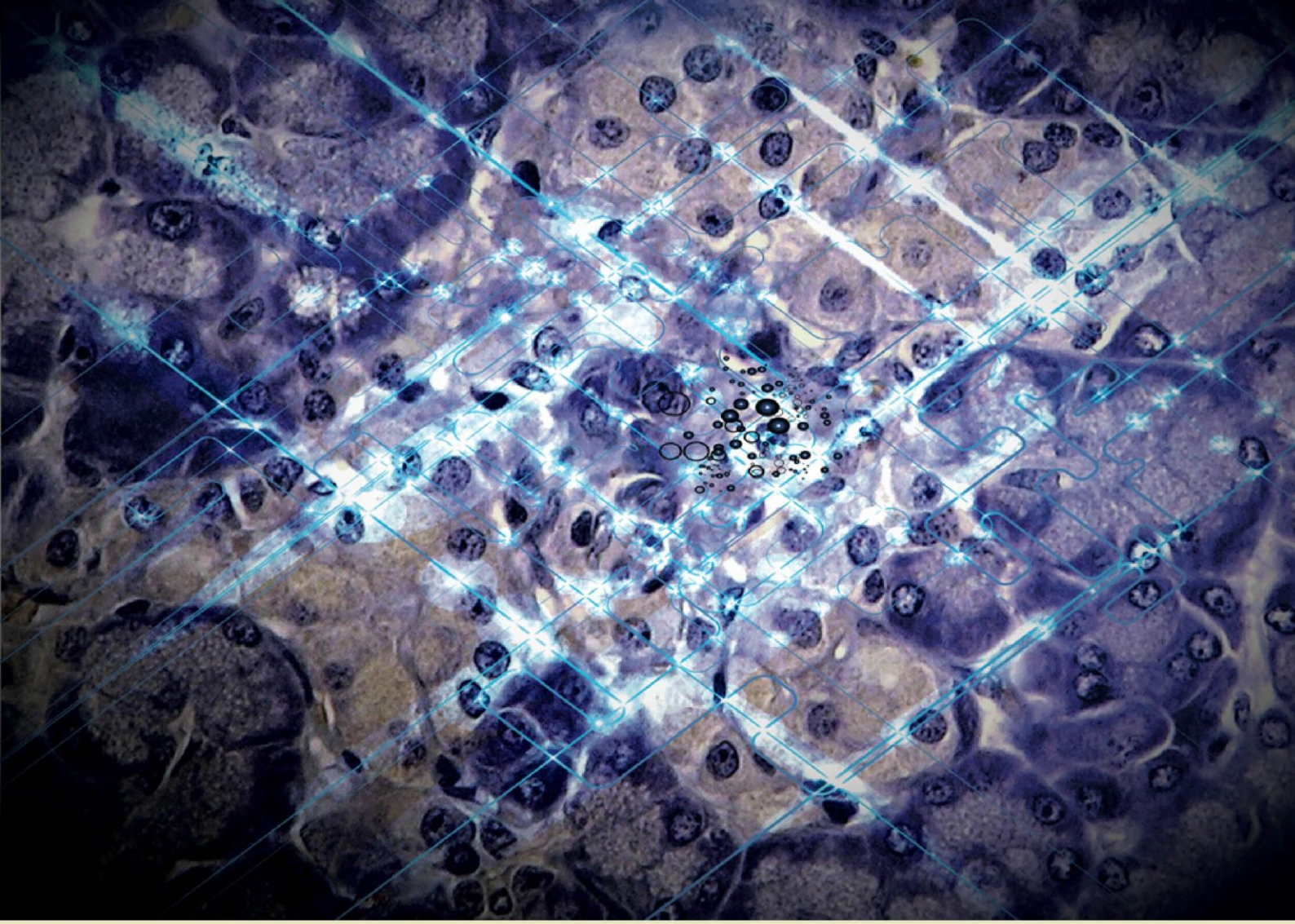

Ana Cristina Pais Mega de Andrade

EFFECTS OF SITAGLIPTIN THERAPY ON THE EVOLUTION OF PANCREATIC AND RENAL LESIONS IN TYPE 2 DIABETES - EXPERIMENTAL STUDY IN THE ZUCKER DIABETIC FATTY RAT MODEL

Doctoral Thesis in Health Sciences, supervised by Doctor Flávio Reis, Doctor Rosa Fernandes and Professor Edite Teixeira de Lemos, presented to the Faculty of Medicine, University of Coimbra.

February, 2017

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Universidade de Coimbra

Ana Cristina Pais Mega de Andrade

Effects of sitagliptin therapy on the evolution of pancreatic and renal lesions in type 2 diabetes - experimental study in the Zucker Diabetic Fatty rat model

Efeitos da terapêutica com sitagliptina na evolução das lesões pancreáticas e renais na diabetes tipo 2 - Estudo experimental em ratos *Zucker Diabetic Fatty*

Doctoral Thesis in Health Sciences, supervised by Doctor Flávio Reis, Doctor Rosa Fernandes and Professor Edite Teixeira de Lemos, presented to the Faculty of Medicine, University of Coimbra.

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ABBREVIATIONS AND ACRONYMS

ACTB	actin Beta
ADA	American Diabetes Association
AIDS	acquired Immunodeficiency Syndrome
AIHW	Australian Institute of Health and Welfare
AGE	advanced glycation end-products
Akt	protein kinase B *alias* PKB
AMI	acute myocardial infarction
ASP	acylation-stimulating protein
Bax	Bcl-2 associated X Protein
Bcl-2	B-Cell CLL/Lymphoma 2
BID	BH3 interacting-domain death agonist
BMI	body mass index
BP	blood pressure
BSA	bovine serum albumin
BUN	blood urea nitrogen
BW	body weight
cAMP	cyclic adenosine monophosphate
CB	capsule of Bowman
CKD	chronic renal disease
CNS	central nervous system
CVD	cardiovascular disease
CRP	C-reactive Protein
DAPI	4',6-diamidino-2-phenylindole
DM	diabetes mellitus
DN	diabetic nephropathy
DNA	deoxyribonucleic acid
DOC	deoxycholate
DPP-	4 dipeptidyl-peptidase 4
sDPP-4	soluble dipeptidyl-peptidase 4
DPP-8	dipeptidyl-peptidase 8
DPP-9	dipeptidyl-peptidase 9
EASD	European Association for Study of Diabetes EASD
EDTA	ethylene-diaminetetra-acetic acid
EGTA	ethylene glycol tetra-acetic acid
EMA	European Medicines Agency
ESRD	end-stage renal disease
EXAMINE	Examination of Cardiovascular Outcomes with Alogliptin versus Standard of Care in Patients with Type 2 Diabetes Mellitus and Acute Coronary Syndrome
FDA	Food and Drug Administration
FFAs	free-fatty acids
FPG	fasting plasma glucose

G0	grade 0
G1	grade 1
G2	grade 2
G3	grade 3
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GBM	glomerular basement membrane
GI	gastrointestinal
GIP	gastric inhibitory polypeptide / glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
GLP-1	glucagon-like peptide 1
GLP-1R	GLP-1 receptor
GLUT-1	glucose transporter 1
GLUT-2	glucose transporter 2
GLUT-4	glucose transporter 4
GPCRs	G-protein-coupled receptors
HbA1c	glycated haemoglobin
HDL	high density lipoproteins
HE	haematoxylin and eosin
HFD	high fed diet
HGP	hepatic glucose production
HF	heart failure
H2O2	hydrogen peroxide
HOMA-IR	homeostatic model assessment – insulin resistance
HSL	hormone-sensitive lipase
IAD	iodoacetamide
IDF	International Diabetes Federation
IFTA	interstitial fibrosis plus tubular atrophy
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL-1β	Interleucin-1-beta
IL-6	Interleucin-6
Ki-67	nuclear protein/antigen KI-67
KW	kidney weight
LDL	low density lipoproteins
LPL	lipoprotein lipase
LSAB	labelled streptavidin-biotin-peroxidase method
MACE	major adverse cardiovascular events
MCP-1	monocyte chemotactic protein-1
MDA	malondialdehyde
MO	microwave oven
MS	metabolic syndrome
NCD	Non-Communicable Diseases
NF-κB	nuclear factor kappa B
NICE	National Institute for Health and Care Excellence NICE
NIDDM	non–insulin-dependent diabetes

NPY	neuropeptide Y
OCT	optimal Cutting Temperature compound
PAI-1	plasminogen activator inhibitor-1
PAS	Periodic acid of Schiff
PBS	phosphate buffered saline
PCNA	cell proliferating nuclear antigen
PCR	polymerase chain reaction
PCT	proximal convoluted tubule
PI3K	phosphoinositide 3-kinase
PMSF	phenylmethylsulfonyl fluoride
PPG	2-hours postprandial glucose
PST	proximal straight tubule
PVDF	polyvinylidene difluoride
PYY	pancreatic peptide YY
Q	quick score
RAAS	renin-angiotensin-aldosterone system
RAGE	receptor for advanced glycation end products
mRNA	messenger ribonucleic acid
RIPA	radioimmunoprecipitation assay buffer
ROS	reactive oxygen species
RPL13	ribosomal protein L13'
RT-qPCR	real-time reverse transcription polymerase chain reaction
SAVOR-TIMI 53	Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SGLT1	sodium/glucose cotransporter-1
SGLT2	sodium/glucose cotransporter-2
SPSS	statistical package for the social sciences
TAS	serum total antioxidant status
TBA	2-thiobarbituric acid
TBARs	thiobarbituric acid reactive-species
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TECOS	Trial Evaluating Cardiovascular Outcomes with Sitagliptin
TAG	triacylglycerol
TBM	tubular basement membrane
TGs	triglycerides
TMP	1,1,3,3-tetramethoxypropane
TNF-α	tumor Necrosis Factor-alpha
Total-c	total cholesterol
TOP1	topoisomerase (DNA) I
TRIB3	tribbles pseudokinase 3
TUNEL	deoxynucleotidyl transferase-mediated dUTP nick-end labeling
USA	United States of America
USPSTF	United States Preventive Services Task Force

dUTP	2´-Deoxyuridine, 5´-Triphosphate
VCAM-1	vascular cell adhesion molecule-1
VP	vascular pole
VEGF	vascular endothelial growth factor
VLDL	very low density lipoproteins
WHO	World Health Organization
ZDF	Zucker Diabetic Fatty

ABSTRACT

Diabetes mellitus (DM) is a leading cause of reduced life expectancy, disability, blindness, chronic renal disease and diminished quality of life, as well as, of personal, familial and economic losses. Type 2 diabetes (T2DM) constitutes about 90% of the total of DM cases, and the prevalence numbers are increasing worldwide, including in Portugal. Regardless of the advances in the pharmacological management of DM, many patients still have poor glycaemic control and register progression of several associated complications, namely diabetic nephropathy (DN). DN is the main cause of end-stage renal disease (ESRD) and a major cause of patients' disability. Therefore, new therapeutic approaches are needed to better control the disease and delay the evolution of diabetic complications. The incretin-based therapies, including the dipeptidyl peptidase-4 (DPP-4) inhibitors, have shown ability to improve glycaemic control and ameliorate dysfunction of diabetes-targeted organs, including the pancreas and the kidney. Our group has previously shown that sitagliptin, the first DPP-4 inhibitor, was able to improve glycaemic and lipidic profile in an animal model of T2DM, showing systemic antioxidant and anti-inflammatory properties.

The purpose of this study was to investigate some of the possible mechanisms underlying the protective effects of sitagliptin on pancreatic and renal tissues, in an animal model of T2DM, focusing on apoptosis, oxidative stress, inflammation, angiogenesis and proliferation mediators and markers. Male obese diabetic Zucker Diabetic Fatty (ZDF) rats, aged 20 weeks, were treated with sitagliptin (10 mg/kg BW/day) during 6 weeks and compared to lean ZDF littermates.

Sitagliptin treatment during 6 weeks was able to ameliorate all the metabolic (glycaemic, lipidic and insulinaemic) parameters in the ZDF rats. In addition, evolution of endocrine and exocrine pancreas lesions was prevented by sitagliptin treatment. This was accompanied by a reduced pancreas Bax/Bcl-2 ratio and IL-1β expression, suggestive of an antiapoptotic and anti-inflammatory effect, respectively. Furthermore, sitagliptin promoted a significant overexpression of PCNA and VEGF, indicating pro-proliferative and pro-angiogenic properties, respectively.

In the diabetic kidney, sitagliptin prevented the aggravation of renal damage, including glomerular, tubulointerstitial and vascular lesions, as well as, decreased renal

lipid peroxidation, IL-1β and TNF-α levels. Moreover, kidney Bax/Bcl-2 ratio, Bid protein levels and TUNEL-positive cells were decreased after sitagliptin treatment. Altogether, these data indicate protective effects against oxidative stress, inflammation and the pro-apoptotic state in the kidney of diabetic rats.

In conclusion, in this animal model of obese T2DM (ZDF rat), besides improving glycaemic control, sitagliptin exerted beneficial effects on pancreas and kidney. This study provides new insights into the cytoprotective actions of sitagliptin, namely its anti-apoptotic, antioxidant, anti-inflammatory and pro-proliferative properties. These findings might contribute to the development of novel approaches to manage T2DM and DN.

Keywords: type 2 diabetes, sitagliptin, cytoprotective properties, pancreas, kidneys, Zucker Diabetic Fatty rat.

RESUMO

A diabetes mellitus (DM) é uma das principais doenças responsáveis por incapacidade física, cegueira, insuficiência renal crónica e pela redução da qualidade e da esperança de vida, bem como de elevadas perdas pessoais, familiares e económicas. A DM tipo 2 (*T2DM*) representa cerca de 90% dos casos de DM, estando a sua prevalência a aumentar a nível mundial, incluindo em Portugal. Apesar dos avanços farmacológicos no tratamento da *T2DM*, muitos doentes ainda apresentam um controlo glicémico deficiente, assim como um agravamento da doença e das suas complicações, nomeadamente da nefropatia diabética (*DN*). A *DN* constitui a principal causa de doença renal terminal (*ESRD*), sendo uma das principais causas de incapacidade física dos doentes. Perante este quadro, são necessárias novas abordagens terapêuticas para controlar melhor a evolução da *T2DM* e retardar a progressão das complicações associadas. As terapias baseadas em incretinas, incluindo os inibidores da dipeptidil peptidase-4 (*DPP-4*), demonstraram capacidade para melhorar o controlo glicémico e contribuir para melhorar a disfunção de órgãos alvo da diabetes, entre os quais se encontram o pâncreas e o rim. O nosso grupo demonstrou previamente que a sitagliptina, o primeiro inibidor da DPP-4, foi capaz de melhorar o perfil glicémico e lipídico num modelo animal de *T2DM*, exibindo ainda propriedades antioxidantes e anti-inflamatórias a nível sistémico.

O objectivo deste estudo foi investigar alguns dos possíveis mecanismos subjacentes aos efeitos protectores da sitagliptina no tecido pancreático e renal, num modelo animal de *T2DM*, focando os mediadores e marcadores de apoptose, stresse oxidativo, inflamação, angiogénese e proliferação. Ratos obesos e diabéticos *Zucker Diabetic Fatty* (*ZDF*), do sexo masculino, com 20 semanas de idade, foram tratados com sitagliptina (10 mg/kg /dia) durante 6 semanas e comparados com os seus controlos, ratos ZDF magros.

O tratamento com sitagliptina, durante as 6 semanas, melhorou todos os parâmetros metabólicos (glicémicos, lipídicos e insulinémicos) nos ratos diabéticos *ZDF*. Adicionalmente, a sitagliptina evitou o agravamento das lesões pancreáticas endócrinas e exócrinas, apresentando simultaneamente uma redução, da expressão de *Bax/Bcl-2* e de *IL-1β* no pâncreas, sugerindo um efeito anti-apoptótico e anti-

inflamatório, respectivamente. Além disso, a sitagliptina promoveu um aumento significativo da expressão de *PCNA* e de *VEGF*, indicando propriedades pró-proliferativas e pró-angiogénicas, respectivamente.

No rim diabético, a sitagliptina preveniu o agravamento das lesões renais, nomeadamente das glomerulares, túbulo-intersticiais e vasculares, e diminuiu a peroxidação lipídica e os níveis de *IL-1β* e de *TNF-α*. Concomitantemente, o tratamento com sitagliptina reduziu a razão *Bax/Bcl-2*, os níveis de proteína *Bid* e as células *TUNEL*-positivas no rim dos ratos diabéticos. Em conjunto, estes dados indicam efeitos protectores contra o stresse oxidativo, a inflamação e o estado pró-apoptótico no rim de ratos diabéticos.

Em conclusão, neste modelo animal obeso de *T2DM* (o rato *ZDF*), a sitagliptina, para além de melhorar o controlo glicémico, exerceu efeitos benéficos ao nível do pâncreas e do rim. Este estudo oferece uma nova perspectiva sobre as acções citoprotectoras da sitagliptina, nomeadamente em relação às suas propriedades anti-apoptóticas, antioxidante, anti-inflamatória e pró-proliferativa. Estas descobertas podem contribuir para o desenvolvimento de novas abordagens para o tratamento da *T2DM* e da *DN*.

Palavras-chave: diabetes mellitus tipo 2, sitagliptina, propriedades citoprotectoras, pâncreas, rim, ratos *Zucker Diabetic Fatty*.

PART I:

INTRODUCTION AND AIMS

Chapter 1

The global burden of type 2 diabetes

"I like beautiful melodies telling me terrible things." Tom Waits (Musician)

1.1 OVERVIEW

Diabetes mellitus (DM) is a heterogeneous group of chronic, serious disorders, characterized by a defect in insulin secretion and/or peripheral insulin resistance (in muscle, liver and fat cells). Both phenomena isolated or in combination, reduce glucose entry into cells, contributing to persistent hyperglycaemia, which seriously raises the risk of microvascular (nephropathy, retinopathy and neuropathy) and macrovascular (ischaemic heart disease, stroke and peripheral vascular disease) complications. DM is associated with diminished quality of life and reduced life expectancy (Cernea et al., 2013; Forbes et al., 2013).

Diagnostic criteria for DM and intermediate hyperglycaemia (also known as prediabetes) have been established by the World Health Organization (WHO), the International Diabetes Federation (IDF), as well as by regional diabetes associations, such as the European Association for the Study of Diabetes (EASD), the American Diabetes Association (ADA) and the American Association of Clinical Endocrinologists/American College of Endocrinology (AACE/ACE) (WHO, 2006; EASD, 2013; IDF, 2015; AACE/ACE, 2016; ADA, 2017).These criteria are based on fasting plasma glucose (FPG) levels, on 2-hour plasma glucose (2-h PG) levels following a 75g oral glucose load in an oral glucose tolerance test (OGTT) and on glycated haemoglobin (HbA1c), as represented on Table 1.1 regarding ADA guidelines. Individuals with glucose levels above the normal range, but which do not yet meet the criteria for the diagnosis of diabetes are said to have intermediate hyperglycaemia, which is a state in which a person has impaired glucose tolerance (IGT) or impaired fasting glucose (IFG). IGT and IFG are not clinical entities, but rather risk factors for development of T2DM and cardiovascular disease (CVD).

Table 1.1 – Diagnostic criteria for intermediate hyperglycaemia and diabetes

Diagnostic criteria	NORMAL	INTERMEDIATE HYPERGLYCAEMIA		DIABETES
		IGT	IFG	
FPG	< 5.5 mmol/l (100 mg/dl)	< 7.0 mmol/l (126 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	< 7.8 mmol/l (140mg/ dl)	≥ 11.1 mmol/l (200 mg/dl)
HbA1c	<5.5%	5.5 to 6.4%	5.5 to 6.4%	≥6.5%

FPG - fasting plasma glucose; 2-h PG - 2-hour plasma glucose levels; HbA1c – glycated haemoglobin A1c; IGT - impaired glucose tolerance and IFG - impaired fasting glucose. * Fasting is defined as no caloric intake for at least 8 h. Adapted from ADA (2017).

DM is expressed in many disease formats, being type 1 (T1DM) and T2DM the two most common forms. Both are caused by a combination of genetic and environmental risk factors (Thanabalasingham et al., 2011). T1DM is caused and initiated by the autoimmune destruction of pancreatic β-cells, and represents approximately 10% of all cases of DM. In contrast, T2DM is the most common form of the disease, accounting for approximately 90 % of all affected individuals (Lyssenko, 2013; ADA 2017). It is mostly initiated by a peripheral insulin resistance and evolves to a relative impaired insulin secretion, which, combined, evolve to persistent hyperglycaemia, β-cell impairment and all of its associated micro and macrovascular complications. It was previously designated as non–insulin-dependent diabetes (NIDDM) or adult-onset diabetes. T2DM is a metabolic disorder with a multifactorial aetiology, with involvement of oxidative stress and inflammation, characterized by disturbances in energy metabolism. There seems to be a genetic component with many genes involved, but their link to the development of the disease is difficult to clarify due to the heterogeneity of the concurring environmental agents, such as lifestyle and neonatal environment during gestation (Baig, 2008; Ozougwu et al., 2013).

Although there is some disparity regarding the many reasons for the development of T2DM, most physicians and scientists have agreed, over the last four decades, that T2DM develops when a diabetogenic lifestyle acts in conjunction with a predisposed genotype, in which the majority of patients exhibit an obese physical profile (Sanghera et al., 2012; IDF, 2014; ADA, 2016). Thus body mass index (BMI), excess weight duration, and distribution of corporal adiposity provide reliable indicators that can be associated to the risk level of an individual developing T2DM. This risk is co-dependent on age (over 45 years), gender (women previously diagnosed with gestational diabetes), ethnicity (African American, Latino, Native American, Asian American, Pacific Islander), high-energy diets, sedentary life, family history of T2DM with an affected first-degree relative, IGT, IFG, hypertension and dyslipidaemia (hypertriglyceridaemia and/or hypercholesterolaemia) are also predisposing factors for the development of T2DM (ADA, 2017). Remarkably, long term consumption of high-energy diets constitutes an independent risk factor, not needing to be related to the obesity baselines for the development of T2DM (Jerant et al., 2015; Nuttall, 2015). Though the main age risk group lies in the population over 45 years, clinically based reports and regional studies suggest that T2DM is being more frequently diagnosed in children and adolescents with a consumption of high-energy diets, a sedentary lifestyle and with an affected first-degree relative (Sahoo et al., 2015; Atkin et al., 2016).

1.2 EPIDEMIOLOGICAL DATA

The United Nations has declared that the global burden of chronic Non-Communicable Diseases (NCDs) constitutes one of the major challenges/obstacles for progress in the 21st century, particularly in developing countries. DM, along with cancer, CVDs and chronic respiratory diseases, represents about 80% of NCD mortality (WHO, 2013).

Diabetes epidemiological studies are performed every year, worldwide, considering the total population in the age group between 20 and 79 years. IDF has updated its 7th edition report and the latest register for 2015 displays 415 million

people with diabetes worldwide, paralleling to one adult in eleven, representing a prevalence of 8.8%, which expresses an increase of 28 million more cases and a rise of 0.5% in prevalence, in comparison to the 2014 IDF data (IDF 2014; IDF, 2015). About 75% of affected people live in low and middle income countries. Demographic distribution shows that Western Pacific and South-East Asia are the most afflicted regions, with 153.2 million and 78.3 million people affected by DM, respectively. Diabetes and its complications accounted for 5.0 million deaths in 2015, representing 14.5% of global all-cause mortality among people in this age group, in which 46.6% were individuals under 60 years old. This death-toll is higher than the combined number of deaths from main infectious diseases in 2013 (1.5 million deaths from HIV/AIDS, 1.5 million from tuberculosis and 0.6 million from malaria) (WHO, 2013; IDF, 2015). At the same time, it is estimated that 193 million people are still undiagnosed worldwide and that every six seconds a person dies from diabetes (IDF, 2015). If these trends continue, by 2040 some 642 million people, or one adult in ten, will have diabetes. The largest increase will take place in the regions where economies are moving from low-income to middle-income levels.

