathophysiological settings (Asahara *et al.*, 1999; Costa *et al.*, 2007).

The angiogenic process accomplishes several events including the degradation of the adjacent basement membrane, detachment of the surrounding pericytes and remodeling of the involving extracellular matrix (ECM) by the action of metalloproteinases (MMP), particularly MMP-2 and MMP-9. These allow local EC migration, proliferation, invasion and

formation of tubular structures, promoting the branching and anastomosis to assemble a vascular structure (Carmeliet *et al.*, 2011). Afterwards, the new vessel becomes lumenized, with the formation of a new basement membrane, and covered with mural cells to become functional (Carmeliet *et al.*, 2011).

The EC phenotype changes in the sequential stages of vessel growth. In response to stimuli, EC switch from a quiescent state to a highly migratory tip cells phenotype. Tip cells are at the forefront to guide the extending sprout through the ECM. The activation of pro-angiogenic signaling programs in tip cells, induce the expression of delta-like 4 (DII4), that acts in neighboring cells through binding the Notch receptor (De Smet *et al.*, 2009; Potente *et al.*, 2011). Once activated, Notch signaling inhibits a tip cell phenotype in these adjacent cells yielding them to adopt a proliferative stalk-like phenotype, responsible for branch elongation. These EC could dynamically switch between both tip and stalk phenotypes during vessel growth, in a competitive and dynamic manner, to reach the tip position (Jakobsson *et al.*, 2010; Potente *et al.*, 2011). Phalange cells line the established perfused vessels and acquire a quiescent state (Hellstrom *et al.*, 2007; Yoon *et al.*, 2014). The delicate balance between the dynamic process involving tip, stalk and phalanx EC phenotype via cell-to-cell signaling is necessary during vascular formation (Gerhardt *et al.*, 2003; Hellstrom *et al.*, 2007; De Smet *et al.*, 2009).

The angiogenic process is orchestrated by an intricate balance between pro-angiogenic factors such as, VEGF-A, placental growth factor (PIGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF), platelet derived-endothelial growth factor (PD-ECGF) and angiopoietins, and anti-angiogenic molecules including pigment epithelium-derived factor (PEDF), thrombospondin-1 (TSP)-1, PAI-1, angiostatin and endostatin (Yancopoulos *et al.*, 2000; Presta *et al.*, 2005; Ferrara *et al.*, 2005; Folkman, 2007).

Despite the contribution of the aforementioned promoters to vessel growth, there is consensus that VEGF-A is the most powerful pro-angiogenic factor and it is secreted by a wide variety of cells, activating EC (Ferrara *et al.*, 2003). VEGF-A stimulates EC through binding to VEGFR-2, conveying signal transduction pathways that regulate distinct cellular responses related to vascular physiology, metabolism and homeostasis. The VEGF family in mammals comprises five structurally-related members: VEFG-A to VEGF-D and PlGF, that exert its biological functions through binding to VEGFR-1, VEGFR-2 and VEGFR-3 (Rahimi, 2006; Koch *et al.*, 2012). VEGF-A binds and activates VEGFR-1 and VEGFR-2 on EC surface. Although VEGF-A has more affinity to VEGFR-1, the intracellular signal transduction is weak (Robinson *et al.*, 2001). The potent pro-angiogenic effect of VEGF-A is mediated by its binding to VEGFR-2, triggering dimerization and transautophosphorylation of cytoplasmatic tyrosine residues associated with distinct steps of the angiogenic process and constitutes a major regulator of vessel physiology (Ferrara *et al.*, 2005; Koch *et al.*, 2012). VEGF-A stimulates EC permeability and survival through the activation of PI3K activity, with subsequent increase in AKT and eNOS. Additionally, it potentiates the activation of PKC and mediates the activation of extracellular signal-regulated kinases (ERK)1/2, a pathway responsible for EC proliferation, migration, tubulogenesis and leukocyte adhesion (Meadows *et al.*, 2001; Zachary *et al.*, 2001; Fearnley *et al.*, 2014). Current knowledge about the role of VEGF-B on angiogenesis has led to controversial results, since some studies demonstrated a pro-angiogenic effect, whereas others observed an anti-angiogenic potential in several experimental models (Silvestre *et al.*, 2003; Kearney *et al.*, 2004; Li *et al.*, 2009). VEGF-B and PlGF bind to the same receptor, VEGFR-1 and NP-1. Surprisingly, however, it has been postulated that VEGF-B activates a signaling cascade related to lipid metabolism. PlGF signaling is involved in inflammation and pathological angiogenesis and also in embryogenesis (De Falco, 2012). VEGF-C and VEGF-D

bind preferentially to VEGFR-3 and VEGF-D and its expression in the is primarily on lymphatic vessels, regulating lymphandothelial function (Cebe-Suarez *et al.*, 2006; Koch *et al.*, 2012).

When a major disequilibrium between angiogenic stimulators and inhibitors arises, the angiogenic process becomes deregulated and is associated with multiple diseases (Folkman, 2007). Excessive angiogenesis is implicated in cancer and several chronic diseases namely, atherosclerosis, diabetic retinopathy, rheumatoid arthritis and age-related macular degeneration. On the contrary, insufficient vessel growth is found in chronic non-healing wounds, ulcers, ischemic heart disease and other pathological situations (Carmeliet *et al.*, 2011). In the pathogenesis of T2DM, angiogenesis plays an ambiguous role, with increased vascularization in certain organs and reduced in others, in the same organism, in the so-called angiogenic paradox.

1.4.3. Angiogenic paradox in diabetes *mellitus*

T2DM is characterized by endothelial dysfunction, resulting in increased inflammation and oxidative stress, impaired vasodilation and perfusion due to the imbalance in VEGF and NO production, and a pro-thrombotic state (Bierhansl *et al.*, 2017; Shi *et al.*, 2017). The heterogeneity of T2DM-related vascular complications is partially explained by the nature of micro- and macrovascular beds, the structure of the endothelium in each tissue, and the local microenvironment surrounding vessels (Waltenberger, 2007). Microvascular disease affects small vessels, including small resistance arteries, venules and capillaries and clinically results in retinopathy, nephropathy and neuropathy that contribute to blindness, kidney failure, limb amputations and nerve damage (Khazaei, 2011; Cheng *et al.*, 2015; Bierhansl *et al.*, 2017). Macrovascular disease occurs in large vessels, often leading to atherosclerosis, thromboembolism, cerebrovascular disease, ischemic heart disease, peripheral artery disease

and stroke. These vascular complications are the leading cause of morbidity and mortality among patients T2DM (Domingueti *et al.*, 2016; Bierhansl *et al.*, 2017).

Chronic hyperglycemia can lead to long-term tissue damage. Together with hyperinsulinemia, glycation and lipoxidation end products, oxidative stress, low-grade inflammation and hypoxia, it potentiates vascular complications associated with T2DM. The exposure of EC to abnormal levels of growth factors, pro-inflammatory cytokines and ROS during the course of T2DM, clearly impairs endothelial signaling pathways, affecting the balance between kinases and phosphatases that alter protein function. These alterations are accompanied by increased ECM synthesis and basal membrane thickness, which affect blood flow and vessel permeability. T2DM impairs the nutritive primary function of EC since the vasculature loses the ability to grow and regress in accordance to tissue metabolic demands (Soares *et al.*, 2009; Chawla *et al.*, 2016).

As mentioned, in T2DM the angiogenic process is affected in a tissue-dependent manner. Excessive angiogenesis occurs in organs, namely kidney and retina, whereas a marked decrease in neovessel formation is found in coronary artery disease and peripheral vascular disease. Patients with diabetic retinopathy exhibit elevated vitreous and aqueous VEGF-A and hypoxia inducible factor (HIF)-1α levels, together with VEGFR-2 overexpression, which ends up in increased neovessel formation (Betts-Obregon *et al.*, 2016). However, the newly formed vessels have less pericyte coverage, are leaky and dysfunctional. This leads to hemorrhages, exudates and edema and enhances diabetic retinopathy, a major cause of blindness (Betts-Obregon *et al.*, 2016; Grigsby *et al.*, 2016). Regarding diabetic nephropathy, diabetic mice exhibit higher renal VEGF-A levels and VEGFR-2 expression when compared to healthy controls. Additionally, diabetic animals exhibited overexpression of ECM components in renal cortex and glomeruli and increased permeability to macromolecules (Chen *et al.*, 2008). Conversely, reduced neovascularization is also a feature of T2DM. Several studies reported a

marked decrease in VEGF-A levels in diabetic dermal wounds and diabetic foot ulcers (Lerman *et al.*, 2003). Since angiogenesis is critical to guarantee normal resolution of wound healing, the reduction in vessel formation and VEGF-A levels, decreases the recruitment of several cell types to the injury site, which compromises the re-epithelization and collagen deposition. Altogether this promotes the occurrence of chronic, non-healing ulcers that frequently result in amputations (Bao *et al.*, 2009). In a study conducted with diabetic patients with coronary heart disease, high cardiac VEGF-A levels but reduced VEGFR-2 expression was found, preventing efficient angiogenic signaling (Sasso *et al.*, 2005). Other studies reported that the myocardium of diabetic patients exhibits reduced levels of both VEGF-A and its receptor VEGFR-2 as well as the downstream effectors AKT and eNOS, together with higher angiostatin production (Chou *et al.*, 2002; Weihrauch *et al.*, 2004; Sasso *et al.*, 2005). This deregulation favoring the anti-angiogenic in detriment of pro-angiogenic factors, leads to an insufficient angiogenesis in diabetic patients with ischemic cardiomyopathy.

Despite several experimental and clinical studies that have addressed the angiogenic paradox, the underlying molecular mechanisms remain to be explained. The present main challenge in T2DM consists in the discovery of tissue-specific therapeutic approaches.

1.4.4. Endothelial cell metabolism as a promising target for vascular disease

The EC metabolic pathways and their regulation during vessel formation and function have only been recognized in the last few years. The in-depth understanding of EC metabolism is crucial to identify potential targets to treat vascular complications.

Tip cells essentially rely on glycolysis to produce ATP for rapid generation of energy to migrate and proliferate towards avascular areas, in response to angiogenic stimuli (De Bock *et al.*, 2013; Eichmann *et al.*, 2013). In avascular areas, oxygen becomes limiting. In order to compensate for the oxygen deficit, EC are activated by HIF-1α and stimulate several

angiogenic factors, glucose transporters and glycolytic enzymes, stimulating the glycolytic pathway (Obach *et al.*, 2004; Cantelmo *et al.*, 2015). The EC glucose uptake is mediated through the activation of the PI3K-AKT pathway, which induces cell surface expression of glucose transporters, mainly GLUT-1 (Yeh *et al.*, 2008). In EC, glucose is metabolized into pyruvate through the glycolytic pathway (De Bock *et al.*, 2013). It has been reported that the 6-phosphofructo-2-kinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3) enzyme expression is induced by hypoxia in several cell lines (Atsumi *et al.*, 2005; Payne *et al.*, 2005) and animal models (Minchenko *et al.*, 2003). PFKFB3 is a bifunctional enzyme abundant in EC, that plays an important role in ensuring the high glycolytic flux, essential for vessel growth (De Bock *et al.*, 2013; Eichmann *et al.*, 2013; Eelen *et al.*, 2013; Zecchin *et al.*, 2015; Smith *et al.*, 2015). This enzyme contains both kinase and phosphatase activities but, under hypoxia conditions, its kinase activity is more active than the phosphatase one, favoring the fructose-2,6-bisphosphate (F-2,6-BP) synthesis. F-2,6-BP is the most potent allosteric activator of the key glycolytic enzyme 6-phosphofructo-1-kinase (PFK-1), and thereby sustains a high glycolytic rate (Van Schaftingen *et al.*, 1982; Eichmann *et al.*, 2013). Recently, Schoors and collaborators demonstrated the therapeutic potential of endothelial PFKFB3 regulation (Schoors, Cantelmo*, et al.*, 2014). The authors used a small molecule 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) to inhibit PFKFB3 *in vitro* and silencing PFKFB3 *in vivo* and the resultant effect was a 30-40% decrease in the glycolytic pathway. Consequently, there was a partial and transient glycolysis inhibition, sufficient to reduce EC migration, proliferation and sprouting (Schoors, De Bock*, et al.*, 2014; Zecchin *et al.*, 2015). Additionally, experiments in mice and zebrafish with a genetic deletion of *Pfkfb3,* showed a reduction in EC sprouting, rendering a stalk phenotype (De Bock *et al.*, 2013; Schoors, Cantelmo*, et al.*, 2014). These data suggest that anti-angiogenic therapies could be based on the inhibition of this metabolic target, starving pathological vessels. In contrary, if there is overexpression of PFKFB3 in stalk EC, they switch its angiogenic

properties acquiring a tip cell phenotype (De Bock *et al.*, 2013; Eelen *et al.*, 2013). Thus, glycolysis is induced by PFKFB3 overexpression and drives angiogenesis, independently of VEGF-A signaling, being sufficient to overcome Notch signaling that would normally promote a stalk phenotype (Schoors, Cantelmo*, et al.*, 2014). Hence, PFKFB3 expression appears as an important regulator of the endothelial phenotype, postulating that a metabolic switch induces the angiogenic switch.

Moreover, recent research demonstrates the role of endothelium in regulating FA transport and uptake for internal use or to fuel perivascular cells (Hagberg *et al.*, 2010). As mentioned above, the best characterized endothelial FA transporters are CD36 and members of the FATP family (Kazantzis *et al.*, 2012). Thus, targeting these lipid transporters is a promising therapeutic strategy for the management T2DM vascular complications.

Interestingly, as already reported, emerging research revealed the interesting role of VEGF-B signaling in the endothelial uptake of circulating FA, through the stimulation of VEGFR-1 and co-receptor NP-1. VEGFR-1 activation enhances the expression of FATP-3 and FATP-4. Herein, the activation of these transporters in EC promotes endothelial lipid uptake and FA delivery in peripheral tissues, to be metabolized (Hagberg *et al.*, 2010; Eichmann *et al.*, 2013). Recently, it was demonstrated that EC treated with VEGF-B increase gene and protein expression of several FATP without affecting the expression of FAT/CD36 or fatty acid binding protein (FABP) (Hagberg *et al.*, 2010). Studies performed in mice fed with HFD and treated with antibodies against VEGF-B showed improvements in insulin sensitivity and glucose tolerance due to decreased endothelial-to-tissues transport of FA. (Hagberg *et al.*, 2010; Carmeliet *et al.*, 2012; Hagberg *et al.*, 2012). These findings are of special importance since the reduction of VEGFB-VEGFR1 signaling could decrease ectopic lipid deposition, a major contributor for the pathogenesis of T2DM.

Angiogenesis seems to be coordinated not only by the well-known angiogenic factors but also by metabolic mediators. Given its pivotal location and function, the endothelium has vital roles in non-vascular systems regulating a wide variety of cell types and tissues, highlighting its therapeutic potential. Metabolism appears therefore as an interesting target to modify EC structure and function, particularly in diseases such T2DM in which angiogenesis is compromised. Therapeutic interventions focused on the modulation of the VEGF-VEGFR axis and EC metabolic molecules, could lead to a better management and improvement of T2DM outcomes.

2. Polyphenols

2.1. Polyphenols in plants and nutritional bioavailability

Polyphenols are phytochemicals and secondary metabolites of plants, abundant in fruits, vegetables, cacao, and beverages, such as wine, tea and beer, widely consumed in Western diets. As secondary metabolites, polyphenols are very important to plant defense mechanisms and to its survival, propagation and development, contributing to their colors, aroma, flavor, bitterness, astringency and antioxidant protection (Manach *et al.*, 2004; Stevenson *et al.*, 2007).

Thus far, thousands of polyphenols have been identified with distinct chemical structures. They are mainly characterized by the presence of several hydroxyl groups covalently linked to an aromatic ring (Manach *et al.*, 2004). Structural differences of polyphenols result in distinct bioavailability due to differences in digestion, gut absorption and rate of metabolism leading to diverse biological activities and therapeutic value. The polyphenol content and chemical structure are distinct according to the climatic conditions, food sources, some of which contain a wide mixture of polyphenols (Manach *et al.*, 2004). However, it has been estimated that the

average dietary intake of these compounds is about 1 g per day, depending on nutritional habits and preferences (Scalbert *et al.*, 2000).

2.2. Health promoting properties

During the last decades, epidemiological and experimental studies have revealed health beneficial effects associated with the consumption of polyphenols (Stevenson *et al.*, 2007; Magalhães *et al.*, 2009). through the regulation of various biochemical, physiological and endocrinological pathways, highlighting their potential in maintaining health and preventing disease (Fraga *et al.*, 2010).

In general, beneficial effects of polyphenols in human's health have been associated to their antioxidant activity as free radical scavengers (Stevenson *et al.*, 2007). However, they also seem to have pleiotropic effects on biological systems, modulating signaling transduction, and gene expression (Santangelo *et al.*, 2007; Fraga *et al.*, 2010). Thus, polyphenols induce, simultaneously, more than one biological effect with distinct but sometimes overlapping mechanisms of action.

Based on several epidemiological findings, animal studies and clinical trials, polyphenols have been implicated in improving glucose homeostasis, reducing insulin resistance and decreasing inflammation (Santangelo *et al.*, 2007). This is particularly important when considering that obesity and metabolic diseases are associated with increased insulin resistance, endothelial dysfunction and dyslipidemia. These metabolic derangements are, in turn, associated with a higher risk for developing hypertension and cardiovascular diseases. Therefore, there is clear evidence to support the role of polyphenols as potential agents for the prevention and treatment of T2DM and its metabolic-associated abnormalities.

2.3. Xanthohumol and 8-prenylnaringenin in hops and beer

The female hop plant (*Humulus lupulus L.*) is a plant of the Cannabacaea family that contains large amounts of relevant secondary metabolites. Its flowers, hops, have been used since ancient times for traditional medicinal purpose. These include treatment of sleep disturbances, depression and management of menopausal symptoms. Nowadays, due to an increase of beer consumption worldwide, especially among young people, hops are almost exclusively used to confer aroma and bitterness in the beer production industry. (Zanoli *et al.*, 2008).

