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COIMBRA

Adelaide Catarina Campos Barbosa

RENAL EFFECTS OF BLUEBERRY JUICE IN A PREDIABETIC RAT MODEL INDUCED BY HYPERCALORIC DIET

**Dissertação de Mestrado em Investigação Biomédica,
da Faculdade de Medicina da Universidade de Coimbra,
realizada sob orientação do Doutor Flávio Nelson Fernandes Reis
e da Doutora Sofia Andreia Domingues Viana.**

Dezembro de 2019

Mestrado em Investigação Biomédica

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Acronyms and abbreviations

ADA - American Diabetes Association

AGE - Advanced Glycation Endproducts

BCA - Bicinchoninic Acid Assay

BJ - Blueberry Juice

BMI - Body Mass Index

BUN - Blood Urea Nitrogen

CD36 - Cluster of Differentiation 36

cDNA - Complementary Deoxyribonucleic Acid

CKD - Chronic Kidney Disease

COAPE - Cooperativa Agropecuária dos Agricultores de Mangualde

Cr - Creatinine

CVD - Cardiovascular Disease

DBP - Diastolic Blood Pressure

DEPC - Diethyl Pyrocarbonate

DGS - Direção Geral de Saúde

DKD - Diabetic Kidney Disease

DM - Diabetes Mellitus

DN - Diabetic Nephropathy

DR - Diabetic Retinopathy

ECL - Chemiluminescence Substrate

ECM - Extracellular Protein Production

ESRD - End-Stage Renal Disease

ESRF - End-stage Renal Failure

ET - Endothelin

FATPS - Fatty Acid Transport Proteins

FFA - Free Fatty Acids

FPG - Fasting Plasma Glucose

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

GFR - Glomerular Filtration Rate

GTT - Glucose Tolerance Test

H2O2 - Hydrogen Peroxide

H&E - Hematoxylin and Eosin

HbA1c - Glycated Hemoglobin

HPRT - Hypoxanthine-guanine Phosphoribosyltransferase

HR - Heart Rate

hs-CRP - High-sensitivity C-Reactive Protein

HsuHF - High sucrose + High Fat

IDF - International Diabetes Federation

IFG - Impaired Fasting Glucose

IFTA - Interstitial Fibrosis and Tubular Atrophy

IGT - Impaired Glucose Tolerance

iNOS - Inducible Nitric Oxide Synthase

IL-6 - Interleukin 6

IR - Insulin Resistance

ITT - Insulin Tolerance Test

KITT - Glucose Disappearance Rate

LPS - Lipopolysaccharide

MBP - Mean Blood Pressure

MDA - Malondialdehyde

MMP2 - Matrix Metallopeptidase 2

NaCl - Sodium Chloride

NF-κB - Nuclear Factor kappa B

NO - Nitric Oxide

OGTT - Oral Glucose Tolerance Test

RAGE - Receptor for Advanced Glycation Endproducts

RAS - Renin-Angiotensin System

RNA - Ribonucleic Acid

ROS - Reactive Oxygen Species

RPGN - Rapidly Progressive Glomerulonephritis

RT - Room Temperature

SEM - Standard Errors of the Mean

SBP - Systolic Blood Pressure

SREBPs - Sterol Regulatory Element-Binding Proteins

T1DM - Type 1 Diabetes Mellitus

T2DM - Type 2 Diabetes Mellitus

TBA - Thiobarbituric Acid

TG - Triglyceride

TLRs - Toll-like Receptors

TLR-4 - Toll-like Receptor 4

TNF-α - Tumor Necrosis Factor Alpha

WHO - World Health Organization

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Abstract

Prediabetes, also known as intermediate hyperglycaemia, is a condition in which patients have higher blood glucose levels than normal but not higher enough to be classified as overt diabetes. Prediabetes has been recognized as a risk factor for the development of type 2 diabetes mellitus (T2DM), a condition that is associated with the development of serious microvascular complications, including diabetic nephropathy (DN), the main cause of end-stage renal disease. Unhealthy lifestyle habits, including hypercaloric diets, have been documented as major contributors to the increasing incidence of prediabetes. In this sense, early therapeutic strategies, particularly those based on natural products, could contribute to prevent the evolution of renal impairment to advanced stages with more serious consequences. We hypothesize that blueberry juice (BJ), due to its notable prebiotic, hypoglycemic, anti-inflammatory and antioxidant activities, could be a promising nutraceutical strategy to prevent the progression of prediabetes as well as the putative early renal impairment.

Thus, the main goals of this dissertation are twofold: i) to characterize the degree of renal impairment in a stage of prediabetes induced in the rat by an hypercaloric diet and ii) to evaluate the metabolic and particularly the renal effects of long-term BJ supplementation.

In order to achieve these goals, we have used an experimental model of prediabetes induced by a hypercaloric diet [high-sucrose (Hsu) - 35% - and high-fat (HF) - 60%] in adult male Wistar rats through the ingestion of Hsu for 9 weeks supplemented by HF for additional 14 weeks (HsuHF group, n=16). After 9 weeks, half of the former rats received BJ orally (25g/kg of body weight, HsuHF+BJ group). Control animals (n=8) received standard diet during the entire protocol. Glycaemic, insulinaemic and lipidic profiles were evaluated throughout the protocol, as well as renal function, using serum and urinary measures of creatinine, uric acid and blood urea nitrogen and calculating the glomerular filtration rate (GFR). Histological characterization of the kidney was

performed by H&E and lipid deposition by Oil Red O staining and quantification of serum triglycerides. Serum and renal tissue inflammatory markers were evaluated by ELISA (hs-CRP) and by RT-qPCR and/or WB (IL-6, TNF-α, iNOS, MMP2, TLR-4 and RAGE).

We found that this model of prediabetes presents the main features of this stage of the disease, with fasting normoglycaemia and mild postprandial hyperglycaemia, glucose intolerance and insulin resistance, hypertriglyceridaemia, as well as increased body weight. In addition, prediabetic rats displayed a significant reduction of GFR along with unchanged classical serum and urine markers of renal function. Histomorphologically, animals under hypercaloric diet presented some glomerular lesions, being of note the glomerular crescent-like ones, which have been associated with DN. BJ treatment was able to ameliorate hypercaloric diet-induced glucose intolerance, insulin insensitivity and plasma hypertriglyceridaemia. In addition, in the HsuHF+BJ rats there was a trend to alleviate the reduction of GFR found in the HsuHF animals, as well as the early glomerular lesions. However, this nutraceutical intervention was unable to halt or slow down glomerular crescent-like lesions, renal lipidosis and the overexpression of renal IL-6 found in the HsuHF-treated animals.

In conclusion, in opposition to previous data from the group strongly suggesting beneficial effects of BJ against liver damage in this rat model of prediabetes, this nutraceutical intervention with BJ presented very modest renoprotective effects, despite the metabolic improvement viewed by the amelioration of glucose intolerance, insulin insensitivity and hypertriglyceridaemia.

Resumo

A pré-diabetes, também conhecida como hiperglicemia intermédia, é uma condição na qual os indivíduos têm níveis de glucose no sangue mais elevados que o normal, mas não suficientemente altos para serem classificados como diabetes. A pré-diabetes é reconhecida como um fator de risco para o desenvolvimento de diabetes mellitus tipo 2 (T2DM), uma condição que está associada ao desenvolvimento de complicações microvasculares sérias, incluindo a nefropatia diabética (DN), que é a principal causa de doença renal terminal. Estilos de vida pouco saudáveis, incluindo dietas hipercalóricas, têm sido reconhecidos como importantes contribuidores da crescente incidência de pré-diabetes. Neste sentido, estratégias terapêuticas precoces, particularmente as baseadas em produtos naturais, poderão contribuir para a prevenção da evolução da lesão renal para estágios mais avançados com consequências graves. A nossa hipótese de trabalho é a de que o sumo de mirtilo (BJ), devido às suas notáveis atividades pré-bióticas, hipoglicémicas, anti-inflamatórias e antioxidantes, possa ser uma estratégia nutracêutica promissora na prevenção da evolução da pré-diabetes, bem como da putativa lesão renal precoce.

Tendo isto em conta, esta dissertação apresenta dois objetivos principais: i) caracterizar o grau de lesão renal num estágio de pré-diabetes induzido em ratos através de uma dieta hipercalórica e ii) avaliar os efeitos metabólicos e, em particular, renais da suplementação com BJ a longo prazo.

Com vista a atingir estes objetivos, utilizamos um modelo experimental de pré-diabetes induzido por uma dieta hipercalórica [alto teor de sacarose (Hsu) - 35% - e alto teor de gordura (HF) - 60%] em ratos Wistar machos adultos, através da ingestão de Hsu durante 9 semanas, com suplementação de HF por 14 semanas adicionais (grupo HsuHF, n=16). Após 9 semanas, metade dos ratos deste último grupo receberam BJ oralmente (25g/kg de peso corporal, grupo HsuHF+BJ). Os animais controlo (n=8) receberam uma dieta padronizada durante todo o protocolo. Os perfis glicémico,

insulinémico e lipídico foram avaliados ao longo do protocolo, bem como a função renal, através de medidas séricas e urinárias de creatinina, ácido úrico e ureia e do cálculo da taxa de filtração glomerular (GFR). A caracterização histológica do rim foi realizada através da coloração com Hematoxicilina e Eosina e a deposição lipídica por coloração de *Oil Red O* e quantificação de triglicerídeos no soro. Os marcadores inflamatórios séricos e no tecido renal foram avaliados através de ELISA (hs-CRP) e de RT-qPCR e/ou WB (IL-6, TNF-α, iNOS, MMP2, TLR-4 and RAGE).

Constatámos que este modelo de pré-diabetes apresenta as principais características deste estágio da doença, com normoglicemia em jejum e hiperglicemia pós-prandial ligeira, intolerância à glicose e resistência à insulina, hipertrigliceridemia, bem como aumento de peso. Além disso, os ratos pré-diabéticos apresentaram uma redução significativa da GFR, sem alteração dos marcadores clássicos séricos e urinários da função renal. Histomorfologicamente, os animais sujeitos à dieta hipercalórica apresentaram algumas lesões glomerulares, destacando-se as lesões glomerulares do tipo "*crescentes*" , que têm vindo a ser associadas a DN. O tratamento com BJ foi capaz de amenizar a intolerância à glucose induzida por uma dieta hipercalórica, a redução de sensibilidade à insulina e a hipertrigliceridemia. Ainda nos ratos do grupo HsuHF+BJ foi verificada uma tendência para a atenuação da redução da GFR encontrada nos animais HsuHF, bem como das lesões glomerulares precoces. Contudo, esta intervenção nutracêutica foi incapaz de travar ou abrandar os crescentes glomerulares, a lipidose renal e o aumento da expressão renal de IL-6, encontrados nos animais do grupo HsuHF.

Em conclusão, em contraste com dados anteriormente obtidos pelo grupo de investigação, que sugerem fortemente a existência de efeitos benéficos do BJ a nível da lesão hepática neste modelo de pré-diabetes em rato, esta intervenção nutracêutica com BJ apresentou efeitos renoprotetores muito modestos, apesar da melhoria metabólica verificada pela atenuação da intolerância à glucose, da insensibilidade à insulina e da hipertrigliceridemia.

INTRODUCTION

1. DIABETES MELLITUS

1.1. Overview

Diabetes mellitus (DM) is characterized by elevated levels of blood glucose, according to World Health Organization (WHO) (WHO, 2016). Glucose belongs to a group of biological macromolecules called carbohydrates (or sugars). It is the most abundant monosaccharide (*i.e.* any class of sugars that cannot be hydrolysed to give a simpler sugar, which means that glucose is itself a simple sugar) in the blood stream. This abundance is utterly related to its extremely important functions in the human organism, namely energy production and precursor of other important molecules.

When the levels of this molecule become uncontrollably high, it leads to an expressive negative impact in one's health. The persistence of a glucose concentration above normal levels often results in deleterious effects to the tissues of diverse organs, affecting mainly the kidneys, eyes, peripheral nerves and the vascular systems. Complications in these represent the most important, and many times fatal, cases caused by DM. In fact, in many developed countries, DM is actually one of the main reasons of blindness, renal failure and amputation related to the lower limbs. Other main chronic complications caused by DM are cardiovascular disease (CVD) (International Diabetes Federation, 2017). Some of these issues will be detailed further in this dissertation, with special emphasis on renal failure, since renal impairment is intimately related with its objectives.

This chronic disease is intrinsically connected with a crucial hormone for survival – insulin – which is produced by the pancreas. DM can be caused in one (or both) of the following scenarios: the pancreas is unable to produce enough quantities of insulin, and therefore, its concentration in the blood decreases to pathological levels (the relationship between this hormone with glucose will be further detailed in another section) – deficient insulin secretion – or, despite the pancreas being able to produce a

sufficient amount of insulin, the insulin receptors present in many tissues, cannot use it efficiently – deficient insulin action.

1.2. Prevalence

In 2014, 422 million adults worldwide have DM according with the 2016 Global Report on Diabetes from WHO (WHO, 2016). In 2017, the estimated prevalence of DM by the International Diabetes Federation (IDF) was of about 425 million adults or 8.8% of the adult population (with ages between 20-79 years old) and their estimations for 2045 are that 629 million adults around the globe will have the disease (International Diabetes Federation, 2018). Regarding Portugal, the estimated prevalence of DM in 2015, also for people with ages between 20 and 79 years old, was of about 13.3% of this population, which is higher than the estimation for the world's population mentioned in the report above from two years ago. Furthermore, the percentage regarding this parameter for men (about 15.9%) was greater than for women (about 10.9%). It was also noted that prevalence increases with age (Observatório Nacional da Diabetes, 2016).

By looking at the figure below (Figure 1) from IDF, it is visible that DM affects people from all over the world, mainly in the continents of North America, Europe and Asia (represented with the darker blue colours). Portugal, specifically, was included in the range of countries with more than one million patients with DM.

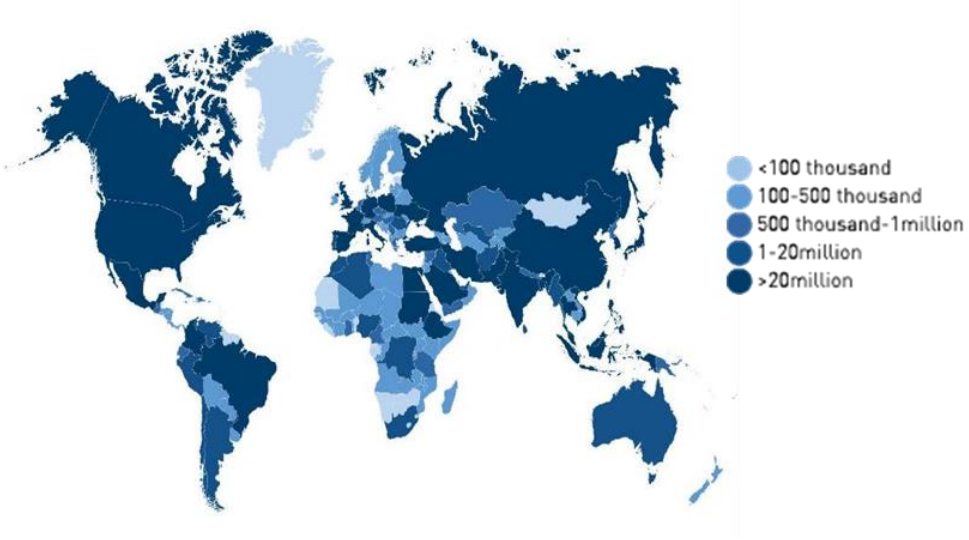

Figure 1. Estimated total number of adults (20-79 years) living with diabetes worldwide, in 2017. Taken from IDF diabetes atlas - 8th edition.

1.3. Diagnosis

According to WHO (WHO, 2016), diabetes can be diagnosed by fasting plasma glucose (FPG) ≥ 7.0 mmol/L (126 mg/dl), 2-h plasma glucose (2 hours after ingestion of 75 g oral glucose load) ≥ 11.1 mmol/L (200 mg/dl) or glycated hemoglobin (HbA1c) ≥ 6.5% (Figure 2).

Type of Diabetes	Normal glucose tolerance	Hyperglycemia		
		Pre-diabetes*	Diabetes Mellitus	
		Impaired fasting glucose or impaired glucose tolerance	Not insulin requiring	Insulin required for control / Insulin required for survival
Type 1				
Type 2				
Other specific types				
Gestational Diabetes				
Time (years)				
FPG	<5.6 mmol/L (100 mg/dL)	5.6–6.9 mmol/L (100–125 mg/dL)	≥7.0 mmol/L (126 mg/dL)	
2-h PG	<7.8 mmol/L (140 mg/dL)	7.8–11.0 mmol/L (140–199 mg/dL)	≥11.1 mmol/L (200 mg/dL)	
HbA1C	<5.6%	5.7–6.4%	≥6.5%	

Figure 2. Glucose homeostasis and diabetes mellitus. Arrows indicate that changes in glucose tolerance may be bidirectional in some types of diabetes. FPG: fasting plasma glucose, 2-h PG: two hours plasma glucose after a glucose challenge; HbA1c: glycated hemoglobin. Taken from Harrison, 2015.

1.4. Types

DM can be classified into four different categories: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes and other specific types (American Diabetes Association, 2018; WHO, 2016).

T1DM

The underlying mechanism of T1DM involves an autoimmune destruction of insulin-producing β cells in the pancreas, which leads to complete insulin deficiency. Thus, people with this condition require regular insulin administration to control their glucose levels (American Diabetes Association, 2018; WHO, 2016). This type of DM most commonly affects children and some of the symptoms include willing to urinate several times, frequent hunger, constant thirst, weight loss, weakness, fatigue, among others (International Diabetes Federation, 2017; Khardori, 1989). T1DM has a very important genetic character, about 90% of cases are hereditary and only 10% are related to changes in lifestyle. In 2015, 3,327 young people aged 0-19 years had T1DM, which corresponds to 0.16% of the Portuguese population in this age group (Observatório Nacional da Diabetes, 2016).