Although the prevalence of T2DM increases with population age, IDF (2015) reports that there are 320.5 million working age (20-64 years) and 94.2 million aged 65-79 people with diabetes. In developing countries, the largest number of people with diabetes lies in the 45 to 64 years age group, while in developed countries the largest number is found in those aged 65 years and older. This discrepancy largely reflects the differences in population age structure between developed and developing countries. For some time now, evidence-based medicine has long been registering a surge of T2DM in worldwide young adult population, and this has urged new epidemiological studies in various countries (AIHW, 2014; Chung et al., 2014). Among people aged 35 - 44 years, which do not come under the recommendation (45 years) for universal screening by ADA, most (71%) were found to be overweight or obese and all had at least one other ADA risk factor. Only 34% of individuals aged ≥ 35 years met United States Preventive Services Task Force (USPSTF) criteria, which means if these guidelines were strictly followed, it would have resulted in the majority (61%) of potential positive test cases being missed (5,508.164 cases in the USA) (Chung et al., 2014). There is little gender difference in the global number of people affected by

diabetes in 2015 and estimated for 2040. There are about 15.6 million more men than women with diabetes (215.2 million men vs 199.5 million women) (IDF, 2015).

The Portuguese situation follows the same trend, as the latest data from 2014 show, in the population between 20 and 79 years (7.7 million people), a 7.4% estimated prevalence of diagnosed diabetes (over 1 million people), with 5.7% of undiagnosed individuals, which is considered by the National Diabetes Observatory Report (2015) to correspond to a total prevalence of 13.1%. Furthermore, 27.2% of the population (2.1 million people) displays intermediate hyperglycaemia, which together with a 13.1% total prevalence, indicates that about 40.3% (3.1 million individuals) of the Portuguese population already have diabetes or is at great risk of developing the disease. As to sex distribution, males have greater prevalence (15.8%) than females (10.8%). In 2013, 4,683 deaths were registered due to diabetes, with the mean age of death being of 80.2 years. As to the main diabetic complications, the prevalence of chronic kidney disease (CKD) was 27.8% in 2014 (Observatório Nacional da Diabetes, 2015).

Summarizing, T2DM is a chronic disease in which aggravation leads to macro- and microvascular complications that result in severe illness and premature death, with elevated personal, familial and economic costs. Aetiology is heterogeneous, presenting a genetic predisposition for which an effective screening method is not yet available, as well as, various civilizational/environmental factors that will be hard to change. These features and the daunting epidemiological scenario demand lifestyle and disease education, earlier screening protocols, improved diagnostic methods and urgent development of effective therapeutic management strategies, as it is necessary to treat the existing and the expected forthcoming millions of diabetic patients.

1.3 MAJOR FEATURES OF T2DM PATHOPHYSIOLOGY

1.3.1 The ominous octet

Although the β-cell is the final common denominator of glucose dysregulation, other mediating pathways of hyperglycaemia, surrounding the β-cell, are also implicated in T2DM pathogenesis. Many of these pathways have already been

considered or are under investigation as therapeutic targets for this disease (Schwartz et al., 2016).

In 1988, DeFronzo called the association of hyperglycaemia, muscle/liver insulin resistance, and β-cell failure, the triumvirate of T2DM's pathophysiological evolution. By 2009, the worldwide increased interest in diabetes research, revealed other key players in T2DM aggravation, which turned the initial triumvirate into De Fronzo's ominous octet (Fig. 1.1)**.** This octet maintains the triumvirate factors, but adds the newly discovered roles played by enhanced lipolysis, hyperglucagonaemia, dysregulation of hepatic glucose production (HGP), brain insulin resistance, increased renal glucose reabsorption, and incretin deficiency (DeFronzo, 2009). These eight factors can be divided into hormonal and organ dysfunctions (Fig. 1.1). Hormonal dysfunction involves decreased insulin and amylin secretion and increased glucagon secretion by the pancreas, as well as, decreased incretin effect (described in Chapter 2). Organ dysfunctions comprise increased HGP, renal glucose reabsorption and lipolysis, as well as, neurotransmitter dysfunction (De Fronzo, 2009).

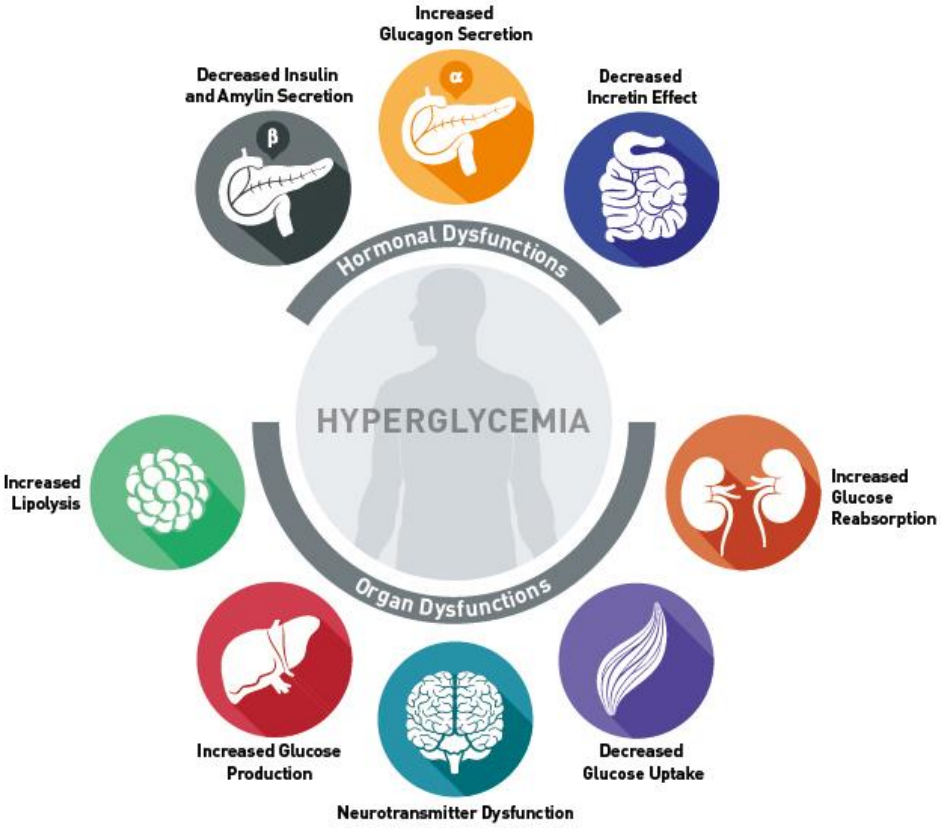


Figure 1.1 – Hormonal and organ dysfunctions that constitute the ominous octet (Source: Battling A1C.com., 2015. Accessed 28th of August, 2016).

1.3.2 Clinical evolution

Many T2DM patients do not present symptoms during the first few years of disease development. Patients are either diagnosed by general routine testing, or when the disease is symptomatic and well advanced and there is already a major loss of β-cell function or mass. This undiagnosed phase, which can last for several years, is characterized by the presence of intermediate hyperglycaemia, which could evolve to overt T2DM. If other dysmetabolic factors are concomitantly present, it is usually called metabolic syndrome (MS)**,** which contributes, and is directly related to the development of T2DM and CVD (King, 2015). Clinically, MS comprehends central obesity and insulin resistance as important causative features with the additional presence of any two of the following four factors: raised triglycerides (TGs), reduced high density lipoproteins cholesterol (HDL-c), raised blood pressure (BP), raised FPG or previously diagnosed T2DM (IDF, 2006).

In T2DM, insulin levels may appear normal, elevated or even reduced. To counterbalance hyperglycaemia, insulin values initially rise, which is viewed as compensatory hyperinsulinaemia. As insulin resistance increases and demands more insulin to maintain euglycaemia, compensatory mechanisms induce β-cell mass to expand through processes of hyperplasia, hypertrophy, as well as, by development of new islets from exocrine pancreatic ducts (Fonseca, 2009; Bonner-Weir, et al., 2012). However, high insulin levels cannot be sustained indefinitely and the progression of the disease, with the relentless effects of chronic hyperglycaemia, start to exhaust the β-cell's functional reserve, which will drop progressively and lead to the collapse of insulin secretory capacity. With disease aggravation, the sustainability of many metabolic factors begins to decline and reach defective levels in these patients, including insulin secretion (Cerf, 2013). Relative insulinopaenia starts when insulin secretion is inadequate to cover the elevated demand created by hyperglycaemia. Eventually, insulinopaenia is aggravated by the state of severe secretory disability of the β-cell. This loss of secretory capacity begins many years before clinical diagnosis. In addition, the molecular mechanisms underlying the development of complications, especially the macrovascular ones, are already in progress many years before the diagnosis, which justifies the classification of diabetes as a progressive (and for many years silent) disease (Zinman, 2006; Teixeira de Lemos et al., 2007; Cornell, 2015).

1.3.3 Effects of gluco-lipotoxicity on the β–cell

Essentially, hyperglycaemia acts in three ways on the β-cell: glucose desensitization, β-cell exhaustion, and glucotoxicity. Glucose desensitization refers to the rapid and reversible refractory phase of the β-cell exocytosis apparatus that occurs after acute exposure to elevated glucose. It is a physiological and reversible state of cellular refractoriness, thus differentiating it from β-cell exhaustion. β-cell exhaustion refers to depletion of the readily releasable pool of intracellular insulin following prolonged exposure to a stimulus (Wilcox, 2005; Fu et al., 2013). The result of this chronic stimulation with glucose is that insulin secretion will not resume until a rest period occurs. The distinction between β-cell exhaustion and glucose toxicity therefore, is that the exhausted islet has no defects in insulin synthesis, and thus cell function fully recovers as it rests. Glucotoxicity is the slow, progressive and irreversible effect of chronic hyperglycaemia on pancreatic β-cell function and survival capacity. Gradual damage occurs to the cellular components involved in insulin production, compromising insulin content and secretion over time. The glucotoxicity state promotes a decline in β-cell function by inducing abnormal insulin gene expression, decreased mitochondrial function, compromised exocytosis mechanisms, and increased apoptosis, thus decreasing insulin secretion and content (Yang et al., 2011). Also, the shorter the period of glucose toxicity exposure, the more likely that full recovery of β-cell function will occur (Gleason et al., 2000). The fact that these associated β-cell defects are reversible up until a certain point in time, and then become irreversible thereafter, suggests a continuum between β-cell exhaustion and glucotoxicity, becoming the latter predominant after prolonged exposure (Moran et al., 1997; Gleason et al., 2000; Fu et al., 2013). The insulin gene is expressed almost exclusively in pancreatic β-cells, making this cell type the key hormonal regulator of glucose homeostasis in the body. Metabolic regulation of insulin gene expression enables the β-cell to maintain adequate stores of intracellular insulin to sustain the secretory demand. Glucose is the major physiologic regulator of insulin gene expression; it co-ordinately controls the recruitment of transcription factors and the stability of insulin mRNA (Jung et al., 2014; Poitout et al., 2002). Glucotoxicity, inflammation and oxidative stress originated by hyperglycaemia, incites an inflammatory process in the pancreas proven by the observation of in *vivo* production

of Interleukin-1 beta (IL-1β) in β-cells of pancreatic tissue sections of T2DM patients, but absent in non-diabetic control subjects. IL-1β inhibits β-cell function and promotes apoptosis; supporting the concept that islet inflammation is present and is detrimental to its function. Besides confirming the inflammatory process, it seems feasible that the IL-1β pathway could be a target to preserve β-cell mass and function (Maedler et al., 2002, Lemos et al., 2008). Interestingly, most of these factors that are involved in this inflammatory process play a role in regulation of β-cell secretory function and cell turnover. β-cell pathophysiology seems to be strongly influenced by the low-grade chronic inflammation state, which is accompanied by an activation of monocytes and other mononuclear leucocytes, as well as, increased levels of other inflammatory markers, like tumor necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6) (Bastard et al., 2006; Shoelson et al., 2006; King, 2008; Poitout et al., 2008; Cerf, 2013).

Hyperglycaemia also creates a systemic state of oxidative stress and an increased production of free radicals, present in diabetic patients and in animal models of T2DM, which occurs in the presence of excessive levels of oxidative stressors or in a state of reduced antioxidant defences (Ceriello, 2003; Fridlyand et al., 2006; Ceriello et al., 2008; Teixeira-Lemos et al., 2011). The major sources implicated in oxidative stress in T2DM, include glucose auto-oxidation, overproduction of reactive oxygen species (ROS) by mitochondria, non-enzymatic glycation that produce advanced glycation end-products (AGEs), and the polyol pathway (Rains et al., 2001; Giacco et al., 2010; Sayed et al., 2011). High intracellular glucose levels intensify oxidative stress by increasing the electron transfer in the electron transport chain in mitochondria, generating a rise in ROS (Rolo et al., 2006; Pitocco et al., 2010). Furthermore, the redox balance is altered and affects redox-sensitive proteins, which also enhances mitochondrial ROS production. Increased mitochondrial ROS, damage mitochondrial components, such as deoxyribonucleic acid (DNA), membrane proteins and lipids, and opens the mitochondrial permeability transition pore (Mantel et al., 2011), releasing pro-apoptotic proteins from the mitochondria, such as cytochrome c, which can lead to induction of cell death. It is believed that ROS generated in the mitochondrial respiratory chain can act as secondary messengers for activation of inflammatory signalling pathways, involving TNF-α and IL-1β (Rolo et al., 2006). Thus, oxidative stress and inflammation seem to generate an endless pathological looping, where one

generates the other, and vice versa, aggravating the initial adverse effects mediated by hyperglycaemia and promoting diabetes evolution.

Damage incited by the binary association hyperglycaemia/glucotoxicity, is supported in disease evolution by another associated binary, hyperlipidaemia/ lipotoxicity**,** being these two pairs crucial sponsors of β-cell death by promoting inflammation and oxidative stress (Fig. 1.2). High levels of glucose and lipids originate an inflammatory response by stimulating pro-inflammatory cytokines and promoting lipid peroxidation, thus contributing to β-cell degradation, particularly by activating apoptosis pathways (Donath et al, 2008).

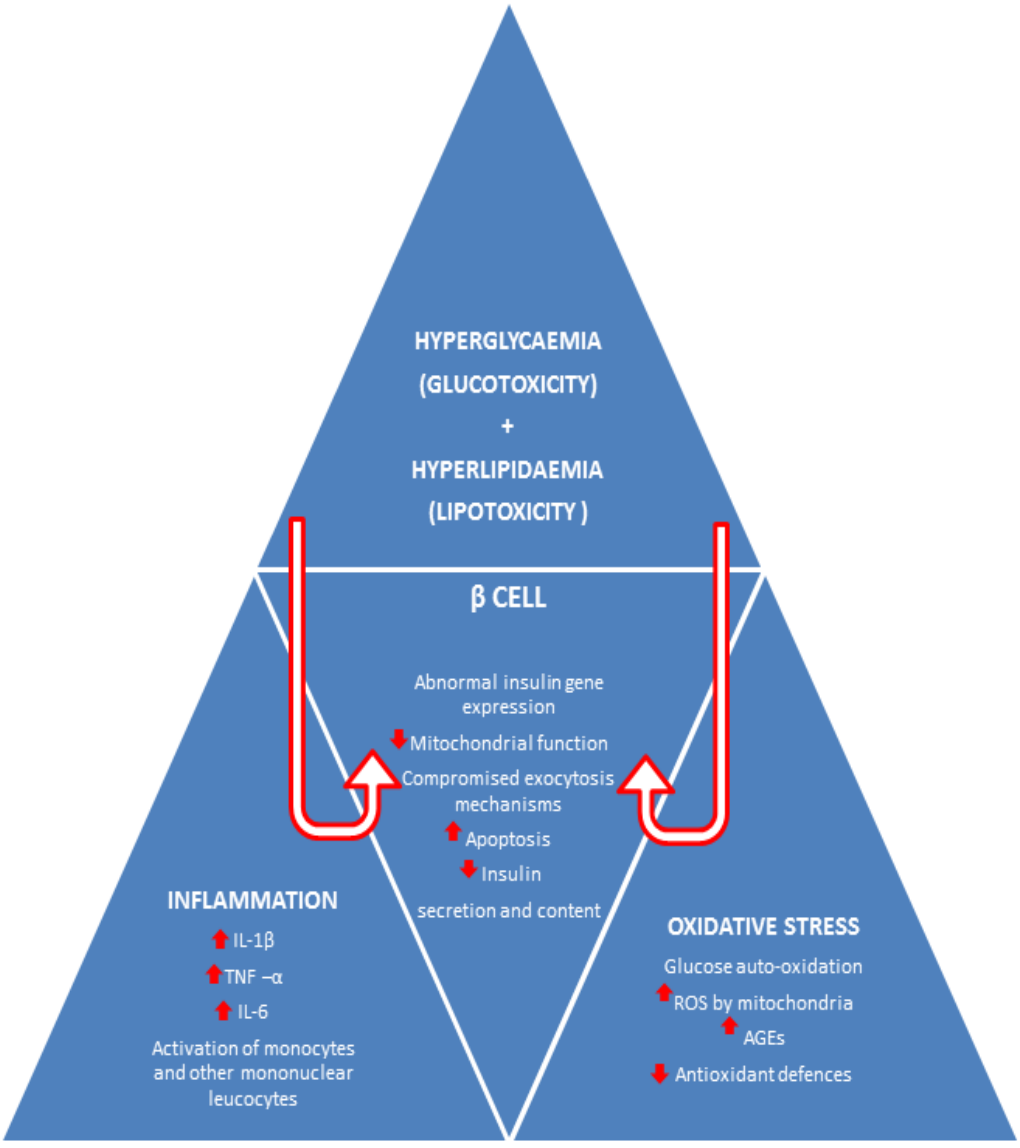


Figure 1.2 – Effects of hyperglycaemia/glucotoxicity and hyperlipidaemia/lipotoxicity on the β-cell. Both factors sponsor insulin secretion dysfunction and β-cell apoptosis through inflammation and oxidative stress. Abreviations: IL-1β – interleukin 1 beta; TNF-α – tumor

factor necrosis alpha; IL-6 – interleukin-6; ROS – Reactive oxygen species; AGEs – Advanced glycation end products.

The adipose tissues of obese and T2DM individuals are in a state of chronic inflammation, infiltrated by mononuclear cells (Furler et al., 2006). These adipocytes and macrophages secrete pro-inflammatory/prothrombotic cytokines, such as, TNF-α, IL-6, resistin, adipsin, acylation-stimulating protein (ASP), plasminogen activator inhibitor-1 (PAI-1) and angiotensinogen, that promote atherogenesis and induce insulin resistance (Teixeira-Lemos et al., 2011) Furthermore, oxidative stress produces ROS and AGE that contribute to β-cell damage and apoptosis. Consequently, lipotoxicity is involved in the modulation of β-cell function and survival (Jung et al., 2014; Sayed et al., 2011; De Fronzo, 2009; Pérez-Matute et al., 2009; Bonora, 2008).

The influence of hyperlipidaemia on the β-cells of individuals will depend on their specific lipid profile, as some free-fatty acids (FFAs) and lipoproteins have shown to be pro-apoptotic for the β-cell, and others, to be protective. Long-term exposure of β-cells to saturated FFAs, such as palmitate, appears highly toxic, while monounsaturated FFAs, such as oleate, protect against both palmitate and glucose-induced β-cell apoptosis (Maedler et al., 2001; Maedler et al., 2003). Lipoproteins may also affect β-cell survival and function in a similar way, whereby very low density lipoproteins (VLDL) and low density lipoproteins (LDL) are pro-apoptotic, high density lipoproteins HDL are protective (Roehrich et al., 2003; Rütti et al., 2007). These lipotoxicity effects are influenced by the prevailing hyperglycaemia, demonstrating that lipotoxicity and glucotoxicity, in concert, determine β-cell failure (Poitout et al., 2002; El-Assaad et al., 2003; Teixeira de Lemos et al., 2012).

The mechanisms regulating β-cell apoptosis and proliferation are inseparable processes in T2DM evolution, as net β-cell mass results from the balance between apoptosis and regeneration or/and proliferation. The greater the apoptosis/ proliferation ratio, the more severe is disease aggravation and the resulting insulinopaenia (Donath et al., 2005). In vivo-stimulated β-cell proliferation seems to be multifactorial and cell proliferating nuclear antigen (PCNA) and nuclear protein/antigen Ki-67 (Ki-67) seem to be involved (Kulkarni et al.,2004; Zhou et al., 2004; Hussain et al., 2006). Islet vascularization is crucial for pancreatic development

and maintenance, largely via vascular endothelial growth factor (VEGF)-A mediated signals. In addition to providing an outlet for blood flow, intra-islet endothelial cells directly enhance insulin transcription and secretion and stimulate β-cell proliferation (Nikolova et al., 2006). VEGF-A expression in islets is further upregulated by hypoxia and glucose, being important in the maintenance of β-cell mass and revascularization of islets following transplantation or regeneration (Zhang et al., 2004; Xiao et al., 2013). However, in diabetic patients, exposed chronically to hyperglycaemia, VEGF production in response to hypoxia is decreased (Thangarajaha et al., 2009).

1.3.4 Insulin resistance

In T2DM, disruption of the normal relationship between β-cell function and insulin sensitivity/resistance is central to the pathogenesis of hyperglycaemia and is well established by the time the patient's abnormal glycaemic levels are discovered. Normal glucose regulation is dependent on a feedback loop among the liver, peripheral tissues (primarily muscle), and pancreatic islet cells. In individuals with normal islet function, the pancreatic islet β-cells adapt to the reductions in insulin sensitivity, insulin resistance**,** in the hepatic and peripheral tissues by increasing insulin secretion, and thereby, preventing the development of fasting hyperglycaemia. Continuous exposure to insulin causes a reduction in the number of insulin receptors exposed on the cell surface of these tissues by promoting internalization, as well as, degradation of hormone-occupied receptors, which further aggravates insulin resistance (De Fronzo, 2009).

Obesity or excess adipose tissue is recognized as one of the most important factors in the genesis of insulin resistance, particularly when a visceral pattern distribution of adiposity is present, with large adipocytes that are resistant to insulin-induced lipolysis suppression, contributing to a chronic increase in FFAs and glycerol due to the uninhibited lipolysis (Ginsberg, 2000; Maedler et al. 2001; Yano et al., 2004; Laakso, 2010; Teixeira de Lemos et al., 2012). Chronically increased plasma FFAs and intramyocellular levels of toxic lipid metabolites play a role in the pathogenesis of muscle/liver insulin resistance, which activate isoforms of protein kinase C, blocking cellular insulin signalling in these tissues (Cushman et al., 2002; Wellen et al. 2003;

Belfort et al., 2005). The increase of intracellular FFAs leads to a decreased translocation of glucose transporter 4 (GLUT-4) to the plasma membrane, contributing to insulin resistance in muscle and adipose tissue (Talior et al, 2003; Teixeira-Lemos et al., 2011). The adipocytes, besides producing abnormal inflammatory adipocytokines, also fail to secrete normal amounts of insulin-sensitizing adipocytokines, namely adiponectin (Bays et al., 2004; Furler et al., 2006).

Besides peripheral insulin resistance, T2DM also exhibits central insulin resistance in the central nervous system (CNS) where insulin signalling seems to be a crucial regulator of energy homeostasis (Hayes et al., 2014).

1.4 MAJOR COMPLICATIONS OF T2DM – FOCUS ON DIABETIC NEPHROPATHY

Diabetes is recognised as being a group of chronic diseases characterized by hyperglycaemia where the importance of protecting the body from excessive glucose circulation cannot be overstated. The direct and indirect effects of hyperglycaemia on the vascular system constitute a major source for complications (Bae, 2016). The long-term multiple complications of T2DM are shared with T1DM, although timing and mechanisms may somewhat differ between these two forms of diabetes (IDF, 2014).

Generally, diabetic complications are divided into macrovascular (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications [diabetic nephropathy (DN), neuropathy and retinopathy] (Chawla et al., 2016). Diabetic patients develop macrovascular complications at a much faster rate in comparison to non-diabetic individuals, and cardiovascular risk is increased up to tenfold, these include myocardial infarction, stroke and limb amputations (Gray et al., 2014).Diabetic neuropathy is responsible for neuropathic pain and loss of motor and sensory nerve function in somatic and visceral organs. Amputation of limb extremities, due to necrosis via vascular and neuropathic alterations, is also a dramatic outcome of the disease (Whaley-Connell et al., 2014).Diabetic retinopathy is a leading cause of blindness, being diabetic macular oedema responsible for most of the visual loss

experienced in T2DM (Lee et al., 2015).DN originates insidious CKD, which leads progressively to end-stage renal disease (ESRD), a condition often requiring dialysis or transplantation as last therapeutic options (Narres et al., 2016; ADA, 2017).