Hops and beer contain several polyphenols that have been studied by various research groups. The chalcone xanthohumol (XN) is the main hops prenylflavonoid. Its content varies from 0.2 to 1.1% (dry weight) in hop cones (Stevens *et al.*, 1999). A small amount of desmethylxanthohumol (DMX) can also be found in lupulin glands of hops. Hops chalcones (XN and DMX) may be accompanied by other prenylated flavanones such as isoxanthohumol (IXN), 8-prenylnaringenin (8PN) and 6-prenylnaringenin (6PN). As represented in Figure 2.1, XN is mainly converted into IXN, due to thermal isomerization during extraction of hop cones and wort boiling in beer process. However, hops' DMX has two free hydroxyl groups that can participate in cyclization and ring closure, leading to two flavanones 8PN and 6PN. 8PN, a potent phytoestrogen, can also be formed *in vivo* by *O*-demethylation of IXN by liver microsomes and intestinal bacteria metabolism (Stevens *et al.*, 1999; Yilmazer *et al.*, 2001; Nikolic *et al.*, 2005).

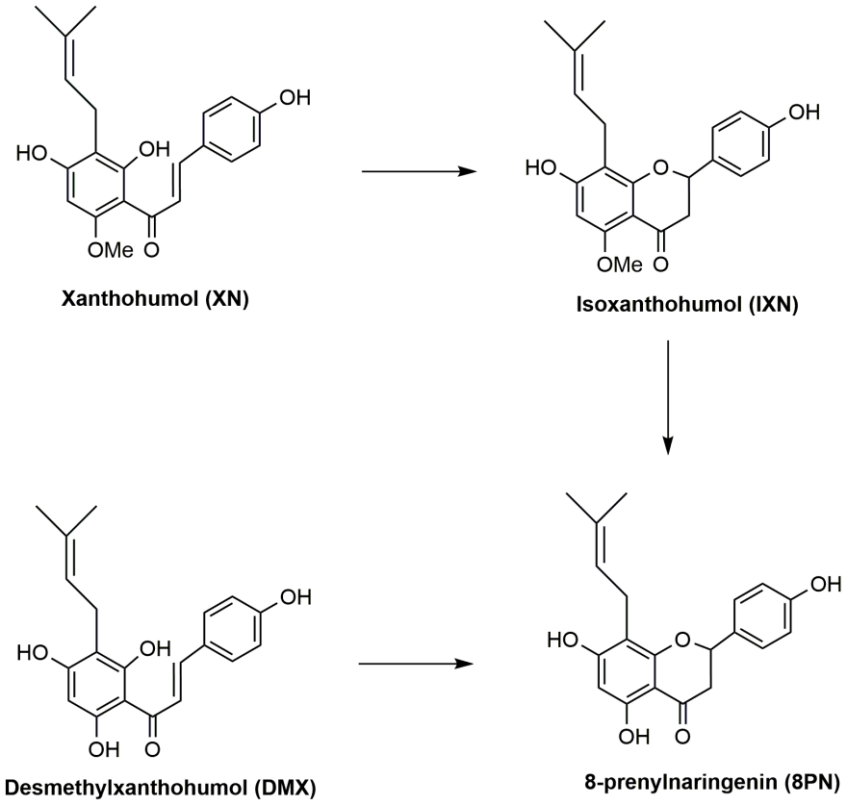

Figure 2.1 - Chemical structures of hop prenylflavonoids. By thermal isomerization, the chalcones xanthohumol (XN) and desmethylxanthohumol (DMX) are converted into the flavanones isoxanthohumol (IXN) and 8-prenylnaringenin (8-PN), respectively. 8-PN could also be formed by *O*-demethylation of IXN *in vivo.*

2.4. XN and 8PN as health-promoting compounds

Beer is an unique source of hop polyphenols in human diet and XN has received special attention, due to its antioxidant behavior and potential biological properties that may have therapeutic interest (Zanoli *et al.*, 2008; Magalhães *et al.*, 2009). Several studies suggest that the consumption of XN is associated with lower risk of development and progression of oxidative stress-related diseases, such as chronic diseases (Nozawa, 2005; Costa *et al.*, 2013; Legette *et al.*, 2013; Liu *et al.*, 2014). It has also been reported that XN possesses a broad-spectrum of anti-infection activity against several microorganisms (antifungal, antibacterial, antiviral and antimalarial) (Gerhauser, 2005b; Nowakowska, 2007; Zanoli *et al.*, 2008).

Additionally, it has significant anti-proliferative and cancer chemopreventive effects, through regulation of mechanisms involved in carcinogenesis, induction of apoptosis and due to its anti-angiogenic potential (Stevens *et al.*, 2004; Gerhauser, 2005a; Zanoli *et al.*, 2008; Monteiro *et al.*, 2008; Negrão *et al.*, 2010; Negrão *et al.*, 2012; Costa *et al.*, 2013). XN also prevents atherosclerosis, by inhibiting cholesterol accumulation in atherogenic regions (Hirata *et al.*, 2012; Doddapattar *et al.*, 2013). XN has strong *in vitro* antioxidant properties and improves plasma antioxidant capacity (Ghiselli *et al.*, 2000). This remarkable antioxidant behavior of XN and other prenylated hop flavonoids is very important in the context of T2DM, since they readily scavenge ROS and RNS , therefore preventing LDL oxidation and protecting blood platelets from oxidative and/or nitrative modifications, thus preventing vascular thrombosis and inflammation (Olas *et al.*, 2011). Experimental data has shown that XN exerts anti-obesity activities through the inhibition of pre-adipocyte differentiation and adipogenesis. This is achieved by decreasing PPARγ and FABP expression in adipocytes, as well as its lipid content (Yang *et al.*, 2007; Kiyofuji *et al.*, 2014).

We have previously shown that beer polyphenols, particularly XN and 8PN, exert distinct effects on angiogenesis, in EC cultures and animal models. It was observed that XN exerted anti-angiogenic effects, whereas 8PN stimulated angiogenesis (Negrão *et al.*, 2010; Negrão *et al.*, 2012). Using a T1DM rat model, we demonstrated that the intake of XN-fortified beverages, led to a reduction of angiogenesis, serum inflammatory markers and tissue oxidative damage together with the enhancement of endogenous antioxidant defenses (Costa *et al.*, 2013). The antidiabetic effect of XN has also been recently investigated. Recent studies revealed that the ingestion of a XN-rich extract by diet-induced obese rats resulted in reduction of body weight gain and decreased liver and plasma TG levels (Yui *et al.*, 2014). An interesting study indicated that XN-fed diabetic mice had decreased plasma glucose and TG levels, reduced adipose tissue mass and increased levels of adiponectin (Nozawa, 2005).

Moreover, Legette and colleagues reported that the XN oral administration ameliorates glucose metabolism and reduces body weight in obese rats (Legette *et al.*, 2013). The mechanisms sustaining the aforementioned antidiabetic activities of XN include the selective inhibition of α-glucosidase, the decrease of intestinal carbohydrate digestion and its absorption (Liu *et al.*, 2014), the inhibition of intestinal FA absorption, the regulation of hepatic FA metabolism, (Yui *et al.*, 2014) and its action as a ligand of the farnesoid X receptor, involved in lipid and lipoprotein metabolism (Nozawa, 2005). Altogether, these findings clearly suggest that XN is a promising molecule to treat obesity and T2DM-metabolic disturbances.

Compelling evidence suggests that dietary phytoestrogens, namely isoflavones from cocoa are able to improve insulin sensitivity in diabetic postmenopausal woman, suggesting a positive effect in diabetes prevention (Curtis *et al.*, 2012). Over the years, it has been suggested that hops have a powerful estrogenic activity mainly attributable to 8PN. Numerous *in vitro* and *in vivo* studies in ovariectomised rats and postmenopausal women, have been conducted to clarify the estrogenicity potential of beer prenylflavonoids. In the breast cancer cell line MCF-7 and in the rat mammary gland, 8PN stimulates cell proliferation by binding to the estrogen receptor (ER), mainly through interaction with ERα, responsible for the proliferative effects of estrogens (Helle *et al.*, 2014). In accordance, 8PN demonstrates higher affinity to ERα when compared to ERβ, that counteracts the ERα-dependent responses, triggering a greater estrogenic activity when compared to other estrogenic compounds, such as coumestrol and genistein albeit with a lesser potency than the natural ligand 17β-estradiol (Milligan *et al.*, 1999; Schaefer *et al.*, 2003; Overk *et al.*, 2008). The pharmacokinetics and metabolism of 8PN have been investigated in animal models and then confirmed in human studies. In these trials, 8PN was orally administered to postmenopausal women, exerting systemic endocrine effects (Rad *et al.*, 2006). Estrogens are important regulators not only in the reproductive system but also in other target tissues including cardiovascular and central

nervous systems and bones (Cos *et al.*, 2003). Furthermore, 8PN signaling mediated by ERα was shown to be more selective than 17β-estradiol in bone tissue (Rad *et al.*, 2006; Luo *et al.*, 2014). These results suggest that 8PN could be used as alternative estrogen replacement therapy in menopause, being also promising to ameliorate osteoporosis. 8PN could be used in nutrition or pharmacological industries as a natural selective ER modulator (Simons *et al.*, 2012).

A previous study performed in ovariectomized rats demonstrated the beneficial effects of 8PN in lipid metabolism. The dietary intake of 8PN led to decreased LDL levels and an overall anti-atherosclerotic effect, suggesting its potential role in cardiovascular disease (Bottner *et al.*, 2008). Nevertheless, little is known about the effect of 8PN in lipid metabolism and metabolic diseases, such as T2DM. Furthermore, the apparent antagonic effect of XN and 8PN in angiogenesis, a deregulated process in T2DM, deserves to be explored. Accordingly, nutritional or pharmacological supplementation with these polyphenols, which distinctly affect the vasculature and metabolism, may potentially ameliorate T2DM-related vascular complications by targeting the “angiogenic paradox”.

XN and 8PN effects deserve to be better studied as they may be very promising compounds with great benefit for public health, particularly when prevention of highly prevalent diseases is mandatory.

II

Research work

Scope of the thesis

The incidence of T2DM has dramatically increased worldwide over the past decades. T2DM-related metabolic and vascular comorbidities have been postulated to be the major causes for the increased morbidity and health care costs associated with this disease. Intensive efforts have been done to develop novel strategies to counteract the T2DM comorbidities. We propose to explore the use of dietary natural compounds as a promising approach to this problem. Two distinct polyphenols have been investigated due to their interesting opposing effects in angiogenesis. XN has been recently reported to be a promising molecule in the treatment of metabolic diseases, given its effects in diabetic-associated metabolic disturbances. Yet, relatively little is known about the potential benefits and risks of its chronic consumption and even less is known about its metabolite, 8PN, as well as about the underlying mechanisms of action of both polyphenols *in vivo*. Herein, the present work aims to unravel the potential modulatory effect of XN and 8PN on T2DM-related complications in a high fat diet (HFD)-induced diabetes animal model. The research presented here focuses on the metabolic and angiogenic signaling pathways that are deregulated in T2DM and highlights the potential preventive role of polyphenols' consumption. The specific aims are:

- To evaluate the effect of XN and 8PN consumption on glucose and lipid metabolic deregulations in a T2DM animal model, by studying metabolic pathways in liver and skeletal muscle;
- To examine the effect of XN and 8PN consumption on angiogenic pathways, in the kidney and left ventricle, tissues associated with the angiogenic paradox, in a HFD-induced T2DM animal model. This can potentially elucidate the role of these polyphenols as tissue-specific vascular modulators in T2DM.

3. Xanthohumol and 8-prenylnaringenin ameliorate diabetic-related metabolic dysfunctions in mice

3.1. Background

The prevalence of obesity and T2DM has dramatically increased over the past decades, and their associated metabolic complications are considered a major health problem affecting more than 400 million adults worldwide (Shaw *et al.*, 2010). As a hallmark sign of T2DM, hyperglycemia is one of the main causes of glucose control and lipid metabolism impairments, early outcomes of insulin resistance. The available treatment strategies for diabetes management are not completely efficient, as highlighted by the markedly increased morbidity and mortality rates in diabetic patients. According to this, lifestyle modification and improved pharmacological preventive and therapeutic approaches for T2DM are needed. In recent years, studies using novel pharmacological treatments and functional foods to regulate energy metabolism have been conducted in order to control T2DM (Tiwari, 2015). Growing evidence indicates that polyphenols, apart from antioxidant activity, possess health-promoting properties associated with the prevention and therapeutic approaches being associated with low risk for the development and progression of chronic diseases namely diabetes and cardiovascular disease (Rains *et al.*, 2011; van Dam *et al.*, 2013). Several epidemiologic studies suggest that a polyphenol-enriched diet is an important strategy to prevent obesity and related chronic diseases namely T2DM. Both XN and 8PN beer-derived polyphenols have been shown to possess interesting biological effects namely antidiabetic, anti-inflammatory and anticarcinogenic (Stevens *et al.*, 2004; Gerhauser *et al.*, 2005; Zanoli *et al.*, 2008; Miranda *et al.*, 2016b). However, the underlying mechanisms of metabolic action of XN and 8PN in lipid and glucose pathways have not been thoroughly investigated.

The present study aims to identify the pathways involved in the metabolic disarrangements and to address the putative role of polyphenols in counteracting the development of T2DM. Our attention has focused in both liver and skeletal muscle, important metabolic tissues in the regulation of energy metabolism and body homeostasis. AMPK is a key metabolic regulator present in both tissues and plays an important role in the control of glucose and lipid metabolism (Hardie, 2013; Ramesh *et al.*, 2016). Evidence suggests that a decrease in AMPK activity may cause metabolic disorders. Thus, modulation of this regulatory enzyme could be a promising target for treating metabolic disturbances. Once activated, AMPK suppresses the expression of SREBP-1c, an important transcription factor involved in the FA biosynthesis and lipid uptake (Lopez *et al.*, 1996; Magana *et al.*, 1996; Goldstein *et al.*, 2006; Sato, 2010; Xiao *et al.*, 2013). SREBP-1c primarily regulates lipogenic enzymes such as ACC and FAS, which in turn inhibits lipid biosynthesis and stimulates FA β-oxidation (Horton *et al.*, 2002; Abu-Elheiga *et al.*, 2005; Hardie, 2013). Hence, AMPK-dependent phosphorylation of SREBP-1c might be a potential approach to treat lipid disarrangements as in T2DM and other metabolic diseases (Li *et al.*, 2011; Xiao *et al.*, 2013; Choi *et al.*, 2014).

FFA are taken up through specific transporter proteins, being the CD36 the best characterized FAT (Goldberg *et al.*, 2009). CD36 was linked to lipid metabolism accounting for the increased rate of FA transport into several tissues of HFD-fed animals (Goldberg *et al.*, 2009; Su *et al.*, 2009). Increased expression of hepatic and muscle CD36 protein in response to a HFD is sufficient to exacerbate TG storage and secretion, contributing to the dyslipidemia associated with T2DM development (Kelley *et al.*, 2001; Goldberg *et al.*, 2009; Su *et al.*, 2009). Some studies have demonstrated that polyphenols down-regulate CD36 gene expression displaying lipid-lowering effect (Aoun *et al.*, 2011). However, the potential of XN and 8PN in this transporter expression has never been addressed. Hagberg and colleagues have recently proposed the involvement of VEGFR-1 and its ligand, VEGF-B, in the lipid transport and uptake

from EC to tissues (Hagberg *et al.*, 2010; Hagberg *et al.*, 2012). Moreover, it is well-documented that the deregulation on glucose uptake by skeletal muscle in T2DM is primarily modulated by insulin-sensitive GLUT-4 through PI3K/AKT pathway (Klip, 2009). In order to understand the involvement of polyphenol consumption in glucose uptake, AS160, an AKT substrate was assessed in the present work as a mediator of the GLUT-4 translocation. Furthermore, the expression of an important glycolytic regulator also involved in insulin signaling, PFKFB3 was also assessed in our study. PFKFB3 regulates glycolysis through the production of Fru-2,6-BP, which is a potent allosteric activator of PFK-1, the glycolysis rate-limiting step (Domenech *et al.*, 2015; Trefely *et al.*, 2015). A study conducted by Trefely and co-workers demonstrated that the *in vitro* inhibition of PFKFB3 suppressed insulin-stimulated glucose uptake and GLUT-4 translocation, impairing insulin signaling and exacerbating insulin resistance (Trefely *et al.*, 2015).

Herein, we evaluated the diabetic-preventive effect of XN and 8PN using an HFD-induced diabetic mice model by exploring the molecular mechanisms associated with diabetic metabolic dysfunction.

3.2. Materials and methods

3.2.1. Animals and experimental mice treatment

Thirty 6-week old male C57Bl/6 mice (Charles-River, Spain) were randomly divided into 5 experimental groups (n=6), and fed *ad libitum* with: Standard diet (C); HFD (obtained from Research diets, #D12451, New Jersey, USA) (DM); HFD plus 0.1 % ethanol in drink water (DM-Ethanol); HFD and 10 mg/L XN (Hopsteiner, Germany) in 0.1 % ethanol (DM-XN); HFD plus 10 mg/L 8PN in 0.1 % ethanol (DM-8PN) during 20 weeks. The 8PN was synthetized according to previously described procedures (Zierau *et al.*, 2002). This mouse strain is prone to develop T2DM under HFD ingestion, displaying significant dyslipidemia, insulin resistance and glucose

intolerance, as demonstrated by others (Blake *et al.*, 2014). During the treatment period, body weight and glycemia were monitored weekly, food and beverage intake were controlled every two days. Beverages were renewed every two days and were kept in dark bottles to avoid compound degradation. After 20 weeks of treatment, animals were then sacrificed and skeletal muscle and liver were frozen at -80 ºC for molecular analyses or fixed in 10% neutral-buffered formalin, dehydrated, and paraffin-embedded for histological assays. Three micrometer-thick tissue sections were used for hematoxylin-eosin histological staining. Blood was also collected for biochemical analyses. Animals were maintained under controlled conditions of temperature (23 ± 5 °C), humidity (35 ± 5 %), and 12 h light/dark cycles. All animal experiments were conducted at the animal house located at the Faculty of Medicine, University of Porto, and were carried out by trained technicians in accordance with the European Community policy for Experimental Animal Studies [European Community law dated from November 24th 1986 (86/609/CEE) with addendum from June 18th 2007 (2007/526/CE)].