T2DM

T2DM is the most common form of diabetes and, on contrary to what happens in T1DM, there is no lack of insulin production by pancreatic β cells, but instead a condition of insulin resistance, at least in the initial stages. Some people with this condition are overweight, which in itself contributes to insulin resistance (American Diabetes Association, 2018; WHO, 2016). Unlike T1DM, about 90% of T2DM cases are due to unhealthy lifestyle habits and only around 10% represent genetic causes. The symptoms are similar to T1DM including frequent urination, tiredness, increased thirst and dry mouth, slow healing wounds. T2DM most often affects older people, but with

decreasing exercise, poor diet and consequently obesity, its prevalence begins to increase in children (International Diabetes Federation, 2017). Throughout this dissertation, the hypercaloric diet effects will be addressed in more detail, as it is a risk factor for T2DM and the way to promote the development of the experimental model to be used.

Gestational diabetes

Gestational diabetes corresponds to any degree of glucose metabolism abnormality first documented during pregnancy. Although it is a temporary condition, carries long-term risk of T2DM (American Diabetes Association, 2018; Observatório Nacional da Diabetes, 2016; WHO, 2016). In 2015, gestational diabetes's prevalence was of about 7.2% of the pregnant population and it increases with age, reaching an alarming 15.9% in women older than 40 years old (Observatório Nacional da Diabetes, 2016). Although the majority of women returns to normal blood glucose levels after childbirth, they are still at high risk (about 35 to 60% of probability) of developing DM in the following 10 to 20 years (Harrison, 2015).

The diagnostic criteria of gestational diabetes differ from those of DM, being the following: FPG ≥ 92 mg/dl (5,1 mmol/l) starting on the first medical consultation of the pregnant woman and 1-h plasma glucose ≥ 180 mg/dl (10 mmol/l) or 2-h plasma glucose ≥ 153 mg/dl (8,5 mmol/l) (1 and 2 hours after ingestion of 75 g oral glucose load, respectively), between the first 24 and 28 gestational weeks (Observatório Nacional da Diabetes, 2016).

Other specific types of diabetes

Other specific types of diabetes can be related to other causes, for instance, genetic defects, namely on the beta cell development or function and regarding insulin action and other rarer genetic syndromes; diseases which affect the exocrine pancreas (such as cystic fibrosis and pancreatitis); endocrinopathies, which are endocrine gland-

related diseases and, therefore, lead to hormonal issues; drug- or chemical-induced diabetes (such as with glucocorticoid use or after organ transplantation); infections and uncommon forms of immune-mediated diabetes (American Diabetes Association, 2018; Harrison, 2015; WHO, 2016).

1.5. Diabetes' socioeconomic costs and complications

Besides the reduction in the quality of life caused by the numerous health complications related, directly or indirectly, with diabetes, this chronic disease has a huge economic impact in the health systems of many countries and, mainly, to the individuals with the disease as well as their families. In fact, in 2017, diabetes-related expenses worldwide, for people aged 20-79 years, were of about USD 727 billion (approximately, 657 billion euros) (International Diabetes Federation, 2017). This economic costs can be divided into two groups (Harrison, 2015): 1) Direct medical costs, which include costs with prevention and treatment of the disease itself and of their associated complications, inclusively; 2) Indirect medical costs, which are associated with the reduction or ceasing, in the most serious cases, of work productivity, for example caused by a premature death; these costs, therefore, can have a very negative impact on nations' gross domestic product.

The diseases related with DM can be divided into two different categories, which applies both for T1DM and T2DM: nonvascular and vascular complications. Nonvascular issues include several pathologies, such as: gastroparesis, condition in which the stomach cannot empty its food content properly, as it would under normal conditions; infections; eye-related problems, namely, cataracts and glaucoma; significant hearing loss; among others. The vascular category is still further divided in microvascular pathologies, such as nephropathy, retinopathy and neuropathy, and macrovascular complications, namely CVD. It's important to highlight that the microvascular issues are diabetes-specific, whereas macrovascular ones are similar to those that occur in people who do not

possess DM; however, their frequency increases abruptly in the DM's population (Harrison, 2015).

Below can be found a summarized description of some of the pathologies mentioned earlier.

Cardiovascular disease

CVD is a complication in which the elevated glucose blood levels make the coagulation system more active, increasing the risk of formation of blood clots. The elevated glucose levels when present in combination with high blood pressure and unhealthy levels of cholesterol lead to complications such as coronary artery disease, myocardial infarction, stroke, cerebrovascular disease and peripheral arterial disease. People with DM have a probability three times higher to develop CVD, when comparing with healthy individuals (International Diabetes Federation, 2017).

Diabetic retinopathy

Diabetic retinopathy (DR) is a vascular-related complication which affects the sensorial part of the eye – retina (area of the eye responsible for converting the light that goes through the eye in electrical waves, which can be, on their turn, processed by the brain). This can lead to progressive vision loss and even blindness. Approximately one in each three people (around 33%) of the individuals with DM have, at least, some degree of DR and one in each ten (around 10%) is likely to develop a vision threatening form of the disease (International Diabetes Federation, 2017).

Diabetic neuropathy

Diabetic neuropathy is related with lesions in the nerves all over the body, which lead to negative alterations in their autonomic, sensitive and motor functions. This pathology can cause, on its turn, ulcers, infections and, in more serious cases, amputations. Furthermore, these lesions in the nerves can often be very difficult to

detect and diagnose. Peripheral neuropathy is the most common form of diabetic neuropathy in individual with DM and affect the nerves that are headed to the inferior limbs, particularly the nerves that descend to the foot, which can lead to one of the most widely known DM's complications – the diabetic foot. Amputations in affected individuals is 10 to 20 times more common, when compared with non-diabetic people (International Diabetes Federation, 2017).

Diabetic nephropathy

The kidney is an organ that can be greatly affected by hyperglycaemia. Since the kidney is the main focus of the thesis, this issue will be further detailed in another topic.

1.6. Treatment

The main aim in DM's treatment, since this disease is characterized by hyperglycaemia, is to maintain the glucose blood levels as close to the normal ranges as possible. It is important to note that, since glycaemia levels vary according to several factors, namely age, physical activity, eating habits or other pathologies, the normal range for the glucose levels in the blood is defined individually (WHO, 2016).

With regards to T1DM, the optimal treatment includes preventive measures, such as a balanced and proper nutrition, physical exercise, the education of the patient on DM, especially on self-control and self-surveillance of the disease and pharmacological options, being exogenous insulin mandatory (WHO, 2016).

Concerning T2DM the first steps are to change one's diet and food habits, as well as, complement those changes with regular physical exercise. Moreover, in many cases, for this type of DM, solely this lifestyle modification and consequent weight loss (in those cases which excessive weight is identified) can be sufficient to manage DM. However, when caloric restriction and physical exercise are not enough, it is imperative to include drugs in the treatment plan and, for the most severe cases, exogenous insulin (WHO, 2016). The first line of pharmacological treatment for DM is an oral antidiabetic

medication known as Metformin. It belongs to the biguanide group, which acts by simultaneously decreasing hepatic glucose production and increasing insulin sensitivity, that is, improves the cell uptake of this molecule, which leads, on its turn, to a great uptake of glucose.

Besides biguanides, there are many other important pharmacological groups for the treatment of DM, which: stimulate insulin secretion such as sulfonylureas (glibenclamide, glyburide, glipizide, glimepiride, gliclazide and gliclazide LA), benzoic acid derivative (repaglinide) and phenylalanine derivative (nateglinide); decrease hepatic glucose production and increase insulin sensitivity (similarly to the biguanides), namely thiazolidinediones (rosiglitazone and pioglitazone); delay carbohydrate absorption in the intestines, for example α-glucosidase inhibitors (acarbose miglitol). Other drugs which have a pivotal role in DM and have emerged in the last few decades are the incretin-based therapy (DPP-4 inhibitors and GLP-1 analogues) and SGLT2 inhibitors.

When the monotherapy with a biguanide proves to be insufficient to control glucose blood levels, the next step is to adopt a combined therapy with some of the drugs mentioned above, according to the physician's best decision, which should take into account several individual factors (Duarte et al., 2018)

2. PREDIABETES

2.1. Overview

Prediabetes, also named as intermediate hyperglycaemia, is a condition in which individuals also have higher blood glucose levels than normal but not higher enough to be classified as diabetes (Observatório Nacional da Diabetes, 2016).

Prediabetes is twice more common than DM and has been recognized as a risk factor for the development of T2DM (Observatório Nacional da Diabetes, 2016). In fact, for patients with prediabetes, the probability of developing DM in the 10 years following diagnosis is very high (Melsom et al., 2016a). Prediabetes progression to T2DM is about 5-10% per year; however, it differs depending on population characteristics and definition of prediabetes (Tabák, Herder, Rathmann, Brunner, & Kivimäki, 2012).

The pathophysiology of prediabetes is globally related to changes in glucose concentration, being characterized by a balance between the entry and exit of glucose into the cells, and consequent concentration in the blood. Insulin resistance may occur due to excessive intake or decreased glucose clearance in the blood, which may arise from problems such as desensitization of glucose receptors in the cells. Regardless of the underlying cause of the pathology, this has several consequences, notably the progression to T2DM and renal damage, as will be clarified ahead in this dissertation.

Some risk factors for prediabetes could be modifiable by changing lifestyle habits, such as body weight, healthy diet, physical activity, stress, ingestion of alcohol and administration of tobacco, among others ("Prediabetes Modifiable Risk Factors | American Heart Association," 2015). Unhealthy lifestyle habits, including hypercaloric diets, have been recognized as a major contributor to the increasing prevalence of prediabetes.

2.2. Prevalence

Prediabetes' prevalence varies according with the criteria selected for its evaluation (ADA or WHO) and also with the population in study. As stated by IFD, there are 352.1 million people worldwide corresponding to 7.3% of adults (between the ages of 20 and 79 years), who are estimated to have impaired glucose tolerance (IGT). By 2045, the estimation is that the number of people between the ages of 20 and 79 years with IGT will rise to 587 million, corresponding to 8.3% of adult population (International Diabetes Federation, 2017).

In Portugal, the estimated prevalence of prediabetes in 2015 was 27.4%, which include people with impaired fasting glucose (IFG), with a prevalence of 10.4%, IGT with 14.3% or both with 2.7% (Observatório Nacional da Diabetes, 2016) (Figure 3). These conditions will be explained below in more detail.

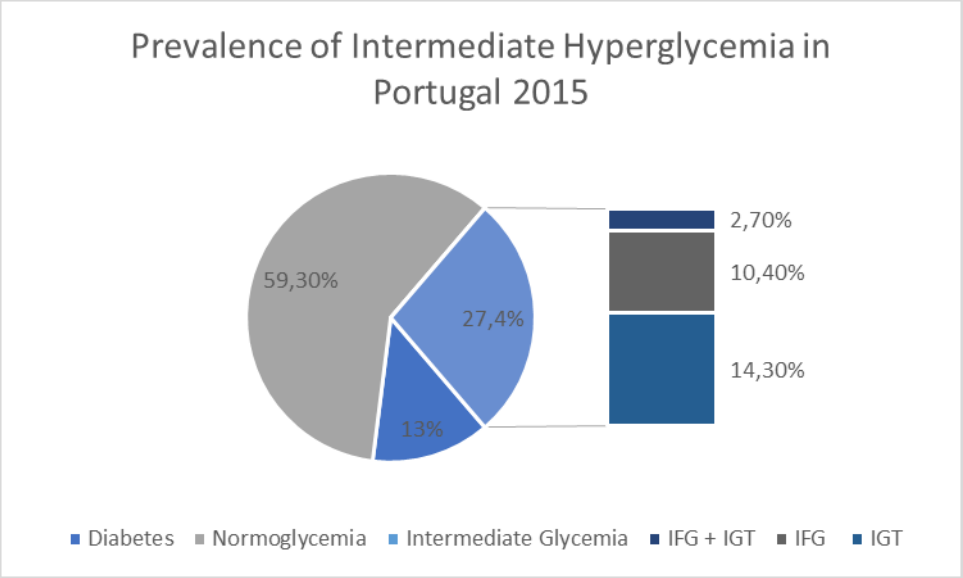

Figure 3. Prevalence of intermediate hyperglycaemia in Portugal (2015), adjusted to the estimated population distribution. Adapted from Observatório Nacional da Diabetes, 2016.

2.3. Diagnosis

Prediabetes can be diagnosed by using IFG, IGT and HbA1c levels, with slight variations between distinct Organizations, as further summarized in Table 1.

Prediabetes can be diagnosed by IFG (≥ 110 and < 126 mg/dl or ≥ 6.1 and < 7.0 mmol/l) or IGT (glucose two hours after an ingestion of 75 g of glucose ≥ 140 and < 200 mg/dl or ≥ 7.8 and < 11.1 mmol/l) according to the Portuguese DGS (*Direção Geral de Saúde*/Directorate-General of Health) Norm N.º 2/2011 (Observatório Nacional da Diabetes, 2016).

In consonance with the American Diabetes Association (ADA), people with prediabetes have IFG levels between 100 mg/dl (5.6 mmol/l) and 125 mg/dl (6.9 mmol/l) and IGT – 2-h values in the oral glucose tolerance test (OGTT) between 140 mg/dl (7.8 mmol/l) and 199 mg/dl (11.0 mmol/l). HbA1c between 5.7 to 6.4% (39–47 mmol/mol) is also a parameter for assessing prediabetes according to ADA. However, WHO does not consider HbA1c as a parameter for prediabetes diagnosis. For IFG, FPG levels are between 6.1 to 6.9 mmol/L (110 mg/dl to 125 mg/dl) and for IGT, FPG are < 7.0 mmol/L (126 mg/dl) and 2-h plasma glucose (2 hours after ingestion of 75 g oral glucose load) are ≥ 7.8 and < 11.1 mmol/L (140 mg/dl and 200 mg/dl). IFG and IGT are related to obesity, hypertension and dyslipidaemia and are major risk factors for other diseases.

Table 1. Comparison of prediabetes diagnosis criteria between ADA, WHO and DGS Norm.

	IFG (mg/dl)	IGT (mg/dl)	HbA1c (%)
ADA	100 – 125	140 – 199	5.7 – 6.4
WHO	110 – 125	140 – 199	–
DGS Norm	110 – 126	140 – 200	–

2.4. Main pathologic features of prediabetes

Insulin resistance

Insulin is the main hormone that regulates and maintains glucose homeostasis, promoting glucose uptake by the cells. Once inside the cells, glucose is consumed. If the glucose molecules are not utilized for energy purposes, they will be converted into glycogen (glucose polysaccharide) – through the process denominated glycogenesis – or it originates fat molecules – through the process of lipogenesis.

Pancreas' beta cells, located in the islets of Langerhans, are sensitive to the glucose blood concentrations. Therefore, whenever this concentration is at high levels, these cells secrete insulin into the blood stream. Coherently, when glucose levels are low, insulin secretion is inhibited. With this being said, whenever glucose concentration is high and insulin is not produced in sufficient quantities by the pancreas' beta cells, or when its action is compromised, leads to the occurrence of hyperglycaemia.

Insulin resistance (IR) is defined as decreased glucose uptake by insulin-dependent tissues, especially muscle and adipose tissue, which may start at very early stages of prediabetes (Del Prato, 2009; Maria da Silva Azevedo, 1993; Wasserman, Wang, & Brown, 2018) IR could not be clinically obvious because plasma glucose levels are preserved at near normal levels by a compensatory increase in circulating insulin levels (Del Prato, 2009). Studies suggest that a high caloric / high fat diet contributes to insulin resistance and impaired glucose tolerance (Deng, Shivappa, Tang, Mann, & Hebert, 2017; Shevalye et al., 2012).

According to the Figure 4 below, when a person develops an increasingly resistance to insulin (which is represented in the graph as the transition between point A to point B), the insulin secretion also increases. When this compensation mechanism, characterized by an increment in insulin secretion, is not sufficient to restore glucose to normal levels, IGT arises, in an initial phase – point C – followed by T2DM, as this mechanism keeps on proving to be unsuccessful (Harrison, 2015).

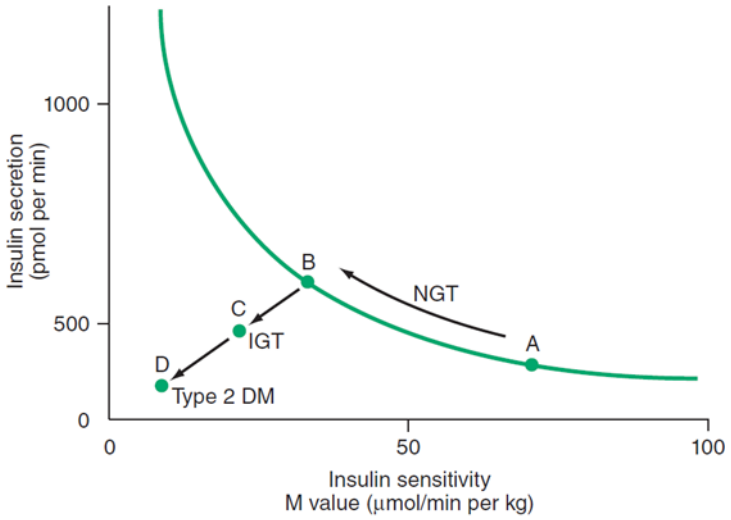

Figure 4. Metabolic changes during the development of T2DM. NGT: normal glucose tolerance; IGT: impaired glucose tolerance; Type 2 DM: type 2 diabetes mellitus. Taken from Harrison, 2015.

The reduction in insulin response to their target tissues constitutes a major risk factor for the development of T2DM (Whaley-Connell & Sowers, 2017). Insulin by itself can have several consequences in the kidney, affecting many aspects of renal function including renal hemodynamics, podocyte viability and tubular function (Artunc et al., 2016). In fact, insulin has several actions on renal tissue such as growth regulation, hypertrophy, as well as fibrotic and microcirculatory pathways, which in turn affect glomerular filtration (Whaley-Connell & Sowers, 2017). It also disturbs vasculature leading to its dysfunction, concomitant with hyperglycaemia (Wasserman et al., 2018).