1.4.1 Diabetic nephropathy

DN is now the single commonest cause of ESRD worldwide and one of the main causes of death in diabetic patients. It is also acknowledged as an independent risk factor for CVD. In recent years, new pathways involved in the development and progression of diabetic kidney disease have been elucidated and accumulated data have emphasized the critical role of many factors in the pathogenesis of DN (Laakso et al., 2010; Laakso et al., 2011; Chawla et al., 2016).

The kidney, besides contributing to the aggravation of hyperglycaemia in T2DM through gluconeogenesis (Gerich et al., 2010) and glucose reabsorption, does not remain unscathed through diabetic evolution, developing progressive lesions and functional impairments that lead to DN (Nauck, 2014). There is emerging evidence that microvascular kidney disease begins prior to the onset of diabetes, and this occurs with microalbuminuria and decreased renal function (Jefferson et al., 2008).

Haemodynamic and metabolic factors, with a central role for chronic hyperglycaemia, have key roles in the pathophysiology of DN (Fig.1.3). In recent years, new pathways involved in the development and progression of diabetic kidney disease have been elucidated and have emphasized the critical role of reactive oxidative stress (Cao et al., 2011) and inflammation (Duran-Salgado et al., 2014) in the pathogenesis of DN. Expression of cell adhesion molecules, growth factors, chemokines and pro-inflammatory cytokines are increased in the renal tissues of diabetic patients (Fig. 1.3). Thus, metabolic and hemodynamic abnormalities seem to be involved in stimulating these pathways in DN. The consequences of molecular activation and inhibition of the various pathways lead to functional and structural changes that clinically manifest as DN, characterized by increasing albuminuria and declining renal function (Duran-Salgado et al., 2014).

Hemodynamic factors (Morano et al., 2008; Chawla et al., 2010) predominantly mediated by angiotensin II, play a role via over-activity of the renin-

angiotensin-aldosterone system (RAAS) and VEGF deficiency. Aggravation of haemodynamic factors and the contribution of metabolic alterations, induce glomerular histological abnormalities in glomerular basement membranes (GBM), endothelial and mesangial cells (Bortoloso et al., 2004; Marshall, 2004) and podocytes (Fig. 1.3) (Baelde et al., 2007; Li et al., 2007), tubulointerstitial lesions are involved too (Nangaku, 2006).

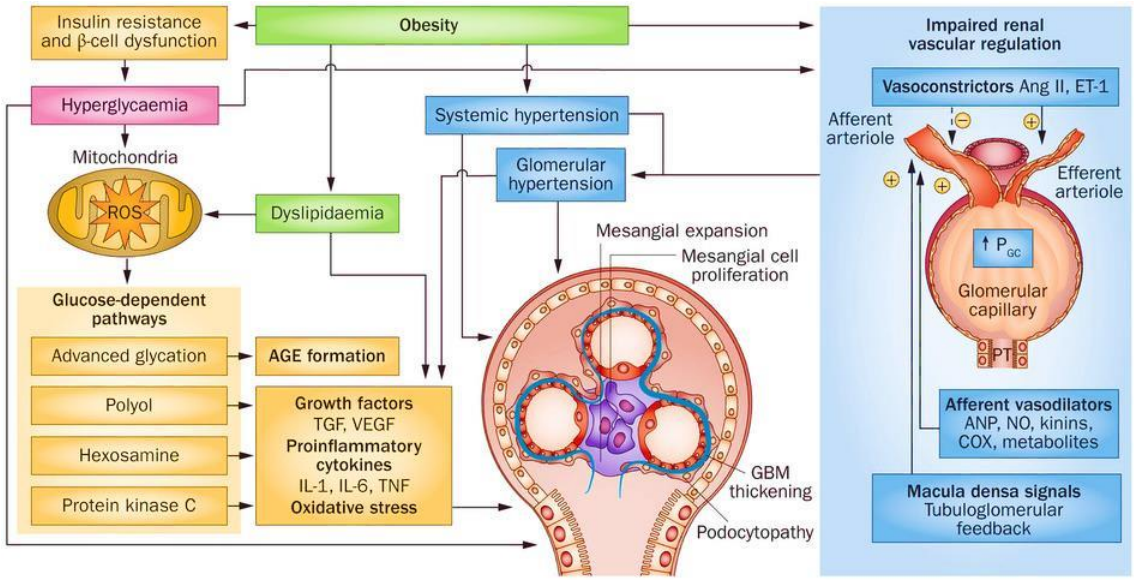


Figure 1.3 – Interconnection between metabolic and hemodynamic abnormalities involved in the pathophysiology of diabetic nephropathy. Dyslipidaemia and hyperglycaemia, both activate advanced glycation, oxidative stress and inflammation. Hemodynamic factors are mediated by angiotensin II and VEGF deficiency. AGE - advanced glycation end products; Ang II - angiotensin-2; ANP - atrial natriuretic peptide; COX - cyclooxygenase; ET-1 - endothelin-1; GBM - glomerular basement membrane; IL - interleukin; NO - nitric oxide; PGC - glomerular capillary hydraulic pressure; PT - proximal tubule; ROS - reactive oxygen species; T2DM - type 2 diabetes mellitus; TGF - transforming growth factor; TNF - tumour necrosis factor; VEGF - vascular endothelial growth factor A (Source: Muskiet et al., 2014).

Obesity and chronic hyperglycaemia alter vasoactive regulating mechanisms of afferent and efferent arteriolar tonus, leading to increased glomerular capillary hydrostatic pressure (P_{GC}), hyperperfusion and hyperfiltration. These early renal haemodynamic changes, combined with systemic hypertension, are important in the development and progression of renal disease in T2DM (Muskiet et al., 2014). Dysfunction of the incretin system also seems to be implicated in alteration of vascular tonus, natriuretic and diuretic properties (Yu et al., 2003).

An increase in glomerular filtration, in association with albuminuria, has been a hallmark of early DN. Albuminuria in DN is mostly glomerular in origin and albumin must cross the glomerular filtration barrier, which consists of fenestrated glomerular endothelial cells, the glomerular basement membrane, and the glomerular epithelial cell or podocyte. Alterations of this barrier, like increased intraglomerular pressure, loss of negatively charged glycosaminoglycans in the basement membrane, and further in the disease process, the increase in basement membrane pore size, all contribute to albuminuria (Marshall, 2004).

Podocytes (or visceral epithelial cells) are highly specialized cells of neuro-epithelial origin that wrap around the capillaries of the glomerulus and between these processes are small slits which contain a slit diaphragm. An increasing number of proteins have been identified to be present in these foot projections of podocytes. Nephrin is a zipper-like protein that plays a functional role in the structure of the slit diaphragm. The spaces between the teeth of the zipper allow, in a selective way, small molecules, such as glucose and water, to traverse, but do not allow large proteins to cross. Evidence suggests that nephrin could play a key role in the glomerular filtration barrier and the development of proteinuria as it is found to be under-expressed in kidney failure and in diabetic rats (Cao et al., 2011). In diabetes, early flattening and retraction of the foot processes are associated with thickening of the glomerular basement membrane. Thickening of the glomerular basement membrane, accumulation of mesangial matrix, and increased numbers of mesangial cells are present as initial microscopic abnormalities. As disease advances, there is a close relationship between mesangial expansion and declining glomerular filtration. Mesangial expansion also correlates inversely with capillary filtration surface area, which itself correlates to glomerular filtration rate (Marshall, 2004).

Additionally, chronic hyperglycaemia and dyslipidaemia induce mitochondrial oxidative stress, which activates several metabolic pathways, specifically, protein kinase C (Noh et al., 2007), non-enzymatic glycosylation (Tanji et al., 2000), acceleration of the polyol pathway, hexosamine biosynthetic pathway and oxidative stress (Forbes et al., 2008; Singh et al., 2011; Singh et al., 2014).

Evidence also points to a crucial role of the inflammatory process in the development and progression of DN (Saraheimo et al., 2003; Dalla Vestra et al., 2005;

Rivero et al., 2009). This inflammatory response is mediated by diverse inflammatory cells, including macrophages, monocytes, and leukocytes, as well as other molecules, such as chemokines, adhesion molecules, and inflammatory cytokines, namely, TNF-α and IL-1β (Rivero et al., 2009; Kanasaki et al., 2013). Besides altering glomerular haemodynamics and promoting increased vascular endothelium permeability, when TNF-α binds to its receptors, several signalling pathways are activated leading to apoptosis and necrosis. IL-1β also modifies vascular permeability and increases the expression of chemokines that induce proliferation and synthesis of extracellular matrix in the mesangium (Duran-Salgado et al., 2014). As inflammation persists, certain vascular lesions are exacerbated, such as endothelial dysfunction, tissue damage, mesangial nodule formation (Kimmelsteil-Wilson bodies), renal fibrosis, and apoptotic cell death (Rivero et al., 2009; Kanasaki et al., 2013).

It was also observed that high glucose levels induce an increased ratio of Bax/Bcl-2, associated with cytochrome-c release from mitochondria in renal mesangial cells (Allen et al., 2005; Matough et al., 2012). In addition, BH3 interacting-domain death agonist (Bid), a pro-apoptotic protein member of the Bcl-2 family, has an important role in the mitochondrial cell death pathway (Sanz et al., 2008).

It has been described that glucose-induced ROS production contributes to apoptosis in podocytes (Susztak et al., 2006; Chen et al., 2013; Gao et al., 2015), mesangial (Wagener et al., 2009) and tubular cells (Susztak et al., 2006; Wagener et al., 2009), leading to DN progression. Furthermore, high glucose-mediated oxidative stress in tubular cells has been associated with increased levels of pro-apoptotic proteins (Verzola et al., 2002).

In the tubule-interstitium, tubular hypertrophy and associated basement membrane alterations precede tubulointerstitial fibrosis and tubular atrophy, which accompanies progressive renal dysfunction. Arteriosclerosis is also described. Interstitial enlargement correlates with glomerular filtration, albuminuria, and mesangial expansion. It has been suggested that the accumulation of protein in the cytoplasm of proximal tubular cells causes an inflammatory reaction which leads to tubulointerstitial lesions (White, 2014; Marshall, 2004).

Although DN was traditionally considered a primarily glomerular disease, it is now widely accepted that the rate of deterioration of function correlates best with the

degree of renal tubulointerstitial fibrosis. This suggests that although the primary event is a condition marked by glomerular changes resulting in proteinuria, the long-term outcome is determined by events in the renal interstitium (Eddy; 2009; Tramonti et al., 2011).

Chapter 2

The incretin defect in T2DM pathophysiology

"Glucose homeostasis is like a violin concerto. The vibrant melody is led by Brain, the maestro; solos are played by Pancreas, a virtuoso violinist that excels in every note and blends pungently with a tuned orchestra, in tempo with the rhythm of incretin percussion, forming a true a hormonal masterpiece". CM, 2015

(Text inspired by André J. Scheen who wrote, "The CNS orchestrates energy and glucose homeostasis" and by Mozart, Beethoven, Handel, Grieg and Rachmaninoff, who knew nothing of energy homeostasis, but composed the best concertos in the history of mankind)

2.1 HISTORY OF INCRETINS

The discovery of the islets of Langerhans in 1869 and the role they played in the development of T2DM (1901), allowed the first glimpse into the pathogenesis of the disease. Incretins and their physiological effects, on the other hand, were only established at the end of the 20th century (Fig. 2.1).

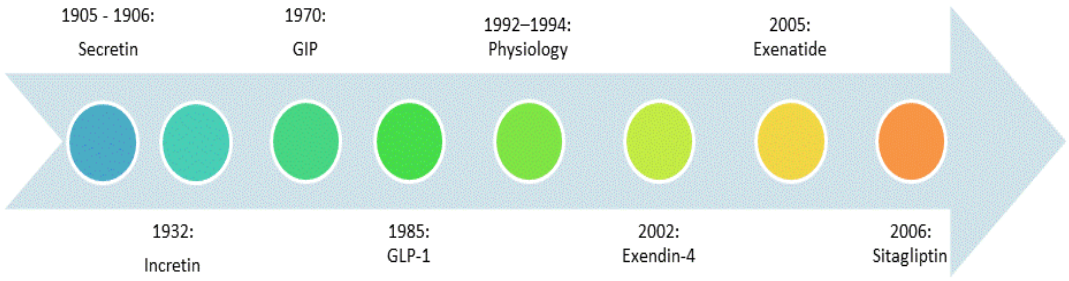


Figure 2.1 –Main historic events that led to the development of incretin based therapies.

In 1905, Bayliss and Starling discovered "secretin", the first identified regulatory peptide by examining the effects of crude intestinal extracts on exocrine pancreatic secretions, and thus, introduced the concept of hormones and their way of action (Creutzfeldt, 2005). These investigators and Moore (1906) hypothesised the role of secretin as a chemical stimulant of internal pancreatic secretion. Between 1906 and 1935, numerous experiments were conducted, testing the effects of injected or ingested duodenal extracts (secretin) on fasting or elevated blood glucose levels of normal or diabetic animals and humans. In one of these studies, La Barre, in 1932, proposed the name “incretin” after inducing hypoglycaemia by intravenous administration of unclean "secretin" and subsequently linking this result to insulin action. Thus, he formed the term “incretin” by adding the fragments of the words: “IN”testin, se”CRET”ion and “IN”sulin. However, after a promising beginning, a series of negative dog experiments considered the existence of “incretin” substances as dubious and research on the subject was interrupted for 20 years (Geelhoed-Duijvestijn, 2007; Menon et al., 2015). The “incretin-concept” was only recovered in 1964 by McIntyre et al., which showed that significantly more insulin was released after oral ingestion of glucose than after an intravenous injection. This fact confirmed

the existence of a link between the intestine and the endocrine pancreas, indicating that gastrointestinal (GI) hormones and peptides could exert some action on insulin secretion (Geelhoed-Duijvestijn, 2007; Ranganath, 2008a). Subsequently to this discovery, for a given hormone to be included in the incretin group it should meet two essential principles (Ranganath, 2008): be released in response to oral glucose intake and be able to achieve physiological concentrations resulting in insulin release. The first substance to be classified as an “incretin” was discovered in 1970 by John Brown, who isolated a hormone composed of 42 amino acids that he later identified as "gastric inhibitory polypeptide" (GIP) (Brown, 1971), which is now also known as “glucose-dependent insulinotropic polypeptide”, due to its ability to stimulate insulin secretion in a glucose-dependent manner (Brown et al., 1978). Yet, the revival of the term incretin was mostly due to Creutzfeldt (1978, 1979, 2005), who highlighted the relationship “glucose - intestine - insulin” in association with the incretin effect, a feature that is of key importance for clinical application. Glucagon-like peptide 1 (GLP-1 was only accepted as a true incretin in 1985 (Geelhoed-Duijvestijn, 2007; Ranganath, 2008b) by genetic studies on pro-glucagon coding sequences, which rendered it as a “glucagon-related molecule” and, as it met the incretin criteria, was classified as one, too (Lund, 2005). In 1986, Nauck et al. found that the level of incretin secretion was dependent on the amount of ingested glucose and that incretins were responsible for approximately 75% of the insulin response after the ingestion of 50 g of glucose. The more precise physiological functions of incretin hormones were only discovered in the 1990s, specifically those concerning regulation of glucose homeostasis and their possible use as therapeutic targets for obesity and diabetes. Since then, incretin based therapies have been a mainstay of diabetic research (Troke et. al., 2014; Tan et al. 2013; Ahrén, 2010; Drucker, 2007; Drucker, 2006; Gallwitz, 2005; Deacon et al., 2001; Nauck et al., 1993; Creutzfeldt, 1992).

2.2 THE ROLE OF NATURAL INCRETINS IN GLUCOSE HOMEOSTASIS

Digestion and absorption of nutrients are associated with increased secretion of multiple gut hormones that act on distal targets. More than fifty GI hormones and peptides are synthesized and released by specific enteroendocrine cells found in the epithelium of the stomach, small and large intestine, which makes the gut the largest hormone producing organ in the body. This assembly of regulatory hormones and peptides convey information not only to the intestine and associated organs, but to other organic systems, such as the CNS.

Glucose is the vital metabolic fuel substrate for all mammalian cells and is the main carbohydrate presented to the cells for energy production and many other anabolic requirements (Triplitt, 2012a). External and endogenous sources of glucose contribute to maintain blood glucose homeostasis. During fasting, glycaemia is mainly sustained by hepatic glucose production (HGP), which accounts for about 85% of endogenous production, while the kidney upholds the remaining 15% (De Fronzo, 2004; Marsenic, 2009). Until some years ago, glucose homeostasis was considered to be under bi-hormonal regulation, regarding insulin and glucagon as sole regulators of glucose metabolism, and thus, the main therapeutic targets. Currently, a more complex regulation is recognized and glucose homeostasis is governed by precise neuronal and hormonal regulation through the interplay of the central nervous system, insulin, glucagon, amylin, somatostatin and GI hormones, more specifically, incretin hormones (Fig. 2.2), which constitute our main focus (Aronoff et al., 2004). This intricate regulation system acts on the rate of absorption of dietary carbohydrates by the intestinal mucosa, glucose consumption by peripheral tissues (muscle and adipose tissue), removal of glucose through the renal tubules and also, hepatic glucose uptake and release (De Fronzo, 2009) .

Incretins are gut hormones that are secreted from enteroendocrine cells into the blood within minutes after eating a meal. GIP and GLP-1 share many common actions in the pancreas but have distinct actions outside of the pancreas. Current knowledge allocates incretin secretion to the intestinal mucosa, where GIP is secreted

from K-cells (enterochromaffin cells), located mainly in the stomach, duodenal mucosa and the proximal jejunum, while GLP-1 is produced in L-cells found more distally in the ileum and colon (Drucker, 2006). To exert their effects, incretins bind to specific receptors of target organs, the GLP-1 receptor (GLP-1R) and GIP receptor (GIPR), which are G-protein-coupled receptors (GPCRs). GLP1R exist in β-cells of the pancreatic islets, the kidney, lung, heart and nervous system. GIPR are found in β and α-cells of pancreatic islets, adipose tissues, heart and brain (Tasyurek et al., 2014). Both peptides are rapidly inactivated ($t_{½}$ ≈ 2 minutes) (Deacon et al., 1995) via N-terminal degradation by the ubiquitous enzyme dipeptidyl peptidase-4 (DPP-4), a specialised enzyme located on the cell surface enzyme of endothelial, epithelial and some other cell types.

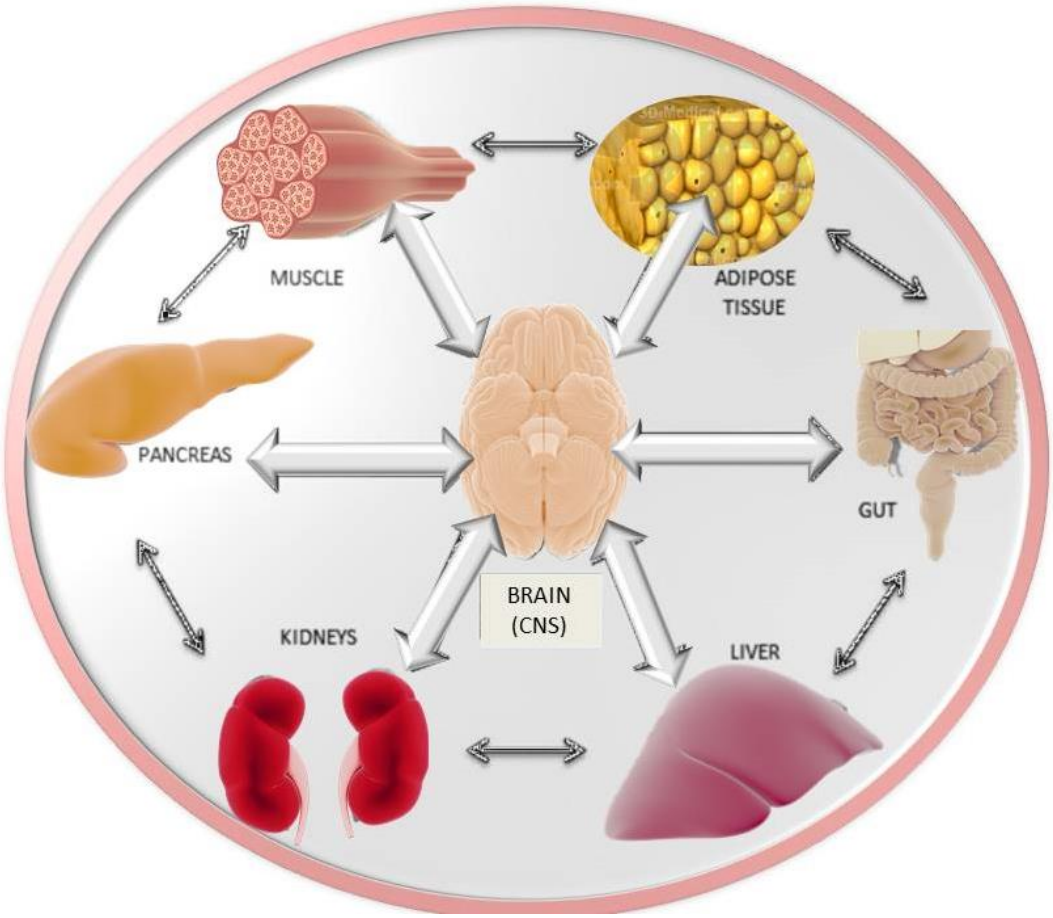


Figure 2.2 – Regulation of energy homeostasis. The brain (CNS) at the centre of energy homeostasis regulation: the brain and other regions of the CNS emit signals to effector organs and, in turn, receive nutrient and hormonal signals that converge and directly act on brain centres. Effector organs are also regulated between them, by reciprocated signalling.

Cleavage by neprilysin (neutral endopeptidase) is also an inactivation route, but is considered to be of minor relevance. Excretion of incretins and its metabolites is done through renal clearance (Mentlein, 2009; Deacon, 2011; Marques et al., 2014). Together, these metabolic processes reduce the high concentration levels of these peptides, present in the postprandial state, to almost undetectable levels during the fasting state. The major biological actions of GLP-1 and GIP in glucose homeostasis are translated into neuroendocrine regulation, insulinotropic and non-insulinotropic effects on pancreatic islet cells, and effects on extra-pancreatic tissues (Phillips et al., 2011; Campbell et al., 2013).

2.2.1 Insulinotropic and non-insulinotropic effects of incretins on pancreatic islet cells

The control of glycaemia by the islet of Langerhans depends on the coordinated secretion of insulin and glucagon by pancreatic β- and α-cells, respectively. Both cell types respond oppositely to changes in blood glucose concentration: whereas β-cells release insulin in hyperglycaemic settings, hypoglycaemic conditions induce α-cell secretion (Quesada et al., 2006). Somatostatin, produced and secreted by the δ-cell population of the islet of Langerhans, besides several other tissues, acts as an inhibitor of both glucagon and insulin release (Hauge-Evans et al., 2009; Meda, 2013).

Insulin secretion is stimulated by metabolic (glycaemia), hormonal and neuronal influences on the endocrine pancreas, which are collectively referred to as the "entero-insular axis" (Ranganath, 2008). Postprandial insulin secretion is directly stimulated through entero-pancreatic nerve activation via chyme and intestinal distension, and by the strong endocrine stimulus mediated by incretin hormones. In minutes after nutrient ingestion GIP and GLP-1 are released into blood circulation and stimulate insulin secretion (McIntosh, 2008). Incretin effects on pancreatic β-cells implicate a series of events that potentiate glucose usage and protect against glucose depletion (Creutzfeldt et al., 1985; Röder et al., 2016), by regulating 30 to 60% of C-peptide and 80 to 90% of insulin response, in a dose dependent manner after an oral

load of glucose (Jones et al., 2013). Accordingly, insulin secretion ceases when euglycaemia is achieved, deeming incretins safe in relation to the risk of hypoglycaemia, which is one of the several features that turn these peptides into attractive potential therapeutic agents (Ahrén, 2014).