3.2.2. Oral glucose tolerance test and intraperitoneal insulin tolerance test

All animals were fasted overnight after 19 weeks of treatment. To perform the oral glucose tolerance test (OGTT), all mice received a glucose solution of 1 g/Kg body weight by oral gavage. To analyze intraperitoneal insulin tolerance test (IPITT), animals were injected intraperitoneal with 0.75 U/Kg body weight of insulin (Sigma, Portugal). Blood glucose concentrations were measured 30 min before, at baseline and thereafter at 15, 30, 60, 90 and 120 min after the glucose or insulin administration, with Precision Xtra Plus test strips and an Optium Xceed device (Abbott Diabetes Care, Ltd., Maidenhead, UK), according to the manufacturer's instructions. Results are expressed as mean±SD of the total area under the curve (AUC) calculated for each measurement.

3.2.3. Systemic biochemical measurements

Plasma biochemical markers were assessed at the Department of Clinical Pathology, São João Hospital Center, using Olympus AU5400® automated clinical chemistry analyzer (Beckman-Coulter®, Izasa, Porto, Portugal). Hepatic function markers were determined through such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) levels and metabolic status was evaluated through glucose, uric acid, TG, total cholesterol, VLDL, LDL, calculated according to Friedewald's equation) and HDL levels. Plasma insulin levels were measured using a rat/mouse insulin ELISA kit (EZRMI-13K; Merck Milipore, Madrid, Spain).

3.2.4. Insulin sensitivity and resistance indexes

Insulin sensitivity and resistance indexes were calculated as follows : quantitative insulin sensitivity check index (QUICKI)=1/[log(I_0) log(G_0)], where I_0 is fasting insulin (U/ml) and G_0 is fasting glucose levels (mg/dL); and homeostasis model assessment (HOMA)=(G_0 I_0)/22.5, where glucose was expressed as mg/dL and insulin was expressed as μU/mL) (Lee *et al.*, 2008).

3.2.5. Extraction and quantification of liver triglycerides and cholesterol levels

TG and cholesterol were extracted from frozen liver samples, and were determined by colorimetric quantification kits for TG (ab65336; Abcam, UK) and cholesterol (ab65359; Abcam, UK), according to the manufacturer's instructions.

3.2.6. Tissue VEGF-B quantification by ELISA

Liver and skeletal muscle VEGF-B concentration was quantified using Quantikine mouse VEGF-B (ABIN869657; Antibodies-online, USA) ELISA kit, using a microplate reader (Thermo Fisher Scientific, USA) in accordance with the manufacturer's instructions.

3.2.7. Western blot analyses for metabolic pathways

Ten to twelve micrograms of total protein were separated by electrophoresis in a 10% SDS-PAGE. Membranes were incubated with antibodies against AMPK (1:1000 dilution; ab80039 Abcam, UK), phospho-AMPK (1:750 dilution; #2531 Cell signalling, UK), ACC (1:1000 dilution; #3662 Cell signalling, UK), phospho-ACC (1:750 dilution; #3661 Cell signalling, UK), SREBP-1c (1:1000 dilution; ab3259 Abcam, UK), CD36 (1:1000 dilution; sc-9154 Santa Cruz Biotechnology, Germany), FAS (1:1000 dilution; GTX50788 Genetex, CA, USA), VEGFR-1 (1:7500 dilution; ab2350 Abcam, UK), phospho-AS160 (1:750 dilution; #8881 Cell signalling, UK), PFKFB3 (1:1000 dilution; #13123 Cell Signalling, UK) and β-actin (1:3000 dilution; ab8227 Abcam, UK), followed by incubation with the respective secondary horseradish-peroxidase (HRP)-coupled antibody (1:10000; anti-rabbit sc-2040 and anti-mouse sc-2060 from Santa Cruz Biotechnology, Germany). The detection was performed using enhanced chemiluminescence (ECL kit; Biorad, USA). Mean relative intensities were quantified by densitometry (Vision Works LS software; UVP Inc., USA) and normalized to the signal intensity of β-actin or the signal intensity of total form for phosphorylated proteins.

3.2.8. Statistical analyses

Every assay was performed at least in three independent experiments. Statistical significance of different groups was evaluated by ANOVA followed by the Bonferroni post-hoc

test, with GraphPad prism version 7.0a software (GraphPad Software, Inc, CA, USA). A difference between experimental groups was considered significant with a confidence interval of 95 %, whenever $p \leq 0.05$.

3.3. Results

3.3.1. XN and 8PN treatments affected body weight and plasma lipid profile

As illustrated in Table 3.1, the mean body weight of DM animals (52.3±2.3 g) and DM-Ethanol group (53.0±1.8 g) was significantly higher when compared to control mice group (32.7±1.9 g). Similarly, blood glucose levels increased 40 % in both DM and DM-Ethanol groups. However, both XN and 8PN treatments significantly suppressed the raise of body weight gain and glycemia, on 15 %.

To characterize the general metabolic status of distinct animal groups, plasma levels of hepatic enzymes were quantified. As expected, both DM and DM-Ethanol groups presented higher AST, AST/ALT ratio, and ALP levels than control group ($p < 0.001$ for DM and $p = 0.01$ for DM-Ethanol). After XN and 8PN consumption, these parameters were attenuated in diabetic animals to levels identical to healthy controls. Plasma uric acid levels did not differ between experimental groups. Diabetic mice drinking water or ethanol exhibited increased levels of TG, cholesterol, LDL, VLDL and a reduced HDL (40 %) and HDL/LDL ratio (3-fold) levels when compared with control group. Remarkably, diabetic animals treated with XN and 8PN showed a lipid profile improvement with a statistical increase in HDL (101.3±6.4 g/L for DM-XN and 99.6±5.6 g/L for DM-8PN vs 77.2±5.6 g/L for DM-Ethanol) and HDL/LDL ratio (3.8±0.9 for DM-XN and 3.7±0.8 for DM-8PN vs 2.1±0.4 for DM-Ethanol) as well as a significant decrease on both TG ($p = 0.04$ for DM-XN and $p = 0.001$ for DM-8PN animals) and total cholesterol ($p = 0.03$ for DM-XN and p=0.002 for DM-8PN) when compared to DM-Ethanol group.

Table 3.1 - Analysis of body weight and plasma biochemical markers in diabetic mice. Control, healthy mice; DM, diabetic animals fed with high-fat diet; DM-Ethanol, diabetic animals drinking 0.1% ethanol in water; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals consuming 10 mg/L 8PN. Evaluated parameters included body weight, hepatic function markers, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) activities and markers of metabolic status including glucose, insulin, uric acid, triglycerides, total cholesterol, very low density lipoprotein cholesterol (VLDL), low density lipoprotein cholesterol (LDL), and high density lipoprotein cholesterol (HDL). Results are expressed as means ± SD (5<n<6). *p < 0.05 vs control, # p < 0.05 vs DM; δ p < 0.05 vs DM-Ethanol.

	Control	DM	DM-Ethanol	DM-XN	DM-8PN
Body weight (g)	32.7±1.9	52.3±2.3*	53.0±1.9*	44.5±3.2*#δ	46.7±4.6*#δ
Glucose (g/L)	154.67±8.77	211.16±8.08*	213.8±6.83*	183.5±11.47*#δ	187.67±6.86*#δ
Insulin (μU/mL)	0.24±0.03	0.61±0.06*	0.62±0.08*	0.43±0.07*#δ	0.48±0.06*#δ
AST (U/L)	75.5±13.7	122.4±7.4*	124.3±9.54*	92.0±7.9#δ	93.40±14.9#δ
ALT (U/L)	59.7±8.1	71.0±4.5	70.5±5.0	65.7±6.5	62.8±16.0
AST/ALT	1.29±0.29	1.73±0.10*	1.77±0.13*	1.41±0.12	1.52±0.24
ALP (U/L)	61.50±17.28	105.00±13.96*	94.00±15.51*	60.00±13.71#δ	66.17±9.06#δ
Tryglicerides (g/L)	59.0±6.6	112.2±12.2*	108.8±20.6*	81.8±13.9#δ	64.3±11.9#δ
Total cholesterol (g/L)	66.17±9.30	135.30±34.66*	127.60±15.53*	90.33±6.83#δ	77.67±15.71#δ
VLDL (g/L)	10.18±2.86	17.40±3.21*	18.00±3.32*	13.27±1.84	13.33±2.82
LDL (g/L)	22.67±3.83	37.83±5.04*	37.40±6.35*	27.67±5.47#	28.33±6.09
HDL (g/L)	119.33±8.52	67.83±3.24*	77.20±15.61*	101.33±6.44*#δ	99.67±5.82*#δ
HDL/LDL	5.37±0.85	1.83±0.43*	2.09±0.45*	3.80±0.88*#δ	3.66±0.81*#δ
Uric acid (mg/L)	1.42±0.26	2.47±0.93	2.53±0.22	1.88±0.57	1.68±0.46

3.3.2. XN and 8PN treatments ameliorated glucose tolerance and insulin sensitivity

To assess glycemic response and insulin sensitivity, an OGTT and an IPITT were performed. As depicted in Figure 3.1 A, DM and DM-Ethanol blood glucose levels following an oral glucose administration were significantly higher reaching the glycemic peak at 30 min, when compared with control group. In contrast, XN and 8PN consumption improved glucose tolerance as shown by the reduced glycemic peak and lower AUC measurement (Figure 3.1 B). Similar results were obtained with IPITT. DM and DM-Ethanol groups exhibited increased blood glucose levels that remained higher at all time-points up to 120 min (Figure 3.1 C), leading to

the highest AUC values, indicating insulin resistance when compared to control group (Figure 3.1 D). XN and 8PN treatments ameliorated insulin sensitivity decreasing blood glucose levels to their own initial levels with significantly lower AUCs ($p < 0.001$ for DM-XN and $p = 0.004$ for DM-8PN).

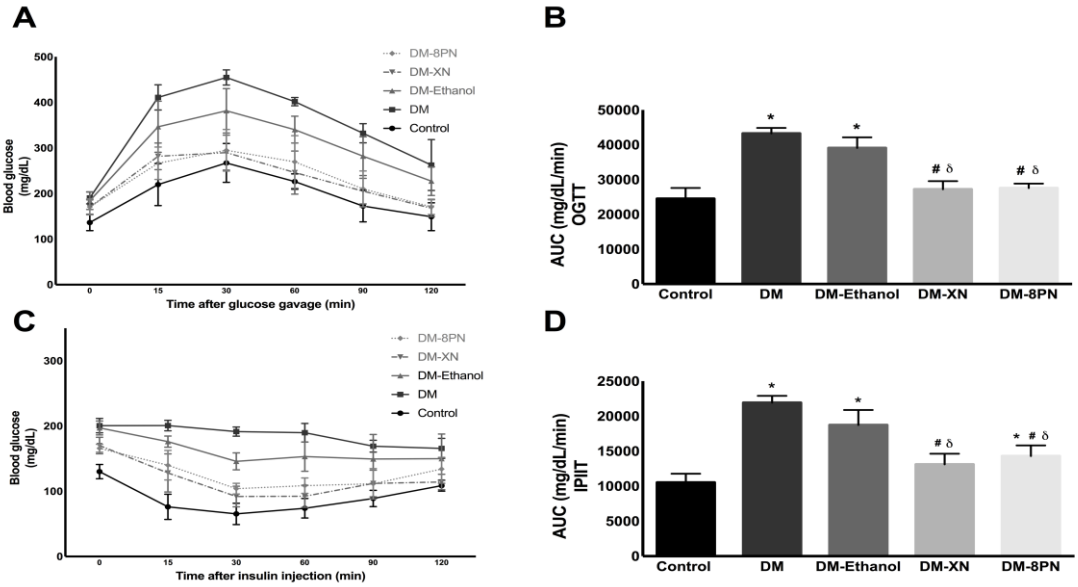

Figure 3.1 - Effects of XN and 8PN treatments in glucose tolerance and insulin sensitivity in diabetic animals. Control, healthy mice; DM, diabetic animals fed with high-fat diet; DM-Ethanol, diabetic animals drinking 0.1 % ethanol in water; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals treated with 10 mg/L 8PN/L. (A) Oral glucose tolerance test (OGTT). All animals were fasted for 6 h, then 1 g/Kg glucose was given by oral gavage and blood glucose was monitored at 0, 15, 30, 60, 90 and 120 min. (B) Quantification of the area under the curve (AUC) from OGTT (C) Intraperitoneal insulin tolerance test (IPITT). The basal blood glucose level (0 min) was quantified before the intraperitoneal insulin injection (0.75 U/Kg). (D) Quantification of the area under the curve (AUC) from IPITT. Blood samples for glucose level determination were taken at 15, 30, 60, 90 and 120 min. Data are expressed as mean ± SD ($5<n<6$). *$p < 0.05$ vs control, # $p < 0.05$ vs DM; δ $p < 0.05$ vs DM-Ethanol.

Accordingly, whereas diabetic animals from DM and DM-Ethanol groups presented around 3.5-fold higher levels of insulin resistance and reduced insulin sensitivity as determined by HOMA (Figure 3.2 A) and QUICKI (Figure 3.2 B) respectively, XN and 8PN treatments reversed both indexes, when compared to DM-Ethanol group.

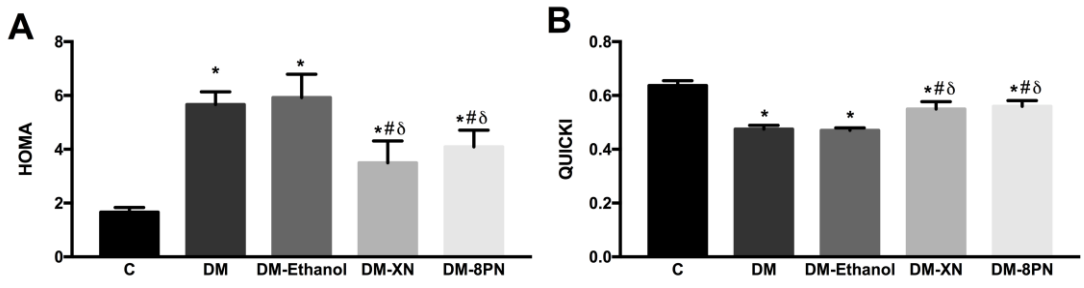

Figure 3.2 - Glucose levels and insulin resistance in diabetic animals treated with XN and 8PN polyphenols. Control, healthy mice; DM, diabetic animals fed with high-fat diet; DM-Ethanol, diabetic animals drinking 0.1 % ethanol in water; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals treated with 10 mg/L 8PN. (A) HOMA at week 20 (B) QUICKI determination for insulin sensitivity assessment. Data are expressed as mean ± SD (5<n<6). *p < 0.05 vs control, # p < 0.05 vs DM; δ p < 0.05 vs DM-Ethanol.

3.3.3. XN and 8PN treatment regulated the expression of AMPK-related metabolic enzymes

To determine whether the effects of XN and 8PN on lipid profile could be mediated by AMPK activation, the expression of AMPK and target proteins was evaluated in mice liver and skeletal muscle. As shown in Figure 3.3 A-D and Figure 3.4 A-D, DM and DM-Ethanol animal groups significantly decreased in about 2-fold the activation of AMPK as revealed by phosphorylated/total ratio expression. This was accompanied by increased expression of SREBP-1c, FAS and a 2-fold decrease in phosphorylation (and subsequent inactivation) of ACC, when compared to control healthy animals in both liver and skeletal muscle. In contrast, this was prevented by XN and 8PN treatments leading to marked enhancement of the phosphorylation of AMPK in the liver (0.97±0.10 for DM-XN and 1.26±0.14 for DM-8PN vs 0.57±0.12 DM-Ethanol) and in skeletal muscle (0.81±0.20 for DM-XN and 1.09±0.20 for DM-8PN vs 0.46±0.11 DM-Ethanol). These findings were accompanied by reduced expression of SREBP-1c and FAS, and increased phosphorylated ACC in both tissues, implying a reduction in lipogenesis by these two compounds.