Hyperglycaemia, hyperlipidaemia, glucotoxicity and lipotoxicity

Small increases in glucose when chronically maintained become damaging due to glucose toxicity (glucotoxicity) impairing both insulin action and insulin secretion, which causes pancreatic cell dysfunction and lesion. Normal pancreatic β cells may adapt to changes in insulin action, this means that a decrease in insulin action is accompanied by an increase in their secretion by pancreatic cells (Del Prato, 2009). This raise in insulin secretion leads to compensatory hyperinsulinaemia and when this adaptive response fails, conducts to hyperglycaemia and then the evolution from normal glucose tolerance

to IGT and, lastly, to T2DM (Del Prato, 2009). Thus, β cell dysfunction is a critical component in the pathogenesis of diabetes. The deleterious effect of chronic hyperglycaemia in both cases (insulin secretion and insulin action) is substantially mediated by activation of oxidative stress as a consequence of increased generation of reactive oxygen species (ROS). In prediabetic conditions, the observed glucose fluctuations can accelerate loss of β-cell function (Del Prato, 2009).

However, diet and physical activity can have influence in this progression. High caloric intake and lack of physical activity conducts to an increase of free fatty acids (FFA) and systemic inflammation that are related with insulin resistance and impaired β-cell function (Del Prato, 2009). Maintained elevation of plasma FFA negatively affects insulin secretion and insulin action (lipotoxicity). In high-risk patients, pancreatic β-cell lipotoxicity may play an important role in the evolution of normal glucose tolerance to hyperglycaemia, which then helps to amplify the process, by activating a vicious cycle that is responsible for maintaining and worsening the diabetic state (Del Prato, 2009). FFA in excess can lead to damage in podocytes as well as in the proximal tubular epithelial cells, due to the following factors: ROS production, lipid peroxidation and tissue inflammation (Gai et al., 2019). One of the glycoproteins which is responsible for the uptake of FFA is CD36 (cluster of differentiation 36), also known as platelet glycoprotein IV and fatty acid translocase. CD36 is the main FFA uptake system in the kidneys and may induce inflammation and oxidative stress. Elevated CD36 levels are associated with kidney damage in humans and animals (Gai et al., 2019).

Regarding the adipose tissue (AT), it is known that an uncontrolled growth of AT is an important risk factor for diseases related with obesity, such as T2DM and hypertension (Zhu & Scherer, 2018). There are three types of adipocytes (cells which constitute the adipose tissue): white adipocytes – characterized by a substantial amount of lipid content and store energy through triglycerides (TGs); brown adipocytes – dissipate energy through heat and contain a vast number of mitochondria; beige adipocytes – contribute to thermogenesis.

There are several FFA transporters, such as the fatty acid transport proteins (FATPs). The levels and function of these receptors relates to the lipid homeostasis, which, if unbalanced, may cause DM. It is important to note that the FATP1, FATP2, and FATP4 receptors are vastly expressed in the kidney and have different roles, including: FATP1, a perimembranous protein which approximates to the cell surface in response to insulin; FATP2, which regulates lipid synthesis in the kidney, and FATP4, associated with insulin resistance and obesity in humans.

Reducing the excessive intake of lipids is beneficial for the prevention of renal changes, since it ameliorates blood pressure and insulin sensitivity and, also, through enhanced AT function associated with an improved adipokine profile. Additionally, the adipose tissue contributes to the release of cytokines which have a crucial role in the immunologic response and, also, in the renal function (Zhu & Scherer, 2018). Renal alterations, on their hand, have a measurable impact in the AT, stimulating inflammation. Two types of macrophages can be found in the adipose tissue: M1-like macrophages, associated with a pro-inflammatory profile and M2-like macrophages, related with a non-inflammatory profile. The M1-like macrophages stimulate the production of high levels of tumour necrosis factor (TNF) and inducible nitric oxide synthase (iNOS) (Zhu & Scherer, 2018).

Studies demonstrate that renal disease could be related to AT and that they are reciprocally regulated. It has been suggested that the AT modulation towards an anti-inflammatory phenotype may improve renal dysfunction (Zhu & Scherer, 2018).

Oxidative stress

Oxidative stress is a condition in which occurs an imbalance between the production of free radicals (namely ROS) and their removal. This means that may occur an increase in ROS production or a decrease in antioxidant levels (Mahat, Singh, Rathore, Arora, & Yadav, 2019; Priyatharshini, Muraliswaran, Kanagavalli, & Radhika, 2017). Hyperglycaemia contributes to higher ROS production and in turn high levels of these

may contribute to cell damage and IR (Al-Waili, Al-Waili, Al-Waili, & Salom, 2017; Mahat et al., 2019; Priyatharshini et al., 2017). The increase in the level of ROS above the physiological limit may induce conformational alterations in lipids, proteins, carbohydrates and nucleic acids (Miranda-Díaz, Pazarín-Villaseñor, Yanowsky-Escatell, & Andrade-Sierra, 2016). Since ROS are very reactive and have a short half-life, it is more adequate to estimate oxidative stress through the measurement of the oxidation target products, including lipid peroxidation, oxidized proteins, and oxidative nucleic acid damage (Bigagli & Lodovici, 2019). In a diabetic condition there is a high production of ROS and a failure in antioxidant mechanisms and oxidative stress is involved in the progression of T2DM (Bigagli & Lodovici, 2019; Priyatharshini et al., 2017). Several studies show that prediabetes is associated with oxidative stress (Agarwal et al., 2016; Mahat et al., 2019; Priyatharshini et al., 2017).

Inflammation

Inflammation is the normal, protective and frequently temporary reaction of the body to an infection or tissue injury and its related to pathogenesis of T2DM (Bharara, M. et al., 2009; Priyatharshini et al., 2017). However, inefficient regulation or recurrent stimuli lead to chronic inflammation (Joseph, Edirisinghe, & Burton-freeman, 2014). Besides classic inflammatory stimuli – such as bacteria –, inflammatory stress may be due to excess body fatness and poor eating habits (Joseph et al., 2014). The inflammatory response is characterized by the production of proinflammatory molecules and anti-inflammatory cytokines (Joseph et al., 2014). Nuclear factor kappa B (NF-κB) is essential in the inflammatory response; when activated stimulates the production of genes which are responsible for the production of cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), chemokines, adhesion molecules, among others. One of ways to activate NF-κB is through the activation of receptor for advanced glycation endproducts (RAGE) (Brahimaj et al., 2017). Toll-like receptors (TLRs) are also important inflammatory mediators (Joseph et al., 2014).

RAGE is a member of the immunoglobulin superfamily and is involved in the tissues' homeostasis but also with the aging process. It can be expressed in two different ways: full-length (non-soluble) and in various soluble forms. The soluble isoforms lack the transmembrane and cytoplasmic domains but preserve the extracellular domain (Sparvero et al., 2009). RAGE activation through the binding of a ligand may lead to chronical inflammation and oxidative stress, which are both characteristic features of DM (Biswas et al., 2015). RAGE is highly expressed in inflammation areas. In fact, its binding with ligands can contribute to podocyte stress and endothelial dysfunction in DN's pathogenesis (Di Pino et al., 2016; Sparvero et al., 2009).

TLR-4 belongs to the TLR family and has a relevant role in innate immunity, by identifying different pathogens. Following their activation, most frequently by bacterial lipopolysaccharide (LPS), they mediate the production of pro-inflammatory cytokines and interferons. By doing so, they enable the initiation of the inflammatory and immune responses (Kuzmich et al., 2017).

IL-6, an important pro-inflammatory cytokine, contributes to the initiation and progression of chronic low grade inflammation, which results in endothelial dysfunction and can contribute to micro and macrovascular complications in diabetic patients (Agarwal et al., 2016; Wegner, Araszkiewicz, Piorunska-Stolzmann, Wierusz-Wysocka, & Zozulinska-Ziolkiewicz, 2013). IL-6 is also related with the body mass index (BMI), waist circumference and waist-to-hip ratio in prediabetes subjects, which suggests that an increase in body weight contributes to a state of chronic inflammation (Agarwal et al., 2016). Studies suggest that overexpression of renal IL-6 is associated with diabetic kidney disease, namely due to an increase in the proliferation of mesangial cells that interferes with the extracellular dynamics associated with matrix formation in the podocytes (Wegner et al., 2013).

TNF-α – which is also a pro-inflammatory cytokine – is an important factor in IR associated with both obesity and T2DM pathogenesis (Hossain et al., 2010). It is produced by monocytes, macrophages and T cells and, also, intrinsic kidney cells. This

cytokine can cause cytotoxicity in renal cells, which may lead to direct renal injury, apoptosis and necrotic cell death in the kidney (Pérez-Morales et al., 2018).

iNOS is an enzyme which leads to the production of nitric oxide (NO). It is constitutively expressed in the kidney and in pathological situations this expression is elevated. Despite this enzyme having important functions in physiologic conditions – such as host defence mechanisms and blood pressure regulation –, it also plays a demarked role in pathophysiological conditions – namely inflammation (when aggravated), infection and diabetes (Lechner, Lirk, & Rieder, 2005). The expression of iNOS requires the exposure to inflammatory stimuli, like cytokines and/or LPS (Joles, Vos, Gröne, & Rabelink, 2002).

Matrix metallopeptidase 2 (MMP2) has relevant functions in the collagen type IV – which is an important constituent of the basal membranes – breakdown. Besides that, it also intervenes, in a significant manner, for the inflammatory response onset (Genetics Home Reference).

Low-grade inflammation, which is promoted by IR, among other factors, is present years before the detection of DM, this means that is present in prediabetes (Gemma Currie, Gerard McKay, 2014; Pérez-Morales et al., 2018). Studies suggest that chronic hyperglycaemia and inflammation stimulates endothelial dysfunction. Therefore, low-grade inflammation is present in prediabetes and has a role in the progression for advanced diabetic states (Bharara, M. et al., 2009; Dandona, Aljada, & Bandyopadhyay, 2004; Mahat et al., 2019; Priyatharshini et al., 2017).

3. RENAL IMPAIRMENT IN PREDIABETES

3.1. Kidney anatomy and functions - overview

Kidneys are the main excretory organs of the human body, being responsible for eliminating harmful substances and molecules, which result from the ingestion of food or from one's metabolism (Harrison, 2015; Seeley, Tate, & Stephens, 2011).

The kidneys can be divided in cortex (external section) and medulla (internal section), which can be seen through a longitudinal cut. The medullar region is constituted by the cone-shaped renal pyramids, in which the base of each pyramid delimits the separation between the cortex and the medulla. The pyramids' vertexes – renal papillas – are directed towards the renal sinus, which ultimately originates the ureter – that is connected with the bladder.

These anatomical features can be seen in the picture below (Figure 5).

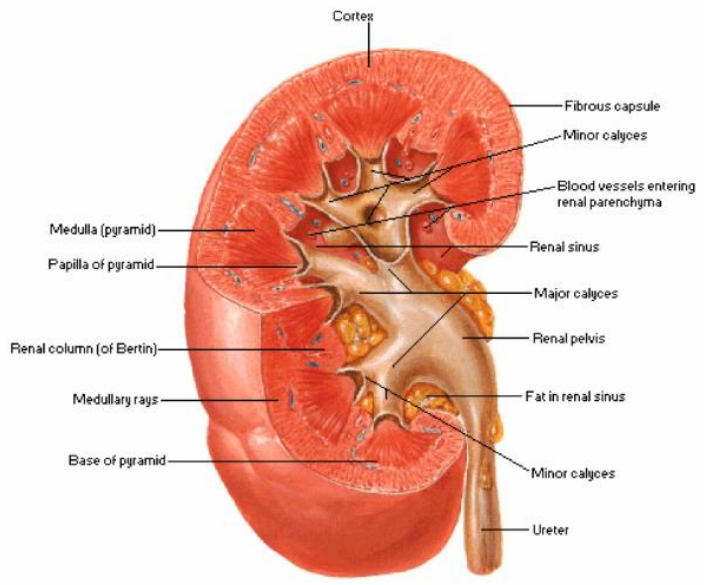

Figure 5. Kidney where it is visible the cortex (external section) and medulla (internal section). Adapted from Netter, 5th edition.

The basic functional and histological unit of the kidney is the nephron (Figure 6). It consists in a tubular structure constituted by (Harrison, 2015): 1) Bowman's capsule (terminal dilated portion) – has a visceral layer (inner), which is formed by podocytes (specialized cells) that surround the glomerular capillaries and a parietal layer (outer); 2) Proximal convoluted tubule – responsible for reabsorbing, approximately, 60% of filtered NaCl (sodium chloride) and water, around 90% of filtered bicarbonate and, also, critical nutrients, namely glucose and amino acids; 3) Loop of Henle – consists of three major segments: descending thin limb (highly water permeable), ascending thin limb and ascending thick limb (responsible for the reabsorption of, approximately, 15–25% of filtered NaCl) and 4) Distal convoluted tubule – responsible for reabsorbing, approximately, 5% of the filtered NaCl and has little water permeability.

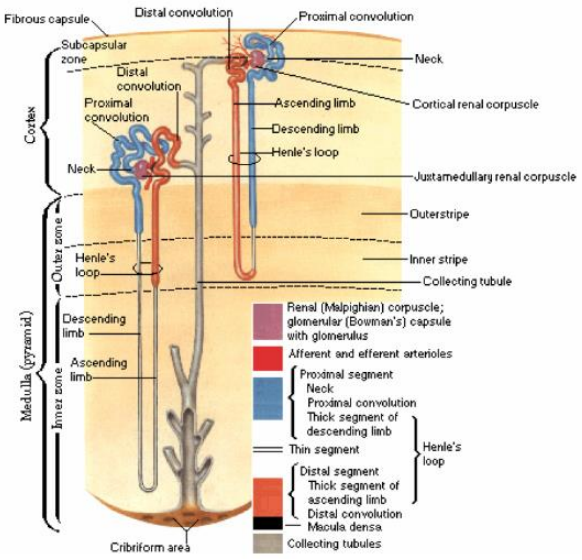

Figure 6. Nephron where it is visible the Bowman's capsule, proximal convoluted tubule, loop of Henle, distal convoluted tubule. Adapted from Netter, 5th edition.

Overall, the kidneys have exocrine, endocrine and metabolic functions, which can be divided and summarized as follows (which will be further detailed in this thesis): 1) Blood filtration, *i.e.* removal of undesired particles from the blood stream, which is intimately related to their excretion role; 2) Regulation of blood volume and solute concentration in the blood; 3) Regulation of pH value of the extracellular fluid; 4)

Regulation of the erythrocytes synthesis, through the synthesis of the hormone erythropoietin by the kidney; 5) Vitamin D synthesis, which greatly contributes to a higher calcium intestinal absorption and 6) Glycogenesis, i.e. the glycogen synthesis process, in which glucose molecules are added sequentially, forming glycogen chains for storage in the cells; (Seeley et al., 2011).

Exocrine, endocrine and metabolic function

In order to maintain the body's homeostasis, the kidneys are in charge of many activities, namely the excretion of excessive water, electrolytes and endogenous substances such as urea and other toxic substances, through the processes of filtration, secretion and reabsorption (DiPiro et al., 2008).

Glomerular filtration is a passive process through which water, ions and low molecular weight molecules (<5 to 10 kDa) cross the basal glomerular membrane into the Bowman's capsule and enter the proximal convoluted tubule. Most proteins in circulation have a very substantial molecular weight (>60 kDa) and, for that reason, are not filtrated and continue in the blood stream. Glomerular filtration rate (GFR) is an important indicator of renal function, since it estimates the amount of blood flowing through the glomeruli each minute; GFR is obtained through creatinine measurement. Another way of evaluating renal health conditions is via the quantification of blood urea nitrogen (BUN), which allow to understand if the kidney is functioning properly, because nitrogen is one of the waste products eliminated from the blood by the kidney.

Secretion is an active process which facilitates the elimination of several compounds from the renal circulation. It consists in an uptake into the lumen of the distal convoluted tubule, predominantly.

Reabsorption of water and solutes occurs throughout the entire nephron, mainly in the proximal convoluted tubules (DiPiro et al., 2008).

The kidney has the capability to synthesize and secrete several crucial hormones for the maintenance of fluids and electrolytes' homeostasis, such as erythropoietin, calcitriol and renin. In addition, in conditions of reduced oxygen pressure in the blood, the kidney is able to, firstly, sense this diminished pressure and to respond by producing erythropoietin, an hormone that is essential to the production of additional red blood cells (which help in low oxygen pressure conditions).

Furthermore, the kidney also produces an important hormone in the regulation of the volume extracellular fluid and arterial vasoconstriction, by the juxtaglomerular apparatus – renin. This hormone is a fundamental part of the renin–angiotensin–aldosterone system, one of the body's main system to control blood pressure (DiPiro et al., 2008).

The kidney has an important role in various metabolic functions, such as: gluconeogenesis, i.e. the endogenous synthesis of glucose – despite being mainly performed by the liver, it also occurs, with a lower but important expression, in the kidney cortex and metabolism of endogenous substances, namely insulin, steroids and xenobiotics.

3.2. Diabetic nephropathy

Diabetic nephropathic (DN) or diabetic kidney disease (DKD) is a sclerotic lesion associated with the thickening of the glomerular basal membrane, mesangial expansion and glomerular sclerosis (Lin, Chang, Yang, Wu, & Chu, 2018). It is defined by macroalbuminuria (>300 mg in a 24-h collection) or microalbuminuria, abnormal renal function obtained by GFR and serum and clearance creatinine (Ahmad, 2015). It derives from long term effects resulting from hyperglycaemia (Harrison, 2015). DN is associated with chronic kidney disease (CKD) and end-stage renal disease (ESRD) and is related with increased albumin excretion, glomerular lesions, reduced GFR and an increase in morbidity and mortality in diabetic patients (Cao & Cooper, 2011; Lin et al., 2018). The

risk factors associated with DN – besides hyperglycaemia – are: elevated blood pressure, dyslipidaemia, obesity, insulin resistance, gestational diabetes, ethnicity and family history (genetics) (Sulaiman, 2019).

Taking into account the degree of renal impairment and the presence of albuminuria, DN can be classified in several stages. Although a rigorous control of glycaemia and blood pressure, as well as the use of angiotensin-converting enzyme (ACE) inhibitors and/or angiotensin II receptor blockers slowing DN's progression down, these measures do not impede it.