Both GIP and GLP-1 exert their insulinotropic effects on pancreatic β-cells (Fig. 2.3) by binding to specific receptors, GIPR and GLP-1R, respectively, where they induce a cascade of events that culminate in stimulation of insulin secretion in a glucose-dependent manner and insulin biosynthesis (Phillips et al., 2011).

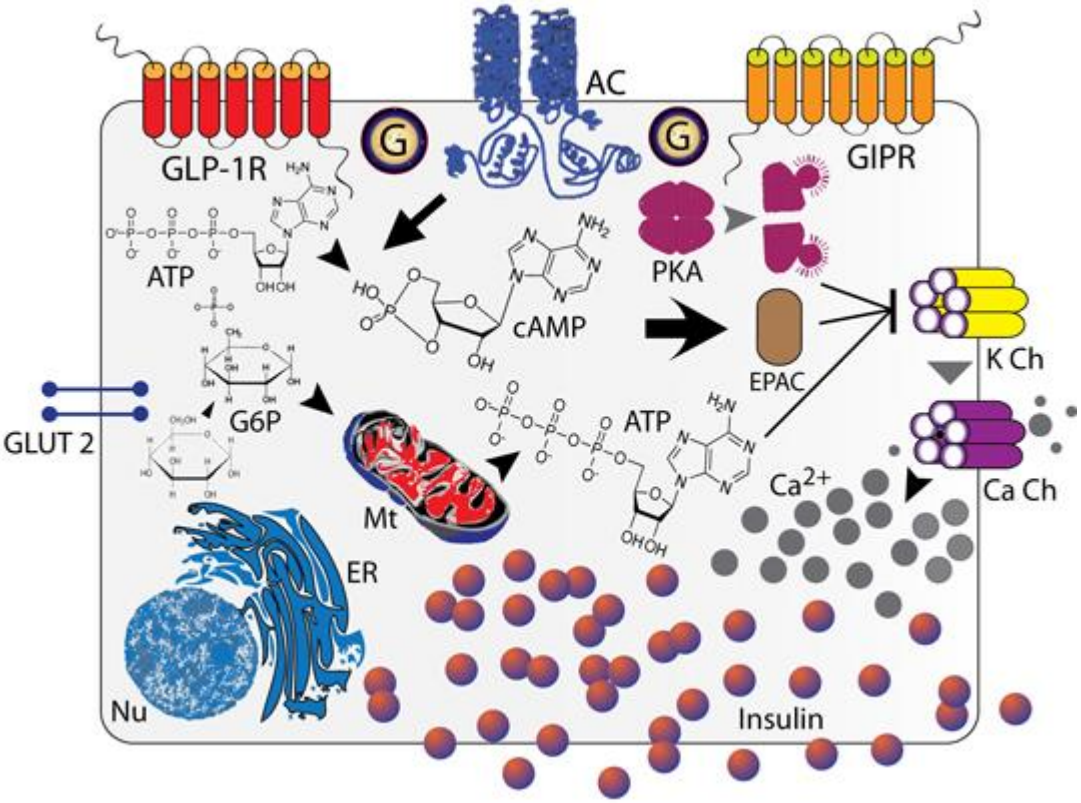


Figure 2.3 – Glucose-mediated insulin secretion and insulinotropic effects of GLP-1 and GIP. Binding of GLP-1 and GIP with their receptors, GLP-1R and GIPR, results in the activation of adenylate cyclase (AC) by way of G proteins (G) leading to increase in intracellular cAMP levels. Activation of PKA and EPAC2 (cAMP-GEFII) closes KATP channels (K Ch) facilitating membrane depolarization resulting in the opening of the voltage gated Ca^{2+} channels (Ca Ch) and influx of Ca^{2+} into pancreatic β-cells. Increase in cytoplasmic Ca^{2+} not only stimulates fusion of insulin-containing cytoplasmic granules leading to insulin secretion from pancreatic β-cells but also promotes transcription of proinsulin gene renovating insulin depots. Glucose-mediated insulin secretion is also depicted in the figure: glucose enters into the cell through Glucose Transporter 2 (GLUT2), is phosphorylated to glucose 6 phosphate (G6P) by glucokinase (GK). Glycolysis increases the ATP/ADP ratio leading to closure of K^+ channels (K Ch) inducing membrane depolarization. Other abbreviations: Nu - nucleus; ER - endoplasmic reticulum, Mt - mitochondria (Source: Tasyurek et al., 2014).

Incretin binding to these receptors leads to activation of adenylate cyclase and subsequent elevation of intracellular cyclic adenosine monophosphate (cAMP) levels, which then triggers a signalling cascade that causes the increment of intracellular Ca^{2+} concentrations eliciting the fusion of insulin-containing granules with the plasma membrane and insulin secretion from the β-cells. Increased Ca^{2+} levels also promote transcription of the proinsulin gene, thereby increasing the insulin content of the β-cell (Seino et al., 2010). Both peptides are rapidly inactivated, circa 2 minutes, by DPP-4 (Deacon et al., 1995), which reduces their high concentration levels present after a meal, to almost untraceable levels during fasting stages.

As incretins, GIP and GLP-1 share common properties, but also possess different biological characteristics. GLP-1 acts in a positive way on the β and δ cells, whereas GIP acts preferentially on the α- and β-cells (Ranganath, 2008). The effects of GLP-1 on pancreatic islet cells include increased insulin secretion by β-cells in a glucose-dependent manner (Fig. 2.4), increased secretion of somatostatin by δ-cells and reduced secretion of glucagon by the α-cells. These events contribute to a decrease in hepatic glucose output. In contrast to GLP-1, GIP has been shown to stimulate pancreatic glucagon secretion. Recent human data suggest that the glucagonotropic effect of GIP is strictly glucose-dependent with no effect during hyperglycaemic conditions. Thus, GIP seems to act as a blood glucose stabilizer with inverse glucose-dependent effects on pancreatic insulin and glucagon secretion, respectively (Meier et al., 2003; Walker et al., 2011; Godoy-Matos et al., 2014).

Although the primary role of GIP and GLP-1 is that of an incretin, presently it is acknowledged that GIP and GLP-1 exert non-insulinotropic actions on the pancreas, such as regulating pancreatic β-cell proliferation and survival. Both hormones seem to be associated with anti-apoptotic and pro-proliferative effects on pancreatic β-cell (Fig. 2.4), but the signalling cascades involved display some differences (Yabe et al., 2011; Deacon et al., 2011; Campbell et al., 2013; Pappachan et al., 2015). Anti-apoptotic function of GLP-1 requires the presence of phosphoinositide 3-kinase (PI3K), which is not required by GIP to exert the same function, being the physiological impact of this

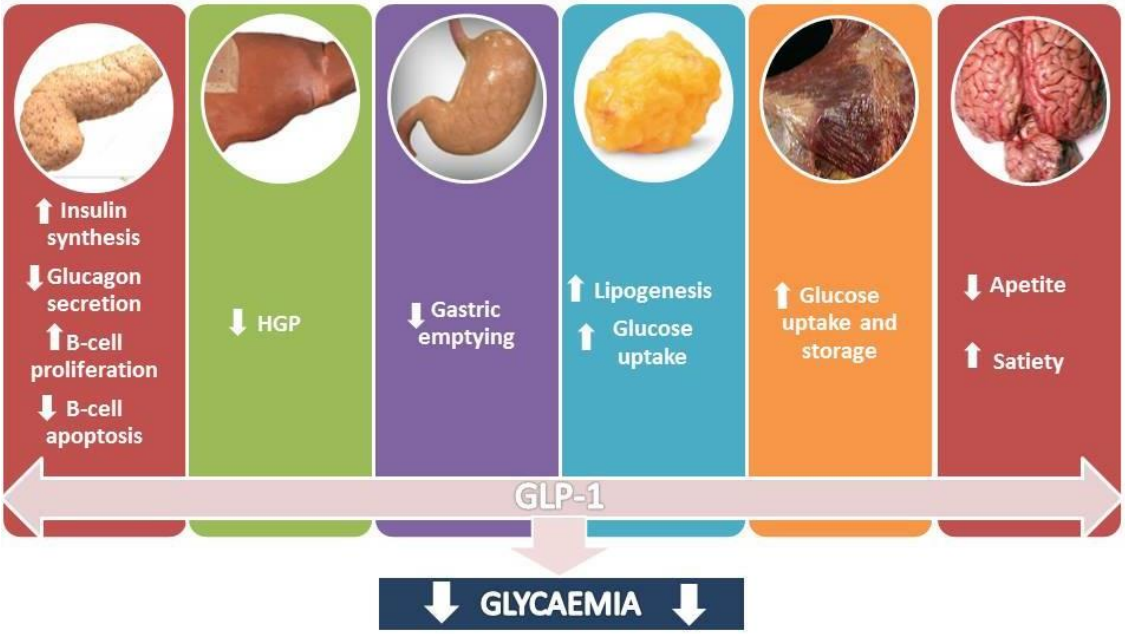


Figure 2.4 – Pancreatic and extrapancreatic ffects of GLP-1 on glucose control. GLP-1 lowers glycaemia through its insulinotropic and non-insulinotropic effects on pancreatic islet cells and by direct and indirect actions in extrapancreatic organs.

difference in the signalling cascade not fully understood (Maida et al., 2009). Another remarkable aspect of incretins is the stimulating effect on proliferation of β-cells and/or progenitor cells (Xu et al., 1999; Bocker et al., 2001; Trumper et al., 2001). Stimulation of β-cell proliferation and inhibition of apoptosis promotes cell expansion, increases β-cell mass and pancreatic islet neogenesis (Wideman et al., 2004; Drucker et al., 2006; Herbach et al., 2011). GLP-1 has a trophic action on β-cells promoting amplification of insulin synthesis and β-cell hypertrophy. GLP-1 also promotes cell differentiation, from exocrine ductal cells or immature islet stem cells, towards a greater degree of differentiation (Drucker, 2003). An increase in the number and mass of β-cells has been demonstrated by direct action of GIP (Drucker, 2003; Drucker et al., 2006; Garber et al., 2011). Further elucidation is needed on the precise molecular mechanisms underlying the effects of GIP and GLP-1 on β-cells to fully reveal the potential therapeutic targets to increase β-cell mass by inhibiting apoptosis and/or stimulating proliferation.

GLP-1 and GIP have opposite effects on glucagon secretion in pancreatic α-cells. GLP-1 suppresses glucagon secretion (Fig. 2.4) when plasma glucose levels are

above fasting level (Nauck et al., 2002; De Marinis et al., 2010), which is clinically important because GLP-1 loses its inhibitory effect on glucagon secretion at hypoglycaemic levels and does not decrease the counter-regulatory responses to hypoglycaemia. It has recently been reported that insulin stimulation and glucagon inhibition contribute equally to the effect of GLP-1 on glucose turnover in T2DM patients (Hare et al., 2010). Although there are no GLP-1 receptors on α-cells, insulin released by β-cells, in response to GLP-1, has an inhibitory action on the physiological secretion of glucagon. Therefore, there is an improvement in the insulin/glucagon ratio, which improves glucose uptake by the liver and peripheral tissues. It seems that somatostatin-28 (S-28) is indirectly involved in the inhibition of glucagon secretion due to its regulatory effect on GLP-1 release through a feedback loop system and mediated by somatostatin receptor subtype 5 in the intestinal mucosa. Contrary to GLP-1, GIP has shown to counteract suppression of glucagon secretion by glucose during euglycaemia, but not so during hyperglycaemia (Meier et al., 2003; Chia et al., 2009).

2.2.2 Extrapancreatic effects of incretins

GIP has been proposed to have a physiological role on nutrient uptake into adipose tissue, thereby linking overnutrition to obesity. GIP levels are high in obese T2DM patients and lipids strongly enhance GIP secretion (Carr et al., 2008). The fact that GIP plays a role on adipose tissue has, only lately, been revealed. The first evidence came from the observation that GIP induced fatty-acid incorporation into rat epididymal fat pads in the presence of insulin (Beck et al., 1983), but was decreased or inhibited in the presence of somatostatin, in the same settings (Beck et al., 1988). The evidence that GIP influences fatty-acid incorporation into adipocytes is supported by GIPR expressed in adipose tissues (Yip et al., 1998), and posteriorly reinforced by studies on the genetic ablation of GIPR (Miyawaki et al., 2002). GIP levels are high in obese T2DM patients and the presence of lipids strongly enhances GIP secretion, apparently making the link between overnutrition and obesity (Carr et al., 2008; Gögebakan et al., 2015).

Although GIP has shown to increase the activity of lipoprotein lipase (LPL), which hydrolyses lipoprotein-associated triglycerides (TGs) to produce FFAs available for local uptake (Miyawaki et al., 2002), the molecular mechanism by which GIP acts on adipocytes is still largely unknown.

Unlike GIP, GLP-1 does not show any role in fat accumulation. Although GLP-1R is expressed in adipocytes (Seino, 2010), activation of GLP-1R affects none of the aforementioned signalling molecules and does not increase LPL activity in adipocytes (Kim et al., 2007a; Kim et al., 2007b). However, insulin secretion evoked by GLP-1 and consequent suppression of FFAs release is probably the most dominant effect observed on adipose tissue (Fig. 2.4).

GLP-1, released after meal ingestion, inhibits hepatic glucose release by the dual islet effects, stimulating insulin secretion and inhibition of glucagon secretion, depressing HGP (Fig. 2.4). A recent study performed by Jun et al. (2015) has consolidated the existence of an islet-independent mechanism. This neuroincretin mechanism of GLP-1 seems to restrain HGP by a direct liver effect and/or an indirect effect through neural afferents passing through the CNS (Katsurada et al., 2016).

GIP and GLP-1 receptors are present in the stomach. Although GLP-1 inhibits gastric emptying (Fig. 2.4) (Tong et al., 2014), GIP has been shown to have little effect on gastric emptying in humans and mice (Chia et al., 2009; Little et al., 2005; Meier et al., 2004; Meier et al., 2003). Activation of GLP-1 receptors in the pyloric sphincter causes a deceleration of gastric emptying and reduces postprandial blood glucose. Delaying gastric emptying and maintaining subsequent distension of the stomach, also affects peripheral satiety signals (Tambascia et al., 2014).

Incretins also seem to contribute to neuroendocrine regulation of energy homeostasis by direct and indirect pathways (Fig. 2.4). Directly, by intestinally-derived GLP-1, which stimulates vagal afferent CNS signalling that modulates suppressive effects on food intake (Hayes et al., 2014; Holst, 2007). Also, GPL-1R are present in the CNS and apparently mediate some effects in glycaemic regulation, such as suppression of HGP, increase in β-cell insulin secretion and reduction of gastric emptying (Hayes et al., 2014). Indirectly, by its insulinotropic action on insulin secretion, playing the latter, an important role in glucose homeostasis in the CNS where it acts on food intake control (Pénicaud, 2010; Rodriguez et al., 2010; Laron, 2009). Besides gut secretion,

GLP-1 seems to be produced by a specific neuronal population in the brainstem, which might be specifically implicated in the control of short-term and long-term energy balance (Hayes et al., 2014).

Cumulative evidence from functional and mechanistic studies supports a role of a renal incretin system in the modulation of Na^+ and water homeostasis and kidney function. GPL-1 affects renal haemodynamic and participates in renal functions that are crucial in glucose homeostasis. The kidney affects glycaemia by three main mechanisms: glucose reabsorption, glycogenolysis and gluconeogenesis (Triplitt, 2012b). Under normal circumstances and in healthy individuals, the kidney filters and reabsorbs 100% of glucose (Prié, 2014). Filtered glucose is 80 - 90% reabsorbed by high capacity Na^+/glucose cotransporter-2 (SGLT2) in the proximal convoluted tubule (PCT), and the remaining 10% by the Na^+/glucose cotransporter-1 (SGLT1) in the straight segment of the descending proximal tubule (PST). The net result is that glucose does not appear in the normal urine (DeFronzo et al., 2012; Abdul-Ghani et al., 2008). The apical transporters SLGT1 and SLGT2 are Na^+ and energy dependent, but in the basolateral domain, the glucose carriers expressed are GLUT-1 and GLUT-2, that do not require energy, Na^+, or any other ion (Brown, 2000).In the kidney, GLP-1R are present in endothelial cells and in the proximal renal tubules, which play a role in regulating the composition of urine. Stimulation of the GLP-1R in blood vessels results in relaxation of smooth muscle and increased renal blood flow. The natriuretic and diuretic properties of GLP-1 were proved in infusion studies in a rat model of salt sensitivity by chronic intravenous infusion of GLP-1 (Yu et al., 2003). Stimulation of GLP-1R in the proximal tubules results in increased loss of salt, water and electrolytes in urine. The latter occurs, as the GLP-1R situated in PCT of the kidney, is functionally linked to the Na^+/H^+ exchanger isoform 3 (NHE3) transporter. NHE3 is a membrane pump that retrieves Na^+ and other electrolytes from the tubular fluid (and thus from urine), thereby returning them into the circulation. Activation of the GLP-1 receptor by GLP-1 results in inactivation of this membrane pump, which leads to increased Na^+ loss in urine and consequentially, through osmotic effects, to increased fluid loss in urine (Salles et al., 2015).

Incretins also seem to have important effects on other organs and tissues, which likewise express GIPR and GLP-1R, however, by not being directly related to our work, will not be focussed.

2.3 THE INCRETIN DEFFECT IN T2DM

Some authors have shown that incretin pathways play important roles on β-cells (insulinotropic action) and on α-cells (glucagonotropic action) and in many other effector organs that are involved in the progression of T2DM, which are described in this section (De Fronzo, 2009; Drucker et al., 2006). GLP-1 is responsible for most of the incretin effect in normal individuals. The incretin defect in T2DM seems to have two main causes (Holst et al., 2004): defective secretion of GLP-1 and pronounced impairment of the insulinotropic effect of GIP. Nauk (2004) also proposed accelerated incretin metabolism and a defective responsiveness to both hormones, as other possible causes. Studies conducted on incretin secretion, especially on GLP-1, found that within 4 hours after a meal, a significant reduction of GLP-1 response was observed in T2DM patients when compared with normal individuals (Toft-Nielsen et al., 2001).

Several observations suggest that the abnormal GLP-1 secretion is most likely a consequence rather than a cause of diabetes (Knop et al., 2007). While GIP concentration is normal or modestly increased in patients with T2DM, its insulinotropic actions are significantly diminished. This implies that a defect exists at the physiologic or even supra-physiologic levels in patients with T2DM in response to GIP. The impaired responsiveness to GIP may suggest a possible link to GIPR down-regulation or desensitization (Geelhoed-Duijvestijn, 2007).

In contrast to GIP, the secretion of GLP-1 is reduced in obese subjects without diabetes, suggesting that incretin secretion is altered in the early stages of diabetes (Phillips et al., 2011). Insufficiency of GLP-1 contributes to a defective first phase of insulin secretion (Leech et al., 2010). In these patients, GIP secretion is normal, but its insulinotropic effect is markedly reduced, while GLP-1 secretion is reduced but

preserves its insulinotropic action, meaning that it can still effectively stimulate insulin secretion if present (Nauck et al., 2004). The cause for the different properties of GIP and the GLP-1 incretin effect in relation to changes in T2DM is not fully understood. The finding that T2DM patients have low circulating levels of GLP-1 with preservation of endogenous insulin secretion supports the therapeutic potential of GLP-1 treatments. Thus, while GIP has a low potential as a drug therapy, GLP-1, on the other hand, has a therapeutic potential as a promising pharmacological tool for the treatment of T2DM, already proposed in the 1990s, when the incretin effect was reviewed (Holst, 1999).

In addition to the pancreas, GIP and GLP-1 receptors are expressed in a wide variety of organs, including kidneys. Incretin defect also seems to play a role in the pathophysiology of DN. Both GLP-1R and DPP-4 are expressed in the kidney of various species, including humans and rats (Von Webskya et al., 2014). The expression of DPP-4 seems to be up- regulated in cultured human renal glomerular epithelial cells during inflammation (Stefanovic et al., 1993) and in a rat model of T2DM (Yang et al., 2007), as well as, in glomeruli of patients with DN, but not in healthy kidneys (Von Webskya et al., 2014). Increased DPP-4 activity has found to be present in human glomerular diseases (Kanwar et al., 2008; Mitic et al., 2008). DPP-4 is expressed in association with the NHE3 in the cells affecting NHE3 activity and the excretion of Na^+, HCO_3^-, and water reabsorption in the PCT (Von Websky et al., 2014). Although glycaemic levels affect renal pathophysiology, the previously mentioned effects of incretin protection appear to be independent of these levels, although the underlying mechanisms remain to be clarified (Saha et al., 2008, Avogaro et al., 2014).

Chapter 3

Incretin-based therapies - focus on sitagliptin

"Declare the past, diagnose the present, and foretell the future." Hippocrates

3.1 MAIN CLINICAL FEATURES OF SITAGLIPTIN

3.1.1 Pharmacodynamics

T2DM can generally be prevented with diet, physical activity, but individuals with impaired glucose tolerance should be treated with antidiabetic drugs (Donath et al., 2008; Rejeski et al., 2012; ADA, 2017). Currently, T2DM is managed with antidiabetic agents from nine pharmacological classes. These include agents that increase insulin secretion, improve insulin action, and delay absorption of carbohydrates. The ground-breaking incretin based therapies, address a previously unmet need in diabetes by modulating glucose supply (Cefalu, 2010; Riddle 2005).

Sitagliptin is a potent and highly selective DPP-4 competitive inhibitor that does not inhibit the closely related enzymes DPP-8 or DPP-9 at therapeutic concentrations (Kim et al., 2005; Mulvihill et al., 2014). Various studies in healthy volunteers, patients with type 2 diabetes and non-diabetic obese individuals showed that sitagliptin significantly ($p < 0.05$ vs. placebo) and dose-dependently inhibited DPP-4 enzyme activity by ≥ 80 % (at 12 h post-dose for 50 mg/ day; at 24 h post-dose for ≥ 100 mg/day) and increased mean postprandial active GLP-1 and GIP levels by approximately 2- to 3-fold (Bergman et al., 2006; Herman et al., 2006a; Herman et al., 2006b; Herman et al., 2005).

As T2DM patients exhibit relative resistance to the actions of GIP (Baggio et al., 2007), the main goals of DPP-4 inhibitors are to prolong the beneficial effects of endogenous GLP-1 (Gautier et al., 2008) in order to maintain its insulinotropic activity (Deacon et al., 2001).

Sitagliptin acts by inhibiting DPP-4 enzymes (Fig. 3.1) that rapidly hydrolyse GLP-1, preventing its inactivation. This increases plasma concentrations of the active form of GLP-1, allowing the consequent stimulation of insulin synthesis and its release from pancreatic β-cells (Fig. 3.1) in a glucose-dependent manner (Inzucchi et al., 2008; Ahrén, 2007; Campbell, 2007; Rosenstock et al., 2007).