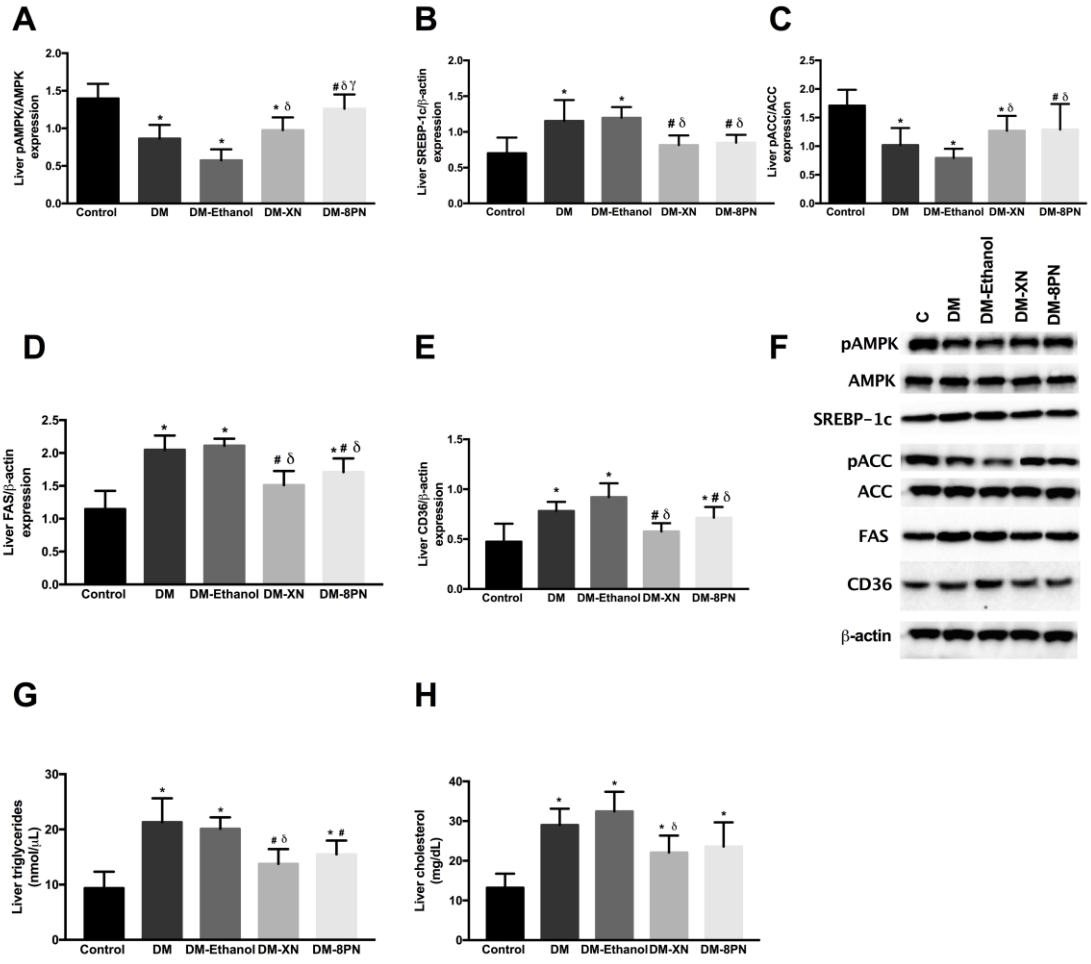

Figure 3.3 - Hepatic lipogenic enzymes and fatty acid transporter protein expression on diabetic animals treated with polyphenols. Control, healthy mice; DM, diabetic animals fed with high-fat diet; DM-Ethanol, diabetic animals drinking 0.1 % ethanol in water; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals treated with 10 mg/L 8PN. Expression of **(A)** pAMPK/AMPK ratio **(B)** SREBP-1c **(C)** pACC/ACC ratio **(D)** FAS **(E)** fatty acid transporter CD36, and **(F)** representative blots of immunoblotting obtained by western blotting analysis. Quantification of **(G)** triglycerides and **(H)** cholesterol content in hepatic tissue. Data are expressed as mean ± SD (5<n<6). *$p < 0.05$ vs control, # $p < 0.05$ vs DM; δ $p < 0.05$ vs DM-Ethanol.

CD36 immunoblotting (Figure 3.3 E-F and Figure 3.4 E-F), responsible for the transport of LCFA to tissues, revealed that the hepatic and skeletal muscle protein expression was also significantly down-regulated in diabetic animals that ingested XN (30 % in liver and 30 % in skeletal muscle) and 8PN (30 % in liver and 20 % in skeletal muscle), in comparison with DM-Ethanol.

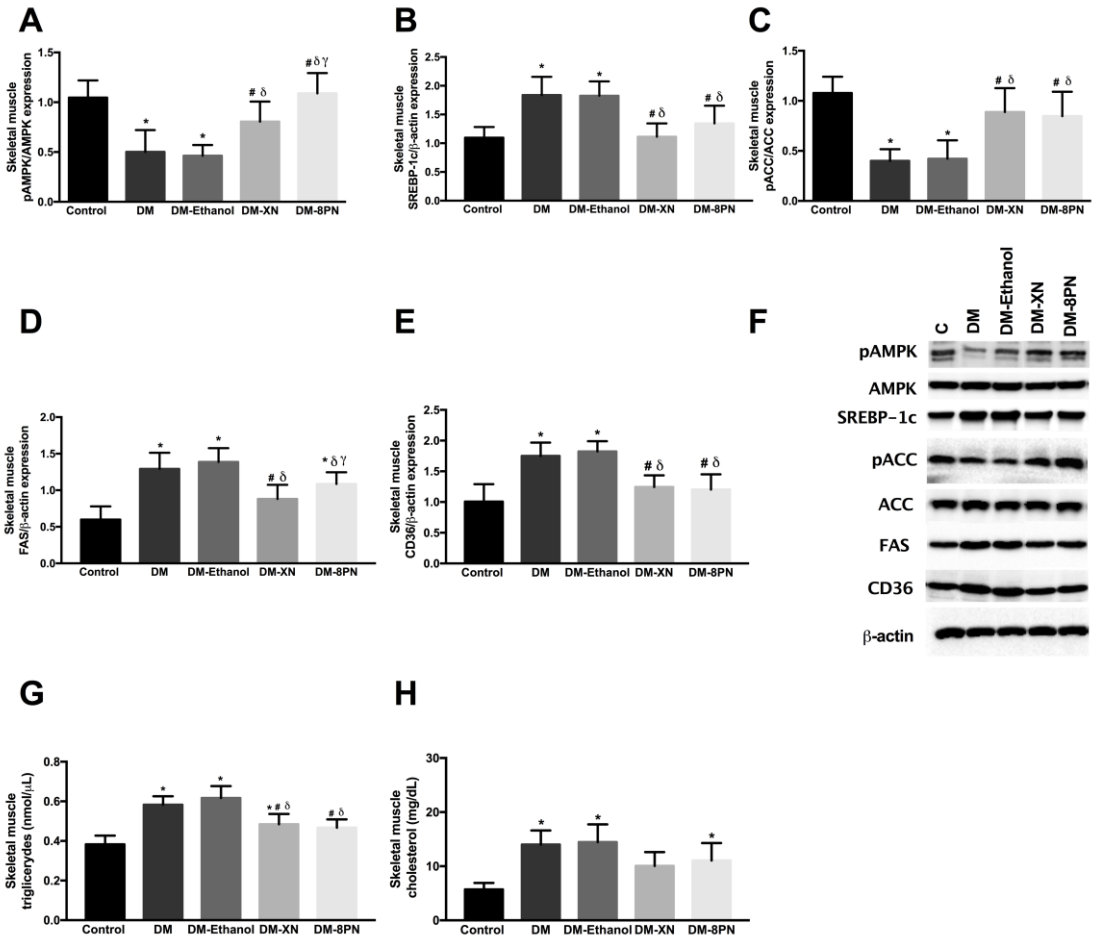

Figure 3.4 - Skeletal muscle lipogenic and β-oxidation enzymes and fatty acid transporter protein expression on diabetic animals treated with polyphenols. Control, healthy mice; DM, diabetic animals fed with high-fat diet; DM-Ethanol, diabetic animals drinking 0.1 % ethanol in water; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals treated with 10 mg/L 8PN. Expression of **(A)** pAMPK/AMPK ratio **(B)** SREBP-1c **(C)** pACC/ACC ratio **(D)** FAS **(E)** fatty acid transporter CD36, **(F)** representative blots of immunoblotting obtained by western blotting analysis. Quantification of **(G)** triglycerides and **(H)** cholesterol content in skeletal muscle tissue. Data are expressed as mean ± SD (5<n<6). *p < 0.05 vs control, # p < 0.05 vs DM; δ p < 0.05 vs DM-Ethanol.

TG and cholesterol levels were quantified in liver and skeletal muscle as indicators of lipid accumulation. Regarding TG levels (Figure 3.3 H and Figure 3.4 H), diabetic animals drinking water and ethanol displayed higher TG content comparing to control group (around 2-fold in liver and 1.5-fold in skeletal muscle). The levels of cholesterol were also higher in diabetic animals (2-fold in liver and 3-fold in skeletal muscle) comparing to control animals (Figure 3.3 I

and Figure 3.4 I). However, a significant decrease of 30 % on cholesterol and TG content was observed in the liver of animals treated with XN and a 23 % reduction of liver TG in DM-8PN animals, comparing to DM-Ethanol group. An identical profile was observed in skeletal muscle. A significant decrease in TG levels was observed after XN (0.48±0.05 nmol/μL) and 8PN (0.46±0.04 nmol/μL) consumption, when compared to DM-Ethanol group (0.61±0.05 nmol/μL). Moreover, a tendency to reduced cholesterol levels after XN and 8PN treatments was also observed.

3.3.4. Polyphenols modulated lipid uptake in diabetic mice

Activation of VEGFR-1 by VEGF-B results in endothelial FA transport and uptake by peripheral tissues. Since this pathway is upregulated in diabetic tissues, we evaluated whether these polyphenols were able to prevent lipotoxicity. As depicted in Figure 3.5, DM and DM-Ethanol groups exhibited 2-fold increased levels of VEGFR-1 in liver and 1.5-fold increase in skeletal muscle. VEGF-B levels were also approximately 2-fold higher in both tissues. However, the expression of VEGFR-1 and VEGF-B content were significantly reduced in the liver of diabetic animals treated with XN and 8PN by around 40 % (comparing to DM-Ethanol mice group).

A significantly lower expression of both receptor and ligand was also found in skeletal muscle of diabetic mice upon XN or 8PN ingestion, being XN treatment more effective in decreasing VEGFR-1 than 8PN (0.78±0.10 for DM-XN and 1.01±0.08 for DM-8PN group).

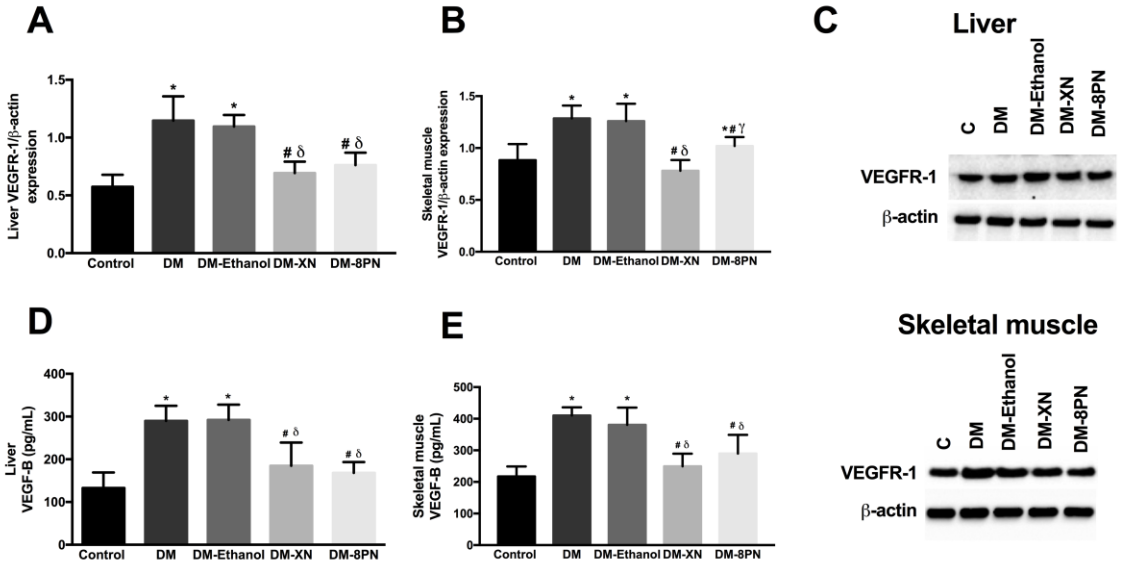

Figure 3.5 - The VEGFR-1 expression in (A) liver and (B) skeletal muscle on diabetic animals treated with polyphenols. Control, healthy mice; DM, diabetic animals fed with HFD; DM-Ethanol, diabetic animals drinking 0.1 % ethanol in water; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals treated with 10 mg/L 8PN. (C) Representative blots of immunoblotting obtained by Western blotting analysis are shown. Determination of VEGF-B by ELISA assay in (D) liver and in (E) skeletal muscle. Data are expressed as mean ± SD (5<n<6). *p < 0.05 vs control, # p < 0.05 vs DM; δ p < 0.05 vs DM-Ethanol.

3.3.5. XN and 8PN treatments affected glycolysis and glucose uptake

As an effective activator of the glycolytic pathway, the expression of PFKFB3 and its modulation by polyphenol's consumption is shown in Figure 3.6. An increase of 1.5-fold was observed in this glycolytic activator in DM and DM-Ethanol groups in both liver and skeletal muscle, compared to control animals. In contrast, XN consumption prevented this effect with a significant decrease in PFKFB3 expression in the liver (0.81±0.06) and in skeletal muscle (0.80±0.20) in comparison to DM-Ethanol group (1.12±0.09 and 1.13±0.19, respectively). Diabetic animals treated with 8PN exhibited a significant reduction in PFKFB3 expression only in skeletal muscle (0.97±0.15 vs 1.30±0.19 for DM-Ethanol).

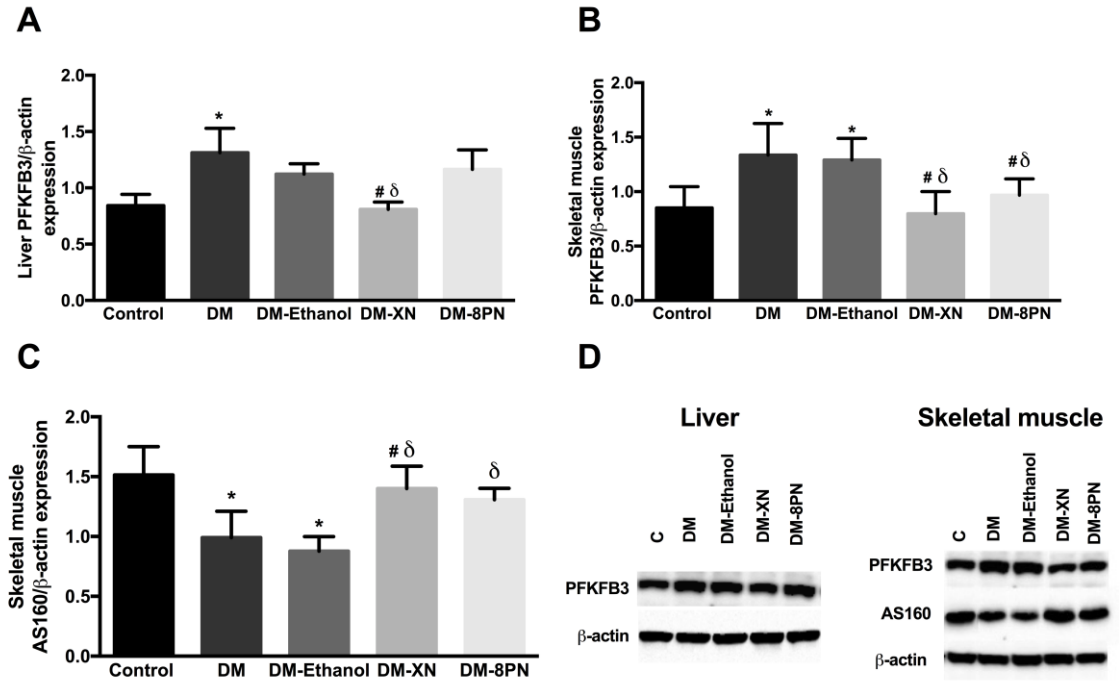

Figure 3.6 - Glycolytic regulator PFKFB3 protein expression in (A) liver and in (B) skeletal muscle and (C) phospho-AS160 protein expression in skeletal muscle, involved in the translocation of GLUT4 to the skeletal muscle plasma membrane, on diabetic animals treated with polyphenols. Control, healthy mice; DM, diabetic animals fed with high-fat diet; DM-Ethanol, diabetic animals drinking 0.1 % ethanol in water; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals treated with 10 mg/L 8PN. (D) Representative images of immunoblotting obtained by Western blotting analysis are shown. Results are expressed as means ± SD (5<n<6). *$p < 0.05$ vs control, # $p < 0.05$ vs DM; δ $p < 0.05$ vs DM-Ethanol.

These findings led us to quantify AS160 in order to evaluate whether XN and 8PN treatment could ameliorate glucose uptake in skeletal muscle. As illustrated in figure 6 C-D, DM and DM-Ethanol groups downregulated phospho-AS160 expression (0.99±0.22 for DM and 0.88±0.12 for DM-Ethanol) in comparison to control group (1.51±0.24). Normal levels of phospho-AS160 expression were restored upon XN (1.40±0.18) and 8PN consumption (1.31±0.09) (Figure 3.6 C and D), suggesting an increase in glucose uptake in diabetic muscle by these two compounds.

3.4. Discussion

T2DM is characterized by hyperglycemia, insulin resistance, dyslipidemia, oxidative stress, chronic inflammation and angiogenesis. Our previous work focused in XN and 8PN modulation of oxidative stress, inflammation and angiogenesis, three intermingled processes implicated in DM (Negrão *et al.*, 2010; Costa *et al.*, 2013). In the present study, C57Bl/6 mice fed with HFD were used to address the effects of XN and 8PN treatments in diabetic-related metabolic complications. Corroborating recent evidence regarding XN effects in metabolism (Miyata *et al.*, 2015), the present work further highlights novel targets for therapeutic interventions, and reports for the first time the metabolic effects of 8PN.

Besides lowering body weight gain and fasting hyperglycemia in diabetic mice, these findings showed that XN and 8PN also ameliorated plasma lipid profile, reducing total cholesterol and TG levels, comparatively to DM-Ethanol animal group. Moreover, OGTT and IPITT assays revealed that both polyphenols counteract HFD-induced glucose intolerance and insulin resistance in diabetic mice, which are corroborated with HOMA and QUICKI determinations of insulin resistance and sensitivity, respectively. In agreement with this, other authors reported that polyphenols consumption improves cardiovascular health, blood pressure, LDL levels and insulin resistance (Yui *et al.*, 2014; Grassi *et al.*, 2015).

Concomitantly, XN and 8PN consumption modulated metabolic-related protein expression in diabetic mice hepatic and skeletal muscle. In line with the effects observed by other polyphenols, the current study demonstrated that both XN and 8PN activated AMPK, a key energy sensor involved in the regulation of energy metabolic pathways (Sears *et al.*, 2012; Guo *et al.*, 2015). Once activated, AMPK inhibits SREBP-1c expression, as well as its downstream lipogenesis enzyme targets, ACC and FAS. These findings are in accordance with the systemic lipid profile improvement, body weight gain, enhancement in insulin sensitivity and glucose tolerance observed.