In epidemiological terms, about 30% of people recently diagnosed with diabetes already have some degree of kidney disease, suggesting that hyperglycaemia may have an impact on the kidney before diabetes glycaemic levels (Echouffo-Tcheugui, Narayan, Weisman, Golden, & Jaar, 2016; Markus et al., 2018). DN affects, approximately, a third of the population with T1DM and T2DM (Reutens & Atkins, 2011).

DN is characterized by the interaction of metabolic components, namely glucose-dependent pathways, advanced glycation end-products (AGEs) and their receptors – such as components of the renin-angiotensin system (RAS). The blockage of RAS has a fundamental role in DM, since it attenuates the renal damage. These components interact with the ROS pathways, which on their turn are implied in DN's pathogenesis (Figure 7) (Cao & Cooper, 2011). In the presence of hyperglycaemia and hypertension there is an excessive production of ROS, which can damage the renal interstitium and, therefore, increase vascular permeability, renal vascular sclerosis and cause structural and functional deleterious effects in the kidney. The main sources of ROS are the activation of NADP+ (nicotinamide adenine dinucleotide phosphate) and protein kinase C, the increased formation of AGEs and the polyol pathway. ROS triggers several downstream mediators, such as ERK (extracellular regulated protein kinases), p38 MAPK (p38 mitogen-activated protein kinases), NF-κB (nuclear factor kappa B) and activator protein-1, whose actions contribute to DN (Tojo, Asaba, & Onozato, 2007).

In the early stages of DN, there is a tubular hypertrophy, followed by a tubular atrophy; interstitial fibrosis occurs in parallel with arteriolar hyalinosis (Lim, 2014). Oxidative stress and inflammation contribute to kidney fibrosis, which is represented by the thickening of the basal membrane, glomerular hypertrophy and expansion of the mesangial matrix. Persistent damage induced by hypoxia, oxidative stress and/or inflammation promote the release of profibrotic cytokines by the proinflammatory cells that leads to excessive production and deposition of extracellular matrix in the renal tissue (Tojo et al., 2007).

In advanced cases, there is a reduction in the endothelial fenestration and podocyte loss. In these cases, there is also a decrease in GFR and an increase in proteinuria (Lim, 2014).

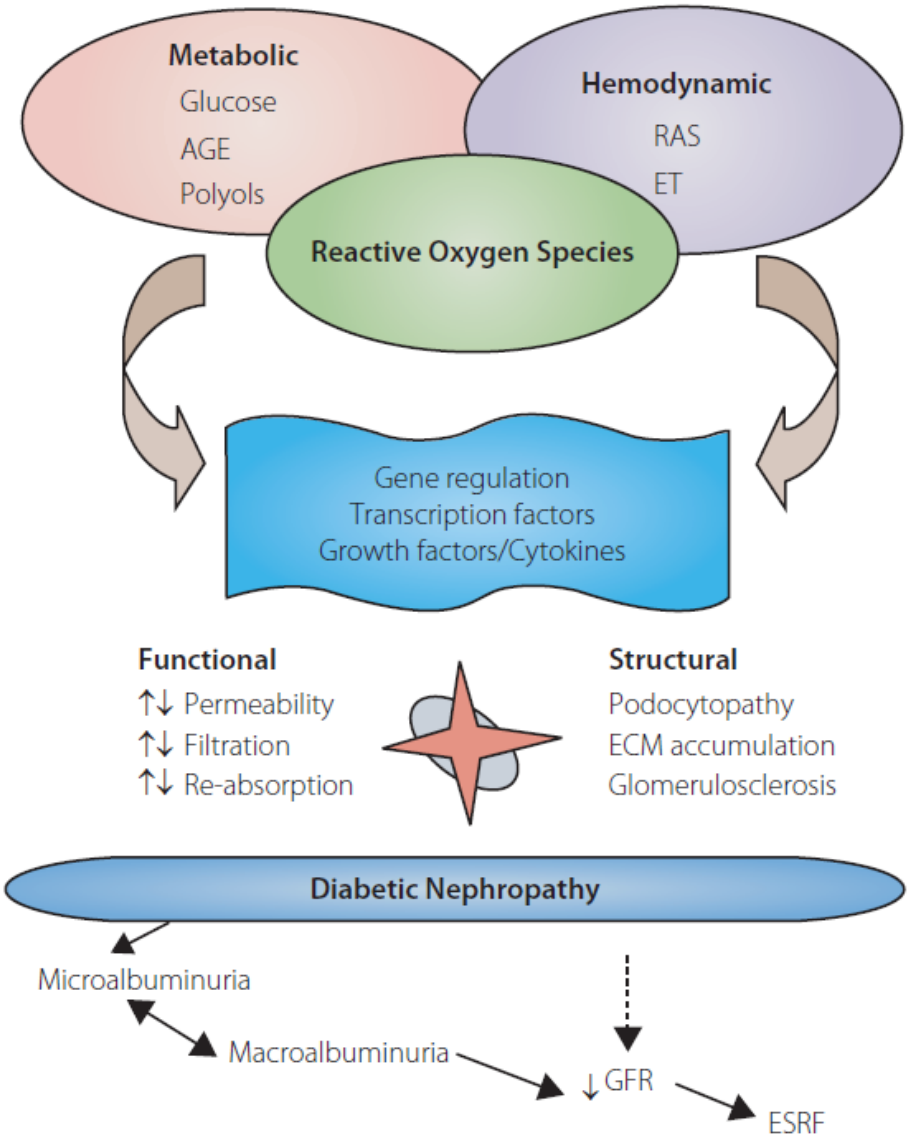

Figure 7. Pathogenesis of diabetic nephropathy. Metabolic and hemodynamic abnormalities, interacting with each other and with reactive oxygen species. AGE, advanced glycation end-products; ECM, extracellular protein production; ESRF, end-stage renal failure; ET, endothelin; GFR, glomerular filtration rate. Adapted from Cao & Cooper, 2011.

3.3. Early renal impairment in prediabetes? When and how?

There is evidence that prediabetes has a negative impact in the kidney, namely by worsening or causing renal disfunction, glomerular hyperfiltration and an increase in the amount of protein released from the body (mostly albumin – albuminuria) (Ferrannini, Gastaldelli, & Iozzo, 2011). More specifically, prediabetes can predict a higher GFR or hyperfiltration, which, on its turn, may occur due to a maladaptive response to metabolic

changes (Melsom et al., 2016b). This hyperfiltration can lead to progressive kidney disease and to morphologic alterations, which then can contribute to podocyte damage and loss of filtration surface (International Diabetes Federation, 2017). Glomerular hyperfiltration is an early renal change in diabetes and hypertension; because it is recognized at an early stage has the potential for reversible kidney damage (Okada et al., 2012).

Albuminuria is a characteristic feature of DN and could be present even before the onset of diabetes, further becoming gradually more prevalent with worsening of glucose tolerance (Lim, 2014; Shevalye et al., 2012). According with the previous studies, DN is associated with an abnormal deposition of lipids in the kidney, called renal steatosis and it is implicated in pathological alterations in renal parenchyma, such as glomerular sclerosis, tubular damage and interstitial fibroses, which in turn has consequences for renal function. Patients with T2DM and microalbuminuria have a higher lipid accumulation than both those with T2DM with normorglycaemia and people without diabetes (Wang, Feng, Lu, & Ju, 2018). High-caloric/high-fat diets in rats has shown an increase in body weight – which, in more severe cases, leads to obesity –, impaired glucose tolerance and insulin resistance. These conditions, on their turn, can affect the kidney, namely by increasing renal weight, reducing GFR and raising albuminuria (Wang et al., 2018).

In accordance with *Gemma Currie* and *Gerard McKay (2014)*, patients with DN have increased TNF-α values in the both urine and serum, when in comparison with controls or patients with normoglycaemia. The same authors state that the progression to DM is associated with a low grade inflammation condition, several years before clinical manifestation, that is, in an early stage (Gemma Currie, Gerard McKay, 2014).

3.4. Measures to prevent kidney disease in prediabetes

Several studies have shown that a lifestyle intervention has a positive effect in the progression of DM, with special emphasis on T2DM, reducing its prevalence. Therefore, strategies for controlling DM are, very often, related to changes in lifestyle habits, namely physical exercise and a balanced diet (Knowler et al., 2008, 2011; Ramachandran et al., 2006). However, since DM patients' lifestyle is, usually, demarked by lack of physical exercise, the therapy for DM requires, very often, pharmacological intervention, which leads to high economic costs. Since prediabetes precedes DM, an intervention on patients' diet could bring many benefits, both at the economical level as well as preventing progression of this chronic disease to more damaging stages.

It is known that the regular consumption of food that is either rich in fat and/or in carbohydrates induces acute inflammatory stress of both overweight and normal weighted people (Joseph et al., 2014). On the other hand, diets that have a heavy fruit and vegetable component are inversely correlated with stress. The ingestion of large amounts of these types of food are associated to a lesser prevalence of T2DM, cancer, CVD and Alzheimer's disease (Joseph et al., 2014). These accentuated benefits can be explained by the presence, in both fruits and vegetables, not only of large amounts of essential vitamins and minerals, but also of active non-biologic compounds such as phenolic acids and polyphenols, which have important antioxidant and anti-inflammatory properties (Joseph et al., 2014). Fruits like berries, namely blueberries, are particularly prone in reducing oxidative stress and inflammation, as it will be shown below.

Another important standpoint is that the available therapeutic strategies for renal diseases are more focused on controlling the associated risk factors and improving the daily lives of the patients, rather than to specifically target the mechanisms underlying kidney disease onset and progression, including DN. In fact, some of the drugs clinically used can ameliorate hypertension (such as the diuretics and the RAS modulators) and decrease uric acid (Alopurinol) while others are basically antioxidant drugs. Thus, there is a clear need to find better renoprotective strategies, which could be not only

additional drugs but, and especially, nutraceutical options to be used at initial stages of the renal disease. An earlier intervention through a nutraceutical option might be extremely interesting in the prevention of renal pathologies, namely by using diets which have high contents of cytoprotective components, such as the blueberries.

4. ARE BLUEBERRIES A PROMISSING RENOPROTECTIVE OPTION?

Blueberries can be classified as lowbush ("wild") or highbush (*Vaccinium corymbosum* and *Vaccinum angustifolium*), depending the size of the shrubs. The chemical and nutritional composition of blueberries varies depending on several factors including variety, cultivation practices, climatic aspects, among others. This fruit has a high amount of water (84%), carbohydrates (9.7%), proteins (0.6%) and fat (0.4%) (Michalska & Łysiak, 2015).

Blueberries have attracted remarkable attention during the last decade due to their distinct protective properties which may be of great interest for human healthy, including hypoglycaemic, antioxidant, anti-inflammatory, antiproliferative, anti-obesity, as well as neuroprotective (Lacombe et al., 2013; Norberto et al., 2013; Vendrame, Del Bo', Ciappellano, Riso, & Klimis-Zacas, 2016). This broad spectrum of beneficial effects are mainly attributed to its enriched content in polyphenols. These compounds comprise a variety of classes, being the most popular the flavonoids, and particularly anthocyanins, which are responsible for the intense red colour. The chronic consumption of anthocyanins have been associated with a decreased risk of developing T2DM, hypertension, myocardial infarction and cancer (Joseph et al., 2014). Besides anthocyanins, there are other compounds that contribute to the antioxidant and anti-inflammatory potential, namely procyanidins (monomeric and oligomeric form), flavonols (kaempferol, quercetin, myricetin), phenolic acids (mainly hydroxycinnamic acids) and ellagitannins (Michalska & Łysiak, 2015). Polyphenols may influence glycaemia levels through the inhibition of glucose absorption in the gut (Guasch-Ferré, Merino, Sun, Fitó, & Salas-Salvadó, 2017) and could also play a role in the improved insulin resistance, namely by ameliorating insulin secretion and sensitivity (Yang et al., 2017). Some metabolites of blueberries mitigate the endothelial inflammation as a result of restoring cell surface glycosaminoglycans and help to fight obesity by promoting the anti-

inflammatory phenotype of adipocytes (Ma, Sun, Zeng, Luo, & Yang, 2018). Supplementation with blueberries improve metabolic impairment in high-fat-diet rats, including amelioration of gut microbiota dysbiosis, low grade inflammation and insulin signalling defects (Ma et al., 2018).

Regarding the kidney, it is acknowledged that inflammation is associated with various kidneys disorders, including DN, as well as with hypertension and renal failure (Nair, Masson, Ebenezer, Del Piero, & Francis, 2014). Studies suggest that in a rat model of hypertension a blueberry-enriched diet has the capacity to decrease blood pressure, maintain renal hemodynamics and improve the reduction-oxidation balance in the kidneys (Elks et al., 2011; Shaughnessy, Boswall, Scanlan, Gottschall-Pass, & Sweeney, 2009). In another study, in which LPS was administered in order to understand the role of TLR-4, rats previously treated with blueberries had better levels of GFR, higher renal blood flow, less vascular resistance and a reduction in the production of ROS. In addition, reduced gene and protein expression of TLR-4 was found when compared with the animals that have not been pre-treated with blueberries (Nair, Masson, et al., 2014).

Although there are some evidences of beneficial effects of blueberries on the kidney, the potential for exert renoprotection in prediabetes remains to be elucidated. Our group have recently described protective effects of BJ against liver steatosis evolution in a rat model of prediabetes induced by hypercaloric diet. Whether a similar influence also holds truth in early renal impairment remains to be addressed.

OBJECTIVES

We hypothesized that prediabetes induced by a long-term hypercaloric diet might be associated with early renal impairments. Moreover, taking into account our previous observations of blueberries' ability to ameliorate experimental prediabetic hepatic steatosis, we secondly hypothesized whether an early nutraceutical strategy based on BJ chronic supplementation would also improve prediabetic early renal impairment.

Thus, the main goals of this dissertation were twofold: i) to characterize the degree of renal impairment (functional, molecular and structural) in experimental diet-induced prediabetes and ii) to evaluate the putative beneficial effects of long-term BJ supplementation on prediabetic renal impairment.

To accomplish these purposes, the following parameters were assessed:

- Evolution of animals' body weight and caloric intake;
- Metabolic characterization through the evaluation of glycaemic, insulinaemic and lipidic profiles;
- Serum oxidative/inflammatory balance;
- Renal (dys)function, evaluated by serum and urinary levels (and clearances) of creatinine, uric acid and BUN, as well as by GFR;
- Kidney histomorphological characterization;
- Renal lipid deposition;
- Renal inflammatory status, assessed by IL-6, TNF-α, iNOS, MMP2, TLR-4 and RAGE.

MATERIALS AND METHODS

1. Animal groups, treatments and in vivo monitoring

1.1. Experimental design

In this study, 16-week-old male Wistar rats obtained from Charles River Laboratories (Barcelona, Spain) were used. Rats were housed two per cage in the animal facility of iCBR (Coimbra Institute for Clinical and Biomedical Research), Faculty of Medicine, University of Coimbra, under controlled temperature (22±1°C) and a 12-h light 12-h dark cycle and relative humidity (50-60%). All procedures involving animals were performed according to the National and European Communities Council Directives of Animal Care. The project received an approval (9/2018) by the iCBR ORBEA (Animal Welfare Body).

After an adaptation period of 1 week, rats were randomly divided into four groups and submitted to a 23-week protocol (Figure 8): group 1 received standard chow and tap water (Control); group 2 received standard chow and 25g/kg BW/day of BJ (in water) from week 9 (BJ); group 3 received 35% sucrose (Hsu - S0389; Sigma-Aldrich, St. Louis, MO, USA) in the drinking water plus standard chow until week 9, further supplemented by High Fat Diet (HF, 60% kcal from fat) until week 23 (HsuHF); group 4 were submitted to the same dietary regimen of group 3 but received 25g/kg BW/day of BJ (in 35% sucrose solution) from week 9 (HsuHF+BJ).

Standard rat chow contained 60% of carbohydrates, 16.1% of protein, 3.1% of lipids, 3.9% of fibers, and 5.1% of minerals (AO4 Panlab, Barcelona, Spain). Animals consumed food and water/BJ *ad libitum* (except during fasting periods). Blueberries (*Vaccinium corymbosum L.* from Cultivar: Liberty) were supplied by the Cooperativa Agropecuária dos Agricultores de Mangualde (COAPE).

Glycaemic (glucose levels, HbA1c and glucose tolerance test - GTT), insulinaemic (insulin levels and insulin tolerance test - ITT) and lipidic (triglycerides) profiles were assessed at week 23.

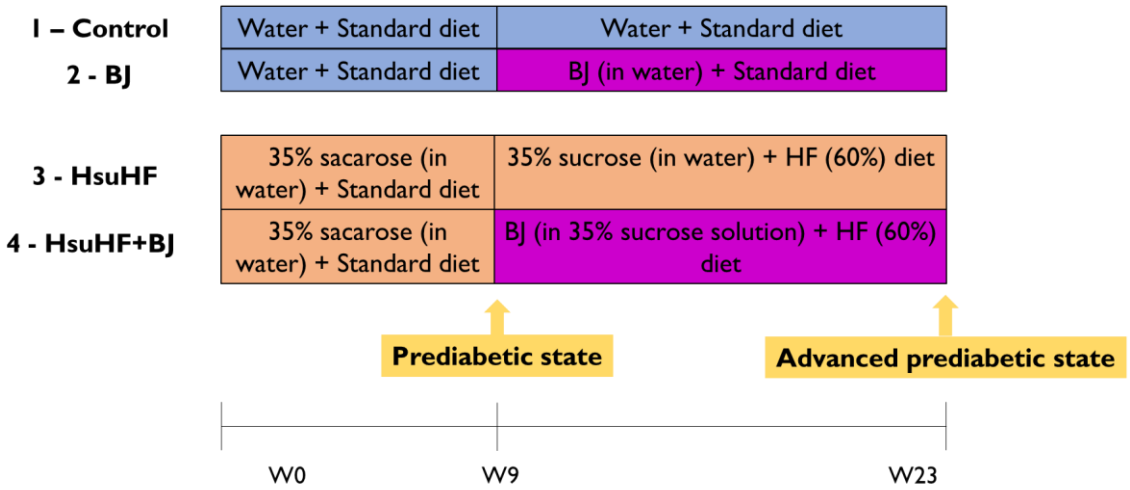

Figure 8. Experimental protocol of the *in vivo* study.