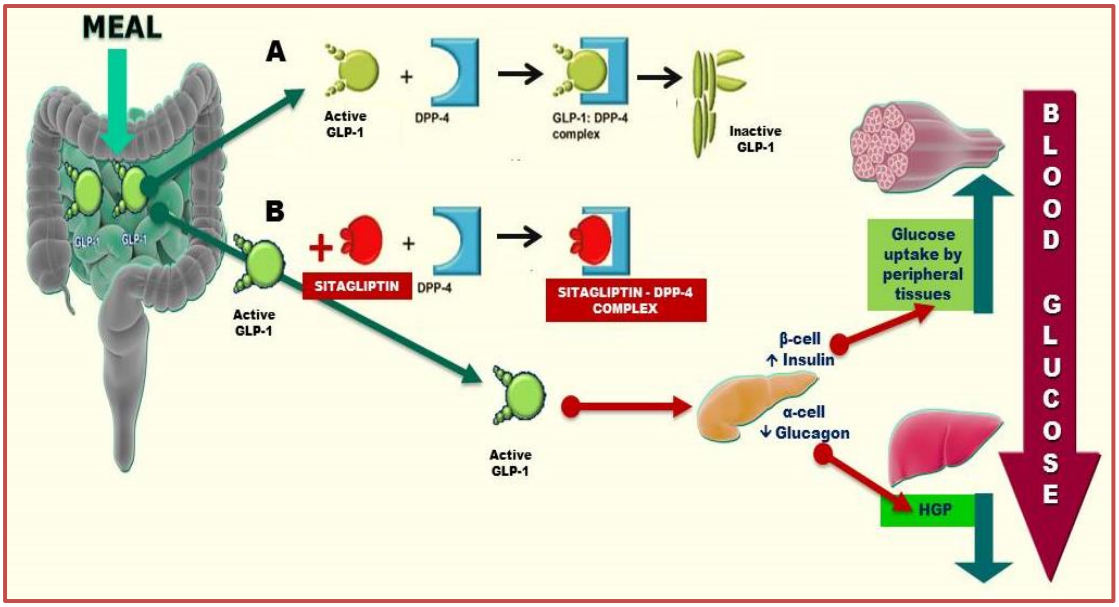


Figure 3.1 – Hypoglycaemic actions of sitagliptin. GLP-1 is produced in the L cells of the ileum, in a glucose dependent manner, and posteriorly degraded by DPP-4 enzyme. Binding to DPP-4 will cause GLP-1 to be inactivated, after which it is liberated in its inactive form. Sitagliptin delays GLP-1 inactivation by competing with GLP-1 for the DPP-4 catalytic site and thus preventing GLP-1 inactivation. The increase in GLP-1 plasma concentration stimulates β- and α-cells with consequent insulin synthesis and glucagon suppression, respectively. Insulin promotes glucose uptake by peripheral tissues (e.g. muscle) and glucagon inhibits HGP. DPP-4 - dipeptidyl peptidase-4; GLP-1 - glucagon-like peptide-1; HGP – hepatic glucose production.

Glycaemic levels are further regulated by the resulting higher insulin levels and glucagon suppression from the direct action of GLP-1 on pancreatic α-cells (Fig. 3.1). Postprandial insulin and C-peptide levels are also found to be increased and glucose excursions after an oral glucose tolerance test reduced (Herman et al., 2006a).

3.1.2 Pharmacokinetics

Sitagliptin promotes around 97% of DPP-4 inhibition (Deacon, 2011) and reduces blood glucose levels, either in the postprandial or the fasting state. It works differently from the previous drugs available for diabetes treatment and is orally active (Drucker, 2003). Its bioavailability is 87% and its oral absorption is not affected by food, with a recommended dose of 100 mg once daily. The fraction of sitagliptin reversibly bound to plasma proteins is 38% (Plosker, 2014).

Hepatic metabolism of sitagliptin is minimal, mainly by cytochrome P450 3A4; and 70-80% is excreted by the kidney in its unchanged form, exhibiting a half-life of around 12.4 hours and renal clearance is approximately 350 ml/min (Herman et al., 2005). In general, the pharmacokinetic profile of sitagliptin is similar in healthy volunteers and patients with T2DM. The pharmacokinetic properties of the drug have also been evaluated in special patient populations with varying grades of hepatic and renal dysfunction. As a result of its metabolism and elimination route, dose adjustment is only required in patients with severe renal impairment, but not in those with mild or moderate renal or hepatic impairment (Bergman et al., 2007; Migoya et al., 2009; Arjona et al., 2013). No dosage adjustment is necessary related to age, gender, race or BMI. Sitagliptin also has a low propensity for pharmacokinetic drug interactions (Plosker, 2014)

3.1.3 Clinical efficacy

Sitagliptin was the first drug of the DPP-4 inhibitors class, also known as gliptins, approved by the FDA, in 2006, for use in patients with T2DM to prevent the inactivation of the incretin hormones GLP-1 and GIP by the ubiquitous DPP-4 enzyme, thereby attenuating postprandial glucose elevations and consequently lowering HbAc1 (0.5 – 1.0%) (Plosker, 2014).

The first clinical efficacy studies with sitagliptin were performed in 2001 by Ahrén et al., still in the premarketing phase and confirmed significant effects in the reduction of PPG, FPG and HbA1c values after daily oral administration, during 4 weeks, in patients with T2DM (Ahrén et al., 2002).

Many post marketing trials have, since then, demonstrated the efficacy of sitagliptin in terms of improving glycaemic control in T2DM patients, either used as monotherapy, initial combination therapy (usually with a fixed-dose combination of sitagliptin/metformin), or add-on therapy to metformin or to other antihyperglycaemic drugs, with or without metformin. Sitagliptin showed efficacy in decreasing HbA1c, FPG and PPG levels, also increasing the proportion of patients achieving target HbA1c levels (<7.0%), as shown in several clinical studies (Scheen et al., 2010; Engel et al., 2013; Garg et al., 2013; Esposito et al., 2014; Plosker, 2014). Most studies also

reported that patients also received counselling on exercise and diet (Dungan et al., 2016).

Overall, gliptins are efficient at reducing plasma glucose, similarly to other therapeutic groups, and its use can be made in a combined form with other antidiabetic agents with distinct mechanism of action, in particular with metformin, the most widely used combination (Raz et al., 2008; Kadowaki et al., 2013).

Collateral beneficial therapeutic actions, including reduction in BP and amelioration of the lipid profile, have also been described for sitagliptin (Scheen, 2010; Campbell et al., 2013; Deacon et al., 2013). Concerning the impact of sitagliptin on lipid profile, the majority of studies reported a beneficial effect on TGs, HDL-c and LDL-c, as concluded in a systematic review and meta-analysis of 14 trials with incretin therapy in patients with T2DM (Amori et al., 2007). In addition, some studies suggested a reduction of BP in patients under sitagliptin treatment (Mistry et al., 2008; Ogawa et al., 2011; Subbarayan et al., 2011; Kawasaki et al., 2015).

3.1.4 Tolerability and safety

Sitagliptin is generally well tolerated by patients and the most common adverse events reported include upper respiratory tract infection, nasopharyngitis and headache. The overall incident rates for specific gastrointestinal events, including a composite of abdominal pain, nausea and vomiting, were similar between sitagliptin and non-exposed groups, with the exceptions of a higher rate of constipation in the sitagliptin group (Engel et al., 2013). Concerning body weight, several clinical trials showed that sitagliptin, generally, has a neutral effect (Bergenstal et al., 2010; Arechavaleta et al., 2011; Barzilai et al., 2011).

Hypoglycaemia seems to be of concern only when sitagliptin is used as add-on therapy to a sulfonylurea or insulin, otherwise the risk is low due to sitagliptin's glucose dependent mechanism of action (Vilsboll et al., 2010; Deacon et al., 2012; Engel et al., 2013; Round et al., 2013). Furthermore, recent recommendations for diabetics during Ramadan fasting encompass a stratifying of patients in relation to their risk of hypoglycaemia and/or the presence of complications prior to the beginning of fasting. Patients at high risk of hypoglycaemia and with multiple diabetic

complications are advised against prolonged fasting, while others should be medicated with agents such DPP-4 inhibitors, which are safe for avoiding hypoglycaemia and do not need major dose adjustments (Mahmoud et al., 2015).

Sitagliptin has proven to be safe to use in patients with mild or moderate renal or hepatic impairment, but an adjustment in dosage is recommended for patients with severe renal insufficiency (Neumiller et al., 2015; Betônico et al., 2016).

The putative association of DPP-4 therapy with development of pancreatitis and pancreatic cancer is not yet completely elucidated, but recent studies have been more reassuring in relation to safety concerning these two aspects. Nonetheless, current evidence is not definitive and vigilance is mandatory (Dore et al., 2009; Garg et al., 2010; Pappachan, 2015).

Concerns on the effects of gliptins on cardiovascular safety, led to evaluation of the following off-target end-points: body weight, BP, lipid profile, albuminuria, major adverse cardiovascular events (MACE), heart failure (HF), acute myocardial infarction (AMI) and β-cell function, where randomised controlled trials were prioritised as the primary source of information. Studies on cardiovascular safety for incretin-based therapies revealed that DPP-4 inhibitors were neutral in terms of CV events (Dicker, 2011) and that the benefits of GLP-1-related therapies far outweigh the potential risks (Deacon et al., 2013; Nauck, 2013). Monami et al., in 2013, revealed that DPP-4 inhibitors may have a protective effect in reducing MACE, as well as, a reducing AMI in greater proportion than expected (based on conventional risk factors) and in all-cause mortality in T2DM. More recently, , and after the revelation of results from the TECOS, McGuire et al. (2016) have reported similar results, deeming sitagliptin safe to be used by diabetic patients with at high cardiovascular risk without increasing rates of cardiovascular complications. To date, only saxagliptin signalled increased hospitalisations for HF, warranting continued evaluation and vigilance in high-risk patients. Moreover, evidence from published clinical trials suggest that incretin-based therapies have shown beneficial effects on off-target CVD risk factors, specifically, body weight, BP, lipid profile, albuminuria and do not increase MACE (Rotz et al., 2015; McGuire et al., 2016).

Also noticeably, a large, retrospective, population based cohort study showed that sitagliptin was not associated with any increased risk of all-cause hospital

admission or death compared with other antidiabetic agents in newly treated patients with T2DM (Eurich et al., 2013).

3.2 CYTOPROTECTIVE EFFECTS OF SITAGLIPTIN BEYOND GLYCAEMIC CONTROL

Sitagliptin was originally developed to lower plasma glucose levels, but accumulating evidence shows additional protective effects in the pancreas and other organs in relation to the diabetic state. Prior treatments for T2DM do not seem to address the progressive decline in β-cell function and patients continue to advance in their disease state (Raz et al., 2006). Streamlining on the natural incretin effect, gliptins could theoretically preserve and even reverse the progressive loss of insulin secretory capacity and consequently, avoid or delay diabetic complications (Raz et al., 2006; Gupta et al., 2011; Russell-Jones et al., 2012).

DPP-4 inhibition by sitagliptin has shown to have cardiovascular protective properties in which sitagliptin improved the myocardial response to dobutamine stress in a study of patients with coronary artery disease (Read et al., 2010). It was found to attenuate atherosclerotic lesions in diabetic models, such as Zucker Diabetic Fatty (ZDF) rats and high-fat diet (LDL)-receptor-deficient mice, via downregulation of oxidative stress and inflammation (Shah et al., 2011). In a study by Bose et al. (2005), GLP-1 demonstrated to directly protect the heart against ischemia/reperfusion injury. In a previous work by our group, sitagliptin lowered known CVD risk factors, such as serum levels of triglycerides, BP, heart lipid peroxidation and serum inflammatory markers in Zucker diabetic fatty rats (Ferreira et al. 2010). DPP-4 inhibition also appears to improves endothelial function in patients with T2DM, in both GLP-1-dependent and -independent manners (Van Poppel et al., 2011; Yoon et al., 2011). Specifically, sitagliptin was able to increase endothelial progenitor cell (EPC) levels in human diabetic patients (Fadini et al., 2010).

Besides increasing hepatic insulin sensitivity, sitagliptin seems to also prevent steatosis (Shirakawa et al. 2011b) through GLP-1R signalling in the liver and reduction

of endoplasmic reticulum stress (Oyadomari et al. 2008). GLP-1R have been found to be expressed in human hepatocytes (Oyadomari et al., 2008), but in contrast, other investigators have failed to detect GLP-1R mRNA transcripts in human, rat, or mouse liver (Flock et al. 2007; Lee et al. 2007; Aviv et al. 2009). Anti-apoptotic effects in human hepatoma cells by DPP-4 inhibition have also been identified (Gaetaniello et al. 1998).

Treatment of non-obese diabetic mice with sitagliptin, not only prevented linoleic acid-induced adipose tissue hypertrophy, but also protected against adipose tissue inflammation (Shirakawa et al. 2011a).

DPP-4 inhibition seems to be associated with the prevention of peripheral nerve degeneration, found in diabetic animal models, through GLP-1 and GLP-1R signalling (Jin et al. 2009), which has later been reinforced by recognition of GLP-1R expression in the sciatic nerve and skin of STZ-induced diabetic rats (Liu et al. 2011).

In the work conducted by Mu et al. (2006 and 2009), sitagliptin significantly restored β- and α-cell mass as well as α/β-cell ratio and significantly increased islet insulin content and improved glucose-stimulated insulin secretion in isolated islets.

In this context, the research of the underlying signalling pathways involved in apoptosis and in other pathophysiological pathways (such as those observed in other organs), like oxidative stress, advanced glycation end products, inflammation and the capacity for regeneration of pancreatic β-cells, could eventually provide knowledge for improved protection of the pancreas and thus, delay diabetic evolution.

The involvement of the incretin system in DN has been suggested by changes in the expression levels of DPP-4, GLP-1, and GLP-1R following injury. The expression pattern of GLP-1R protein in the human and porcine kidneys was identified, by immunohistochemical analysis, predominantly in the proximal tubules (Schlatter et al., 2007). DPP-4 was found to be upregulated in several tissues, including the kidney, in high-fat diet–fed rats (Kirino et al., 2009). DPP-4 upregulation was also observed during inflammation in cultured glomerular epithelial cells (Stefanovic et al., 1993) which has been associated with the development of glomerulosclerosis in DN. Anti-DPP-4 antibody inhibited the glomerular deposition of complement on the mesangial cell surface, indicating a probable protective role for the neutralizing antibody of DPP-4 against complement-mediated tissue injury (Shinosaki et al., 2002). However, it is still

not elucidated whether similar protection might be conferred by the enzymatic inhibition of DPP-4 using sitagliptin, or other DPP-4 inhibitors. Jost et al. (2009) showed that DPP-4 inhibition influenced collagen metabolism in the kidney, thereby leading to the depletion of collagen-derived peptides, which suggests that extracellular collagen may be an in vivo substrate for DPP-4. In this framework, it would be interesting to know whether the inhibition of DPP-4 activity might affect extracellular matrix remodeling during the development and evolution of DN. It is also known that inflammation promotes development and progression of diabetic microangiopathy, which is subjacent to the most severe diabetic complications, nephropathy, retinopathy, and neuropathy (Kern, 2007; Navarro-González et al., 2008; Vincent et al., 2011). The identified incipient protective effects of sitagliptin, through the reduction of oxidative stress, inflammation, anti-apoptotic and pro-proliferative actions on various organs and tissues, suggests that these could be researched in the kidneys by investigating the effects of sitagliptin on renal diabetic lesions and pathophysiological signalling pathways.

This emerging knowledge makes sitagliptin, as a DPP-4 inhibitor, an interesting target to study regarding its glycaemic and non-glycaemic actions (Durinx et al., 2000; Boonacker et al., 2003; Gorrell, 2005).

Chapter 4

Aims

T2DM is a metabolic disease characterized by chronic hyperglycemia resulting from the combination of insulin resistance, deficient insulin secretion and dysregulation of glucagon secretion. Given that insulin is either not sufficient or the body is unable to recognize and use it properly, glucose cannot be used by the cells and builds up in the bloodstream. Over time, the high levels of blood glucose will damage pancreatic β-cells and other organs of the body. DN, one of the major microvascular complications of diabetes, is the major cause of end-stage renal disease, often requiring dialysis or transplantation.

In the last decade, a new class of anti-hyperglycemic agents, based on incretins, has enriched the therapeutic arsenal for T2DM treatment. The incretin-based therapies comprise DPP-4 inhibitors and GLP-1 receptor agonists. Besides improving glycaemic control, they have shown the possibility to change the evolution of T2DM and its complications, which might be due to beneficial effects on the pancreas, as well as, on diabetes-target tissues, including the kidneys'.

We have previously reported that sitagliptin, the first DPP-4 inhibitor approved for clinical use in T2DM patients, is able to prevent pancreas alterations induced by chronic hyperglycemia in T2DM animal models. In addition to improved glycaemic dysmetabolism, sitagliptin also showed a positive impact on lipid peroxidation and prevented hypertension development (Ferreira et al, 2010).

Based on previous claims for effects of incretin peptides in distinct tissues, we hypothesize that sitagliptin is able to preserve pancreatic and kidney functionality, not only by the improvement of the glycaemic profile and insulin resistance, but also by other effects. Our main goal was to investigate the mechanisms underlying the putative protective effects of sitagliptin on pancreatic and renal tissues. This was pursued by using an animal model of T2DM (the ZDF rat).

In Chapter 5, we assessed the beneficial effects of sitagliptin on endocrine and exocrine pancreas lesions and dissected some of the mechanisms known to underlie pancreatic dysfunction and diabetes evolution, such as apoptosis, inflammation, angiogenesis and proliferation. The extra-pancreatic effects of sitagliptin, specifically its putative ability to halt the progression of DN, are described in Chapter 6, focusing on antioxidant, anti-inflammatory and anti-apoptotic cytoprotective properties.

PART II:

EXPERIMENTAL WORK

Chapter 5

Sitagliptin prevents aggravation of endocrine and exocrine pancreatic damage in the Zucker Diabetic Fatty rat - focus on amelioration of metabolic profile and tissue cytoprotective properties

5.1 BACKGROUND AND AIMS

T2DM prevalence and incidence is increasing rapidly in many countries, including in Portugal. It is predicted, according to the latest estimates of WHO, that diabetes will be the 7th leading cause of death in 2030 (WHO, 2013). T2DM is a chronic disease leading to macro- and microvascular complications, which results in severe illness and premature death, with elevated personal and economic costs (WHO, 1999; Stumvoll et al., 2004).

The central features of T2DM are a defect in insulin resistance and/or insulin secretion, which lead to hyperglycaemia; disruption of the normal relationship between insulin sensitivity and pancreatic β-cell function, which is a hallmark of T2DM progression (Virally et al., 2007). In fact, degeneration of Langerhans islets with β-cell loss is secondary to insulin resistance and is regarded as the most important lesion for progression of the disease (Kahn et al., 2000; Marchetti et al., 2010). As β-cell function declines, the impairment of insulin action becomes more important. Hyperglycaemia, *per se*, may have a detrimental effect on secretory function, – "glucotoxicity" –, which induces increased apoptosis in pancreatic islets; in addition, the abnormal lipid profile commonly observed in these subjects may be associated with functional impairment of the islet – "lipotoxicity" (Marchetti et al., 2010; Del Prato, 2009; Poitout et al., 2002; Kahn et al., 2000).

Current knowledge adds further complexity in the picture of T2DM pathogenesis by including the role of incretin hormones. Incretins (GIP and GLP-1) are regulatory peptides secreted in the gastrointestinal mucosa after meal ingestion. These are released in response to oral glucose intake and are able to attain physiological concentrations causing insulin release, which is called the "incretin effect" (McIntyre et al., 1964; Creutzfeldt, 1979; Creutzfeldt, 2005; Ranganath, 2008). GLP-1 acts in a positive way on the β and δ cells, whereas GIP acts preferentially on α and β cells. These peptides are almost undetectable during fasting and exist only in high concentrations in the postprandial state, since they are rapidly metabolized (Deacon et al., 1995) by the ubiquitous enzyme DPP-4 to inactive metabolites, which are eliminated by urine (Ranganath, 2008). The incretin effect is responsible for about

60% of the secretion of postprandial insulin, which is decreased in T2DM (Gallwitz, 2005). In these patients, the incretin effect is stifled, producing an "incretin defect". This condition occurs as a result of reduced secretion of GLP-1, accelerated metabolism of GLP-1 and GIP, as well as, a defective response to both hormones, particularly to the insulinotropic effect of GIP (Holst et al., 2004; Nauck et al., 2004). The key mechanisms by which these factors exert their action on β-cells are not yet completely elucidated, but currently lie on metabolic processes such as apoptosis and inflammation, among others also putatively involved.

Low-grade inflammation has been viewed as a major player in insulin resistance development and T2DM evolution. Indeed, hyperglycaemia seems to induce the production of acute phase reactants from the adipose tissue, while obesity, present in many diabetic patients, is in itself, characterized as a state of low grade inflammation (Lin et al., 2001). T2DM is found to display increased concentrations of C-reactive protein (CRP) and pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, which are implicated in instigating metabolic insulin resistance (Hotamisligil et al., 1999). Additionally, although the loss of β-cell mass is not yet completely clarified, apoptosis seems to be involved, as previously observed in the pancreas at autopsy and isolated islets from people with T2DM (Huang et al., 2007; Laybutt et al., 2007). Based on these assumptions, it is becoming clear that T2DM management, namely by using pharmacological agents, must envision not only glycaemic control but also, and particularly, the mechanisms behind progression of pancreatic deterioration and underlying evolutional complications. In fact, T2DM therapeutics should be able to preserve β-cell mass as the mainstay of disease control, by addressing the mechanisms implicated in diabetic pathogenesis, including apoptosis, inflammation or even an added capacity for cell proliferation.

Enhancing the incretin effect is now a possible therapeutic target in T2DM, using GLP-1 analogues or DPP-4 inhibitors. Sitagliptin belongs to a class of oral antidiabetic drugs, the gliptins, which inhibit the enzyme DPP-4 that degrades incretins, prolonging the physiological actions of GLP-1 (Knøp et al., 2007; Seshadri et al., 2009). GLP-1, a prominent active compound of the incretin family, modulates many processes in the pancreatic islet: it potentiates insulin synthesis and secretion by the β-cells (Holst et al., 2009), inhibits glucagon secretion in α-cells (Toft-Nielsen et al.,

2001), increases islet cell proliferation, and decreases cell apoptosis (Farilla et al., 2002; Li et al., 2003).

Our group has previously shown that sitagliptin is able to ameliorate dysmetabolism, insulin resistance, inflammation and oxidative stress in an animal model of T2DM, the ZDF rat (Ferreira et al., 2010). Thus, the purpose of the current study was to investigate some of the possible mechanisms underlying the protective effects produced by chronic sitagliptin treatment on pancreatic tissue in the ZDF rat, focusing on apoptosis, inflammation, angiogenesis and proliferation mediators.

5.2 METHODS

5.2.1 Animals and experimental design

Male ZDF rats (ZDF/Gmi, fa/fa) and their littermates (ZDF/Gmi, +/+) were purchased from Charles River Laboratories (Barcelona, Spain) with 6 weeks of age and kept in our animal facility until they reached 20 weeks of age. Rats were properly housed, handled daily, and kept at a controlled standard temperature (22-23°C), humidity (60%) and light–dark cycles (12/12 h). Throughout the experiment, the animals were provided with distilled water *ad libitum* and rodent maintenance chow (A-04 Panlab, Barcelona, Spain, containing 15.4% of protein and 2.9% of lipids). The chow was adapted to the animal's body weight (BW): 100 mg/g. Animal experiments were conducted according the European Council Directives on Animal Care and the National Laws. Along the text and in order to simplify the description of the animals, the ZDF/Gmi, fa/fa rats will be designated as diabetic rats, and, when under sitagliptin treatment, as sitagliptin-treated diabetic rats. The ZDF/Gmi, +/+ rats will be designated as lean control or control rats. The initial groups were established as 24 diabetic rats and as 16 lean control rats. When aged 20 weeks, 8 obese diabetic and 8 lean control rats were sacrificed for blood and tissue collection, in order to establish the basal levels. The remainder lean control rats followed to week 26, as well as, the diabetic rats which were divided in two sub-groups (n = 8 each). The sitagliptin-treated group received by oral gavage, once a day (6:00 PM), during 6 weeks, 10 mg/kg/ body weight

(BW) of sitagliptin dissolved in orange juice (vehicle) and the diabetic untreated group received, in the same conditions, only the vehicle (orange juice). Food intake and BW were measured each day before treatment and expressed as weekly average values. The same procedures were adopted with the lean control rats. Since the ZDF (+/+) control group under sitagliptin treatment showed no relevant differences when compared with the ZDF (+/+) control rats under vehicle, the results were excluded from tables and figures in order to facilitate data comparison and interpretation. At 26 weeks of age, the animals were sacrificed by anaesthetic overdose, blood and tissues were collected for different analyses.

5.2.2 Sample collection and preparation

Blood

When aged 20 weeks (T0) and at the end of the experience (26 weeks -Tf) the rats were subjected to intraperitoneal anaesthesia with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal) and blood samples were immediately collected by venipuncture from the jugular vein into syringes without anticoagulant (for serum samples) or with the appropriate anticoagulant: ethylene-diaminetetraacetic acid (EDTA)-2K for HbA1c measurement.