In order to generate energy, liver and skeletal muscle oxidize LCFA transported from the blood into tissues mainly through CD36, which has been shown to be augmented in T2DM, leading to increased uptake of FFA (Miquilena-Colina *et al.*, 2011). In fact, increased hepatic and muscle CD36 expression plays a causative role in the pathogenesis of T2DM contributing to the diabetes-associated dyslipidemia (Koonen *et al.*, 2007; Goldberg *et al.*, 2009). The expression of CD36 transporter was downregulated in animals treated with polyphenols, which probably results in decreasing FA uptake by the tissues as documented by other authors (Aoun *et al.*, 2011).

In addition, increased hepatic and skeletal muscle VEGFR-1 and its ligand VEFG-B expression was found in our diabetic animals. VEGF-B binds to its receptor, inducing the expression of FATP that are involved in the lipid transport and uptake from EC to tissues (Hagberg *et al.*, 2010). XN or 8PN treatment caused a reduction in both VEGFR-1 and VEGF-B in diabetic mice, suggesting that the intake of these two polyphenols prevents ectopic lipid accumulation. Hagberg and colleagues reported that VEGF-B did not affect CD36 expression (Hagberg *et al.*, 2010), however according to our results both VEGFR-1 and CD36 were increased in diabetic animals and down-regulated by XN and 8PN treatment. These findings suggest that both pathways were independently up-regulated in T2DM, contributing to an increase in TG and cholesterol content in hepatic and skeletal muscle of diabetic animals observed in the current study. Moreover, the XN and 8PN consumption diminished the expression of VEGFR-1/VEGF-B and also CD36 transporter, concomitant with a decreased in TG and cholesterol content within the tissues.

These findings led to the assumption that this signaling pathway is of great interest as a target in metabolic disorders, since it is related with improved glucose tolerance, further restoring insulin sensitivity in T2DM (Hagberg *et al.*, 2010; Hagberg *et al.*, 2012; Carmeliet *et al.*, 2012).

In T2DM, hepatic glucose production is increased as a consequence of an impaired insulin capacity to stimulate glucose uptake by peripheral organs as skeletal muscle (Cryer, 2012). The antidiabetic effect of XN and 8PN was additionally demonstrated through reduced hepatic and skeletal muscle PFKFB3 expression, an important glycolytic regulator, known as a modulator of insulin action (Trefely *et al.*, 2015). Modulation of PFKFB3 activity or glycolysis pathway affects insulin signaling. Thereafter, insulin activates PI3K/AKT signaling pathway, which phosphorylates AS160 downstream substrate, triggering GLUT-4 translocation to cell membrane (Klip, 2009). In the present study, PFKFB3 was identified as a positive regulatory kinase of GLUT-4 translocation as its downregulation by polyphenol's consumption leads to the impairment on AS160 expression. Accordingly, animals fed with HFD displayed an impairment in GLUT-4 translocation to plasma membrane. Our data suggests that XN and 8PN ingestion increased phospho-AS160 expression, which is known to lead to GLUT-4 membrane translocation, improving skeletal muscle glucose uptake. These findings are further corroborated by the reduction in glycemia and increase in insulin sensitivity upon these compounds intake.

Altogether, the present work provides evidence that both XN and 8PN treatments protect mice against the development of T2DM metabolic-related complications. The XN and 8PN consumption exerted their beneficial effects by reducing body weight gain, preventing insulin resistance and modulating lipid and glucose metabolic pathways. They exert beneficial effects by a metabolic switch from FA synthesis to oxidation and promoting muscle glucose uptake. Our findings suggest a potential dietary strategy for preventing common metabolic diseases as T2DM.

XN and 8PN are natural multifunctional compounds that confer multiple health benefits by acting simultaneously in multiple targets. Despite not enough data is available regarding the metabolic effects of 8PN in diabetes, our data highlight the promising benefits of this

compound in the protection against T2DM-related metabolic dysfunctions. Nevertheless, additional experimental studies are required to better understand the underlying mechanisms of potential effects of these two polyphenols.

4. Modulation of VEGF signaling in a mouse model of diabetes by xanthohumol and 8-prenylnaringenin: Unveiling the angiogenic paradox and metabolism interplay

4.1. Background

The prevalence of obesity and associated T2DM has increased dramatically in the last decades. T2DM is a chronic, multifactorial and progressive disease, which affects more than 300 million people worldwide, being considered a major public health threat. Diabetes is characterized by hyperglycemia due to a deficiency in insulin production and/or its resistance, which contributes to endothelial dysfunction, resulting in macro and microvascular complications (Brownlee, 2001; Blake *et al.*, 2014). Diabetic patients have a high incidence of renal disease, lower limb amputation, and retinopathy that are important causes of morbidity, and portend an increased risk to develop cardiovascular disease responsible for a significant number of deaths (Schnuelle *et al.*, 2011).

T2DM is a paradoxical disease regarding vascular complications. Increased neovascularization in organs such as the kidney and retina, coexist with a marked inhibition of new vessel growth in coronary heart disease and peripheral arterial disease (Soares *et al.*, 2009). Although the angiogenic paradox is well established in diabetes, the molecular mechanisms underlying these distinct vascular phenotypes in different organs remain unclear.

Angiogenic EC relies on glycolysis for rapidly generating energy to migrate and proliferate to avascular areas. Recently, it has been reported that PFKFB3, a bifunctional enzyme abundant in EC, plays an important role in ensuring the high glycolytic flux that promotes vessel growth (De Bock *et al.*, 2013; Eichmann *et al.*, 2013; Zecchin *et al.*, 2015). In fact, its kinase activity is more active than the phosphatase, favoring F-2,6-BP synthesis, a potent

allosteric activator of 6- PFK-1 key glycolytic enzyme (Van Schaftingen *et al.*, 1982). When PFKFB3 is silenced *in vitro* or inactivated *in vivo*, glycolysis becomes partially and transiently suppressed and angiogenesis impaired inducing EC quiescence (Schoors, De Bock*, et al.*, 2014).

The cross talk between angiogenesis and endothelial metabolism also enrolls VEGF signaling pathway. VEGF-A stimulates endothelial response through VEGFR-2 tyrosine kinase activity (Ferrara *et al.*, 2005; Roskoski, 2007; Waltenberger, 2009; Koch *et al.*, 2012), and also has affinity to bind to VEGFR-1 but with poor signal transduction (Rahimi, 2006; Robinson *et al.*, 2001). Conversely, VEGFR-1 and NP-1 co-receptor bind to VEGF-B (Li, 2010; Bry *et al.*, 2010), which has recently been associated to endothelial lipid metabolism. VEGF-B induces the expression of FATP mediating the transport and the uptake of dietary lipids to peripheral tissues through EC (Hagberg *et al.*, 2010; Li, 2010). Blocking VEGF-B signaling has attracted considerable attention in metabolic diseases, as it may prevent ectopic lipid accumulation and restore insulin sensitivity in obesity and T2DM animal models (Carmeliet *et al.*, 2012; Hagberg *et al.*, 2013; Muhl *et al.*, 2016).

Several approaches are used in T2DM management, namely insulin and oral anti-diabetic drugs or a combination therapy of both. However, they often exhibit undesirable side effects and fail to alter the course of long-term complications in many organs and to achieve and maintain long-term glycemic control. The development of alternative therapeutic approaches may include natural bioactive compounds as polyphenols that are present in fruits, vegetables and some beverages, including beer, green tea and wine. Moreover, evidence from animal and clinical studies suggests that the consumption of polyphenol-rich diets provide a variety of health benefits, including antioxidant, anti-inflammatory, antiatherogenic, among others (Vinson *et al.*, 2003; Imhof *et al.*, 2004). In recent years, much attention focused on the promising therapeutic potential of polyphenols in metabolic diseases, namely metabolic

syndrome, a cluster of risk factors that predispose to cardiovascular disease, cancer and T2DM (Rosell *et al.*, 2003; Nozawa, 2005; Gerhauser, 2005a; Legette *et al.*, 2012; Scoditti *et al.*, 2012).

Beer-derived XN, the main hop flavonoid, has substantial health-promoting properties and is mainly converted into IXN and then into 8PN phytoestrogen during beer production and after *in vivo* metabolism by liver microsomes and intestinal bacteria (Nikolic *et al.*, 2006; Possemiers *et al.*, 2008; Possemiers *et al.*, 2009). We previously demonstrated that whereas topic administration of 8PN stimulates the growth of neovessels, XN and IXN exhibit anti-angiogenic and anti-inflammatory properties in a wound healing *in vivo* assay (Negrão *et al.*, 2010). Recently, it was reported that XN exerts anti-obesity activity and affect lipid and glycolic metabolism in rats with diet-induced T2DM (Bobak *et al.*, 2003; Nozawa, 2005; Gorinstein *et al.*, 2007; Legette *et al.*, 2012; Kiyofuji *et al.*, 2014; Legette *et al.*, 2014; Sumiyoshi *et al.*, 2013; Yui *et al.*, 2014; Miranda *et al.*, 2016a). Dietary phytoestrogens consumption also ameliorates insulin sensitivity, energy homeostasis and metabolic complications on animals and in post-menopausal women (Cederroth *et al.*, 2009; Llaneza *et al.*, 2010; Andreoli *et al.*, 2015).

Thus nutritional polyphenols supplementation both acting in the endothelium, and improving metabolic dysfunction are putative tissue specific-target agents, potentially allowing a better resolution of T2DM-related complications. The current study aimed to examine the effect of XN and 8PN in diabetic vascular complications.

4.2. Material and methods

4.2.1. Animals and experimental mice treatment

Thirty C57Bl/6 6-week old male mice (Charles-River, Spain) were randomly divided into 5 experimental groups (n=6), and fed with different diets and beverages during 20 weeks: Standard diet (Control, C); High-fat diet (DM); HFD and 0.1 % ethanol in drink water (DM-Ethanol); HFD and 10 mg/L XN (Hopsteiner, Germany) in 0.1 % ethanol (DM-XN); HFD and 10

mg/L 8PN in 0.1 % ethanol (DM-8PN). 8PN was synthetized according to previously described procedures (Zierau *et al.*, 2002). Briefly, starting from acetylation of commercially available naringenin, the 7,4'-diacethyl-naringenin derivative was *O*-prenylated by the Mitsunobu reaction and following the tandem Claisen-Cope rearrangement of 5-prenyl-7,4-diacetylnaringenin with $Eu(fod)_3$, 8PN was obtained in 32 % yield. XN and 8PN were administered in a dose of 10 mg/L previously studied by our group, equivalent to 1 mg/Kg/day, which is in line with the doses reported previously without displaying hepatotoxic effects (Negrão *et al.*, 2012; Costa *et al.*, 2013). Polyphenols consumption was administered throughout the 20 weeks of experiment, the duration necessary to establish hyperglycemic conditions. A control of diabetic animals with 0.1 % ethanol in water (DM-Ethanol) was included, corresponding to the final ethanol concentration in polyphenols treatment.

HFD was obtained from Research diets, USA. This mouse strain is prone to develop T2DM under HFD ingestion, displaying significant dyslipidemia, insulin resistance, and glucose intolerance (Cong *et al.*, 2008).

During the treatment period, body weight, glycemia, food and beverage intake were monitored. Glycemic control was monitored by measuring blood glucose obtained by pricking the vein from the tip of the tail using a sterile needle once a week with Precision Xtra Plus test strips and an Optium Xceed device (Abbott Diabetes Care, Ltd., Maidenhead, UK), according to the manufacturer's instructions. Plasma insulin levels were measured using a rat/mouse insulin ELISA kit (EZRMI-13K; Merck Milipore, Madrid, Spain).

Beverages were renewed every two days and were kept in dark bottles to avoid degradation. After 20 weeks of treatment, animals were sacrificed and left ventricle and kidney were frozen at -80 ºC for molecular analyses or fixed in 10 % neutral-buffered formalin, dehydrated, and paraffin-embedded for histological assays. Three micrometer-thick tissue

sections were used for histological and immunohistochemistry analysis. Blood was also collected for biochemical analyses.

Animals were maintained under controlled conditions of temperature (23 ± 5 °C), humidity (35 ± 5 %), and 12 h light/dark cycles and access to diet and beverages were allowed *ad libitum*. All animal experiments were conducted at the animal house located at the Faculty of Medicine, University of Porto, and were carried out by trained technicians in accordance with the European Community policy for Experimental Animal Studies [European Community law dated from November 24th 1986 (86/609/CEE) with addendum from June 18th 2007 (2007/526/CE)]. The project was approved by the Portuguese National Authority for Animal Health, DGAV, Portugal.

4.2.2. Microvessel Density Evaluation

Immunohistochemistry was performed in kidney and left ventricle paraffin sections with anti-CD31 antibody (1:100 dilution; ab 28364; Abcam, UK), as previously described (Negrão *et al.*, 2012).

The number of vessels was counted in three tissue sections for each animal and normalized to the total tissue area. A negative control was included. Any positive-stained EC or cluster that was separated from adjacent vessels was considered an individual vessel.

4.2.3. VEGF-A, VEGF-B and phosphorylated VEGFR-2 quantification by ELISA

Plasma and tissue VEGF-A and VEGF-B concentrations were quantified using Quantikine mouse VEGF-A (R&D Systems, UK) and mouse VEGF-B (ABIN869657, Antibodies-online, USA) ELISA kit. Levels of phosphorylated VEGFR-2 in cell lysates were measured using a Duoset

human Phospho-VEGFR-2 (Tyr 1175) ELISA kit (R&D Systems, UK), using a microplate reader (Thermo Fisher Scientific, USA) in accordance with the manufacturer's instructions.

4.2.4. Western blot analyses for angiogenic pathways

Ten to twelve micrograms of total protein were separated by electrophoresis in a 10 % SDS-PAGE. After transfer to a nitrocellulose membrane (Biorad, USA), membranes were incubated overnight with antibodies against VEGFR-2 (1:750 dilution; #2479 Cell signalling, UK), phospho-VEGFR-2 (1:750 dilution; #2478 Cell signalling, UK), AKT (1:1000 dilution; #9272 Cell signalling, UK), phospho-AKT (1:750 dilution; #4051 Cell signalling, UK), ERK (1:1000 dilution; #9102 Cell signalling, UK), phospho-ERK (1:750 dilution; #4370 Cell signalling, UK), VEGFR-1 (1:7500 dilution; ab2350 Abcam, UK), NP-1 (1:1000 dilution; ab25998 Abcam, UK), PFKFB3 (1:1000 dilution; #13123 Abcam, UK) and β-actin (1:3000 dilution; ab8227 Abcam, UK), followed by incubation with the respective secondary horseradish-peroxidase (HRP)-coupled antibody (1:10000; anti-rabbit sc-2040 and anti-mouse sc-2060 from Santa Cruz Biotechnology, Germany), during 2 hours. The detection was performed using enhanced chemiluminescence (ECL kit; Biorad, USA). Mean relative intensities of the different proteins expression were quantified by densitometry (Vision Works LS software; UVP Inc., USA) and normalized with the signal intensity of β-actin or the signal intensity of total form for phosphorylated proteins.

4.2.5. Cell culture and polyphenol treatment of HMECs

Human microvascular endothelial cells (HMEC-1; ATCC, UK) were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS; Invitrogen Life Technologies, UK), 1 % penicillin/streptomycin (Invitrogen Life Technologies, UK), 1.176 g/L sodium bicarbonate, 4.76 g/L HEPES, 10 µg/mL EGF and 1 mg/L hydrocortisone >98 % (Sigma, Portugal), and were

maintained at 37ºC in a humidified 5 % CO2 atmosphere. All experiments were performed between passages 4 and 9. Treatments were performed for 5 min and 24 h in serum-free conditions and with 5.5 mM or 20 mM glucose concentration, to mimic diabetic condition. To evaluate whether polyphenols interact directly with VEGF-A, a pre-incubation of VEGF-A (25 ng/mL; Sigma, Portugal) with polyphenols (XN and 8PN at 1 µM) was performed during 5 min, and then HMECs were washed twice and incubated with the mixed VEGF-A/polyphenol solution during 5 min or 24 h. At the same time-points, HMECs were incubated with polyphenols alone followed by stimulation with 50 ng/mL VEGF-A for 5 min. Control assays included vehicle control (0.1 % ethanol); a positive control (25 ng/mL VEGF-A) and a VEGFR-2 inhibitor was used as a negative control (1 nM cabozantinib, Santa Cruz Biotechnology, Germany). Cells were lysed with RIPA buffer containing protease and phosphatase inhibitors and proteins were extracted and used in phosphorylated-VEGFR-2 ELISA assay as previously described. The results are expressed as percentage change relative to ethanol 0.1 %.

4.2.6. Statistical analysis

Every assay was performed at least in three independent experiments. Statistical significance of different groups was evaluated by ANOVA followed by the Bonferroni post-hoc test. In order to compare body weight and glycemia differences during 20 weeks, were evaluated by repeated measures two-way ANOVA, followed by the Bonferroni post-hoc test. The normality of data distribution was assessed Shapiro-Wilk test and for the homogeneity of variance the Levene's test was used. A difference between experimental groups was considered significant with a confidence interval of 95 %, whenever $p \leq 0.05$.

4.3. Results

4.3.1. Polyphenols reduced body weight gain and glycemia in diabetic mice

At the onset of the studies, the five mice groups revealed similar mean body weight gain (A) and blood glucose levels (B) as illustrated in Figure 4.1. During the 20-week experiment, body weight gain and blood glucose levels of diabetic animals were significantly higher as compared to control group.