1.2. In vivo monitoring

Body weight and food and water consumption

BW measurements were obtained weekly using an analytical balance (KERN CB 6 K1, Germany) and food and water consumption were also monitored weekly.

Blood pressure and heart rate

Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP) and heart rate (HR) values were obtained using a tail-cuff sphygmomanometer LE 5001 (Letica, Barcelona, Spain) in suitable contention cages in conscious rats. Prior to these measurements, animals were warmed at 25-30 °C for 10-20 min to allow the detection of tail artery pulsations and to attain the pulse level ready. BP and HR values were determined through averaging 8-10 measurements. In order to reduce stress-induced fluctuations in BP, all rats were habituated to the appropriate cages and measurements during the previous 2 weeks. The final values were taken by the same person in the similar stress-free environment between 14:00 h and 18:00 h.

1.3. Sample collection

The day before finishing the experiment - at week 23 -, rats were submitted to an intraperitoneal anaesthesia with a 2 mg/kg BW of a 50 mg/kg pentobarbital (Sigma-Aldrich, Portugal) solution and a blood sample was instantly collected by venepuncture from the jugular vein into syringes (for serum samples collection). Thereafter, the rats were sacrificed by cervical dislocation and kidneys were collected and fixed or cryopreserved in liquid nitrogen for further analysis.

2. Metabolic profile

2.1. Glycaemic profile

Glucose tolerance test (GTT)

During week 23, rats were administered intraperitoneally with a glucose bolus of 2 g/kg BW following a 6-hour fasting period. Blood glucose levels were quantified through the tail vein before the injection and 15, 30, 60 and 120 min after, using the portable device One Touch® UltraEasy® glucometer (Lifescan, Johnson and Johnson, Portugal). The area under the curve (AUC) for the GTT was calculated by using the trapezoidal method.

Postprandial glycaemia

The postprandial glucose levels were measured in the sacrifice day, ate the same hour, from a drop of blood collected by venepuncture in the jugular vein with the aid of the One Touch® UltraEasy® glucometer (Lifescan, Johnson and Johnson, Portugal).

HbA1c

Proteins such as hemoglobin can be non-enzymatically and irreversibly glycated, a reaction that is proportional to blood glucose levels over a period of 2 months. Hence,

HbA1c is often used as an indicator of the mean daily blood glucose levels. HbA1c was therefore dosed on the day of sacrifice through a drop of blood placed on an automated analyser (Siemens, DCA Vantage AnalyzerTM, EUA).

2.2. Insulinaemic profile

Insulin tolerance test (ITT) and glucose disappearance rate (KITT)

During week 23, rats were administered intraperitoneally with insulin (I9278, Sigma), 0.75 U/kg BW following a 6-hour fasting period. Blood glucose levels were obtained through a drop of blood from the tail vein before the bolus and 15, 30, 45, 60 and 120 min after, using the portable device One Touch® UltraEasy® glucometer (Lifescan, Johnson and Johnson, Portugal).

The KITT, derived from the ITT, is represented by the slope of the decline in blood glucose. The slope can be calculated by dividing 0.693 by plasma glucose half-time, as shown below (Patarrão, Wayne Lautt, & Paula Macedo, 2014):

$$\text{KITT} = \frac{0.693}{t1/2} \times 100$$

Serum insulin

Insulin levels were measured in serum samples (in postprandial and 6-hours fasting conditions) by using a rat insulin ELISA (Enzyme-Linked Immuno-Sorbent Assay) kit from Mercodia (Uppsala, Sweden). This kit consists of a two-stage solid phase enzyme immunoassay, in which two monoclonal antibodies are directed against certain antigens separate from the insulin molecule. In the incubation phase, insulin molecules in the sample react with peroxidase-conjugated anti-insulin antibodies and the corresponding anti-insulin antibodies fixed in the microtiter well. Unbound enzyme labelled antibody is removed in the washing step. Bound conjugated enzyme is detected by reaction with 3,3 ', 5,5' – tetramethylbenzidine. The reaction ends after the addition of an acidic solution, which leads to the formation of a coloured compound. Colour intensity is assessed by spectrophotometry at 450 nm.

2.3. Lipid profile

At the time of sacrifice/the end of the *in vivo* protocol, blood was collected into syringes without anticoagulant and centrifuged (35000 rpm, 15 minutes at 4°C). Total cholesterol and TG levels were assessed in serum using an automated analyser (Hitachi 717, Roche Diagnostics Inc., MA, EUA) that makes use of Cholesterol RTU reagent and kit TG PAP 1000 (bioMérieux, Lyon, França) for the measurement of cholesterol and TGs, respectively, by colorimetric methods.

3. Renal data

3.1 Renal function

Serum biochemical parameters

The following biochemical parameters were evaluated in serum by validated automated methods and equipment (Hitachi 717, Roche Diagnostics Inc., MA, USA): creatinine, uric acid and BUN.

24-hour urine parameters

During week 23, animals were housed in metabolic cages for 24 hours, receiving water and food *ad libitum*. Urinary glucose, creatinine, uric acid and BUN concentrations were evaluated in 24-hour urine (Cobas Integra 400 plus, Roche®, Amadora, Portugal), and urine volumes were measured to calculate clearances and GFR, according to what was previously described (Pestel, Krzykalla, & Weckesser, 2007). GFR, in particular, was calculated using a formula for conscious rats that makes use of creatinine and BUN clearances (Pestel, Krzykalla, & Weckesser, 2007).

3.2 Renal histomorphology

Hematoxylin and Eosin (H&E) staining

Tissue samples were formalin-fixed and embedded in paraffin wax (n=3 each experimental group). One HE-stained cryosection (5 μm) from each block was reviewed. Tissue sections were deparaffinized in xylene and hydrated to a decrescent series of ethanol until distilled water. Thereafter, the tissue sections were immersed in haematoxylin stain Solution, Gill 1 (Sigma Aldrich; Missouri, USA) for 2 minutes and washed in tap water. Then, they were counterstained with 0,5% aqueous eosin (Sigma Aldrich; Missouri, USA) for 30 seconds and after that dehydrated, cleared and mounted. All samples were examined by light microscopy using a Zeiss microscope Mod. Axioplan 2 (Bancroft & Gamble, 2002).

Oil Red O staining

Tissue sections of fresh frozen tissue were cut to 5 μm thickness, mounted on slides and allowed to dry for 30 minutes. The cryosections were placed in absolute propylene glycol for 2 minutes and transferred to 0.5% red oil in absolute propylene glycol solution for 10 minutes. The sections were differentiated in 85% propylene glycol solution for 2 minutes, washed in distilled water and counterstained in Hematoxylin Stain Solution Gill 1 (Sigma Aldrich; Missouri, USA) for 30 seconds. They were rinsed under running water for 3 minutes and mounted with CC / Mount aqueous mounting medium (Sigma Aldrich; Missouri, USA). The lipids were stained with bright red colour and nuclei with a blue colour. All samples were examined by light microscopy using a Zeiss microscope Mod. Axioplan 2 (Bancroft & Gamble, 2002).

3.3 Molecular analysis

Gene expression

Total RNA extraction: 35 – 50 mg from frozen renal tissue (preserved in RNA later Stabilization Solution, ThermoFisher) were homogenised by mechanical

dissociation using a Potter-Elvehjem (Thomas Scientific, USA) in 1 mL of Trizol (93289, Sigma)and stored overnight at -80°C. After thawing the samples, 200 µL of chloroform were added to each homogenate and centrifuged at 15300 rpm for 15 min at 7°C. After centrifugation, three phases appeared - organic, interphase and aqueous phases. Aqueous phase containing ribonucleic acid (RNA) was collected and 1 µL of glycogen (R0551, Thermo Scientific™, Lithuania) plus 400 µL of isopropanol were added and stored overnight at -20°C for RNA precipitation. After thawing, samples were centrifuged (15300 rpm, 30 min, 4°C), the supernatant was discarded and remaining pellets were washed in 75% ethanol in water with diethyl pyrocarbonate (DEPC) (UltraPure™ DEPC-Treated Water, Invitrogen™, USA). A final centrifugation was performed (9500 rpm, 5 min, 4°C) and then the pellet was dried for one hour inside the chamber. Pellets were dissolved in water with DEPC to inactivate RNase enzymes and RNA concentrations were determined (NanoDrop ® ND-1000 Spectrophotometer). Samples were stored at -80°C until subsequent analysis.

cDNA synthesis: Synthesis of complementary Deoxyribonucleic acid (cDNA) was performed using a Xpert cDNA Synthesis Mastermix (GK81.0100, Lot. 7E2709A, GRISP). For each tube, it was pipetted the volume corresponding to 2µg RNA, 10 µL of Mastermix and water (to a final volume of 19 µL. Then, in the thermocycler (1861096, T100™ Thermal Cycler, Bio-Rad) cDNA was synthesized following the Xpert cDNA Synthesis Mastermix protocol. Samples were stored at -20°C.

RT-PCR: A mixture was prepared containing 10 µL of Sybr Green (iTaq Universal SYBR Green Supermix 1725124, Bio-Rad), 0.4 µL of mix primers (table 2) and 7.6 µL of autoclaved water. 18 µL of this mixture and 2 µL of the sample were transferred into each well. RT-PCR protocol consisted of 1 cycle for initial denaturation (10 min at 95°C), followed by 40 cycles comprising the following steps: 15s, 95°C; 45s, 58 or 60°C; 30s at 72°C. Standardization was achieved with GeNorm algorithm, where gene stability was attained with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Hypoxanthine-guanine phosphoribosyltransferase (HPRT). The relative expression

ratio of each of the target gene was computed on the basis of $\Delta\Delta Ct$ ($2^{-\Delta\Delta Cp}$) values. Results are expressed as percentage of control.

Table 2. Primer sequence used for the RT-PCR analysis.

Gen	Primer Sequence		Temp. (°C)
	Forward	Reverse	
iNOS	AGAGACAGAAGTGCGATC	AGATTCAGTAGTCCACAATAGTA	60
MMP2	GGGTGGTGGTCACAGCTATT	CCCAGCCAGTCCGATTTGAT	60
IL-6	GGAGAAGTTAGAGTCACAGA	GCCGAGTAGACCTCATAG	60
TNF-α	CCCAATCTGTGTCCTTCT	TTCTGAGCATCGTAGTTGT	60
GAPDH	GACTTCAACAGCAACTCC	GCCATATTCATTGTCATACCA	60
HPRT	ATGGGAGGCCATCACATTGT	ATGTAATCCAGCAGGTCAGCAA	58

Protein expression by western blotting

Protein extraction from rat kidney: Kidneys were weighed (150-200 mg) and homogenised by mechanical dissociation using a Potter-Elvehjem in solubilization buffer [Radioimmunoprecipitation assay buffer (RIPA buffer), consisting of 50mM Tris HCl (pH 7.5); 150mN NaCl; 1.0% Triton X-100; 0.5% (w/v) sodium deoxycholate (DOC); 0.1% (w/v) sodium dodecyl sulphate (SDS); 5mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) supplemented with 1 pill of PROTEASE inhibitor cocktail (11836170001, Roche Diagnostics GmbH, Germany), 2 mM of iodoacetamide, 2 mM of phenylmethyldulfonyl fluoride, 30 mM of sodium fluoride and 1 mM of sodium orthovanadate. After solubilization on ice for one hour, samples were sonicated three times in 30-second intervals. Then, samples were centrifuged at 13200 rpm for 15 min at 4°C. The supernatant was collected and stored at -80°C.

The protein concentration was determined using the bicinchoninic acid (BCA) assay (PierceTM BCA Protein Assay Kit, Pierce Biotechnology, Rockfor, IL, USA) using BSA standard solutions. The plate was incubated for 30 min at 37°C and the absorbance was then read at 570 nm using BioTek Synergy TM HT. Considering the calibration

curve obtained through the BCA assay, total protein concentration in each sample was calculated.

Protein lysates denaturation: Samples were denatured at 95°C during 5 minutes in 6x denaturing solution [1g SDS, 0.93g Dithiothreitol, 1 mg blue of bromophenol and dissolved into 7 mL of Tris HCl 0.5 M (pH= 6.3) and 3 mL of glycerol 3% (v/v)].

Polyacrylamide gel electrophoresis and immunodetection: After the system was set up, 80µg of protein were loaded per lane and separated by electrophoresis on SDS-7.5% polyacrylamide gel in running buffer (125mM Tris-base; 100mM glycine and 0.5% (w/v) SDS; pH 8.3), at 100 volts for 20 min and then at 140 volts for ≈ 60 min, depending on the molecular weight of the target protein.. After activation of 0.45µm polyvinylidene difluoride Amersham™ Hybond™ PVDF membranes (GE Healthcare, USA), the transfer step was processed at 300A for 90 min in transfer buffer (100mM CAPS; pH 11). Blocking of the membrane was performed using 5% milk/0.1% PBS-T approximately for 1 hour. Membranes were then incubated overnight with primary antibodies (Table 3) diluted in 1% milk/ 0.1% PBS-T. Then, membranes were washed every 20 minutes with PBS-T for 1 hour and incubated with adequate secondary antibodies (Table 3) diluted in 1% milk/ 0.1% PBS-T with agitation for 1 hour at room temperature (RT). After secondary antibody incubation, membranes were washed again every 20 minutes with PBS-T for 1 hour. Revelation of the membranes required about 500 µl of the enhanced chemiluminescence substrate (ECL) (R-03031-D25, R-03025-D25 WesternBright™ ECL and Peroxide, Advansta, USA) *per* detection step. Membrane revelation was made in ImageQuant™LAS500 (GE Healthcare Life Sciences). To ensure equal protein loading and sample transfer, membranes were re-incubated against loading proteins with mouse anti-GAPDH, mouse anti-tubulin or mouse anti-β-actin antibodies. Optical density of the bands was quantified by densitometry, using ImageQuant TL 5.0. Results were normalized against loading proteins and expressed as percentage of control.

Table 3. Primary and secondary antibodies used for Western Blotting analysis.

Antibody	Dilution	Company /Catalog number
TLR-4	1:500	Novus Biologicals: Ab76B357.1
MMP2	1:1000	Abcam: Ab37150
iNOS	1:1000	Abcam: Ab178945
TNF	1:500	Abcam: Ab66579
RAGE (N-terminal)	1:400	Sta Cruz Biotech: N16
β-actin	1:1000	Sigma-Aldrich: A5316
GAPDH	1:1000	Merck Milipore: MAB374
Tubulin	1:1000	SICGEN: AB0046-200
Goat anti-mouse IgG-HRP	1:10000	Advansta: R-05071
Goat anti-rabbit IgG-HRP	1:10000	Advansta: R-05072

Serum and kidney lipid peroxidation

Lipid peroxidation was determined by assessing malondialdehyde (MDA) production, through the thiobarbituric acid reactive substances (TBARs) test. One hundred µl of renal tissue homogenate (previously centrifuged to remove undesired particles) and one hundred µl of serum were incubated for one hour in a TBA solution at room temperature and in the dark conditions. The samples were then incubated in a 90° C bath for 60 minutes. Afterwards, the tubes were placed on ice and so the reaction ceased.

In this assay, one MDA molecule chemically reacts with two TBA molecules, the final product being a molecule which can be spectrophotometrically quantified at 532 nm (pink pigment). MDA concentration was calculated against a calibration curve using 1,1,3,3-tetramethoxypropane as external standard (range: 0.1-83.5 µM). The results obtained were expressed for kidney in µM/g tissue.

Serum total antioxidant status (TAS)

Serum total antioxidant status (TAS) was quantified using a commercial kit – Total Antioxidant Status (Randox Laboratories Ltd., UK) – and measured on a Hitachi 704 analyzer. This test is based on a calorimetric method and ferric reducing antioxidant potential assay. In this reaction the metmyoglobin, a peroxidase, reacts with hydrogen peroxide (H2O2), and ends with the formation of ferrylmyoglobin. The ferrylmyoglobin then reacts with 2,2'-azinobis (3-ehylbenzothiazoline-6-sulfonate) (ABTS) originating a cationic radical (ABTS•+), which due to its blue-green colour is detected spectrophotometrically at 600nm. The antioxidants in the samples cause inhibition of this colour production to a degree that is proportional to its concentration.

High- sensitivity C-reactive protein

High-sensitivity C-reactive protein (hs-CRP) was measured using a rat-specific Elisa kit (MBS764381, MyBiosource, San Diego, CA, USA).

4. Data processing and statistical analysis

The distribution of continuous variables was analysed using the Kolmogorov-Smirnov test to assess significant deviations from normality. Accordingly, comparisons between 4 experimental groups were performed using the nonparametric test Kruskal-Wallis test (followed by the Dunn's test for multiple comparisons) or the parametric tests One-way or two-way analysis of variance (ANOVA, followed by Bonferroni's test for multiple comparisons). Results were expressed as means ± standard errors of the mean (SEM) with the aid of GraphPad Prism® software, version 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered significant at p values less than 0.05.

RESULTS

1. Effects of BJ in metabolic profile

1.1. Evolution of body weight

Body weight was monitored during 23 weeks of treatment. Until 9 weeks of treatment, similar body weight values were recorded across all experimental groups. After the introduction of the hypercaloric diet, a slight increase in body weight was observed in HsuHF and HsuHF+BJ groups, being statistically significant ($p < 0.05$) when compared to animals of the control and BJ groups, respectively (Figure 9A).

1.2. Caloric intake

Feed intake was evaluated weekly per cage throughout the 23 weeks of treatment, and later estimated by rat. Significantly lower feed intake was observed in rats of HsuHF and HsuHF+BJ groups when compared to animals of the Control and BJ groups ($p<0.0001$). However, animals fed simultaneously with the HsuHF+BJ ingested more than animals fed only the HsuHF diet (Figure 9B).

Regarding drink consumption evolution, a higher value was found in the BJ and HsuHF groups compared to Control and HsuHF+BJ ($p < 0.0001$) groups (Figure 9C).