Tissues

The rats were sacrificed by anaesthetic overdose and the pancreas was immediately removed, placed in ice-cold Krebs' buffer and carefully cleaned of extraneous fat and connective tissue. For histopathological and immunohistochemical studies part of the organ was cross-sectioned, fixed and processed for paraffin embedding in compliance with the histological protocols used. For pancreatic gene (mRNA) expression by RT-qPCR the remaining cleaned portion of the pancreas was immediately placed in preservative RNA later™ solution (Ambion, Austin, TX, USA) and frozen straightaway at −80°C until analysis.

5.2.3 Glycaemic, insulinaemic and lipidic profile assays

Serum glucose levels were measured using a Glucose oxidase commercial kit (Sigma, St. Louis, Mo, USA). Considering the variability of serum glucose levels in the rat, HbA1c levels were used as an index of glucose control, through the DCA 2000+ latex immunoagglutination method (Bayer Diagnostics, Barcelona, Spain). Plasma insulin levels were quantified by using a rat insulin Elisa assay kit from Mercodia (Uppsala, Sweden). The steady state β-cell function of individual animals was evaluated using the previously validated homeostasis model assessment (HOMA) of β-cell function (Wallace et al., 2004). The formula used was as follows: [HOMA-β%] = 360 × fasting serum insulin (mU/l)/ fasting serum glucose (mg/dl) − 63. The values used (insulin and glucose) were obtained after an overnight of food deprivation. Serum levels of TGs and serum total cholesterol (Total-C) were analysed on a Hitachi 717 analyser (Roche Diagnostics) using standard laboratorial methods. TGs kit was obtained from bioMérieux® (Lyon, France).

5.2.4 Endocrine and exocrine pancreas lesions analysis by histopathology

Haematoxylin & eosin staining

Samples were fixed in Bock's fixative embedded in paraffin wax and 3 µm thick sections were stained for routine histopathological diagnosis with haematoxylin and eosin (HE). All samples were examined by light microscopy using a Microscope Zeiss Mod Axioplan 2. Image acquisition was performed with digital microscope camera (Leica DFC450) and image processing was performed with the LAS Advanced Analysis Software Bundle (No: 12730448.). The degree of injury visible by light microscopy was scored in a double blind fashion. *Assessment of mean islet number:* Islets were counted using a 10× magnification, in three different microscopic fields, and the mean number per field was calculated for each study group. Assessment of islet size: Islet dimensions were obtained by measuring its maximum girth with an ocular grid of 1000 µm, using a 10× magnification. The maximum diameter was found by comparing all available radii diameters of each islet, and choosing the greatest. Islets were evaluated in three different microscopic fields and the mean size of the islets from each group of rats was calculated.

Histopathology

Appreciation of islet architecture was based on the uniformity of islet boundaries and classified as regular or irregular. Endocrine pancreatic damage was assessed by evaluating changes in the islets of Langerhans, namely, islet architecture (shape), presence of inflammatory infiltrate, fibrosis, and vacuolization and intra-islet congestion. A semiquantitative rating for each slide ranging from 0 (minimal) to 3 (severe and extensive damage) was assigned to each of the studied components: inflammatory infiltrate, congestion, vacuolization and fibrosis. Each islet was examined and scored. Severity was graded as 0 = absent, 1 = mild, 2 = moderate and 3 = severe. Extension was evaluated by the area occupied by the lesion, an area of < 25% of the islet, was scored as 0, an area 25 - 50% scored as 1, an area 50 - 75% scored as 2, and if detected in an area > 75% scored as 3. The final score of each sample was obtained by the average of scores observed in individual islets. Exocrine pancreatic damage was evaluated according to the presence of congestion, fibrosis and inflammatory infiltrate in the interstitial tissues and graded also, by the same semiquantitative method, considering the entire exocrine parenchyma on the slide, as previously described (Ferreira et al., 2010).

Periodic acid of Schiff staining

Periodic Acid of Schiff (PAS) was used to confirm the levels of islet and exocrine fibrosis. Samples were fixed in neutral formalin 10%, embedded in paraffin wax and 3 µm thick sections were immersed in water and subsequently treated with a 1% aqueous solution of periodic acid, then washed to remove any traces of the periodic acid and finally treated with Schiff's reagent. A semiquantitative rating was set for intensity and extension of staining, ranging from 0 (no staining) to 3 (intense and extensive staining).

5.2.5 Pancreatic protein expression by immunohistochemistry

Formalin-fixed and paraffin embedded tissues were cut into 3 µm sections and deparaffinised in xylene. 3% H_2O_2 was used to remove endogenous peroxidase, and citrate buffered saline (pH 6.0), in MO, was used for antigen retrieval. Sections were pre-incubated with normal rabbit serum to prevent nonspecific binding and then incubated overnight at 4°C with anti-Bax (Δ 21, 1:50, rabbit polyclonal antibody, Santa

Cruz, Biotechnology), Bcl-2 (C 21, 1:50, rabbit polyclonal antibody, Santa Cruz, Biotechnology) and TRIB3 (ab22107, 1:100, rabbit polyclonal antibody, Abcam). The sections were then sequentially incubated at room temperature, with labelled-(strept)avidin-biotin-peroxidase method (LAB-SA) (Histostain®-Plus kit Zymed). Negative controls were included in each staining series, by omission of the primary antibodies. Positive controls were, respectively for Bax, Bcl-2 and TRIB3, canine tonsils, canine breast carcinoma and rat exocrine pancreas. Sections were counterstained with hematoxylin. The results were examined by light microscopy using a Zeiss Axioplan 2 microscope. Image acquisition and processing was performed as described in the previous section. Immunopositivity was scored in accordance to staining intensity (I) and percentage of positive cells (P). Staining intensity was evaluated as 0, undetectable; 1, weak staining; 2, moderate staining and 3, intensive staining. Positive cells were evaluated in all Islets of Langerhans present on the slide. Final scoring for each rat was calculated by the Quick Score (Q) in which the percentage of positive cells (P) is multiplied by the intensity (I), using the formula: $Q = P \times I$, resulting in a score between 0 – 300 (Detre et al., 1995). The final score for each group was found by mean average.

5.2.6 Pancreatic gene (mRNA) expression analysis by RT-qPCR

Total RNA isolation

Samples were removed from the RNA later™ preservation solution and 1200 µL of RLT Lysis Buffer was added to proceed with disruption and homogenization for 2 min at 30 Hz using TissueLyser (Qiagen, Hilden, Germany). Tissue lysate were processed according to the protocol from RNeasy® Mini Kit (Qiagen, Hilden, Germany). Total RNA was eluted in 50 µL of RNase-free water (without optional treatment with DNAse). In order to quantify the amount of total RNA extracted and verify RNA integrity (RIN, RNA Integrity Number), samples were analysed using 6000 Nano Chip® kit, in Agilent 2100 bioanalyser (Agilent Technologies, Walbronn, Germany) and 2100 expert software, following manufacturer instructions. The yield from isolation was from 0.5 to 3 µg; RIN values were 6.0-9.0 and purity (A260/A280) was 1.8-2.0.

Reverse transcription

RNA was reverse transcribed with SuperScript™ III firststrand synthesis system for RT-PCR (Invitrogen, California, USA). One microgram of total RNA was mixed with a 2× First-Strand Reaction Mix and a SuperScript™ III Enzyme Mix [Oligo (dT) plus Random hexamers]. Reactions were carried out in a thermocycler Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA), 10 min at 25°C, 50 min at 50°C and 5 min at 85°C. Reaction products were then digested with 1 µL RNase H for 20 min at 37°C and, finally, cDNA eluted to a final volume of 100 µL and stored at −20°C.

Relative quantification of gene expression

Performed using a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A normalization step preceded the gene expression quantification, using geNorm Housekeeping Gene Selection kit for Rattus norvegicus (Primer Design, Southampton, UK) and geNorm software (Ghent University Hospital, Center for Medical Genetics, Ghent, Belgium) to select optimal housekeeping genes to this study (Vandesompele et al., 2002). Real-time PCR reactions used specific QuantiTect Primer Assays (Qiagen, Hilden, Germany) with optimized primers for Bax, Bcl-2, TRB3, IL-1β, PCNA and VEGF. Endogenous controls were also used: GAPDH, ACTB, TOP1, and RPL13 together with QuantiTect SYBR Green PCR Kit Gene expression (Qiagen, Hilden, Germany) according to manufacturer's instructions. RT-qPCR reactions were carried out with 100 ng cDNA sample, primers (50–200 nM) and 1X QuantiTect SYBR Green PCR Master Mix. Nontemplate control reactions were performed for each gene, in order to assure no unspecific amplification. Reactions were performed with the following thermal profile: 10 min at 95°C plus 40 cycles of 15 s at 95°C and 1 min. at 60°C. Real-time PCR results were analysed with SDS 2.1 software (Applied Biosystems, Foster City, CA, USA) and quantification used the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

5.2.7 Statistical analysis

For all biochemical measurements made over time and treatment effect, independent samples t-Student test was used. For histopathology and immunohistochemistry data: Chi-square test with Monte Carlo simulation or exact test (when contingency tables are 2×2) was performed to find out the differences in lesions

of endocrine/exocrine pancreas between lean control and diabetic ZDF rats at the beginning of the study, (20 week-old); untreated and sitagliptin treated diabetic ZDF and lean control rats at 26 weeks of age. Independent samples t-Student test was used to determine the differences in the number, regularity and size of the pancreatic islets between lean control and diabetic ZDF rats in the pre-therapeutic stage, at 20 weeks; untreated and sitagliptin-treated diabetic ZDF and lean control ZDF rats at 26 weeks of age. Data were analysed using SPSS Statistics 20 (2011). For RT-qPCR data: For statistical analysis, we used the GraphPad Prism, Version 5.0. Comparisons between groups were performed using ANOVA and the post-hoc Bonferroni test. All values are reported as mean ± SEM (standard error of means). Significance level was accepted at 0.05.

5.3 RESULTS

5.3.1 Sitagliptin prevents aggravation of glycaemic, insulinaemic and lipidic profiles

Concerning body weight, no significant differences were encountered between the diabetic and the lean control rats at the beginning of treatments (T0: week 20), despite the obese profile encountered in the diabetic rats between the 8th and the 14th week. At the end of the study (26 weeks), the untreated diabetic rats exhibited a reduction in their BW ($p < 0.001$); nevertheless, the lean control group gained weight. Sitagliptin treatment, during 6 weeks, stabilized the loss of weight in the diabetic ZDF rats and even prevented part of the BW loss when compared with the rats without treatment (Table 5.1). At the beginning of treatments (T0: week 20), fasting blood glucose, HbA1c and TGs were already significantly higher in diabetic rats when compared with their lean counterparts, indicative of a metabolic deregulation. These results were accompanied by a decrease in fasting serum insulin and in the functional ability of the pancreas, which was demonstrated by the reduction of 87.99% in HOMA-beta values. An age-dependent increase in the metabolic deregulation was observed in diabetic untreated ZDF rats presenting augmented levels of glucose,

HbA1c and TG from 20 to 26 weeks old. They also exhibited an aggravation of relative insulinopaenia, as well as a decrease in estimated steady-state β-cell function ($p < 0.001$). Six weeks of sitagliptin treatment was able to significantly ameliorate all the metabolic parameters as shown in Table 5.1. In fact, sitagliptin significantly ($p < 0.001$) prevented the additional increase in blood glucose and serum TG contents (16.54% and 37.63%, respectively, *vs* untreated), while preventing further decrease in serum insulin and enhancing the functional capacity of β-cells (156.28% and 191.74%, respectively, vs untreated) (Table 5.1).

Table 5.1 – Body weight and glycaemic, insulinaemic and lipidic profile in the rats under study at the initial and final times

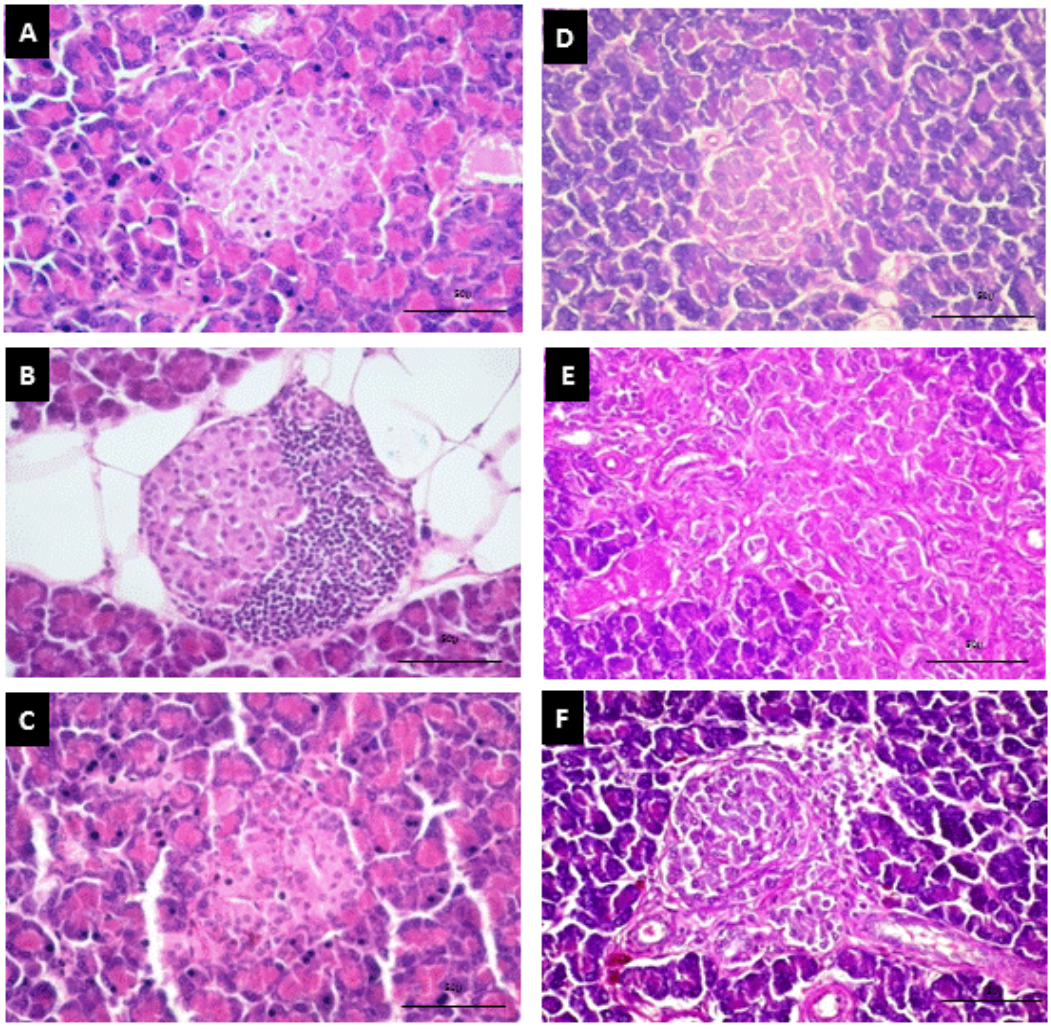
Parameter	Group	20 weeks	26 weeks
BW (g)	Control	406.70 ± 6.83	445.70 ± 8.16
	Diabetic (Vehicle)	388.10 ± 8.87	354.40 ± 8.85^{aaa}
	Diabetic (Sitagliptin)	—	380.00 ± 14.46
Glucose (mg/dL)	Control	131.80 ± 1.20	133.40 ± 2.16
	Diabetic (Vehicle)	512.00 ± 4.,53^{aaa}	623.60 ± 7.30^{bb}
	Diabetic (Sitagliptin)	—	520.44 ± 3.31^{ccc}
HbA1c (%)	Control	3.66 ± 0.15	3.76 ± 0.26
	Diabetic (Vehicle)	9.96 ± 0.86^{aaa}	10.68 ± 0.75
	Diabetic (Sitagliptin)	—	8.68 ± 0.37^{ccc}
Insulin (mU/L)	Control	14.82 ± 3.90	15.13 ± 3.34
	Diabetic (Vehicle)	11.62 ± 1.15^{aaa}	7.16 ± 1.14^{bbb}
	Diabetic (Sitagliptin)	—	11.19 ± 1.73^{ccc}
HOMA-Beta (%)	Control	77.70 ± 2.28	77.52 ± 1.10
	Diabetic (Vehicle)	9.33 ± 0.83^{aaa}	4.60 ± 0.16^{bbb}
	Diabetic (Sitagliptin)	—	8.82 ± 0.32ccc
TGs (mg/dl)	Control	111.60 ± 8.33	158.40 ± 6.80
	Diabetic (Vehicle)	366.00 ± 5.92^{aaa}	409.80 ± 8.04^{bb}
	Diabetic (Sitagliptin)	—	255.60 ± 3.70^{ccc}

Values are means ± SEM of n = 8 rats. Comparisons between groups: aaa = $p < 0.001$ between diabetic versus control groups (20 or 26 weeks); bb and bbb = $p < 0.01$ and $p < 0.001$, respectively, within the diabetic group (26 versus 20 weeks); ccc = $p < 0.001$ between diabetic sitagliptin-treated versus diabetic untreated groups (at 26 weeks). BW: body weight; HbA1c: glycosylated haemoglobin; HOMA: homeostasis model assessment; Total-c: total-cholesterol; TGs: triglycerides.

5.3.2 Sitagliptin prevents aggravation of endocrine and exocrine pancreas lesions

Comparative analysis between the endocrine pancreas of lean control and diabetic ZDF rats of 20 weeks of age revealed a significant increase in inflammation and fibrosis of Langerhans islets in the diabetic group ($p < 0.01$), with no statistically significant differences in other analysed parameters. At 26 weeks of age, endocrine pancreatic inflammation was significantly higher ($p < 0.001$) in the diabetic rats when compared with the lean control animals (Fig. 5.1 A, B and G). Sitagliptin-treated diabetic ZDF rats showed significantly ($p < 0.001$) reduced inflammation when compared with the untreated diabetic rats (Fig. 5.1 B, C and G). A similar profile was encountered concerning endocrine pancreatic fibrosis, which, despite the differences, did not reach statistical significance (Fig. 5.1 D-H).

Endocrine inflammation **Endocrine fibrosis**



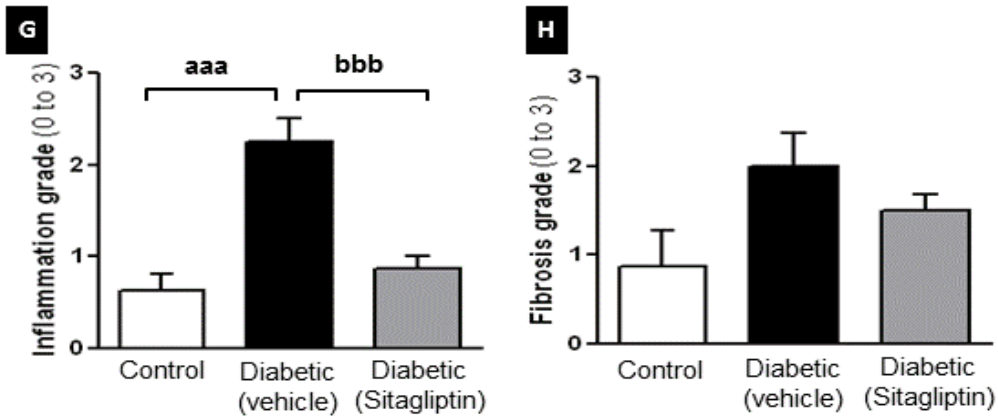


Figure 5.1 - Sitagliptin effects on inflammation and fibrosis of endocrine pancreas of diabetic ZDF rats. Endocrine inflammation in 26 week-old animals (images A-C): (A) Micrograph of normal islet of Langerhans observed in a lean control ZDF rat; (B) Islet of Langerhans of an untreated diabetic ZDF rat showing severe inflammatory infiltrate occupying over 50% of the islet's area (Grade 3 inflammation); (C) A clear regression of inflammation is observed after 6 weeks of sitagliptin treatment in obese diabetic ZDF rat (Grade 1 inflammation) (HE). Endocrine fibrosis observed in 26 week-old animals (images D-F): (D) Normal islet of Langerhans of lean control ZDF rat; (E) Grade 3 fibrosis in an untreated obese diabetic ZDF rat, displaying a large and irregularly shaped islet filled with fibrous tissue, evidenced by intense pink staining; (F) Grade 1 fibrosis in a regular, small islet in sitagliptin treated diabetic ZDF rats (PAS). Semiquantitative evaluation of inflammation (graph G) and fibrosis (graph H): (G) A significantly higher ($p < 0.001$) endocrine inflammation in diabetic rats was recorded when compared to lean control animals. Sitagliptin treatment of diabetic ZDF rats during 6 weeks significantly reduced ($p < 0.001$) inflammation in comparison to untreated counterparts; (H) Data for endocrine fibrosis showed a trend for an increase in diabetic rats when compared to lean control animals, and a trend for improvement with sitagliptin treatment, although without statistical significance. Chi-square test with Monte Carlo simulation or exact test (when contingency tables are 2 × 2) was performed to find out the differences in histomorphological lesions observed in endocrine pancreas; ($p < 0.05$, $p<0.01$ and $p < 0.001$ for one, two or three symbols, respectively; n - 5 per group).

Concerning the exocrine pancreas lesions, in rats aged 26 week-old, while no significant changes were found concerning exocrine pancreas inflammation (Fig. 5.2 A-C and G), fibrosis was significantly ($p < 0.05$) increased in the diabetic ZDF rats, when compared with the lean control, which was prevented in the diabetic animals under sitagliptin therapy (Fig. 5.2 D-H).