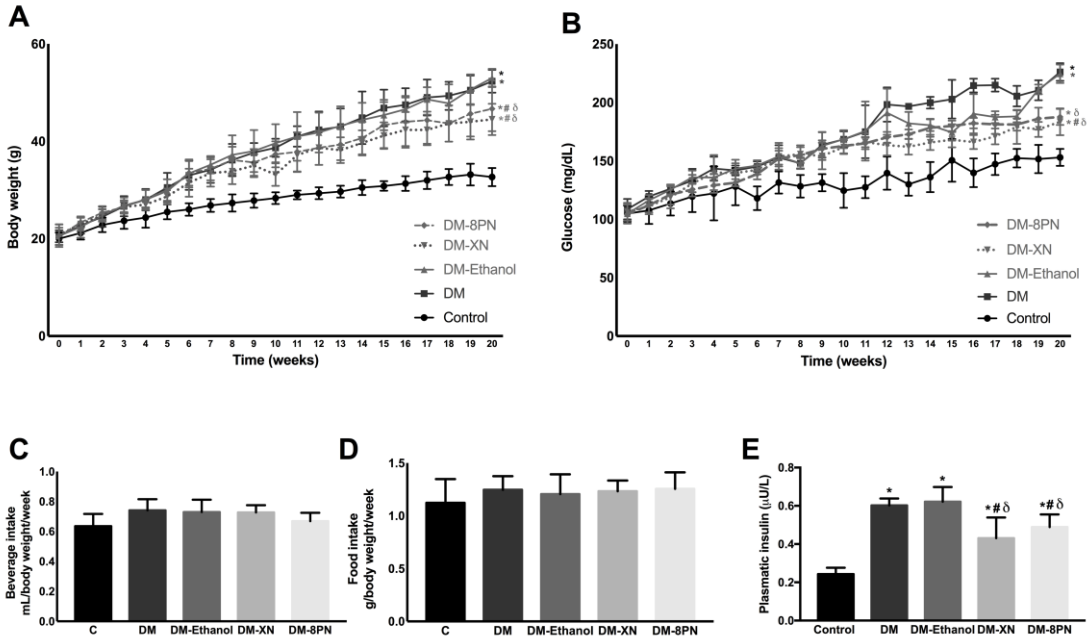

Figure 4.1 - Mean values for body weight (A), blood glucose levels (B), beverage consumption (C) and food intake (D) monitored weekly in mice with distinct treatments during 20 weeks. Quantification of insulin plasmatic levels at 20 weeks (E). Control, healthy (standard diet-fed) mice; DM, diabetic animals (fed with HFD); DM-Ethanol, diabetic animals drinking 0.1 % ethanol; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals consuming 10 mg/L 8PN. Results are expressed as means ± SD (5<n<6). *$p \leq 0.05$ vs control; # $p \leq 0.05$ vs DM; δ $p \leq 0.05$ vs DM-Ethanol at week 20.

No significant difference was observed between DM and DM-Ethanol groups. Interestingly, XN and 8PN treatment significantly suppressed weight gain and glycemia relatively to diabetic ethanol group. To assess if the beverage consumption affects food intake, we monitor both parameters during the 20 weeks. Every experimental group drank the same volume of

beverage (Figure 4.1 C) and consumed the same amount of food (Figure 4.1 D) per body weight.

Insulin plasmatic measurements were also performed at the end of the study to confirm the diabetic condition in our animal model. In accordance to the raised hyperglycemia in DM (0.61±0.03 μU/mL) and DM-Ethanol group (0.62±0.08 μU/mL), insulin plasmatic levels were also higher in DM and DM-Ethanol group when compared to control (0.24±0.03 μU/mL) as depicted in Figure 4.1E. DM-XN (0.43±0.07 μU/mL) and DM-8PN (0.49±0.06 μU/mL) were able to counteract these effect leading to decreased plasmatic glucose and insulin levels as those presented by DM-Ethanol group.

4.3.2. Dietary XN and 8PN affected kidney and left ventricle neovascularization in DM animals

The development of T2DM comprises distinct extracellular matrix remodeling and deregulated angiogenesis in different organs. We next evaluated whether XN and 8PN interfere with the number of vessels in diabetic mice kidney and left ventricle.

A significant increase in the number of CD31 endothelial-stained vessels was found in kidneys of diabetic animals drinking water (127.0±21.8 vessels/mm^2) and 0.1 % ethanol (123.7±23.4 vessels/mm^2) compared to control animals fed with normal diet (68.2±12.5 vessels/mm^2) (Figure 4.2 A and C). Upon polyphenol treatment, the number of vessels decreased to control values, being statistically significant for XN-treated animals (70.7±21.5 vessels/mm^2). Consumption of 8PN did not show significant differences (92.9±10.1 vessels/mm^2), when compared to DM-Ethanol group (δ $p \leq 0.05$ vs DM-Ethanol).

Inversely, in the left ventricle (Figure 4.2 B and C), angiogenesis was impaired with a significant reduction in the number of vessels in DM (154.1±40.2 vessels/mm^2) and DM-Ethanol group (204.0±30.3 vessels/mm^2) when compared to healthy control (297.4±35.1

vessels/mm^2). In contrast, XN (244.0±25.2 vessels/mm^2) and 8PN (353.7±60.9 vessels/mm^2) administration increased microvessel density in comparison to diabetic animals that did not consume polyphenols. Treatment with 8PN reached statistical significance when compared with DM-Ethanol group (Figure 4.2 B and C).

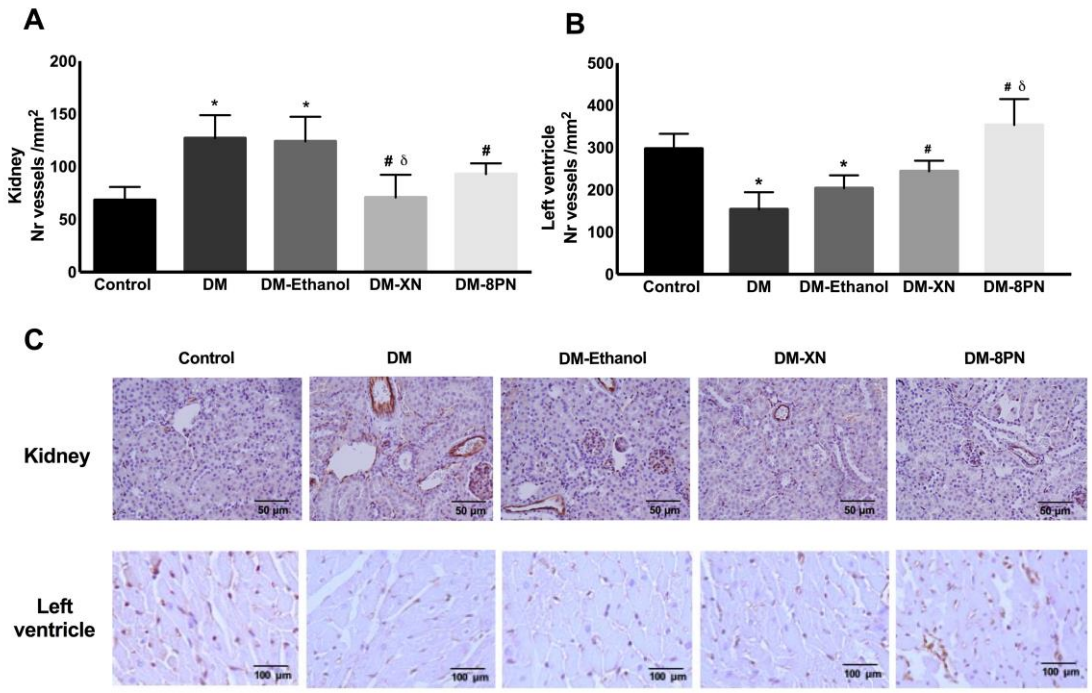

Figure 4.2 - Quantification of blood vessels in kidney (A) and left ventricle (B) in healthy and diabetic animals upon distinct treatments during 20 weeks. (C) Representative images of immunohistochemistry with CD31 staining in kidney and left ventricle. Control, healthy (standard diet-fed) mice; DM, diabetic animals (fed with HFD); DM-Ethanol, diabetic animals drinking 0.1 % ethanol; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals consuming 10 mg/L 8PN. Results are expressed as means ± SD (5<n<6). *$p \leq 0.05$ vs control; # $p \leq 0.05$ vs DM; δ $p \leq 0.05$ vs DM-Ethanol.

4.3.3. XN and 8PN affected systemic and local VEGF-A and VEGF-B levels of DM mice

Polyphenols effect on VEGF-A and VEGF-B concentrations in plasma and kidney and left ventricle tissue lysates were addressed by ELISA assay. As illustrated in Figure 4.3 A, diabetic animals consuming water (82.3±12.7 pg/mL) or 0.1 % ethanol (89.8±10.2 pg/mL) displayed reduced systemic levels of VEGF-A when compared to healthy control group (120.9±16.0

pg/mL). XN did not change this effect (87.6±15.8 pg/mL). Nevertheless, the 8PN group exhibited a significant elevation of plasma VEGF-A levels to control values (142.2±25.9 pg/mL).

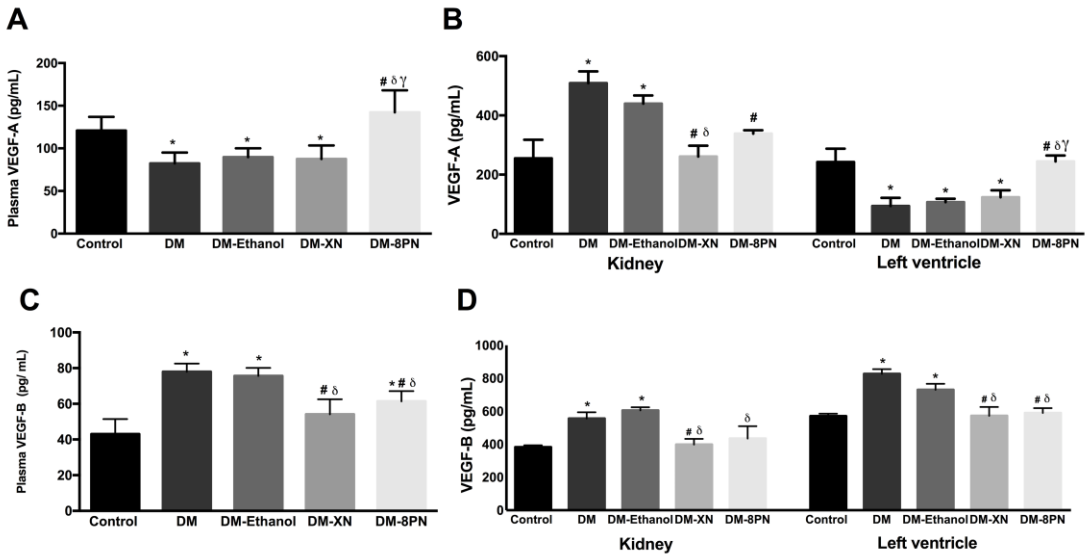

Figure 4.3 - Quantification of systemic and tissue levels of VEGF-A (A and B) and VEGF-B (C and D) in healthy and T2DM animals subjected to distinct treatments during 20 weeks. Control, healthy (standard diet-fed) mice; DM, diabetic animals (fed with HFD); DM-Ethanol, diabetic animals drinking 0.1 % ethanol; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals consuming 10 mg/L 8PN. Results are expressed as means ± SD (5<n<6). *$p \leq 0.05$ vs control; # $p \leq 0.05$ vs DM; δ $p \leq 0.05$ vs DM-Ethanol, γ $p \leq 0.05$ vs DM-XN.

Quantitative analysis of VEGF-A in kidney and left ventricle followed the vascular profile previously observed (Figure 4.2). In kidney (Figure 4.3 B) VEGF-A levels augmented in DM mice (508.9±39.8 pg/mL) and in DM-Ethanol animals (439.9±27.3 pg/mL) when compared to healthy controls (254.9±62.7 pg/mL). This increase was prevented in animals that consumed polyphenols, reaching statistical significance after XN intake (261.2±36.7 pg/mL). An opposite behavior was seen in left ventricle with a significant decrease in VEGF-A in all diabetic animal groups, except for 8PN-treated animals, which led to VEGF-A stimulation (244.4±20.2 pg/mL) to healthy control levels (242.6±45.1 pg/mL).

Given the recently reported role of VEGF-B in endothelial metabolism (Hagberg *et al.*, 2010; Hagberg *et al.*, 2013), VEGF-B was also quantified in these animals. Plasma and tissue

VEGF-B levels (Figure 4.3 C and D) revealed an identical pattern, increasing in diabetic animals subjected to treatment with water (77.9±4.6 pg/mL in plasma; 556.5±37.4 pg/mL in kidney; 826.9±28.5 pg/mL in left ventricle) and 0.1 % ethanol (75.5±4.5 pg/mL in plasma; 605.6±18.8 pg/mL in kidney; 697.3±58.1 pg/mL in left ventricle) when compared to non-diabetic controls (43.0±8.4 pg/mL in plasma; 382.7±11.3 pg/mL in kidney; 604.0±68.7 pg/mL in left ventricle). Inversely, a significant reduction to control values was observed upon XN (54.1±8.5 pg/mL in plasma; 398.1±35.0 pg/mL in kidney; 572.7±53.5 pg/mL in left ventricle) and 8PN (61.4±5.78 pg/mL in plasma; 434.8±74.5 pg/mL in kidney; 656.5±70.1 pg/mL in left ventricle) consumption.

4.3.4. Polyphenols modulated VEGF angiogenic pathway in diabetic mice

Since the angiogenesis are important targets in metabolic disorders, namely cancer and T2DM, the potential preventive activity of dietary polyphenols in the VEGF signaling pathway in diabetic mice was investigated. VEGFR-2 is expressed in EC and is the predominant receptor involved in angiogenic signaling. Increase of VEGFR-2 activation (2.03±0.5 in DM and 1.83±0.1 in DM-Ethanol) and its downstream effectors, pERK/ERK (2.17±0.2 in DM and 2.11±0.4 in DM-Ethanol) and pAKT/AKT (1.19±0.2 in DM and 1.03±0.1 in DM-Ethanol), was observed in kidney lysates (DM and DM-Ethanol) when compared to control (Figure 4.4 A, B, C and G). After XN consumption, VEGFR-2 activation in the kidney was prevented (0.64±0.2) as well as its downstream effectors pERK (1.42±0.3) and pAKT (0.70±0.06), when compared to DM-Ethanol. Strikingly, reduction of this pathway by 8PN treatment only reaches significant relevance for pERK/ERK (1.32±0.24) protein expression.

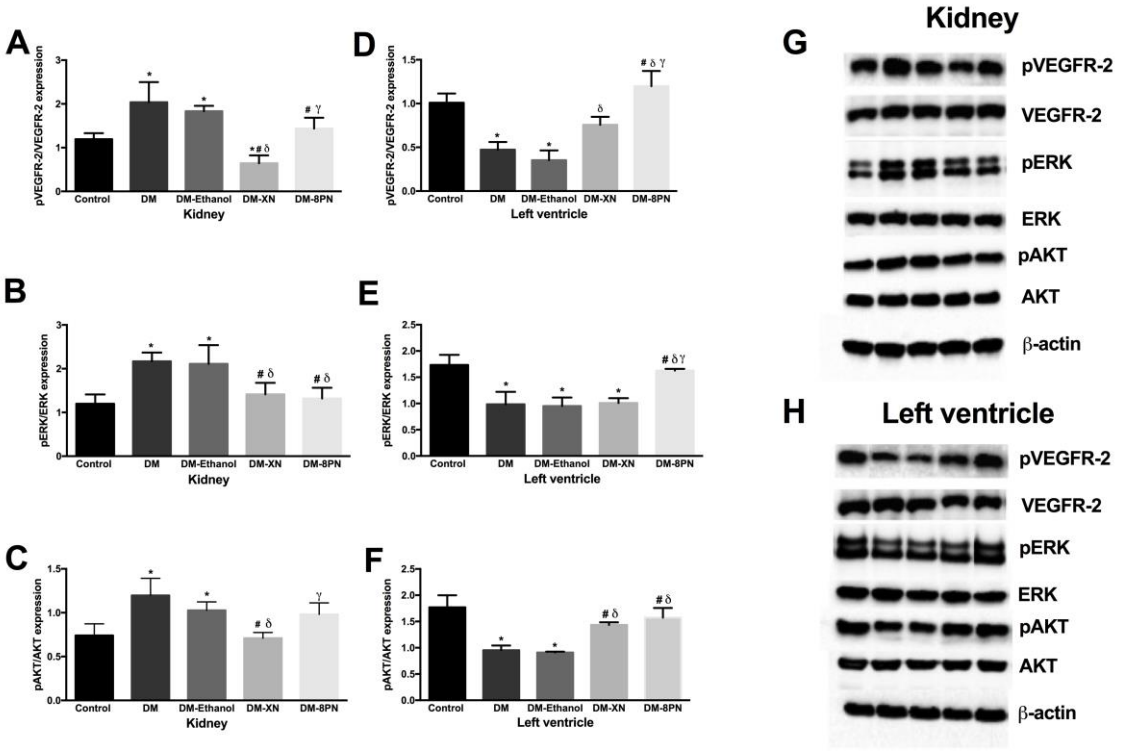

Figure 4.4 - Expression of VEGFR-2 and downstream molecules ERK and AKT in kidney (A-C) and in left ventricle (D-F) by Western blot analyses, in healthy and T2DM animals subjected to distinct treatments during 20 weeks. A representative Western blotting is shown (G and H). Control, healthy (standard diet-fed) mice; DM, diabetic animals (fed with HFD); DM-Ethanol, diabetic animals drinking 0.1 % ethanol; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals consuming 10 mg/L 8PN. Results are expressed as means ± SD (5<n<6). **p* ≤ 0.05 vs control; # *p* ≤ 0.05 vs DM; δ *p* ≤ 0.05 vs DM-Ethanol, γ *p* ≤ 0.05 vs DM-XN.

Conversely, in left ventricle, a significant reduction on phospho-VEGFR-2 and related ERK and AKT activation in DM mice (0.47±0.1; 0.98±0.2; 0.95±0.1, respectively) and DM drinking 0.1 % ethanol (0.35±0.1; 0.95±0.2; 0.91±0.1, respectively) was found when compared to healthy control group (1.01±0.1; 1.73±0.2; 1.77±0.2, respectively) (Figure 4.4 D, E, F, and H). Upon 8PN treatment, the expression of VEGFR-2 (1.20±0.2) and downstream pERK/ERK (1.63±0.1) and pAKT/AKT (1.58±0.2) effectors were significantly activated. Surprisingly, XN was also able to activate VEGFR-2 (0.75±0.1) and AKT (1.43±0.1) in left ventricle when compared to DM-Ethanol group, although, without reaching healthy control levels.