Considering the total caloric consumption, the HsuHF and HsuHF+BJ groups showed a higher caloric intake value compared to the Control ($p < 0.0001$) and BJ ($p < 0.0001$ and $p < 0.001$) groups (Figure 9D).

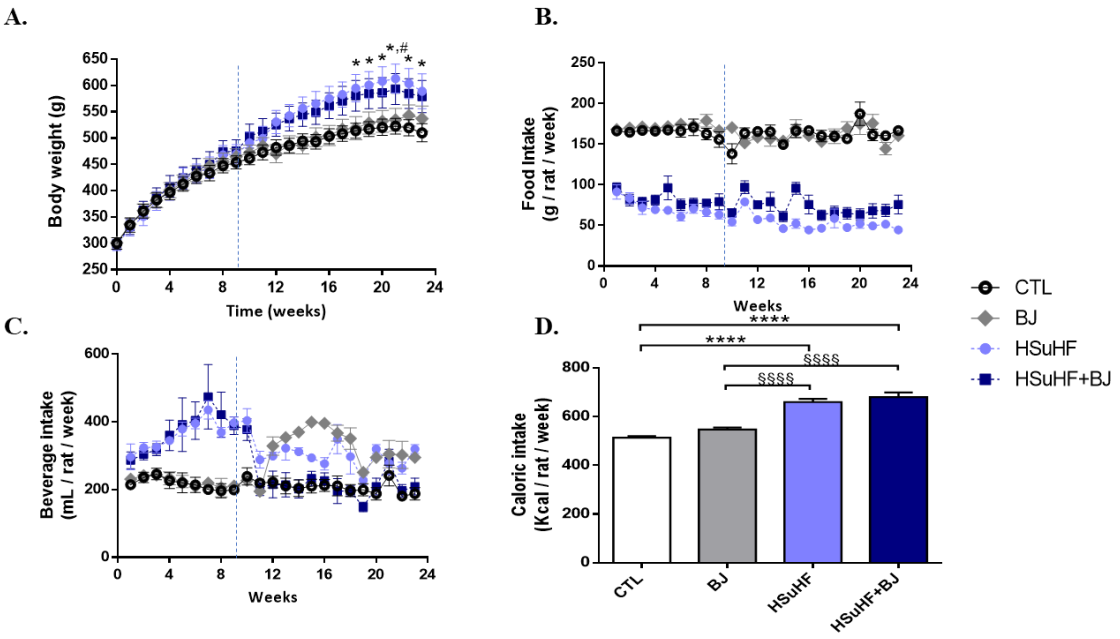

Figure 9. *Body weight and food, beverage and total caloric intake monitoring throughout the 23 weeks of treatment.* **A**. Evolution of body weight. **B**. Evolution of food intake. **C**. Evolution of beverage intake. **D**. Total caloric intake for the 23 weeks of treatment. Two-way analysis of variance (ANOVA) test was used followed by Bonferroni's test for multiple comparisons. Values are expressed as mean ± SEM (*, # p <0.05 vs Control). (n = 8–10 per group; **** p <0.0001 vs Control; §§§§ p <0.001; §§§§ p <0.0001 vs BJ; § p <0.05; §§§§ p <0.0001 vs HsuHF).

1.3. Blood pressure and heart rate (HR)

No significant differences were found in either systolic, diastolic and mean blood pressure. However, regarding HR, HsuHF group had higher values when compared to the Control group (Table 4).

Table 4: Blood pressure and heart rate values in experimental groups.

Parameter	Control	BJ	HsuHF	HsuHF+BJ
SBP (mmHg)	116.27 ± 5.02	119.75 ± 8.55	122.23 ± 6.6	127.38 ± 8.52
DBP (mmHg)	94.21 ± 4.96	86,78 ± 7.47	92.77 ± 4.69	91.2 ± 8.21
MBP (mmHg)	105.51 ± 6.17	97.93 ± 7.54	104.81 ± 18.13	99.9 ± 9.53
HR (beats/min)	346.15 ± 21.02	361.82 ± 19.95	398.43 ± 31.63*	390.61 ± 38.37

* p <0.05 vs Control

1.4. Glycaemic profile

Fasting and fed glycaemia

There were no statistically significant differences between the 4 groups regarding fasting glucose (Figure 10A). Postprandial blood glucose presented a significantly higher value in the HsuHF group compared to the other groups. The addition of blueberry juice to the group of animals fed the high calorie diet (HsuHF+BJ group) had a positive effect on postprandial glucose values. A significant decrease was observed in the HsuHF+BJ group when compared to the HsuHF ($p < 0.01$).

Glycated hemoglobin (HbA1c)

There were no statistically significant differences between the 4 groups regarding HbA1c (Figure 10B).

Glucose tolerance test (GTT)

Initial fasting glucose concentrations were similar in all groups. Peak glucose was reached after 15 minutes for HsuHF group (502.44 ± 21.49 mg/dL) and 30 minutes for the remaining groups. For the HsuHF and HsuHF+BJ groups, the glucose peak and area under the curve (AUC) of the glucose plot between baseline and 120 minutes were significantly higher than the remaining Control and BJ groups. Nevertheless, in the HsuHF+BJ group, AUC was significantly lower ($p<0.05$) compared to the HsuHF group, confirming a partial glucose intolerance in the HsuHF group that was significantly prevented in the HsuHF+BJ one (Figure 10C and D).

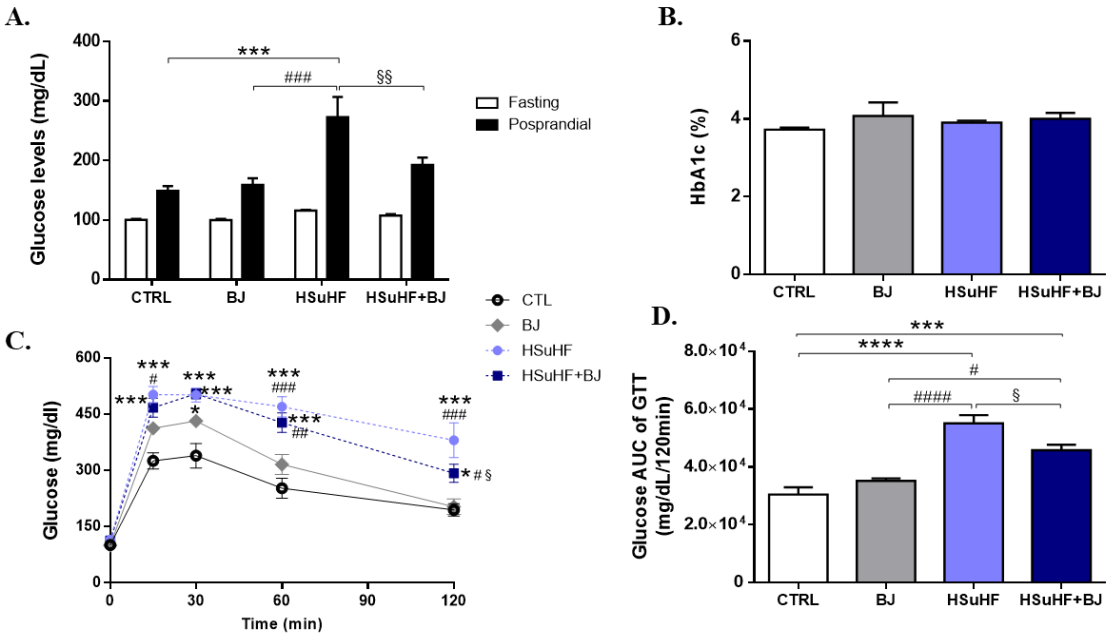

Figure 10. *Glycaemic profile after 23 weeks of treatment*. **A**. Fasting and postprandial blood glucose values. **B**. Glycated hemoglobin (%). **C**, **D**. Glucose tolerance test (GTT): Left -Evolution of glucose values between 0 and 120 minutes after injection of a glucose solution (2 g/kg body weight); Right - Area under the curve (AUC) of blood glucose evolution. Data are expressed as mean ± SEM (n = 8–10 per group; * p <0.05, *** p <0.001, **** p <0.0001 vs Control; # p <0.05, ## p <0.01, ### p <0.001, #### p <0.0001 vs BJ; § p <0.05 vs HsuHF).

1.5. Insulinaemic profile

Fasting insulin levels

Fasting serum insulin values appear to display a tendency to rise in the HsuHF and HsuHF+BJ groups; however, no significant differences were observed between the 4 groups (Figure 11A). Still, in the postprandial state, a significantly higher insulin value was observed in the HsuHF group when compared to the Control (p <0.05); there was a trend to prevent this elevation in the HsuHF+BJ group, despite not reaching statistical significance.

Insulin tolerance test (ITT)

Initial glucose concentrations were higher in the HsuHF (125.00 ± 4.00 mg/dL) and HsuHF+BJ (120.86 ± 2.85 mg/dL) groups compared to the Control (100.88 ± 2.02 mg/dL) and BJ (99.50 ± 2.26 mg/dL) ones (Figure 11B). Forty-five minutes after insulin

injection (0.75 U/kg body weight), glucose levels in the HsuHF (79.44 ± 4.99 mg/dL) group were significantly higher compared to the Control (59.13 ± 2.30 mg/dL) ($p<0.01$). After 120 minutes, the HsuHF group showed significantly higher glucose values than the Control and BJ groups, confirming a decrease in insulin sensitivity in the animals fed with the high calorie diet. In the HsuHF+BJ group, on the other hand, a glucose value significantly lower than in the HsuHF group ($p <0.01$) was observed, reaching values similar to those of the Control and BJ group, suggesting an improvement in insulin sensitivity. The AUC values of the insulin tolerance test confirmed the insulin resistance in the HsuHF group versus the Control group ($p <0.0001$), and an improvement in insulin tolerance in the HsuHF+BJ group ($p <0.01$) (Figure 11C).

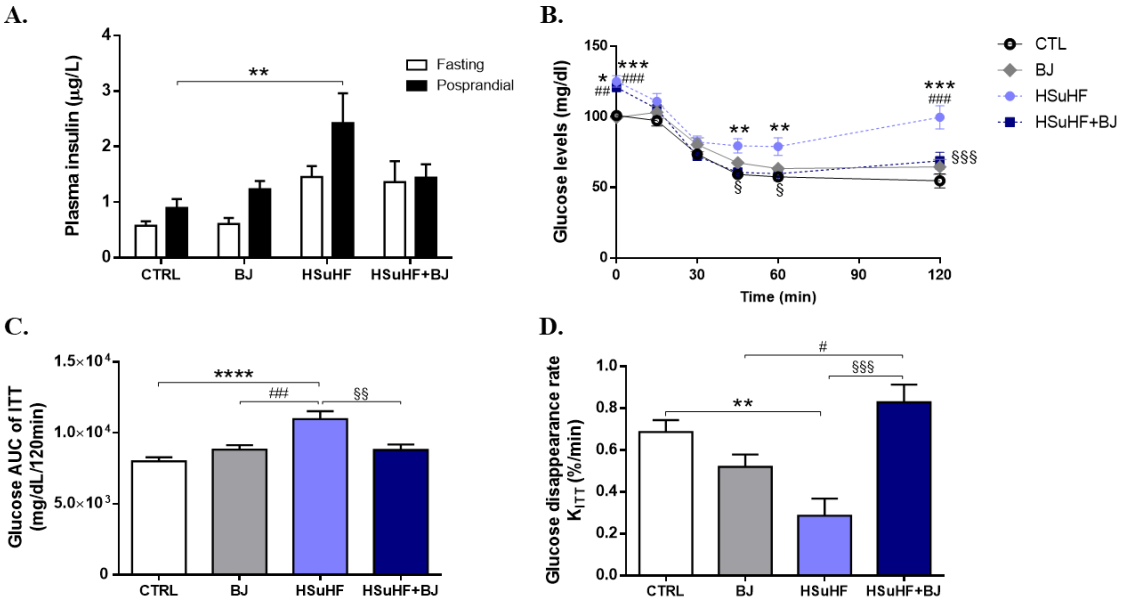

Figure 11. *Insulinaemic profile after 23 weeks of treatment*. **A**. Fasting and postprandial plasmatic insulin values. **B**, **C** - Insulin tolerance test (ITT): **B**. Evolution of glucose levels between 0 and 120 minutes after intraperitoneal injection of insulin (0.75 U / kg body weight); **C**. Area under the curve (AUC) of blood glucose evolution in Control, BJ, HsuHF and HsuHF+BJ. **D**. Glucose disappearance rate. Data are expressed as mean ± SEM (n = 6–9 per group; *** p <0.001 vs Control; ### p <0.001 vs BJ; §§ p <0.01vs HsuHF).

KITT presents statistically significant differences between the following groups (values are presented in brackets): Control (0.6863 %/min) and HsuHF (0.2867 %/min); BJ (0.5200 %/min) and HsuHF+BJ (0.8283 %/min); HsuHF (0.2867 %/min) and HsuHF+BJ (0.8283 %/min) (Figure 11D).

1.6. Lipidic profile

Serum TGs concentration

Regarding postprandial serum TGs, significantly higher concentrations were found in the HsuHF group (182.33 ± 20.33 mg/dL) compared to the Control (103.20 ± 5.52 mg/dL; p <0.05). This effect was prevented by BJ consumption in the HsuHF+BJ group, which presented significantly lower TGs values (86.29 ± 9.97 mg/dL) compared to the HsuHF (182.33 ± 20.33 mg/dL; p <0.001) and BJ (153.13 ± 16.09 mg/dL; p <0.05) groups (Figure 12).

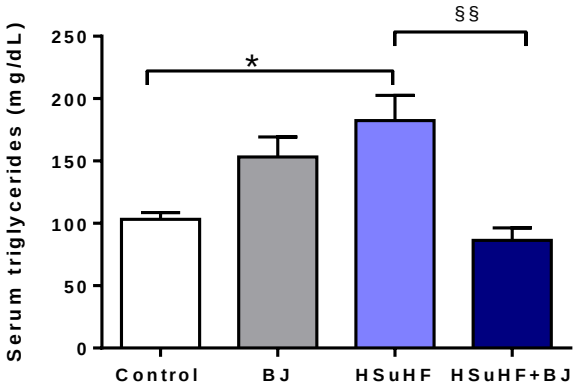

Figure 12. Serum TGs concentration. Data are expressed as mean ± SEM (n=6 per group; * p <0.05 vs Control, §§ p <0.01 vs HsuHF +BJ).

Serum Total-cholesterol concentration

There are no differences between groups regarding serum total cholesterol concentration: Control – 71.25 ± 8.91 mg/dL; BJ – 72.25 ± 13.97; HsuHF – 75.89 ± 15.72; HsuHF+BJ – 74.63 ± 16.42).

1.7. Serum markers of redox and inflammatory status

Serum total antioxidant status (TAS) and MDA levels were used to assess the redox status and hs-CRP to estimate systemic inflammation. TAS was higher in the BJ group, vs the Control, while there were no other relevant changes, including in MDA levels (Figure 13A and 13B). MDA/TAS ratio was significantly higher in the HsuHF group,

an effect that was prevented by BJ treatment (Figure 13C). No statistically significant differences were observed in hs-CRP levels between the four groups (Figure 13D).

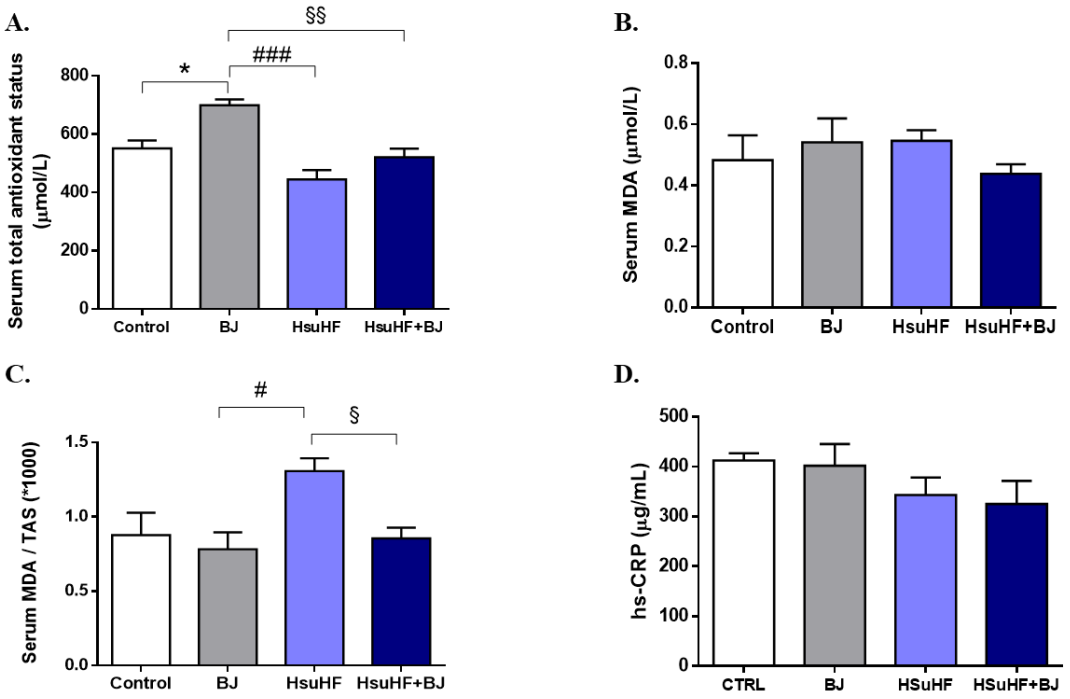

Figure 13. Serum markers of redox and inflammatory status. A. Total antioxidant status; B. MDA concentrations; C. MDA/TAS ratio; D. hs-CRP levels. Data are expressed as mean ± SEM (n=6-9 per group).

2. Renal effects of BJ

2.1. Functional data

Relative kidney weight (in relation to BW) was lower in the HsuHF+BJ group when compared to BJ. Urine creatinine concentration was lower in all groups when compared to the control group, although only significant for the BJ group. Regarding the BUN levels in serum and urine, as well as it's clearance, they were statistically lower in the HsuHF group versus the Control. There were no statistically significant differences regarding uric acid. Concerning glucose, there were no statistically significant differences in serum and clearance, but reduced urine levels were found in the BJ group when compared to control (table 5). GFR decreased in HsuHF group when compared to control one, an effect that tend to be ameliorated by BJ supplementation (Figure 14).