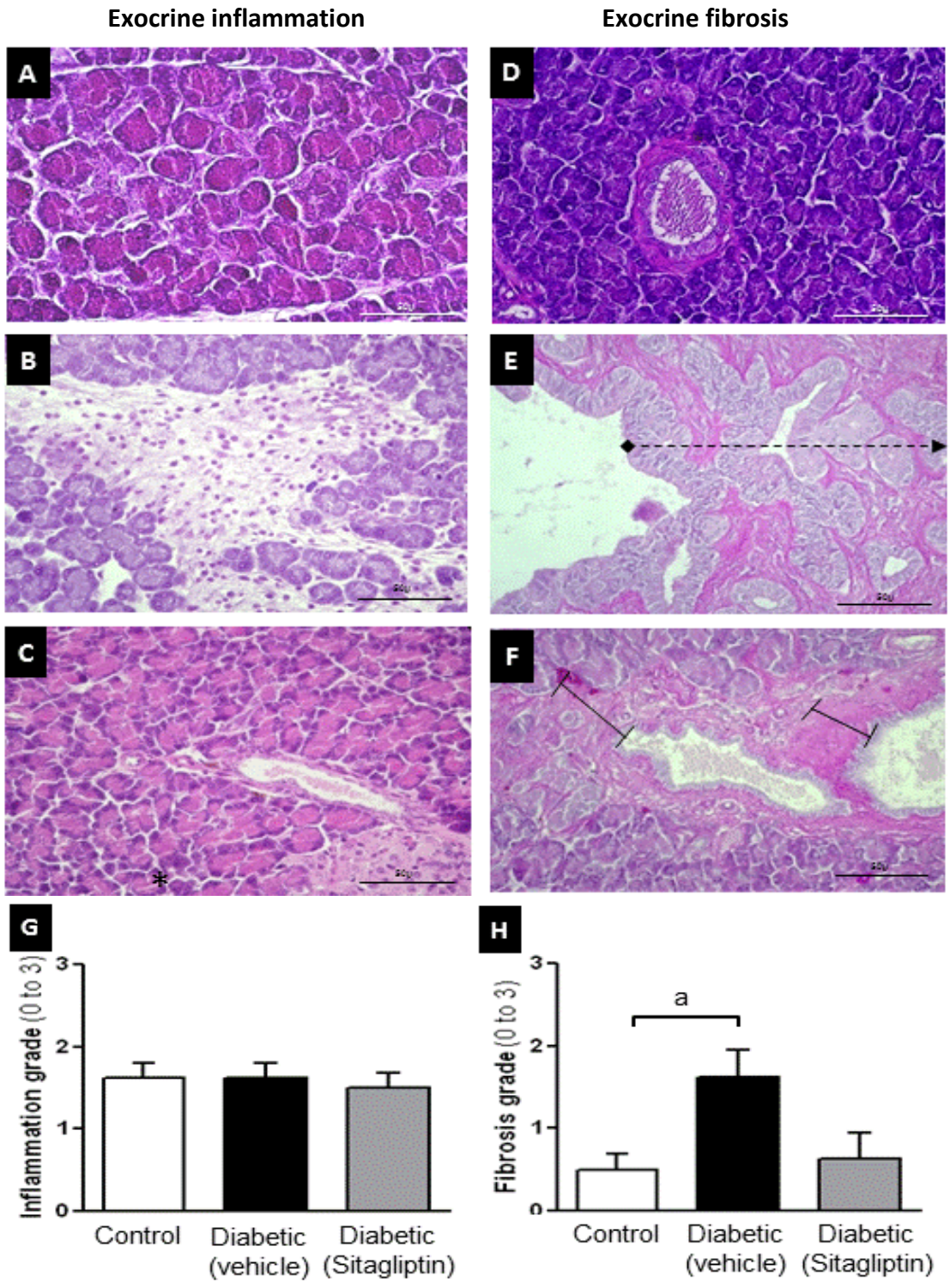


Figure 5.2 - Sitagliptin effects on inflammation and fibrosis of exocrine pancreas of diabetic ZDF rats. Histopathological observation of exocrine inflammation in 26 week-old animals (images A-C): (A) Micrograph of a normal exocrine parenchyma of lean control ZDF rat; (B) The untreated diabetic rats exhibiting severe inflammatory infiltrate, amid the acini of the exocrine parenchyma (grade 3 inflammatory lesions); (C) Normal exocrine parenchyma with no inflammatory signs in the exocrine pancreas of sitagliptin-treated diabetic ZDF rats (HE). Histopathological observation of exocrine fibrosis in 26 week-old animals (images D-F): (D)

Normal pancreatic duct in a lean control ZDF rat; (E) An extremely thickened, grade 3, fibrotic duct, which overextends the microscopic field (interrupted arrow), with numerous neocanaliculi in the duct wall, .present in an untreated diabetic ZDF rat; (F) Improvement of duct fibrosis from grade 3 to grade 1 (full line) in a 6 weeks sitagliptin treated diabetic ZDF rat (PAS). Semiquantitative evaluation of inflammation (graph G) and fibrosis (graph H): (G) Exocrine pancreatic inflammation revealed only a slight descent in sitagliptin treated animals in relation to its untreated counterparts; (H) Sitagliptin presented a trend to decrease the duct fibrosis increment ($p < 0.05$) found in the untreated ZDF rats. Chi-square test with Monte Carlo simulation or exact test (when contingency tables are 2×2) was performed to find out the differences in histomorphological lesions observed in exocrine pancreas ($p < 0.05$, $p< 0.01$ and $p < 0.001$ for one, two or three symbols, respectively; $n = 5$ per group).

5.3.3 Cytoprotective effects of sitagliptin against pancreatic damage progression

Pancreatic tissue mRNA levels of mediators of apoptotic machinery showed a significantly increased ($p < 0.05$) expression of the apoptotic Bax, as well as, anti-apoptotic Bcl-2 in the 26 week-old diabetic ZDF rats (Fig. 5.3 A and B, respectively), when compared with the lean ZDF animals, thus resulting in an unchanged Bax/Bcl-2 ratio (Fig. 5.3 C). In the diabetic rats under sitagliptin treatment, there was an overexpression of the mRNA for both Bax and Bcl-2, favouring a reduced Bax/Bcl-2 ratio (Fig. 5.3 A-C) as a result of a higher increment of mRNA expression of Bcl-2 when compared with Bax.

The pancreatic mRNA expression of Bax and Bcl-2 was accompanied by protein expression studies of immunohistochemistry (Fig. 5.3 D-L). In the untreated diabetic animals there was a significantly ($p < 0.05$) rise in Bax stained cells and unchanged Bcl-2, resulting in a trend to an increased Bax/Bcl-2 ratio, when compared with the controls; sitagliptin-treated diabetic rats presented a trend for increased protein expression of Bax, accompanied by a significantly ($p < 0.001$) increased expression of Bcl-2, which results in a Bax/Bcl-2 ratio identical to that found for the control animals (Fig. 5.3 D-L).

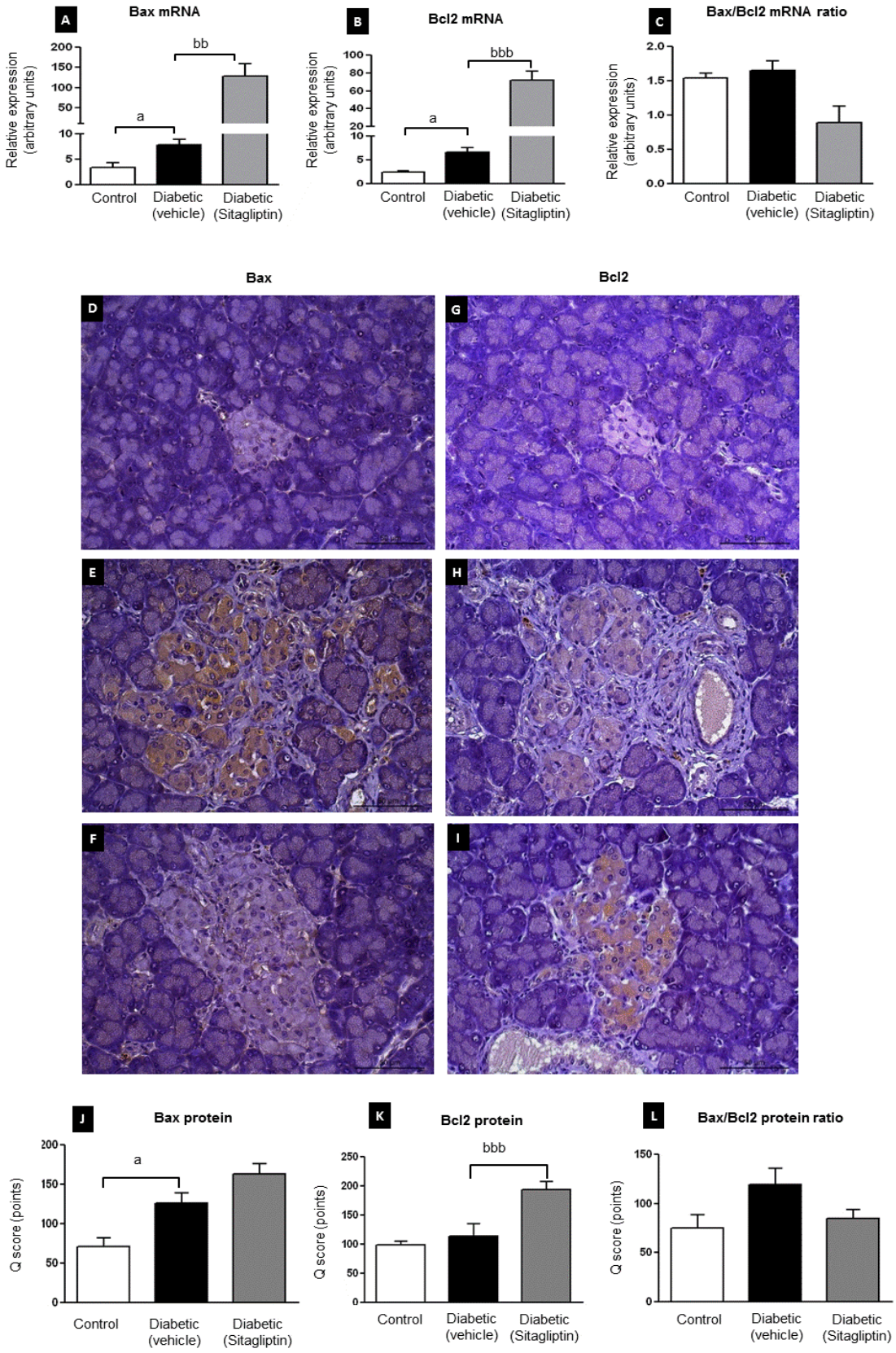


Figure 5.3 - **Sitagliptin protects the diabetic ZDF rats against endocrine pancreas apoptotic cell death.** Upper panel (A-C) - pancreas mRNA expression of Bax and bcl-2 in 26 week-old ZDF rats: A significant increase ($p < 0.05$) in apoptotic protein Bax (A), as well as, in anti-apoptotic Bcl-2 (B) was observed in the untreated diabetic ZDF rats when compared with the lean control

ZDF animals, resulting in an unchanged Bax/Bcl-2 ratio (C). In the sitagliptin-treated diabetic rats an overexpression of the mRNA was registered for both Bax (not statically significant) and Bcl-2 ($p < 0.001$) ensuing a reduced Bax/Bcl-2 ratio (C). Middle panel (D-I) - Immunostaining of pancreas Bax and Bcl-2 in 26 week-old ZDF rats: (D) The expression of Bax protein is not present in the pancreatic islet (grade 0) of a lean control rat; (E) A deeply stained islet (grade 3) revealing Bax protein expression in untreated diabetic ZDF rat; (F) The diabetic sitagliptin-treated rats displayed a light stained islet (grade 1); (G) Expression of Bcl-2 protein not observed in islet (grade 0) of a lean control rat; (H) A moderately stained islet (grade 2) with Bcl-2 antibody in an untreated diabetic ZDF rat; (I) An intensely stained islet (grade 3) in a diabetic sitagliptin-treated rat. Lower panel (J-L) - Quantification of protein expression: Bax protein expression in diabetic untreated rats exhibited a significant increase in relation to lean control rats; in sitagliptin-treated diabetic rats, Bax presented a trend for overexpression (J), accompanied by a significantly ($p < 0.001$) increased expression of Bcl-2 (K), resulting in a Bax/Bcl-2 ratio identical to control animals (L). Statistical comparisons between groups were performed using one-way ANOVA and the post-hoc Bonferroni test ($p < 0.05$, $p < 0.01$ and $p < 0.001$ for one, two or three symbols, respectively; $n = 5$ per group).

Concerning other putative mechanisms behind the protective effects of sitagliptin on the pancreatic tissue, we found that the diabetic rats, aged 26 weeks, presented a significantly increased pancreatic mRNA expression of IL-1β ($p < 0.01$), which was prevented ($p < 0.001$) in the sitagliptin-treated group (Fig. 5.4 A). Sitagliptin was able to promote overexpression ($p < 0.001$) of VEGF and PCNA mRNA when compared with the untreated diabetic rats (Fig. 5.4 B and C, respectively).

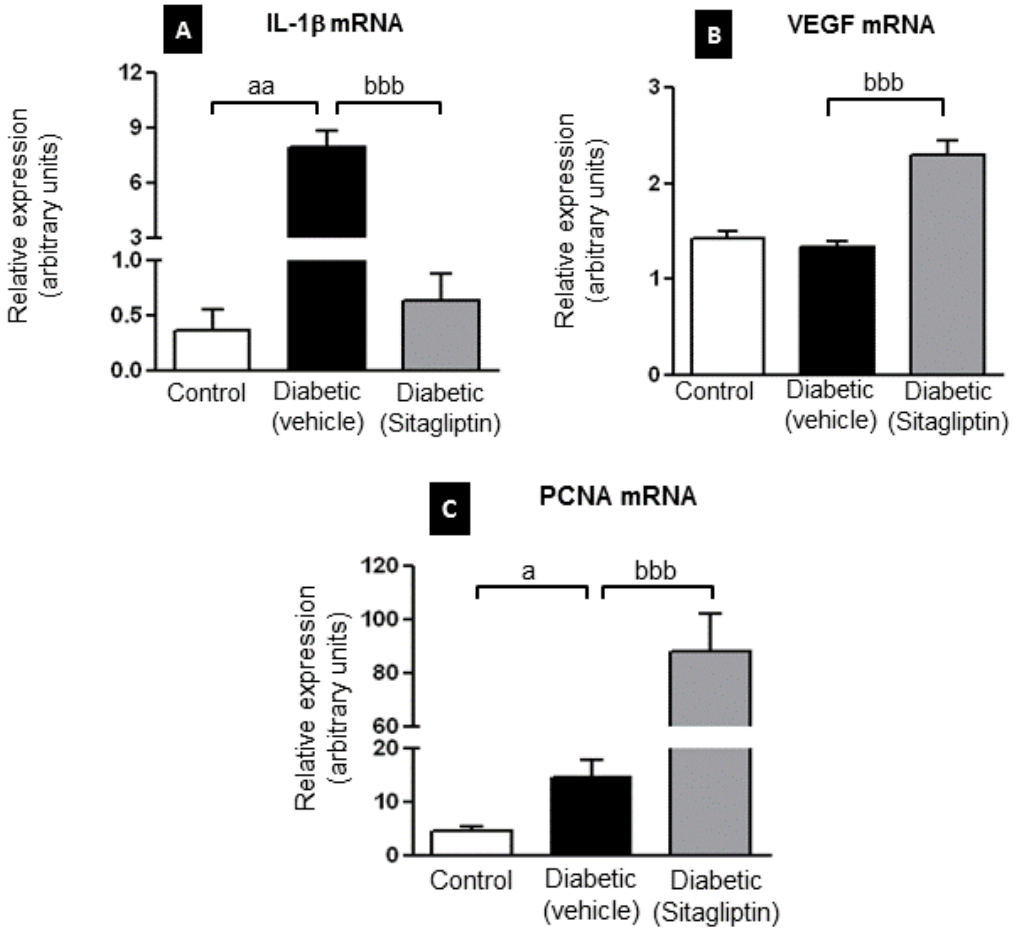


Figure 5.4 - Effects of sitagliptin treatment on pancreatic mRNA expression of mediators of inflammation, angiogenesis and proliferation: IL-1β, VEGF and PCNA. (A) mRNA expression of pancreatic IL-1β in untreated diabetic ZDF rats was significantly increased ($p < 0.01$) in contrast to lean control rats; in the sitagliptin-treated diabetic group, overexpression of IL-1β was significantly prevented ($p < 0.001$). Overexpression ($p < 0.001$) of VEGF (B) and PCNA (C) mRNA in ZDF diabetic rats treated with sitagliptin for 6 weeks when compared with untreated diabetic rats. Statistical comparisons between groups were performed using one-way ANOVA and the post-hoc Bonferroni test ($p < 0.05$, $p < 0.01$ and $p < 0.001$ for one, two or three symbols, respectively; n = 5 per group).

In addition, sitagliptin treatment totally ($p < 0.001$) prevented the diabetes-induced increment ($p < 0.001$) in TRIB3 expression in the pancreatic tissue (Fig. 5.5 A-D).

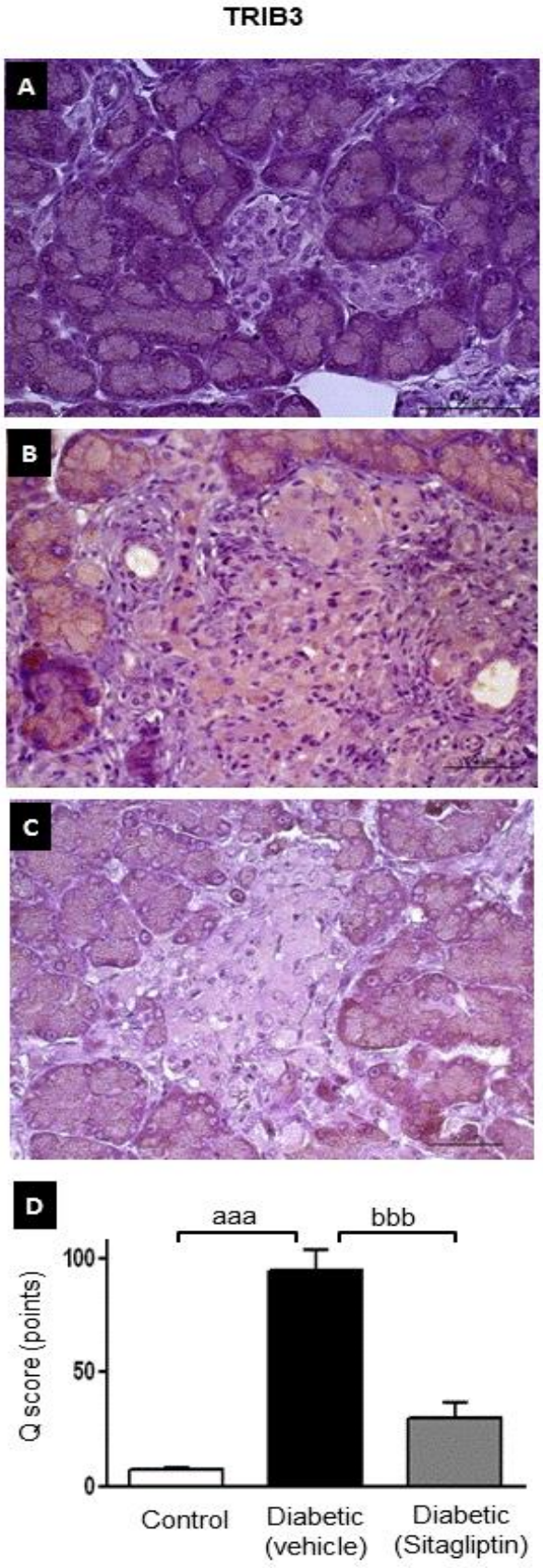


Figure 5.5 - Sitagliptin prevents TRIB3 protein overexpression in pancreas of ZDF diabetic rats. Micrographs of TRIB3 expression by immunostaining in 26 week-old ZDF rats (A-C): (A) Lean ZDF control rat presenting unstained pancreatic islet (grade 0); (B) Untreated diabetic ZDF

rat showing an intensely stained pancreatic islet (grade 3); (C) Sitagliptin-treated diabetic rat for 6 weeks displayed light staining of endocrine cells (grade 1). Exocrine pancreatic tissue presents normal constant staining for TRIB3 in all three groups. (D) Quantification of protein expression by score points: expression of TRIB3 is almost undetectable in control rats, rising very significantly ($p < 0.001$) in untreated diabetic ZDF rats; and declining very meaningfully ($p < 0.001$) in 6 weeks sitagliptin-treated diabetic treated rats. Statistical comparisons between groups were performed using one-way ANOVA and the post-hoc Bonferroni test ($p < 0.05$, $p<0.01$ and $p < 0.001$ for one, two or three symbols, respectively; $n = 5$ per group).

5.4 DISCUSSION

Previous studies propose that a disruption of the normal relationship between insulin sensitivity and pancreatic β-cell function is crucial for the pathogenesis of T2DM (Virally et al., 2007), and that the degeneration of Langerhans islets with β-cell loss is secondary to insulin resistance and may have a key role in the progression of the disease (Kahn et al., 2000; Marchetti et al., 2010). Furthermore, the loss of β-cell mass is not yet completely elucidated, but a possible cause may reside in apoptotic processes and in a lost capacity for pancreatic regeneration (Robertson, 2004; Li et al., 2003; Brownlee, 2003). Previous studies have been suggesting that gliptins are able to preserve both β-cell function and cell mass in animal models of diabetes (Yeom et al., 2011; Ferreira et al, 2010; Matveyenko et al., 2009; Maida et al., 2009) but the mechanisms underlying the protective effects remain to be elucidated.

Consistent with previous reports our study demonstrated that a 6 weeks sitagliptin treatment was able to improve β-cell function as well as preserve pancreatic islet structure. We hypothesize that sitagliptin is able to preserve pancreatic function by improving insulin resistance and by other cytoprotective effects, including anti-apoptotic, anti-inflammatory and pro-proliferative pathways, based on the cytoprotective properties previously reported for incretin peptides in distinct tissues (Mu et al., 2006; Matveyenko et al., 2009; Maida et al., 2009; Ferreira et al, 2010; Nachnani et al., 2010; Yeom et al., 2011; Maiztegui et al., 2011; Gonçalves et al., 2012). In fact, the results presented herein strongly suggest that in diabetic ZDF rats sitagliptin may derive its cytoprotective effects via two different type of influences: directly reducing apoptosis and promoting cell proliferation due to increased incretin availability; indirectly via metabolic effects, including amelioration of chronically

elevated glucose and triglycerides, prevention of insulinopaenia and reduction of inflammation, thus protecting from deleterious effects derived from glucotoxicity, lipotoxicity and insulin resistance.

The histomorphological evaluation of endocrine and exocrine pancreatic tissue shows that the differences between diabetic untreated and sitagliptin-treated animals were striking. In fact, the sitagliptin-treated rats presented an amelioration of inflammation and fibrosis in endocrine and exocrine pancreas. In particular, inflammation was highly reduced in the islets of Langerhans, and the exocrine pancreas of diabetic rats receiving sitagliptin did not present fibrotic changes in the vascular and the ductal walls. The changes described above were repeatedly and systematically observed by two pathologists unaware of the identity of the slides. These findings are in accordance with our preliminary work (Ferreira et al, 2010) but in contradiction with the results obtained by Matveyenko et al. (2009) using a DPP-4 inhibitor in human IAPP transgenic (HIP) rats and by Nachnani et al. (2010) using an injection of GLP-1 agonist, suggesting that the enhancement of endogenous GLP-1 levels could induce undetected low grade asymptomatic chronic pancreatitis. The histomorphological observations were in accordance with an improvement in pancreatic β-cell function as shown by the augmentation in HOMA-beta in diabetic sitagliptin-treated rats. The effects of chronic inhibition of DPP-4 in increasing β-cell mass and function over time could be due, at least in part, by the increase in glucose-stimulated insulin secretion, which is believed to be mediated primarily via stabilization of the incretin hormones, including GLP-1 (Farilla et al., 2002).

It is well established that apoptosis is one of the pathways responsible for the progressive deterioration of β-cells and evolution of diabetes. Our study suggests that sitagliptin is able to promote an anti-apoptotic effect, which is in agreement with other reports (Yeom et al., 2011; Maiztegui et al., 2011; Matveyenko et al., 2009; Maida et al., 2009; Nauck et al., 2009) in the pancreatic tissue. In fact, Matveyenko et al. (2009) reported that sitagliptin therapy led to preservation of β-cell mass in HIP rats as compared with its untreated counterparts, while Maida et al. (2009) reported an increment of percentage of β-cell area in streptozotocin-induced diabetic mice under sitagliptin treatment. The anti-apoptotic properties of sitagliptin is also in agreement with the effects reported in extra-pancreatic tissues, such as the kidney, with

improvement of renal function and reduction of parenchymal damage, due to a decrease in apoptosis, inflammation and an increase of in antioxidant capacity (Maiztegui et al., 2011; Hocher et al., 2012; Joo et al., 2013). Apart from the anti-apoptotic effect suggested by our results, the protective effects afforded by sitagliptin on the pancreas tissue might be the result of other activities previously described for the incretin peptides, including GLP-1 (Deacon, 2002; Ahrén et al., 2004; Holst et al., 2004; Gallwitz, 2005; Green et al., 2006; Darnell et al., 2008; Gallwitz 2014). Anti-inflammatory, pro-angiogenic and pro-proliferative properties are suggested by the reduced expression of IL-1β and by the increased expression of VEGF and PCNA observed in the pancreas tissue of sitagliptin-treated diabetic rats. Inflammation has been associated with the development of insulin resistance, β-cell apoptosis and evolution of diabetes, and IL-1β is one of the main inflammatory cytokines in the process (Maedler et al., 2011). We and other authors have suggested anti-inflammatory properties of gliptins in distinct animal models and tissues, as well as, in T2DM patients (Ferreira et al., 2010; Goçalves et al., 2012; Joo et al., 2013; Lee et al., 2013; Satoh-Asahara et al., 2013; Derosa et al., 2013), in agreement with our hypothesis. VEGF is expressed in the endocrine cells and the increased VEGF expression found in the diabetic rats under sitagliptin treatment might be viewed as an increased capacity for tissue regeneration. The same is true for PCNA, which is an indicator for cell proliferation and has been used in the present work to determine β-cell mass expansion (Duvillié et al., 2002; Li et al., 2006). It could be hypothesized that sitagliptin-evoked increased GLP-1 availability, due to inhibition of its degradation by DPP-4, will favour the development of new cells, via proliferation enhancement of pre-existing cells and induction of islet neogenesis, effects that were previously reported for GLP-1 (Farilla, 2002).