To better understand the dual effect of XN and 8PN in modulating VEGFR-2 phosphorylation, a cell culture assay was performed in the presence or absence of VEGF-A. Incubation of HMEC with XN either for 5 min or 24 h, led to a significant decrease in VEGFR-2 activity (Figure 4.5, assay 1). This effect was even enhanced when XN-treated HMECs were stimulated with VEGF-A for 5 min (Figure 4.5, assay 2).

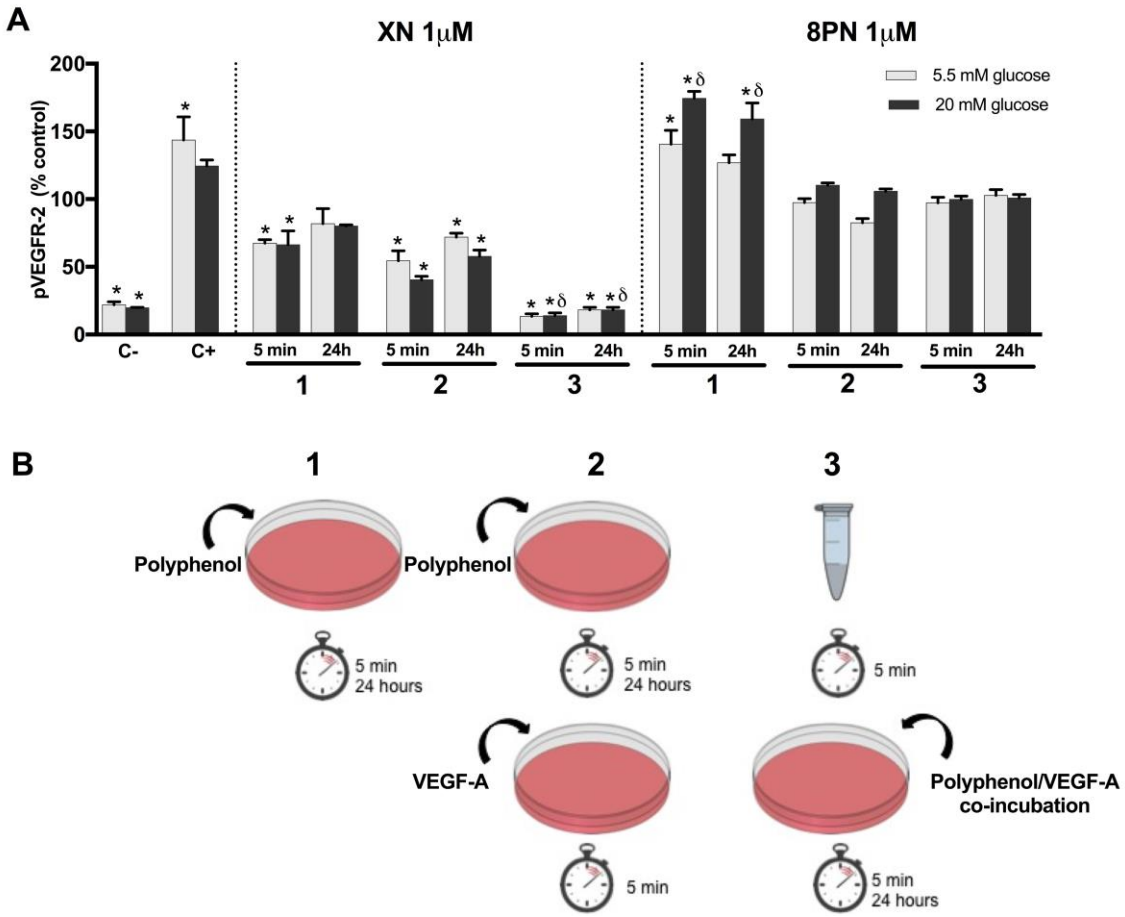

Figure 4.5 - XN and 8PN modulate VEGFR-2 activation in endothelial cells by ELISA assay. (A) HMEC were exposed to normal (5.5 mM glucose) and hyperglycemia (20 mM glucose) conditions. HMEC were incubated with 1 μM XN or 8PN for 5 min and 24 h (1). HMEC were incubated with 1 μM XN or 8PN for 5 min and 24 h and then stimulated with VEGF-A for 5 min (2). HMEC were co-incubated with XN or 8PN and VEGF for 5 min and 24 h (3). (B) Schematic representation of cell culture experimental design. Results are expressed as percentage of ethanol control at each time point and glucose concentration and presented as mean ± SD (n=4) *$p \leq 0.05$ versus control; δ $p \leq 0.05$ versus normal glucose. C-, HMECs treated with VEGFR-2 Inhibitor; C+, HMECs incubated with VEGF for 24h.

As depicted in Figure 4.5 (assay 3) prior co-incubation of XN with 50 ng/mL VEGF-A for 5 min, resulted in an 87 % reduction of VEGFR-2 activation to levels similar to those achieved with VEGFR-2 inhibitor, implying that anti-angiogenic effects of XN may be due to VEGF-A quenching by this polyphenol.

Conversely, HMEC incubated with 8PN exhibited a significant activation of VEGFR-2 only in the absence of VEGF-A (Figure 4.5, assay 1). When HMEC were previously incubated with 8PN and then stimulated with VEGF-A or co-incubated with a mixture of 8PN and VEGF-A, no significant effect on VEGFR-2 activation was detected. These findings indicate that 8PN pro-angiogenic action occurs only when VEGF-A concentrations were low or absent.

4.3.5. Polyphenols modulated VEGF-B signaling and PFKFB3 glycolysis regulator in diabetic mice

As depicted in Figure 4.6, diabetic animals exhibited an increase VEGFR-1 and its co-receptor NP-1 expression in kidney and left ventricle, as well as in VEGF-B (Figure 4.3). VEGF-B binds to VEGFR-1 and NP-1, controlling lipid transport and peripheral tissues uptake (Hagberg *et al.*, 2010; Hagberg *et al.*, 2013). Diabetic animals consuming XN and 8PN, the expression of VEGFR-1 is significantly reduced for both beer polyphenols in kidney (1.41±0.2 for XN and 1.29±0.1 for 8PN) and left ventricle (0.69±0.1 for mice treated with XN and 0.92±0.1 for 8PN), when compared to DM-Ethanol (2.06±0.1 in kidney and 1.70±0.1 in left ventricle) (Figure 4.6 A, D, G, H). NP-1 was also reduced upon treatment of XN (0.73±0.2 in kidney and 0.72±0.1 in left ventricle) and there is a tendency towards reduction for 8PN consumption (0.77±0.1 in kidney and 0.83±0.1 in left ventricle), when compared to DM-Ethanol (1.10±0.2 in kidney and 1.17±0.1 in left ventricle).

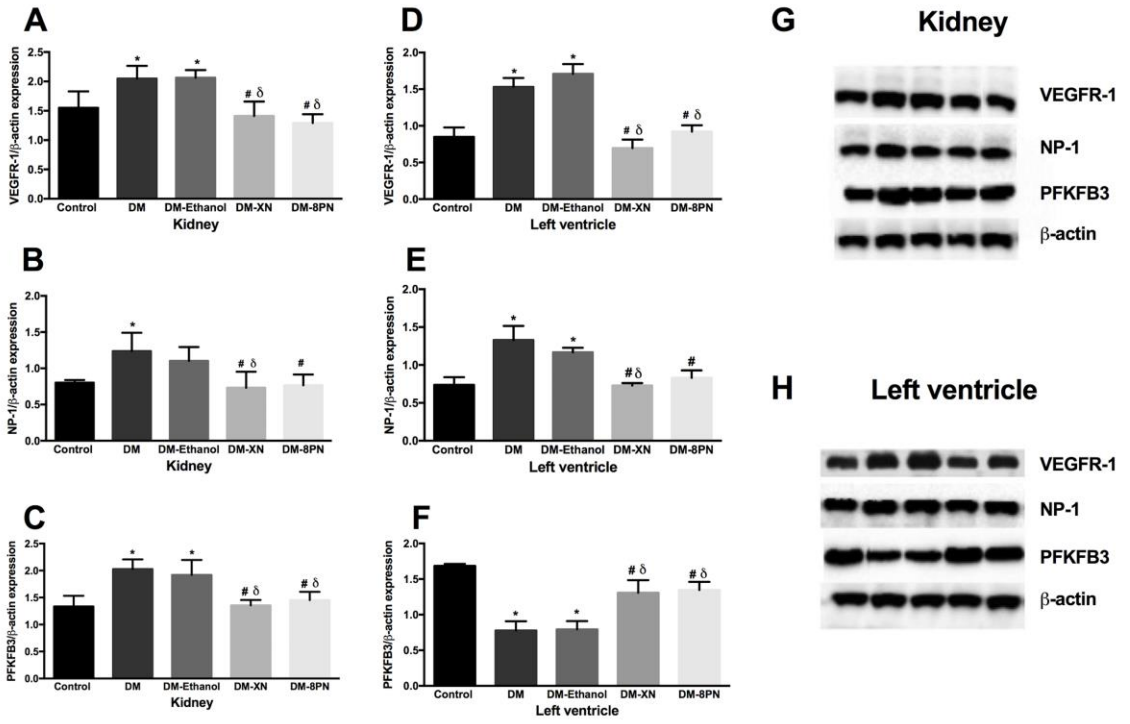

Figure 4.6 - Expression of VEGFR-1, NP-1, and PFKFB3 enzyme in kidney (A-C) and left ventricle (D-F) of healthy and T2DM animals subjected to distinct treatments during 20 weeks. A representative Western blotting is shown from three independent experiments (G and H). Control, healthy (standard diet-fed) mice; DM, diabetic animals (fed with HFD); DM-Ethanol, diabetic animals drinking 0.1 % ethanol; DM-XN, diabetic animals treated with 10 mg/L XN; DM-8PN, diabetic animals consuming 10 mg/L 8PN. Results are expressed as means ± SD (5<n<6). *$p \leq 0.05$ vs control; # $p \leq 0.05$ vs DM; δ $p \leq 0.05$ vs DM-Ethanol, γ $p \leq 0.05$ vs DM-XN.

Since metabolism also mediates angiogenesis, the influence of polyphenols in PFKFB3 expression, an enzyme that enhances glycolysis when the angiogenic process is activated, was evaluated. Interestingly, as shown in Figure 4.5 C and F, PFKFB3 expression profile was identical to the angiogenic receptor pathway expression observed (Figure 4.4). PFKFB3 expression was increased in kidney of diabetic animals (2.03±0.2 and 1.92±0.3, in DM and DM-Ethanol groups respectively) when compared to healthy control (1.34±0.2). This was prevented after XN (1.36±0.1) and 8PN (1.4±0.2) oral administration. Inversely, the expression of this glycolytic activator is downregulated in left ventricle in diabetic animals (0.78±0.1) when compared to healthy control mice (1.68±0.1). XN (1.31±0.2) and 8PN (1.35±0.1) consumption

reversed this effect as shown by the significant increase in PFKFB3 expression in comparison to their ethanol control group (0.79±0.1).

4.4. Discussion

Diabetes-associated vascular complications are highly dependent on organ-specific metabolic disturbances. We addressed the interplay between angiogenesis and metabolism, by examining two organs that display increased (kidney) and impaired (left ventricle) vascularization in T2DM mice fed during 20 weeks with a HFD. Diabetic mice exhibit increased body weight gain and sustained elevated glycemia levels without affecting the amount of food and beverage intake per body weight throughout the study. We showed that oral administration of XN and 8PN lowered blood glucose in diet-induced diabetic mice, improving T2DM conditions. Corroborating our data, there is growing experimental and epidemiological evidence that XN aside from the antioxidant properties, also plays anti-hyperglycemic and anti-diabetes roles (Legette *et al.*, 2013; Yui *et al.*, 2014). But to our knowledge, the effect of 8PN in body weight gain and glycemia in diabetic animals has never been reported before.

The most devastating consequences of diabetes arise from the microvascular complications associated with nephropathy, neuropathy and retinopathy. In agreement with the literature, our results confirm the existence of an angiogenic paradox in diabetic animals (Wirostko *et al.*, 2008). As illustrated in Figure 4.7, an increase in renal neovascularization and an impairment of blood vessel growth in left ventricle assessed by immunohistochemistry stained for the endothelial marker CD31, and corroborated by Western blotting analyses for phospho-VEGFR-2 and downstream effectors, and VEGF-A by ELISA was observed. According to our results, VEGF-A up-regulation in diabetic kidney binds and activates VEGFR-2 in EC, resulting in increased ERK and AKT MAPK downstream effector signaling. This whole mechanism leads to augmented microvessel density. MAPK directly up-regulates pro-

inflammatory mediators such as IL-1β and NF-κB (Kerimi *et al.*, 2016) that are augmented in T2DM. In contrast, these animals exhibit a reduction in the number of vessels in left ventricle, which can be explained by the decreased VEGF-A levels and VEGFR-2 signaling pathway impairment. Circulating VEGF-A levels in these experimental groups are decreased in accordance to other studies (Carneiro *et al.*, 2012; Zakareia, 2012; Costa *et al.*, 2013), but this systemic biomarker is a poor angiogenesis indicator, as the systemic levels did not correspond to the ones quantified in organs from T2DM animals (Figure 4.3).

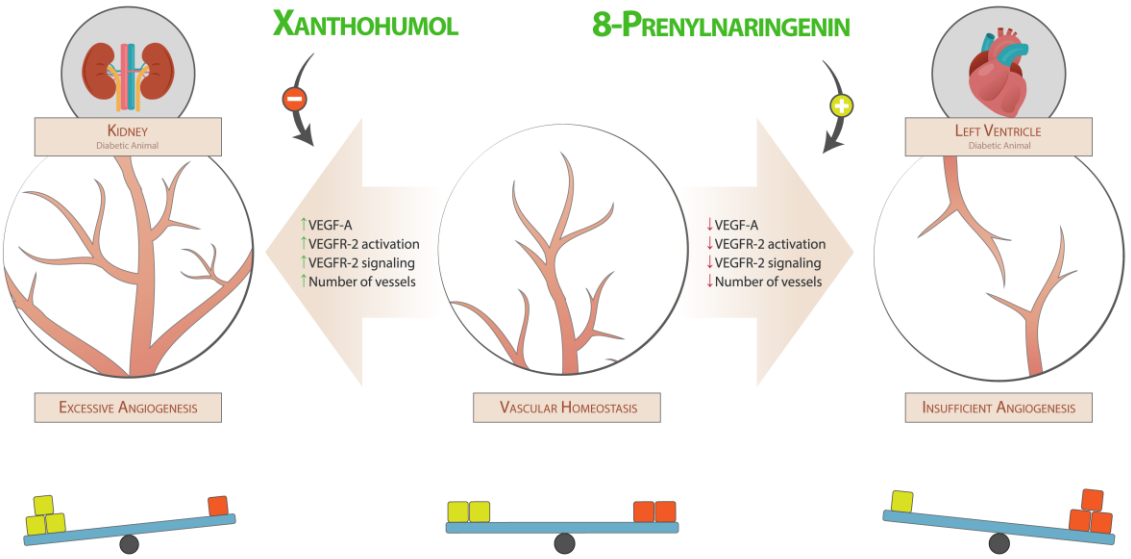

Figure 4.7 - Schematic representation of the angiogenic paradox in HFD-fed mice and its modulation upon XN and 8PN consumption. XN consumption prevents the augment in kidney neovascularization assessed by the VEGF-A levels, VEGFR-2 and downstream signal activation (ERK 42/44 and AKT effectors) and the microvascular density in T2DM-induced mice. 8PN consumption activates the angiogenic process which is impaired in left ventricle of T2DM-induced mice with lower levels of VEGF-A, expression of VEGFR-2 and downstream effectors (ERK 42/44 and AKT) and reduced number of vessels.

In accordance with our previous studies, the current study demonstrated that XN exhibit an anti-angiogenic potential, especially in the kidney, where it significantly reduced VEGF-A levels, VEGFR-2 activation, and microvessel density. 8PN ameliorate the angiogenic paradox as

well, enhancing VEGF-A in diabetic left ventricle where angiogenesis is impaired in T2DM. Corroborating these findings, Yoon and collaborators demonstrate cardio-protective effects in diabetic mice after injection of a plasmid encoding VEGF-A, restoring microvascular homeostasis by improving angiogenic signaling (Yoon *et al.*, 2005). Our group already pointed out 8PN as a pro-angiogenic molecule in diabetic animals (Negrão *et al.*, 2010), possibly due to its estrogen agonist properties, already established as angiogenic promoter (Soares, Guo, Gartner*, et al.*, 2003; Soares, Guo, Russo*, et al.*, 2003),

Interestingly, 8PN led to an augmented VEGF-A serum and left ventricle levels without any significant effect on this growth factor in kidney. Interestingly, despite the opposite effect of both polyphenols concerning angiogenic process, in kidney, 8PN also down-regulates ERK phosphorylation possibly leading to a more controlled endothelial proliferation. In turn, XN up-regulates AKT in left ventricle to levels similar to healthy mice, playing thus a role in survival and also in the insulin signaling cascade by stimulating glucose uptake (Konner *et al.*, 2012). These results suggest that other pathways must be regulated, contributing to the mentioned effects due to the pleiotropic action of polyphenols.

Previous experiments indicated that specific polyphenols inhibit VEGF-induced activation of VEGFR-2 by direct binding to VEGF-A (Cerezo *et al.*, 2015; Moyle *et al.*, 2015). Our cell culture assays show that inhibition of VEGF-induced VEGFR-2 phosphorylation by XN was remarkably increased upon co-incubation of this compound with VEGF-A.

On the other hand, 8PN treatment leads to a significant activation of VEGFR-2 but it only occurs on HMEC-1 without stimulation. These findings suggest that these two polyphenols differently affected VEGFR-2 phosphorylation depending on tissue VEGF-A content. For instance, in kidney from T2DM mice, with increased VEGF-A levels, XN was able to reduce angiogenic process. On the other hand, in left ventricle where angiogenesis is impaired, 8PN

was able to increase angiogenesis. The low VEGF-A levels in this tissue could explain this effect, since 8PN *in vitro* led to a significant VEGFR-2 activation only in the absence of VEGF-A.