Table 5. Serum and urine renal parameters.

Parameters	Control	BJ	HsuHF	HsuHF+BJ
Kidney Weight				
Absolute (g)	2.86 ± 0.13	3.05 ± 0.13	2.90 ± 0.12	2.87 ± 0.13
Vs to BW (g/kg)	5.63 ± 0.22	5.76 ± 0.16	4.92 ± 0.07	**4.92 ± 0.19**$^{\#}$
Creatinine				
Serum (mg/dL)	0.36 ± 0.01	0.36 ± 0.02	0.38 ± 0.02	0.34 ± 0.02
Urine (mg/dL)	100.40 ± 14.78	**48.19 ± 8.28***	61.15 ± 7.84	73.07 ± 15.63
Clearance (mL/h)	200.40 ± 18.55	181.90 ± 17.55	184.4 ± 11.57	209.10 ± 19.43
BUN				
Serum (mg/dL)	14.19 ± 0.58	14.56 ± 1.02	**8.57 ± 0.60*****	11.71 ± 1.02
Urine (mg/dL)	1554.00 ± 132.80	**653.90 ± 69.62*****	**493.00 ± 82.90*****	**472.10 ± 147.30*****
Clearance (mL/h)	92.53 ± 7.24	93.06 ± 8.74	**35.59 ± 4.29****	53.67 ± 8.22
Uric Acid				
Serum (mg/dL)	1.79 ± 0.33	1.99 ± 0.37	2.33 ± 0.52	1.91 ± 0.30
Urine (mg/dL)	12.80 ± 1.74	**6.15 ± 0.97***	9.86 ± 0.69	9.48 ± 1.78
Clearance (mL/h)	6.50 ± 1.10	5.58 ± 0.84	6.00 ± 1.02	5.93 ± 1.65
Glucose				
Serum (mg/dL)	149.1 ± 7.95	159.0 ± 11.15	**273.10 ± 33.53**$^{***, \#}$	192.90 ± 12.24$^{\$}$
Urine (mg/dL)	18.86 ± .2.31	11.00 ± 2.56	**7.29 ± 2.10***	11.00 ± 3.41
Clearance (mL/h)	0.10 ± 0.01	0.10 ± 0.01	**0.04 ± 0.01****	0.06 ± 0.01

Data are expressed as mean ± SEM * p <0.05 vs Control, ** p <0.01 Control, *** p <0.001 vs Control (n=6 per group).

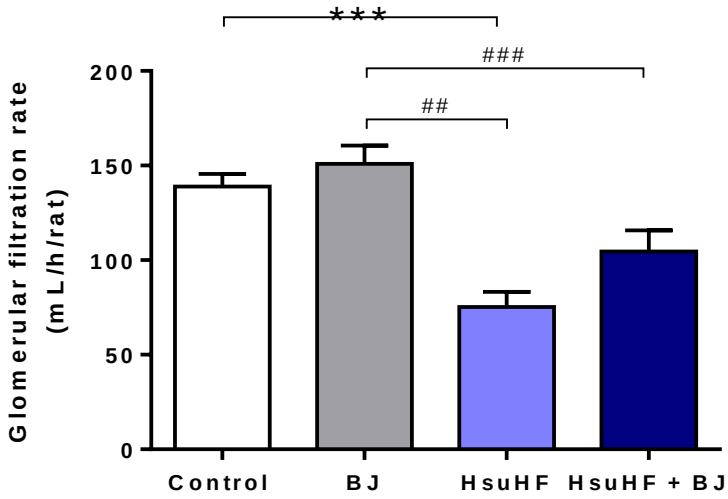

Figure 14. Glomerular filtration rate (GFR). Data are expressed as mean ± SEM(n=6 per group; *** p <0.001 vs Control, ## p <0.01 vs HsuHF +BJ, ### p <0.001 vs HsuHF +BJ).

2.2. Histomorphological data

H&E staining

As depicted in Figure 15 (g,h), glomeruli corresponding to the HsuHF and HsuHF+BJ groups exhibit structural features resembling what has been previously described as glomerular crescents, when compared with glomeruli from the other groups, which was accompanied (data not shown) by other glomerular lesions, mainly middle ones, such as mesangial expansion and thickening of glomerular basement membrane. Regarding tubules (i-l), no structural differences were observed between the groups aforementioned.

Using H&E staining, we performed a quantitative evaluation of the glomeruli displaying glomerular crescent-like lesions (a total of 100 glomeruli per experimental group were analyzed in randomly chosen sections). Figure 16 represents the percentage of glomerular crescents in the four groups. It can be concluded that the results of the HsuHF and HsuHF+BJ groups were higher and statistically significant, when compared to the Control group.

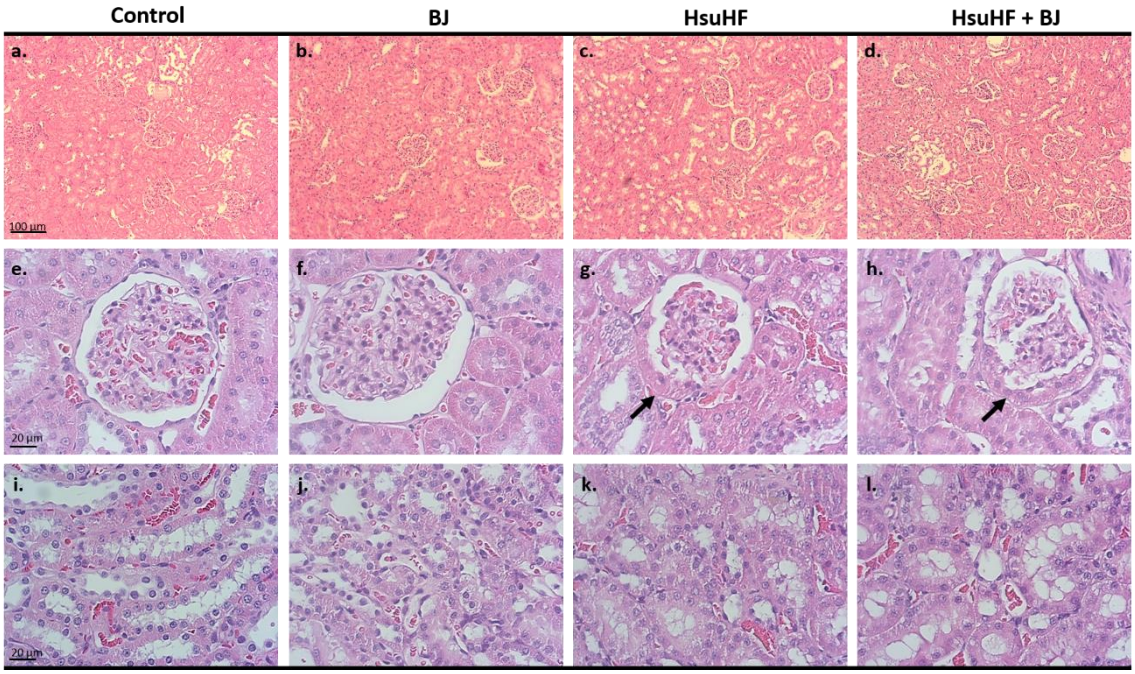

Figure 15. Representative images of renal tissue (a-d, 10x). Morphological features of glomeruli (e-h) and tubules (i-l) are highlighted at 40x magnification. H&E staining.

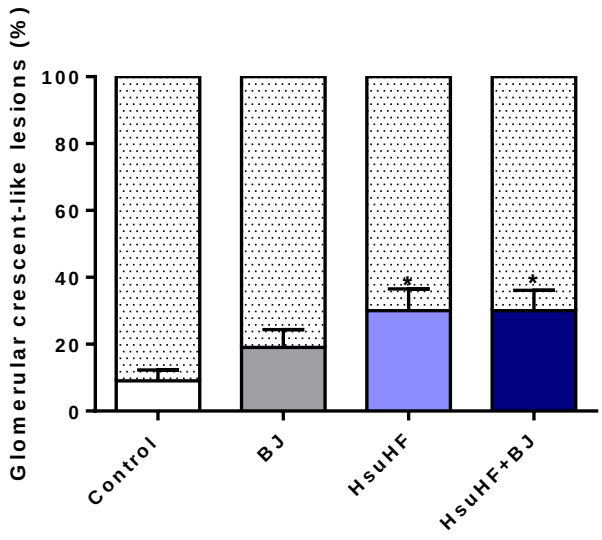

Figure 16. Glomerular crescent-like lesions (full-coloured column) versus non-lesioned glomeruli (black-dot column). * p <0.05 vs Control

Lipid deposition (Oil Red O staining)

Regarding the Oil Red O staining, it can be noted, with a 40x amplification, that there is renal lipid accumulation in the HsuHF and HsuHF+BJ groups, regarding the

higher intensity of lipid red spots when compared with the remaining groups (Figure 17, upper panels).

With a higher amplification level (100x), this difference is even more notorious. In fact, a higher portion of reddish colouring is revealed in the groups which were submitted to a hypercaloric diet. However, in the HsuHF group, the higher intensity spots are more evident and agglomerated (Figure 17, lower panels).

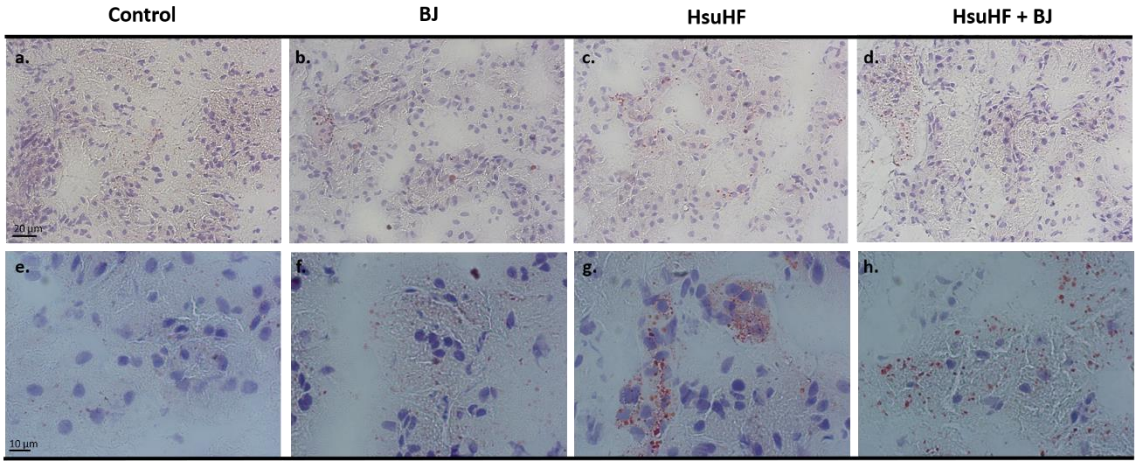

Figure 17. Representative images of renal lipid deposition at 40x (a-d) and 100x (e-h). Oil Red O staining.

2.3. Kidney lipid peroxidation

No statistically significant differences were observed in MDA levels in the kidney tissue (Figure 18) between the four groups.

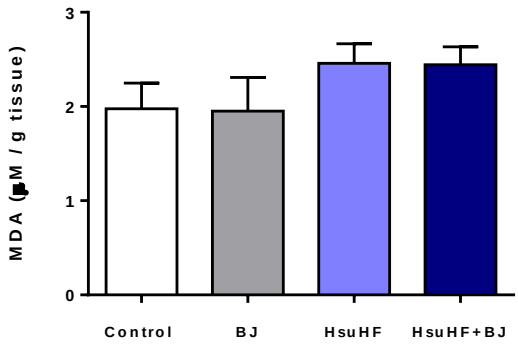

Figure 18. Renal malondialdehyde (MDA) levels in experimental groups (n=6-9 per group).

2.4. Renal inflammatory markers

As noted above, IL-6, TNF-α, iNOS and MMP2 markers were evaluated in the renal tissue by RT-PCR. Additionally, iNOS, MMP2, TNF- α, TLR-4 and RAGE were evaluated by Western Blot.

Regarding IL-6, it presented statistically significant differences between HsuHF and Control and HsuHF and BJ groups.

Concerning TNF-α, iNOS and MMP2 there were no statistically significant differences in genetic and protein levels between the groups, as shown below (Figure 19). Nonetheless, for TNF-α it appears to exist a tendency so that the groups with BJ supplementation have a decrease in its relative expression. Also, in relation to the molecular weight of TNF-α, we noted that – despite its normal value its around 26kDa – the band reached 35kDa. With regards to iNOS, higher values were found in hypercaloric groups, when comparing with the remaining groups – even though these are not statistically significant. The molecular weight of iNOS was measured around 100-150 kDa. MMP2, on its turn, showed more elevated expression levels in the HsuHF and HsuHF+BJ, in comparison with the control and BJ groups, despite this difference also not being considered statistically significant. The molecular weight of MMP2 was measured around 72 kDa.

Equally, taking into account the protein results of TLR-4 and RAGE, no statistically significant differences have been registered. In respect of TLR-4, we noticed that, despite its molecular weight being, usually, between 90 and 100 kDa, it can also be between 36-55 kDa. According with our results, the measured molecular weight was between 35 and 48 kDa. For RAGE, on its turn, the quantification was performed to a molecular weight of 45 kDa.

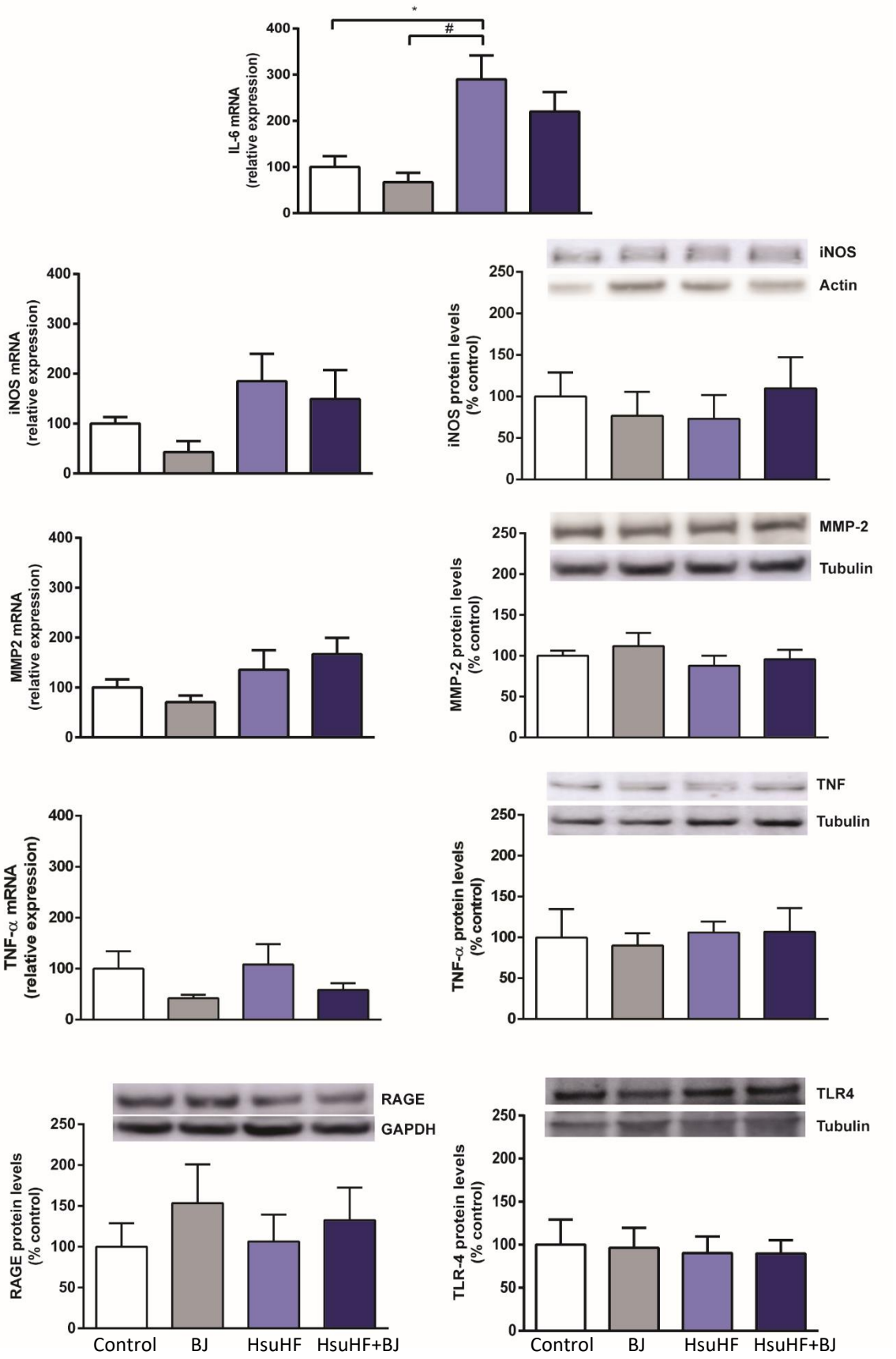

Figure 19. Renal gene and protein expression of inflammatory markers. Data (percentage of controls) are expressed as mean ± SEM (n=4-6 per group; * p <0.05 vs Control, # p <0.05 vs BJ).

DISCUSSION

Currently, diabetes affects millions of people worldwide and the estimations for 2045, made by IDF, are that approximately 629 million adults will have this disease. Regarding prediabetes, about 7.3% of the adult population has IGT and future projections is that this numbers will further increase. Given the serious consequences and complications of diabetes, such as cardiovascular disease, diabetic retinopathy and neuropathy and principally diabetic nephropathy – which is the main focus of this dissertation and the main cause of ESRD, affecting approximately a third of the population with T1DM and T2DM –, an early intervention can be crucial and should be a strategy to favor whenever possible.