A considerable body of evidence indicates that hyperglycemia and associated comorbidities affects EC metabolism. In obesity, the excessive FFA are initially stored in subcutaneous adipose tissue, but once that capacity is reached, they are ectopically accumulated in peripheral tissues and vasculature, leading to lipotoxicity, increased inflammation and immune system activation (Heilbronn *et al.*, 2004; Costanzo *et al.*, 2015). In the current work, VEGF-B and its receptors are overexpressed in HFD-fed animal's kidney and left ventricle. For the first time, our results showed that XN and 8PN modulate the VEGF-VEGFR-1 pathway to healthy controls levels. Other authors demonstrated that when VEGF-B binds to VEGFR-1 and NP-1, the vascular expression of FATP-3 and FATP-4 is increased through a PI3K-dependent pathway (Hagberg *et al.*, 2010), and also by estrogen-related receptor alpha and peroxisome proliferator-activated receptor gamma coactivator 1- alpha induced by AMPK (Villena *et al.*, 2008). Taking these findings into account, our results suggest that both XN and 8PN can be promising agents against pathological lipid accumulation, a characteristic of T2DM patients.

Moreover, the carbohydrate metabolism was also highlighted in the current study. PFKFB3 is a glycolysis activator present in active EC (De Bock *et al.*, 2013). Accordingly, the expression profile of this enzyme accompanied the vascular profile, as diabetic mice presented increased PFKFB3 expression in kidney and reduced in left ventricle. By inducing fructose-2,6-bisphosphate, an allosteric effector of primary control point of glycolysis, PFKFB3 enzyme enhances ATP synthesis, a mainstay for EC growth and migration activity (De Bock *et al.*, 2013). Remarkably, both XN and 8PN attenuated these effects into healthy control values, implying that these compounds also normalized carbohydrate metabolism in vascular endothelium.

Altogether, we demonstrated that XN and 8PN, in a dose of 1 mg/kg body weight/day, reduce diet-induced obesity and diabetes by modulating angiogenesis and metabolic parameters. In addition to well-documented health-promoting effects of polyphenols, the present work demonstrated that both XN and 8PN, possess positive effects on the HFD-fed animal's heart and kidney, ameliorating the angiogenic paradox as illustrated in Figure 4.7. We further described for the first time target molecules mediating its biological actions, including the modulation of glycolytic enzyme PFKFB3 that mediates vessel sprouting and VEGF-B signaling pathway that mediates cellular responses involved in the lipid transport in EC and uptake by peripheral tissues.

Although additional research is needed to better understand the interplay between angiogenesis and metabolism, our findings suggest that beer-derived polyphenols prevent angiogenic impairment and metabolic pathways that are implicated in the pathogenesis of T2DM, being promising compounds to mitigate the increasing number of diabetic patients and inherent health care costs.

III

General discussion

General discussion

During the last decades many efforts have been made to identify and develop novel therapeutic strategies for the management of T2DM. However, the current therapies are not entirely sufficient to maintain sustained glycemic control. Moreover, generalized unhealthy dietary habits and sedentary life style of the western population, contributes to the rising incidence of T2DM, a major public health problem. It is therefore critical to identify new molecular targets in T2DM and to develop new therapeutic approaches that prevent and control T2DM progression. Natural compounds, as polyphenols, are attractive alternatives to prevent the development of T2DM and accompanying cardiovascular disease.

The present study aimed to investigate the potential role of the XN and 8PN polyphenols as modifiers of T2DM physiopathology, using a murine model of HFD-induced T2DM. Specifically, this work explored how XN and 8PN modulate fundamental molecular mechanisms and signaling pathways involved in the development of T2DM and related comorbidities, including metabolic deregulation and vascular deregulation.

The liver is a central organ responsible for whole-body energy metabolism and nutrient homeostasis and the skeletal muscle is an important site for insulin action. Therefore, we focused in these two organs in order to examine the effect of these polyphenols in T2DM metabolism. Consumption of XN or 8PN improved glycemic control as demonstrated by the reduction of glycemia and insulin levels, HOMA and QUICKI, when compared to untreated HFD-fed animals. The improvement of insulin sensitivity could be one of the underlying mechanisms by which both polyphenols reduce the body weight gain during the 20 weeks of intervention. This effect has also been reported by other authors (Kim, Keogh*, et al.*, 2016).

Corroborating our data, there is growing experimental and epidemiological evidence that XN, aside from the antioxidant properties, also plays anti-hyperglycemic and antidiabetic roles

(Legette *et al.*, 2013; Yui *et al.*, 2014). But to our knowledge, the effect of 8PN in body weight gain, glycemia and lipid-lowering capacity has never been reported before.

The present study showed that both XN and 8PN significantly reduced plasma total cholesterol and TG that were elevated after a HFD regimen. These lipid-lowering effects upon oral administration of XN and 8PN could be partially attributable to an inhibition of lipid absorption in the gut, a decrease of FA synthesis, a reduction in the synthesis of cholesterol, an alteration in the metabolism of lipoproteins or an increase of FA oxidation, among other possible mechanisms. In order to better understand whether XN and 8PN improve metabolic parameters, the expression of enzymes that regulate lipid and glucose metabolism was also evaluated together with the energy metabolism master regulator, AMPK. Importantly, several pharmacological agents used to treat T2DM are powerful inducers of AMPK as for instance, insulin-sensitizing activators of PPAR-γ drugs from the thiazolidinedione family, hypoglycemia drug metformin, GLP1 agonists, and DPP4 inhibitors, demonstrating its ability on the prevention of T2DM (Hardie, 2013; Coughlan *et al.*, 2014; Kim, Yang*, et al.*, 2016; Tahrani *et al.*, 2016). We observed that both polyphenols were able to counteract the reduction of AMPK expression caused by HFD in liver and skeletal muscle tissues. The polyphenol-induced activation of AMPK led to a decrease of the expression of the downstream effector SREBP-1c and its target proteins, ACC and FAS. ACC down-regulation led to a decline in the flow of acetyl CoA to malonyl-CoA, the precursor of FA synthesis. This probably resulted in CPT-1 inhibition release, promoting the transfer of cytosolic long-chain fatty acyl CoA into the mitochondria to undergo β-oxidation. The reduction in FAS expression has already been described for other polyphenols and reported as a potential target to treat obesity, metabolic syndrome and cancer, conditions in which this enzyme is over-expressed (Tian, 2006).

Polyphenols consumption was also able to improve the plasmatic lipid profile, reduce the hepatic and skeletal muscle TG and cholesterol content and decrease the lipid uptake. The

tissue uptake of FA is mediated by several facilitated transporters as the CD36, which is upregulated in obesity (Goldberg *et al.*, 2009). Interestingly, the expression of CD36 in HFD-fed animals was reduced and normalized after XN or 8PN consumption. This could be, therefore, one of the mechanisms responsible for the reduction of hepatic and muscular lipid accumulation after polyphenol treatment. Additionally, both polyphenols also modulate the VEGFR-1-VEGF-B axis. VEGF-B levels and the expression of its receptor VEGFR-1 and co-receptor NP-1 in the liver and skeletal muscle were all decreased in HFD-fed animals treated with polyphenols. As this pathway has been recently associated with the expression of FATP (Hagberg *et al.*, 2010), the down-regulation of this signaling pathway suggests that this could be another mechanism by which these polyphenols may prevent ectopic lipid accumulation within these tissues. Other authors have previously demonstrated that VEGF-B does not affect CD36 expression (Hagberg *et al.*, 2010), leading us to hypothesize that the expression of VEGFR-1-VEGF-B axis and CD36 transporter are independently players down-regulated by XN and 8PN in T2DM. These results are in agreement with studies testing other polyphenols, namely grape extract-derived polyphenols, that significantly reduce CD36-mediated lipid transport and increase GLUT-4 expression in the skeletal muscle of high fat and high sucrose diet-fed rats (Aoun *et al.*, 2011).

The associated T2DM-related comorbidities also affect the metabolism of EC. In an attempt to give new insights in the interplay between metabolism and angiogenesis we evaluated the VEGFR-1-VEGF-B axis in the kidney and in the left heart ventricle. We decided to focus in these two organs because in T2DM, angiogenesis is paradoxically increased in the kidney and reduced in the left ventricle, contributing to diabetic nephropathy and heart failure, respectively. This is the so called diabetic angiogenic paradox (Wirostko *et al.*, 2008; Waltenberger, 2009). We observed that both XN and 8PN were also able to counteract the increase of VEGF-B levels and the expression of VEGFR-1 and NP-1, observed in the kidney and

left ventricle of HFD-fed animals. Our results showed that both polyphenols modulate the VEGF-VEGFR-1 pathway, decreasing its signalization. Other authors demonstrated that the activation of this signaling pathway leads to the upregulation of FATP-3 and FATP-4 through a PI3K-dependent pathway promoting the endothelial-to-tissues lipid transport (Hagberg *et al.*, 2010). Blocking VEGF-B signaling has attracted considerable attention in metabolic diseases, as it may prevent ectopic lipid accumulation and restore insulin sensitivity in obesity and T2DM animal models (Carmeliet *et al.*, 2012; Hagberg *et al.*, 2013; Muhl *et al.*, 2016).

Moreover, XN and 8PN were also able to ameliorate glucose homeostasis as demonstrated by the decrease in plasma glucose levels and HOMA results. This could be related to the observed increase in AS160 expression in skeletal muscle. AS160 phosphorylation is related to the translocation of GLUT-4 to the cell membrane and subsequent increase of glucose uptake by the skeletal muscle (Klip, 2009). Furthermore, XN and 8PN also modulated the expression of PFKFB3, an important glycolytic regulator involved in insulin signaling (Trefely *et al.*, 2015), in liver and skeletal muscle. This suggests that the observed polyphenol-induced activation of PFKFB3 may promote glycolysis together with glucose uptake in the skeletal muscle. Apart from its metabolic role, PFKFB3 was also recently found to regulate angiogenesis (Cantelmo *et al.*, 2015). It was shown that during angiogenesis, EC rely on glycolysis for rapid energy generation to proliferate and migrate towards avascular areas. In this process, endothelial PFKFB3 is a key player (Cantelmo *et al.*, 2015). Therefore, we pinpointed the linkage between metabolism and angiogenesis mediated by PFKFB3 in T2DM and questioned whether XN and 8PN could modulate PFKFB3. Indeed, our results demonstrate that PFKFB3 is increased in the kidney and decreased in left ventricle in HFD-fed animals and that remarkably, both XN and 8PN were able to attenuate this imbalance to levels similar to those presented by healthy control group, controlling vascular growth. The results are in agreement with Shoors and co-authors that have demonstrated that when PFKFB3 is silenced *in vitro* or inactivated *in vivo*,

glycolysis becomes partially and transiently suppressed and angiogenesis impaired inducing endothelial cell quiescence (Schoors, De Bock*, et al.*, 2014). Accordingly, the expression profile of this enzyme accompanied the angiogenic pattern observed in the diabetic kidney and left ventricle. Understanding the vascular imbalance observed in distinct organs in diabetes may provide a mean to therapeutically modulate neovascular events with precision in diabetes. Moreover, we confirmed an increase in renal neovascularization and an impairment of angiogenesis in left ventricle of HFD-fed animals, assessed by the expression of endothelial marker CD31 and VEGFR-2-VEGF-A axis and downstream effectors. This results in an augmented microvessel density. In contrast, these animals exhibit a reduction in the number of vessels in the left ventricle, which can be explained by decreased levels of VEGF-A and by an impairment of the VEGFR-2 signaling pathway. In accordance with our previous studies (Negrão *et al.*, 2010; Negrão *et al.*, 2012; Costa *et al.*, 2013), the current study demonstrates that XN has an anti-angiogenic potential, especially in the kidney. Contrarily, 8PN enhances VEGF-A in the diabetic left ventricle where angiogenesis is impaired, without significant effect in kidney, therefore ameliorating the angiogenic paradox as well. Our group had previously shown that in diabetic animals, 8PN is a pro-angiogenic molecule (Negrão *et al.*, 2010), possibly due to its estrogen agonist properties, which are known to promote angiogenesis (Soares, Guo, Gartner*, et al.*, 2003; Soares, Guo, Russo*, et al.*, 2003).

Interestingly, despite the apparently opposing angiogenic effects of both polyphenols in kidney, 8PN also down-regulates ERK phosphorylation possibly leading to a more controlled endothelial proliferation. Also, XN up-regulates AKT in the left ventricle to levels similar to healthy mice, therefore playing a role in cell survival and in the stimulation of insulin signaling and glucose uptake (Konner *et al.*, 2012). These intriguing results led us to investigate the effect of each polyphenol on the VEGF-induced activation of VEGFR-2 in an EC culture, subjected to hyperglycemic conditions. Several studies reported that the angiogenic behavior

of some polyphenols was mediated by direct binding to VEGF-A (Cerezo *et al.*, 2015; Moyle *et al.*, 2015). In agreement, it was demonstrated that XN when co-incubated with VEGF-A led to an increase inhibition of VEGF-induced VEGFR-2 phosphorylation. In contrast, when EC are treated with 8PN there is an activation of VEGFR-2 only in the absence of VEGF-A. XN and 8PN seemed to distinctly affect VEGFR-2 activation depending on the tissue VEGF-A content. While XN counteracted the increase of angiogenesis in the kidney mediated by HFD consumption, 8PN increased vascularization of the left ventricle, where angiogenesis was impaired. The low VEGF-A levels in this tissue could explain this effect, since 8PN promotes the *in vitro* activation of VEGFR-2 only in the absence of VEGF-A.

Despite the well-recognized benefits of daily consumption of fruits and vegetables, the daily dietary intake of polyphenols in Europe is below the recommended to obtain a significant health-promoting effect (Vogiatzoglou *et al.*, 2015). In recent years, the production of polyphenol-enriched foods and beverages becomes a challenge to increase polyphenol consumption. The dietary intake of XN and 8PN only occurs upon beer ingestion, and several studies concerning pharmacokinetics and tissue distribution of these compounds were recently carried out. In the present thesis, the administered dose of XN and 8PN was 10 mg/L, which is equivalent to 1.0 mg/kg body weight/day in mice, in line with our previous studies (Negrão *et al.*, 2012; Costa *et al.*, 2013). The administered doses that attain health benefits are not reached by beer consumption alone emphasizing the importance of supplemented beverages that are already being produced by the brewing industry

Leggete and collaborators tested oral administration of XN in a dose of 16.9 mg/kg body weight that corresponds to 180 mg for 66 kg of body weight in humans, and demonstrated that it appears in the plasma 4 hours post-administration in a concentration of 0.37 μM after intestinal absorption and enterohepatic recirculation. XN isomerization increases the concentration of IXN reaching a peak around 7-8 hours and 8PN only reached the peak

concentration at 15-24 hours due to the hepatic demethylation of IXN into 8PN (Legette *et al.*, 2012).. Later, the same authors described an improvement of DNA stability and health-related biochemical markers after a daily oral consumption of a XN drink or XN pills both with 12 mg/L, reaching 6.13 ng XN/mL in plasma 4 hours after administration (Legette *et al.*, 2014). Recently, in an interesting study in animals fed with a HFD mixed with 60 mg XN/kg body weight/day, a dose that corresponds to 350 mg/kg body weight/day in humans, Miranda C and co-authors demonstrated the anti-obesity capacity of XN by a decrease in body weight, inflammatory mediators and biochemical markers, implicated in insulin resistance of T2DM (Miranda *et al.*, 2016a). 8PN was quantified in the plasma after *in vivo* XN metabolization (Legette *et al.*, 2012), but animal and human studies focusing the non-estrogenic effects of 8PN are scarce. For the first time, the current study reports the effect of 8PN in T2DM.

The study of molecular mechanisms leads to the identification of new molecular targets for XN and 8PN intake. Despite the similar chemical structure, XN and 8PN display opposite effects on angiogenic process and on the regulation of PFKFB3. This cross-talk between metabolism and angiogenesis emerges as an important therapeutic target. New perspectives have arisen regarding the health benefits of polyphenols as promising tissue-specific preventive approaches to ameliorate T2DM-related complications and improve the quality of life of diabetic patients.

IV

Final remarks and future work

Final remarks and future work

The present thesis aimed to explore the potential role of two beer-derived polyphenols, XN and 8PN, in the prevention of metabolic and vascular T2DM-related complications in a HFD-induced T2DM mice model. A particular focus was given to the modulation of fundamental molecular pathways implicated in T2DM deregulation by these polyphenols. The main conclusions of this work are:

XN and 8PN reduced body weight gain and maintained glucose homeostasis in a HFD-diabetes induced *in vivo* model. This was achieved by a reduction of hyperglycemia, an improvement in HOMA index and an increase in insulin sensibility, in part as a result of the modulation of glycolytic pathway through PFKFB3 regulation;

XN and 8PN demonstrated a lipid-lowering effect through the reduction of blood TG and cholesterol, a decrease in lipid transport from the endothelium to tissues, and a reduction of lipogenic enzymes expression, therefore preventing lipotoxicity. Importantly, AMPK/SREBP-1c and target enzymes ACC and FAS, CD36 and VEGFR-1/VEGF-B were identified as targets for these polyphenols;

XN and 8PN seemed to modulate angiogenesis selectively, according to the VEGF-A concentration in the surrounded microenvironment. This observation suggests that both polyphenols can be used as tissue-specific therapies to resolve the so-called angiogenic paradox.

The presented data unravel new insights in the interesting inter-relation between metabolism and angiogenesis, and its modulation by dietary polyphenols rendering them potential preventive approaches for T2DM-related complications.

The work developed in this thesis provides interesting new findings but also opens new questions and suggests future experiments that address the proper scientific validation to

attain safety and efficacy of optimal polyphenols doses. It is also important to discard any possible side effects of their consumption especially regarding the less studied 8PN consumption, which could have undesirable effects mediated by its proestrogenic properties. Clinical trials are also needed to assess the optimal dosage and formulation for both compounds delivery, since the inter-individual variations and gut composition alters the polyphenols bioavailability and the nutritional-drug interactions.

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