As it is widely described in the literature and as it has been extensively explained in this dissertation, T2DM results largely from risk factors associated with lifestyle habits. The lifestyle currently adopted by an increasingly number of people is characterized by the ingestion of highly caloric and fat-rich foods, often associated with lack of physical exercise (sedentarism). Studies suggest that a high caloric/high fat diet contributes to insulin resistance and impaired glucose tolerance (Shevalye et al., 2012) (Deng, Shivappa, Tang, Mann, & Hebert, 2017). This eating behaviour is one of the leading causes of the accelerated increase in the prevalence of insulin resistance, T2DM and associated complications. In fact, the regular consumption of food that is either rich in fat and/or in carbohydrates induces acute inflammatory stress in both average weighted and overweight (Joseph et al., 2014). Therefore, tackling these risk factors, namely with nutraceutical strategies, is the first line of intervention in T2DM treatment, which should be made as early as possible (such as in prediabetes). On the other hand, diets containing equilibrated amounts of both fruits and vegetables are inversely correlated with oxidative stress and to less risk of development of metabolic diseases (Joseph et al., 2014). Red fruits in particular, such as the blueberry, have a high concentration of polyphenols (namely anthocyanins) with strong antioxidant and anti-inflammatory activities, which could help in the prevention of onset and progress of several metabolic pathologies (Huang et al., 2018; Noratto, Chew, & Atienza, 2017; Skates et al., 2018).

In a previous work, our group found that BJ was able to protect against lipid deposition and hepatic mitochondrial dysregulation in a rat model of prediabetes. For the present study, we hypothesize that BJ could be a promising nutraceutical strategy to prevent the progression of prediabetes as well as the putative early renal impairment. Our research group has previously characterized a rat model of prediabetes induced by a sucrose-rich (Hsu, 35%) diet for 9 weeks. The animals under this diet have developed insulin resistance as well as glucose intolerance, even without changes on glucose levels in a fasting state. This model was associated with unchanged body weight and blood pressure. In order to evaluate the possible beneficial effects of BJ, we decided to aggravate the metabolic condition. Furthermore, we were also interested to assess whether or not kidney impairment was already present in a prediabetic state as there is some controversy in the literature about the type of early renal abnormalities found in animal models fed hypercaloric diets. Thus, we fed the animals for further 14 weeks (between weeks 9 and 23) with a Hsu+HF diet.

Metabolic effects of BJ in the diet-induced rat model of prediabetes

We have followed the progression of body weight and characterized the glycaemic and insulinaemic profiles at both fasting and post-prandial states, as well as the lipidic profile.

Regarding body weight monitoring, the results were similar between the Control and the HsuHF-treated groups, until the ninth week. This result validates what was previously obtained by our group - the absence of obesity in rats undergoing this type of diet (Burgeiro et al., 2017; Nunes et al., 2013). From the ninth week onward, a divergence regarding body weight was noted between the HsuHF and HsuHF+BJ groups and the Control and BJ groups, which results from the fat-diet treatment and/or from the animals' preference towards the sugary beverage. In fact, there was an increment in the calories' ingestion by the HsuHF e HsuHF+BJ groups, despite the decrease in the

food intake. However, as presented in the Results section – figure 9 –, even with the mentioned reduction in food intake, there was a rise in beverage intake, as already found in other studies (Soares et al., 2013). Therefore, it can be concluded that despite the lower ingestion of chow than the animals in the Control and BJ groups, the hypercaloric diet causes higher overall caloric intake in the HsuHF fed groups than in the normal chow-treated ones.

We have also observed a reduced beverage consumption in the HsuHF+BJ group starting in the ninth week (coincident with lipid diet beginning); on the other hand, food (chow) ingestion has increased in this group. Despite that, the caloric intake was maintained, since there was a compensation between the amount of carbohydrates and lipids.

Regarding the BJ group, we have noticed a similar caloric intake when compared with the Control. Despite the fact that, from the ninth week on, the beverage intake was superior in the BJ group, the chow intake was similar. However, no differences were observed between both groups regarding body weight.

There are some conflicting data regarding the effects of blueberry treatment on body weight. In fact, some studies have demonstrated beneficial effects of BJ in models of obesity and diabetes (Mykkänen et al., 2014; Vuong et al., 2009), with other reported increased insulin sensitivity in obese rodents following blueberries' consumption, significant impact in body weight and adiposity (DeFuria et al., 2009; Nair, Elks, et al., 2014; Takikawa, Inoue, Horio, & Tsuda, 2010), which is in line with our results.

The glycaemic profile was evaluated using distinct parameters, including fast and postprandial blood glucose levels and a glucose tolerance test. Concerning fasting glycaemia, the HsuHF group presented a trend to higher levels when compared with the remaining groups, despite the differences did not reach statistically significant value. Previous studies showed that blueberries were able to significantly reduce blood glucose levels in a fasting state (Roopchanda, Kuhna, Rojoa, M. Lilab, & Raskin, 2011). Postprandial blood glucose levels were significantly higher value in the HsuHF group,

when compared to the other groups. The addition of BJ to the group of animals fed with the high caloric diet (HsuHF+BJ group) promoted a beneficial effect on postprandial glucose values towards normoglycaemia. These results suggest that using BJ might prevent the progression of prediabetes evoked by a hypercaloric diet.

At the end of the experimental protocol, animals were subjected to a GTT. Since the glycaemia levels were higher in the HsuHF and HsuHF+BJ groups when compared with the Control and BJ ones, we can conclude that the hypercaloric diet promoted reduction in glucose tolerance, in agreement with previous studies with identical diet (Mykkänen et al., 2014). In the HsuHF+BJ group, the recovering of glycaemia levels to normal values was faster than in the HsuHF one, thus suggesting an improvement of glucose tolerance, even though above those found for the Control. These results were corroborated by the AUC of the GTT, strongly suggesting amelioration of glucose intolerance by BJ supplementation. Our results are in disagreement with the study of *Mykkänen et al.* (2014) that was unable to obtain differences in AUC between high-fat animals treated and those untreated with blueberries 5% (w/w) or 10% (w/w) (Mykkänen et al., 2014), suggesting that the type and percentage of blueberry consumption could influence the outcome on glycaemia tolerance.

Concerning the insulinaemic profile, we were able to evaluate insulin levels, in fasting and postprandial conditions, perform an ITT test and estimate AUC and KITT curves, as well as quantify HOMA-IR, an index of insulin resistance. Although no statistically differences were observed regarding fasting serum insulin values, in the postprandial state a significantly higher insulin value was obtained in the HsuHF group when compared to the Control. This effect tended to be prevented in the HsuHF+BJ group, despite no reaching statistically significant differences between the HsuHF and HsuHF+BJ groups. Concerning the ITT, glucose levels in the HsuHF group were significantly higher when compared to those of the Control group, confirming a decrease in insulin sensitivity in the animals fed with the high caloric diet. These effects were prevented in the HsuHF+BJ-treated animals, which presented similar values to those of

the Control and BJ groups, thus suggesting an improvement of insulin sensitivity. These effects were corroborated by the AUC and KITT indexes obtained from the insulin tolerance test. The protective effects of blueberries against insulin intolerance were in agreement with previous studies that showed improved insulin sensibility in insulin-resistant volunteers taken blueberries (in beverages) (Joseph et al., 2014; Roopchanda et al., 2011). In addition, *DeFuria et al.* (2009) have found that C57BL/6 mice under a HF diet (60%) plus 4% of blueberries, during 8 weeks, had a lower AUC of glucose (which translates in an increase in insulin sensitivity), in comparison with the mice that were only given the HF diet (DeFuria et al., 2009). Similar results of improved insulin sensitivity (as evaluated by ITT) were observed in KKAy diabetic mice treated with an blueberry-based diet during 5 weeks (Takikawa et al., 2010). Additionally, according with *Seymour et al*. (2011), in obese Zucker rats with hyperlipidemia, insulin resistance and systemic inflammation, blueberry treatment was able to improve insulin sensitivity and reduce AUC glucose levels (Seymour et al., 2011).

Regarding postprandial serum TGs, significantly higher concentrations were found in the HsuHF group, when compared to the Control one. This effect was prevented by the consumption of BJ (HsuHF+BJ group). Previous studies stated that rats under a high-caloric diet (containing sucrose, among other carbohydrates) developed dyslipidaemia (including hypertriglyceridaemia), hypertension, and impaired glucose tolerance, which corroborates our results (Hafizur, Raza, Chishti, Shaukat, & Ahmed, 2015; Panchal et al., 2011). Impaired glucose tolerance was observed in Wistar rats fed a HF diet since the ninth week of experiment, which was associated with the insulin's incapacity to promote glucose absorption, as well as, with the glucose metabolism by glucose-sensitive tissues (Marques et al., 2016). However, according to *Simental-Mendía et al.* (2015), hypertriglyceridaemia was independently associated with isolated IGT (Simental-Mendía, Rodríguez-Morán, & Guerrero-Romero, 2015). Another study performed in rats fed a blueberry-supplemented diet during 12 weeks showed a reduced serum TGs concentration in comparison with animals without blueberries treatment

(Elks et al., 2016), which is in agreement with our results. Regarding total-cholesterol levels, no significant differences were found between the study groups, which is in line with previous data (Hafizur et al., 2015).

Renal effects of BJ supplementation

There were no significant changes on serum and urine markers of renal function in the HsuHF group versus the Control one, excepting a reduce urine BUN and corresponding clearance. This effect might be explained by the reduce amount of protein consumption in this group, considering the reduced food ingestion previously reported. However, taking into account the accentuated reduction in the filtration capacity of the kidney – as depicted by the demarked reduction in the GRF in the HsuFH group – it can be assumed that the hypercaloric diet has led to some degree of renal lesion. *Wang et al.* (2018) reported in rats under an HF diet an increase in body weight, impaired glucose tolerance and insulin resistance. These conditions were accompanied by kidney impairment, as viewed by the reduced GFR (Wang et al., 2018), which is in accordance with our results. In our study, however, there was no effect of the BJ (HsuHF+BJ group) in the serum and urine renal parameters, except a trend to improve the reduced GFR found in the HsuHF group.

Regarding histological findings, we were able to obtain mild glomerular lesions, such as mesangial expansion and thickening of glomerular basement membrane, which are compatible with initial stages of diabetes. However, there was no inflammatory infiltrate nor interstitial fibrosis and tubular atrophy (IFTA) or other type of tubular lesions. Alternatively, and surprisingly, we observed an accumulation of cells inside the limit of Bowman's Capsule which compress and surround the glomerulus, denominated as glomerular crescent (Otani et al., 2012). These glomerular crescents have been associated to rapidly progressive glomerulonephritis (RPGN) as well as to noninflammatory chronic glomerulopathies (Ryu et al., 2012). In our study, a higher percentage of crescents was found in the HsuHF and HsuHF+BJ groups, when compared

to the Control, strongly suggesting a link between hypercaloric diet and this phenomenon.

Considering that there was an increased serum TGs contents in the HsuHF animals, and that these hypertriglyceridaemia was attenuated by BJ treatment (HsuHF+BJ group), we assessed whether there was (or not) lipid accumulation in the kidney. Indeed, we identified, through Oil Red O, lipid accumulation in the kidney, which corroborates the assumption that, previously to DM establishment (prediabetic state), there are already significant changes as a result of a dyslipidemic profile. However, concomitant BJ treatment was unable to promote significant reduction of renal lipid accumulation, as viewed by the Oil Red O staining, contrasting with the beneficial effect found in serum as well as in the liver tissue (data not shown).

Besides the extra-renal lipid accumulation, namely in the retroperitoneal space, pararenal space, perirenal space and hilar space, there might also be an accumulation in the parenchyma (intra-renal) (Mende & Einhorn, 2019). In DM conditions, the sterol regulatory element-binding proteins (SREBPs) become expressed, which lead to the production of TGs and enhance lipogenic gene expression, causing an excessive lipid accumulation in the kidney (Su, Cao, He, Guan, & Ruan, 2017; Weinberg, 2006). With this being said and since we are dealing with a prediabetic stage in which there is already is lipid accumulation, it would be very relevant, in the future, to mark these proteins – SREBPs – in order to investigate if the activated pathway is the same as in an already established condition (overt diabetes).

Oxidative stress is involved in the progression from prediabetes to T2DM, with a progressive failure of antioxidant mechanisms and a higher production of ROS (Bigagli & Lodovici, 2019; Priyatharshini et al., 2017). Several studies show that oxidative stress is already evident in the prediabetic state (Agarwal et al., 2016; Mahat et al., 2019; Priyatharshini et al., 2017). Thus, we aimed to evaluate if this rat model already presents signs of oxidative imbalance and, in parallel, to investigate the antioxidant effects of BJ. We observed in this early stage of the diabetic disease (prediabetes) the absence of

significant changes regarding lipid peroxidation in the serum and in the kidney, as measured by MDA levels, although there was a trend to increased values in the groups under hypercaloric diet. Studies performed in humans have shown that blueberry consumption leads to an increase in the plasma's antioxidant capacity (Neto, 2007), and our results are in accordance since BJ group had a higher serum TAS, when compared to the other groups. Anthocyanins, one of the main phenolic groups present in the blueberries, have been described as potential inhibitors of lipid peroxidation (Brown & Kelly, 2007), which can explain the fact that, in our results, the serum MDA/TAS ratio is higher in the HsuHF group and ameliorated in the animal treated with BJ.

As mentioned before, inflammation is the normal reaction of the body to an infection or tissue injury (Bharara, M. et al., 2009; Priyatharshini et al., 2017). Nevertheless, inefficient regulation or recurrent stimuli lead to chronic inflammation with increased production of proinflammatory cytokines (Joseph et al., 2014). Considering the close link between hyperlipidaemia, lipotoxicity and inflammation, we wondered whether there were impaired molecular markers of inflammation in serum and kidney tissue. For that purpose, we assessed serum hsCRP and renal gene/protein expression of several relevant molecules, such as IL-6, TNF-α, iNOS, MMP2. RAGE and TLR-4.

We did not observe any significant difference in serum hsCRP levels in any experimental group, hinting for a lack of an overt inflammatory status in this stage of metabolic impairment. Then, we looked for inflammatory related markers in renal tissue. IL-6 levels have been associated with BMI in prediabetes subjects, suggesting that an increase in body weight contributes to a state of chronic inflammation (Agarwal et al., 2016). Our results demonstrated an increased expression of IL-6 mRNA in the HsuHF group, together with increased body weight, which is concordant with *Agarwal et al.* (2016). Considering that IL-6 could act as an anti-inflammatory cytokine, whether this IL-6 increment, also viewed in the HsuHF+BJ group, is a counter anti-inflammatory effect as a result of HF diet should be further dissected. Regarding TNF-α, iNOS, MMP2, RAGE

and TLR-4, there were no significantly differences between the experimental groups. Overall, it seems that in this model of prediabetes induced by a hypercaloric diet, renal inflammation is not as relevant as other possible mechanisms, at least to explain the impaired renal function. Distinct inflammatory mediators, namely those more closely related with lipidosis, deserve to be assessed in the future.

To sum up, we were able to demonstrate that the prediabetic model submitted to 23 weeks of hypercaloric diet – characterized by high levels of carbohydrates and lipids (HsuHF), which mimics the eating habits of a major portion of the current society – experienced body weight gain, hyperglycaemia in a post-prandial state (with normoglycaemia in a fasting state), a reduction of glucose tolerance, an increase in the serum concentration of insulin in a post-prandial state, insulin resistance, an elevation in the serum concentration of TGs and a decrease in the total antioxidant ability in the serum. Concerning renal function and lesions, there was a decrease in GFR, an increase in lipid deposition (verified through the Oil Red O staining) and an early glomerular lesion (evident by the presence of glomerular crescent-like lesions) but without inflammatory markers (except IL-6, which presented significant changes).

The effects of BJ when added to an hypercaloric diet (HsuHF+BJ), could be summarized as follows: BJ was able to improve insulin sensitivity and glucose tolerance, as well as ameliorate hypertriglyceridaemia. Regarding renal function and lesions, BJ was able to partially improve the levels of GFR, although not statistically significant, but had no effect in the lipid deposition, inflammation and in the glomerular crescent-like lesions.

Overall, BJ seems to be an interesting nutraceutical strategy against the progression of glucose tolerance and insulin sensitivity in a prediabetic state, despite very limited renoprotective properties, at least when viewed by the parameters assessed in this work. The underlying mechanisms the evolution of renal dysfunction induced by hypercaloric diet, as well as the metabolic improvement promoted by BJ should be further dissected.

The results of our work raised other questions for future research, namely why is there a decrease in GFR rather than an increase in the HsuHF group; since we are dealing with an early stage (prediabetes), the increase in GFR would be the compensation mechanism that would be expected, in opposition with the observed data, thus suggesting a better clarification of the disease stage, namely regarding renal (dys)function/lesion, in this animal model. Another important topic to dissect is the impact of lipid deposition on renal tissue and whether there is a relationship between lipid accumulation and crescent-like glomerular lesions.

CONCLUSION

The main conclusions to take of this dissertation, regarding the influence of BJ in early renal lesions – in a prediabetic rat model, induced by an hypercaloric diet – can be summarized as follows:

A – Concerning the rat model of prediabetes:

- 23 weeks of hypercaloric diet was able to induce some of the main features of prediabetes, including postprandial hyperglycaemia (with fasting normoglycaemia), glucose intolerance, postprandial hyperinsulinaemia, together with reduced sensitivity to insulin (insulin resistance).
- Despite a moderate effect on body weight (about 10%), there was a clear hypertriglyceridaemia and lipid deposition in the kidney, without obvious signs of systemic and renal inflammation.
- The reduced GFR and the existence of glomerular lesions, including formation of glomerular crescents, suggests the existence of a prediabetic renal dysfunction.

B – Concerning the metabolic and renal effects of BJ supplementation:

- There was a consistent improvement of glucose tolerance and insulin sensitivity, accompanying by correction of hypertriglyceridaemia, without a significant impact on renal lipidosis.
- Regardless of a trend to GFR correction, the impact on glomerular lesions was very modest, without significant effect on markers of inflammation.

Additional studies are required in order to better understand the protective effects of the blueberries towards preventing prediabetes evoked by an hypercaloric diet. For instance, it would be crucial to study the effects of different BJ doses, as well as, obtaining results for other duration of exposure. The analysis of more specific markers for the renal tissue should also be a topic to take into consideration so that new data can be clearer and more specific.

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