The second mechanism involved in the effect of sitagliptin may be related to significant improvement in the metabolic profile, including amelioration of glucose, insulin and TGs levels. We must empathize that the dose of sitagliptin used in our study (10 mg/kg/day) may be considered a low dose as others have used higher doses or a twice a day treatment (Matveyenko et al., 2009; Maida et al., 2009; Maiztegui et al., 2011). Nevertheless, sitagliptin treatment improved hyperglycaemia and hypertriglyceridaemia, thus ameliorating glucolipotoxicity in the diabetic ZDF rats.

Several pathophysiological mechanisms have been identified as potential contributors to β-cell stress and subsequent dysfunction, including glucotoxicity, lipotoxicity, and increased secretory demand resulting from insulin resistance. In addition, disturbances in secretion of various adipose tissue-secreted factors or cytokines derived from the innate immune system might also play a causal role (Lin et al., 2001). Furthermore, both hyperglycaemia and hyperlipidaemia are associated with induction of oxidative stress in β-cells. Previously, we have already demonstrated using this animal model that a low-dose chronic sitagliptin treatment was able to promote a favourable impact on chronic inflammation and oxidative stress (Ferreira et al, 2010). In this context, it should also be noted that the effect of liraglutide upon endoplasmatic reticulum stress, oxidative stress and cell apoptosis in diabetic db/db rats, as well as the results of vildagliptin in diabetic KK-Ay mice, are essentially compatible with those observed in this study (Prudente et al., 2009; Shimoda et al., 2011; Hamamoto et al., 2013).

Malfunctioning insulin secretion and/or insulin resistance are recognized as key factors for the pathogenesis of T2DM (Prudente et al., 2009; Beguinot, 2010); the latter results from anomalies in the insulin signaling cascade, a regulated complex molecular pathway, which may be inhibited and activated by many biochemical mechanisms (Beguinot, 2010). One of the genes implicated in coding inhibitors of insulin signaling and action is TRIB3, a mammalian tribbles homolog that binds Akt inhibiting downstream insulin-signalling cascade (Beguinot, 2010; Prudente et al., 2012). Our current study revealed that 26 week old ZDF diabetic rats showed pancreas overexpression of TRIB3 which, concurrently, showed insulin resistance and relative insulinopaenia. Sitagliptin treatment was able to completely reduce tissue TRIB3 expression, which might be a key mechanism for the decline of insulin resistance and improvement of insulin secretion observed in the diabetic rats under sitagliptin treatment. It has been shown, in cellular and animal models, that changes in TRIB3 expression levels induce systemic insulin resistance (Bi et al., 2008; Wang et al., 2009; Liu et al., 2010). Indeed, increased TRIB3 expression was observed in islets from T2DM donors and high fed diet (HFD) mice (Prudente et al., 2009; Prudente et al., 2012). In humans, TRIB3 has also been associated with insulin resistance and T2DM, accompanied by enhanced inhibition of insulin signalling and AKT/PKB activation in different tissues, including the β-cells (Prudente et al., 2005; Gong et al., 2009;

Prudente et al., 2009). Prior rodent studies (Liu et al., 2010; Bi et al., 2008; Wang et al., 2009; Ostertag et al., 2010), indicate that TRIB3 overexpression plays a major role in modulating whole-body insulin sensitivity and suggest a possible involvement in the pathogenesis of insulin resistance-related metabolic abnormalities. Another pivotal aspect by which TRIB3 seems to be associated with the evolution of insulin resistance and pancreas degradation is its role in inducing apoptosis in pancreatic β-cells and inhibiting cell proliferation; so by downregulating the expression of TRIB3, sitagliptin promotes anti-apoptotic effects and enhance β-cell proliferation, thus contributing to the beneficial effects afforded by this DPP-4 inhibitor in this animal model.

In conclusion, in this animal model of obese type 2 diabetes (the ZDF rat) sitagliptin prevented β-cell dysfunction and evolution of pancreas damage. The protective effects afforded by this DPP-4 inhibitor may derive from improvement of metabolic profile (related to amelioration of glycaemic and lipidic levels and of insulin resistance) and from cytoprotective properties. In fact, sitagliptin was able to reduce Bax/Bcl-2 ratio, suggestive of an anti-apoptotic effect, and completely prevented the increased pancreas overexpression of IL-1β and TRIB3 found in the untreated diabetic animals, thus demonstrating an anti-inflammatory action; in addition, sitagliptin was able to promote overexpression of VEGF and PCNA, suggesting pro-angiogenic and pro-proliferative properties.

Chapter 6

Sitagliptin ameliorates diabetic nephropathy in the Zucker Diabetic Fatty rat due to anti-inflammatory and anti-apoptotic properties

6.1 BACKGROUND AND AIMS

T2DM is an increasing health problem, with increasing prevalence and incidence, according to all estimates worldwide (IDF, 2011). The core pathophysiology of T2DM has been attributed to the classic triad of decreased insulin secretion, increased insulin resistance, and elevated hepatic glucose production. Further mechanisms have also key relevance, including those related with the fat cell (accelerated lipolysis), the gastrointestinal tract (incretin deficiency/resistance), the pancreatic α-cell (hyperglucagonaemia), the kidney (increased glucose reabsorption), as well as the brain (insulin resistance), now referred to as the "ominous octet" (De Fronzo, 2009). The main problem affecting T2DM management is its serious microvascular complications, which include, among others, DN (Gray et al., 2011). The incidence of T2DM is rapidly increasing, as is the prevalence of CVD and CKD resulting from diabetic complications (Stewart et al., 2007; Marchant, 2008; Crawford, 2010). Diabetes remains the single most important cause of kidney failure in different regions of the world. DN is a major microvascular complication of diabetes with progression to ESRD (Wakai et al., 2004; Stewart et al., 2007), accounting for approximately one-third of all cases of ESRD.

There is emerging evidence that microvascular kidney disease begins prior to the onset of diabetes, and this occurs with microalbuminuria and decreased renal function. Experimental and clinical studies showed an adaptive response by the kidney to conserve glucose, which is essential to meet the energy demands of the body (Dominguez et al., 1994; Kamran et al., 1997; Noonan et al., 2001; Lorenzo et al., 2008). In the diabetic patient, instead of dumping glucose in the urine to correct hyperglycaemia, the kidney chooses to hold on to glucose. Even worse, the ability of the diabetic kidney to reabsorb glucose appears to be augmented by an absolute increase in the renal reabsorptive capacity for glucose (Balakumar et al., 2009; Sego, 2007). The hyperglycaemic profile is aggravated by oxidative stress damage and inflammation, as well as by over activity of the RAAS and alteration of the extracellular matrix protein synthesis by glomerular epithelial cells, which contributes to further aggravate DN (Singh et al., 2011; Rivero et al., 2009; Chawla et al., 2010, Morano et al.,

2008). Additionally, accumulating evidence also points to a crucial role of the inflammatory processes in the development and progression of DN (Saraheimo et al., 2003; Dalla Vestra et al., 2005; Nelson et al., 2005). This inflammatory response is mediated by diverse inflammatory cells, including macrophages, monocytes, and leukocytes, as well as, by other molecules, such as chemokines, adhesion molecules, and inflammatory cytokines, namely TNF-α and IL-1β (Nelson et al., 2005; Rivero et al., 2009; Lim et al., 2012). As inflammation persists, certain vascular changes are exacerbated, such as endothelial dysfunction, tissue damage, renal fibrosis, and apoptotic cell death (Rivero et al., 2009; Lim et al., 2012). Apoptosis has been increasingly associated with the development and/or progression of DN (Wagener et al., 2009; Sanchez et al., 2010). It has been described that glucose-induced ROS production contributes to apoptosis in podocytes (Susztak et al., 2006), mesangial (Kang et al., 2003) and tubular cells (Verzola et al., 2002), leading to DN progression. Furthermore, high glucose-mediated oxidative stress in tubular cells has been associated with increased levels of pro-apoptotic proteins, such as Bax (Verzola et al., 2002). It was also observed that high glucose induces an increased ratio of Bax/Bcl-2, associated with cytochrome-c release from mitochondria in renal mesangial cells (Kang et al., 2003).

Evidence is available that long-term maintenance of normal or near-normal glucose levels using pharmacological means is protective in diabetic patients, improving microvascular disease and reducing both morbidity and mortality (Bilous et al., 2006; Bouattar et al, 2009; Krentz et al., 2009). Traditionally, non-insulin dependent T2DM is pharmacologically managed with oral antidiabetic agents from several different classes, which includes agents that increase insulin secretion, improve insulin action and delay absorption of carbohydrates. The more recent incretin-based therapies address a previously unmet need in the diabetic therapeutic approach by modulating glucose supply (Frias et al., 2007; Mizuno et al., 2008; Srinivasan et al., 2008). Their pharmacological action is based on gut incretin hormones (GIP and GLP-1), which appear to be malfunctioning in T2DM and have important effects on insulin and glucagon secretion (Girard, 2008; Gautier et al., 2008). Sitagliptin is the best known incretin enhancer (or gliptin), which increases incretin contents due to inhibition of DPP-4 activity, which is responsible for the degradation of GLP-1

(Srinivasan et al., 2008; Unger, 2010; Abel et al., 2010; Dhillon, 2010). Even though there is a patent association in observational studies between hyperglycaemia and diabetic complications, the benefits of strict glycaemic control on micro- and macrovascular complications have been questioned. Therefore, the benefit of lowering glucose seems to be, at least, partly minimized by the side-effects of the glucose-lowering antidiabetic agents, including hypoglycaemia, weight gain and fluid retention. In this context, new therapeutic options with fewer side-effects are advisory, and the appearance of incretin-based therapies is a hope. However, clinical studies with renal end-points using these agents are lacking, as well as, animal studies assessing the influence of these drugs on renal function and lesion.

Rodent models of T2DM are frequently used to clarify the mechanisms responsible for the pathophysiology of diabetes evolution, as well as its complications. The ZDF rat has a mutation in the gene coding the leptin receptor (fa/fa) that results in obesity, insulin resistance, reduced glucose tolerance, hypertension, and renal and CV diseases, thus developing a phenotype very similar to humans with T2DM, including the existence of DN (Janssen et al., 1999; Phillips et al., 1999; Peterson et al., 1990). Our group has previously reported that a chronic low-dose sitagliptin treatment promotes not only a reduction of hyperglycaemia, but also other protective actions (including antioxidant and anti-inflammatory effects) (Ferreira et al., 2010). Considering the extra-pancreatic effects of incretins, namely GLP-1's ability to positively modulate the function of other tissues (Drucker, 2006b; Holst et al., 2009), it seems important to evaluate the effects of sitagliptin in DN, as well as to characterize the nature of the putative benefit. We and other authors have been exploiting the cytoprotective actions of DPP-4 inhibitors in distinct organs and pathological conditions in pancreas, retina, and heart disorders (Mu et al., 2006; Read et al., 2010; Gonçalves et al., 2012). However, until now, few studies have addressed the putative beneficial impact of these agents, including sitagliptin, on DN (Vaghasiya et al., 2011; Liu et al., 2012). In addition, the impact of sitagliptin therapy on oxidative stress, inflammation and apoptosis underlying DN development remains relatively unexploited. Several experimental studies have shown that different therapeutic strategies prevent the development or ameliorate renal injury in diabetes, suggesting that modulation of oxidative, inflammatory and apoptotic processes is a potential

therapeutic target to prevent renal injury in animal models of diabetes (Yozai et al., 2005; Tone et al., 2005; Ohga et al., 2007). Concerning the management of DN, the ability of antidiabetic drugs to ameliorate renal microvascular disease might be as important as their capability to control glucose. Thus, the present study aimed to evaluate whether sitagliptin can prevent the development of renal dysfunction in diabetic ZDF rats, focusing on antioxidant, anti-inflammatory and anti-apoptotic properties.

6.2 METHODS

6.2.1 Animals and experimental design

The animal protocol (including animals, animal groups, performed treatment and comparison between animal groups) was previously described in Chapter 5.

6.2.2 Sample collection and preparation

Kidneys were collected in the same conditions as described for the pancreas in Chapter 5. For immunohistochemistry and fluorescence microscopy studies the kidneys were embedded in OCT tissue embedding matrix (Thermo Scientific, Waltham, MA, USA) at −50◦C and for immunoblot analysis were frozen in liquid nitrogen and then stored at −80◦C.

6.2.3 Kidney function and trophism

Serum creatinine and blood urea nitrogen (BUN) concentrations were used as renal function indexes, and assessed through automatic validated methods and equipment (Hitachi 717 analyser, Roche Diagnostics Inc., MA, USA). The weights of kidneys (KW) and the ratio KW/BW were measured in all the rats under study in order to be used as renal trophy indexes.

6.2.4 Kidney lipid peroxidation

Kidney lipid peroxidation was assessed by the thiobarbituric acid reactive-species (TBARs) assay, measuring the malondialdehyde (MDA) content, according to

that previously described in (Ferreira et al., 2010). Samples were analysed spectrophotometrically at 532nm using 1,1,3,3-tetramethoxypropane (TMP) as external standard. The concentration of lipid peroxides (in MDA) was expressed as μmol/L.

6.2.5 Histopathological analysis

Haematoxylin and Eosin Staining

Samples were processed as previously described in Chapter 5.

Periodic Acid of Schiff Staining

Samples were processed following the same method elucidated in in Chapter 5. Periodic acid of Schiff (PAS) was used to evaluate and confirm the levels of mesangial expansion, thickening of basement membranes and sclerotic parameters.

Histopathology

Glomerular damage was assessed by evaluating mesangial expansion, glomerular basement membrane and capsule of Bowman thickening, nodular sclerosis, glomerulosclerosis, atrophy, and hyalinosis of the vascular pole. Analysed tubulointerstitial lesions comprised inflammation, presence of hyaline cylinders, tubular basement membrane irregularity, tubular calcification, and the association of interstitial fibrosis and tubular atrophy (IFTA). The evaluation of vascular lesions was concentrated on arteriolar hyalinosis and arteriosclerosis. A semiquantitative rating for each slide ranging from normal (or minimal) to severe (extensive damage) was assigned to each component. Severity was graded as 0-absent/normal, 1-mild, 2-moderate, and 3-severe. Scoring was defined according to the extension occupied by the lesion (% area): 0-normal: <25%; 1-mild: 25–50%; 2-moderate: 50–75% and 3-severe: >75%. The final score of each sample was obtained by the average scores observed in individual glomeruli in the considered microscopic fields. Tubulointerstitial damage was evaluated and graded by the same semiquantitative method, with the exception of IFTA, which was graded as 0-normal, 1-mild, 2-moderate, and 3-severe, if present in <25%, between 25–50%, and over 50% of affected area, respectively. Regarding vascular lesions, arteriolar hyalinosis was scored as 0 if absent, as 1 if one arteriole with hyalinosis was present, and as 2 if more than one arteriole was observed in the entire slide. Arteriosclerosis was scored as 0 if no intimal thickening was present,

as 1 if intimal thickening was less than the thickness of the media, and as 2 if intimal thickening was more than the thickness of the media and considering the worst artery on the slide. When using PAS, the rating was set for intensity and extension of staining, ranging from 0 (no staining) to 3 (intense and extensive staining), respecting tissue specificity scoring when adequate.

6.2.6 Kidney gene (mRNA) expression

Total RNA isolation

The kidneys were stored in RNAlater solution (Ambion, Austin, TX, USA). For RNA extraction the methodology followed is described in in Chapter 5.

Reverse transcription

RNA reverse transcription was executed as in in Chapter 5 with SuperScript III First-Strand Synthesis System for RTPCR (Invitrogen, California, USA). One microgram of total RNA was mixed with a 2 × First-Strand Reaction Mix and a SuperScript III Enzyme Mix [Oligo (dT) plus Random hexamers]. Reactions were carried out in a thermocycler Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA), 10 min at 25◦C, 30 min at 50◦C, and 5 min at 85◦C. Reaction products were then digested with 1 μL (2U) RNase H for 20min at 37◦C and, finally, cDNA was eluted to a final volume of 50 μL and stored at −20◦C.

Relative gene expression quantification

Gene expression was performed using the protocol in Chapter 5. For the kidneys, real-time PCR reactions used specific QuantiTect Primer Assays (Qiagen, Hilden, Germany) with optimized primers for Bax, Bcl-2, IL-1β, and TNF-α. The endogenous controls used were: glyceraldehyde-3-phosphate dehydrogenase, β-actin, and topoisomerase I together with a QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) used according to manufacturer's instructions.

6.2.7 Kidney Immunohistochemistry and fluorescence microscopy

Transverse sections of rat kidneys (6 μm) were fixed with cold acetone for 10 min. The sections were then washed with phosphate-buffered saline (PBS), permeabilised for 30min with 0.25% Tx-100 in PBS, and blocked for 40min with 10%

normal goat serum or with 5% BSA, prior to incubation overnight at 4ºC with primary antibodies goat anti-IL-1β and rabbit anti-TNF-α from R&D Systems (Minneapolis, MN, USA). The sections were rinsed with PBS and then incubated with 4',6-diamidino-2-phenylindole (DAPI) and secondary fluorescent antibodies for 1h at room temperature. After washing, samples were imaged using a confocal microscope (LSM 710, Carl Zeiss, Gottingen, Germany).

6.2.8 Western blotting

Kidney sections were weighed, cut into small pieces, and homogenized by mechanical dissociation using a Potter-Elvehjem, at 4ºC, in 5 volumes of RIPA lysis buffer (150mM NaCl, 50mM Tris (pH 7.5), 5mM ethylene glycol tetra-acetic acid (EGTA), 1% Triton X- 100 (Tx-100), 0.5% sodium deoxycholate (DOC), and 0.1% sodium dodecyl sulfate (SDS), supplemented with 2mM phenylmethylsulfonyl fluoride (PMSF), 2mM iodoacetamide (IAD), 30mM NaF, 1mM sodium orthovanadate, and 1x protease inhibitor cocktail (Roche, Indianapolis, IN, USA). After incubation on ice for 1 h, the lysates were sonicated and then centrifuged at 16,000 ×g, for 15 min, at 4ºC. After centrifugation, the resulting supernatant fractions were used to determine protein concentration using the bicinchoninic acid assay (Pierce, Rockfor, IL, USA) and then were denatured with Laemmli buffer. For the western blot analysis, 40 to 80 μg of protein were loaded per lane on SDS-PAGE. Following electrophoresis and transfer to polyvinylidene difluoride membranes (Immobilon-P PVDF transfer membranes 0.45 μm, Millipore, Billerica, MA, USA), the blots were incubated with mouse monoclonal anti-Bcl-2 and rabbit polyclonal anti-Bax from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and rabbit polyclonal anti-Bid from Millipore and mouse monoclonal anti-β-actin antibody from Sigma-Aldrich. The bands intensity was detected by ECL reagent (Bio-Rad, Hercules, CA, USA) using an imaging system (VersaDoc 4000 MP, Bio-Rad). Densitometric analyses were performed using the ImageJ 1.42n software.

6.2.9 Apoptosis assay

Apoptotic cell death was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, using the *in situ* Cell Death Detection

Kit, Fluorescein (Roche, Basel, Switzerland). Briefly, kidney frozen sections (6μm) were fixed with 1% paraformaldehyde and permeabilised with 1% Tx-100 in 1% sodium citrate (pH6) for 2 minutes on ice. Slides were washed in PBS and then incubated for 60min at 37ºC with TUNEL reaction mix. The nuclei specimens counterstained with DAPI were analysed with the confocal microscope.

6.2.10 Statistical analysis

For the histopathological data the categorical variables are counts of renal lesions severity in scores. Quantitative values are reported as mean ± SEM. Significance level was accepted at 0.05. Data were analysed using SPSS Statistics 18 (2009). Chi-square test with Monte Carlo simulation or exact test (when contingency tables are 2 × 2) was used to find out the differences of severity score distributions in renal lesions at the beginning of the study (20 weeks old) between lean control and diabetic rats and at the end of the study (26 weeks old), between untreated diabetic, sitagliptin treated diabetic and lean control rats. To analyse the influence of sitagliptin treatment in renal lesions after 6 weeks of chronic treatment (final time 26 weeks), we generated two quantitative variables, by averaging the scores of two types of renal lesions: global glomerular lesions comprising mesangial expansion, thickening of GBM, thickening of CB, nodular sclerosis, glomerulosclerosis, glomerular atrophy, and hyalinosis of the vascular pole and global tubulointerstitial lesions comprising hyaline cylinders, TBM irregularity, tubular calcification, IFTA, and tubular degeneration. On these two variables was performed an ANOVA and subsequent LSD post hoc test to find out the differences between untreated diabetic rats, sitagliptin treated diabetic rats, and lean control rats. Regarding the remaining parameters, data are expressed as mean ± standard errors of the mean (SEM). The comparison of values between groups was performed by using analysis of variance (ANOVA) followed by Bonferroni's post hoc test (Graph Pad Prism 5.0 software, La Jolla, CA, USA). Values of $p < 0.05$ were considered statistically significant.

6.3 RESULTS

6.3.1 Sitagliptin treatment partially improves kidney function

At the beginning of the study, 20 weeks, BUN contents were already significantly higher ($p < 0.001$) in the diabetic ZDF rats when compared with the control animals, without significant changes of creatinine (Table 6.1). The diabetic rats treated with sitagliptin showed BUN values identical to those found for the control animals at the final time (26 weeks), contrasting with the higher value ($p < 0.01$) encountered in the diabetic ZDF without treatment (Table 6.1). Concerning kidney trophism, we found that at week 20, there was already kidney hypertrophism, viewed by increased value ($p < 0.05$) of KW and of KW/BW in the diabetic rats when compared with the control animals, which was even increased in the final time. Sitagliptin treatment did not changed kidney trophism parameters in the diabetic animals (Table 6.1).

Table 6.1 - Effects of sitagliptin on renal trophism and function parameters

Parameter	Group	20 weeks	26 weeks
KW (g)	Control	2.39 ± 0.08	2.56 ± 0.04
	Diabetic (Vehicle)	3.25 ± 0.26[a]	3.02 ± 0.09[a]
	Diabetic (Sitagliptin)	—	3.15 ± 0.05
KW/BW (g/Kg)	Control	6.11 ± 0.15	5.71 ± 0.07
	Diabetic (Vehicle)	8.82 ± 0.73[a]	8.42 ± 0.42[aaa]
	Diabetic (Sitagliptin)	—	8.42 ± 0.40
Creatinine (mg/dl)	Control	0.55 ± 0.03	0.53 ± 0.03
	Diabetic (Vehicle)	0.55 ± 0.06	0.54 ± 0.08
	Diabetic (Sitagliptin)	—	0.49 ± 0.04
BUN (mg/dl)	Control	14.35 ± 0.47	15.05 ± 0.54
	Diabetic (Vehicle)	18.15 ± 0.84[aaa]	18.03 ± 1.20[aa]
	Diabetic (Sitagliptin)	—	15.16 ± 0.61[b]

Values are means ± SEM of n = 8 rats. Comparisons between groups: a – Lean control vs Diabetic ZDF rats; b - diabetic untreated versus diabetic sitagliptin-treated rats; .$p < 0.05$, $p < 0.01$ and $p \leq 0.001$ for one, two or three letters, respectively. KW: kidney weight; BW, body weight; BUN, blood urea nitrogen; 20 weeks, initial time; 26 weeks, final time.

6.3.2 Sitagliptin treatment improves kidney lipidic peroxidation

At the initial time (20 weeks), MDA contents were unchanged between the lean control and the diabetic animals. A trend to higher values in the diabetic rats was found at the final time, 26 weeks. This profile was completely reversed by sitagliptin treatment, since the kidney MDA values were substantially ($p < 0.001$) lower than those found in the diabetic untreated animals (Fig. 6.1).

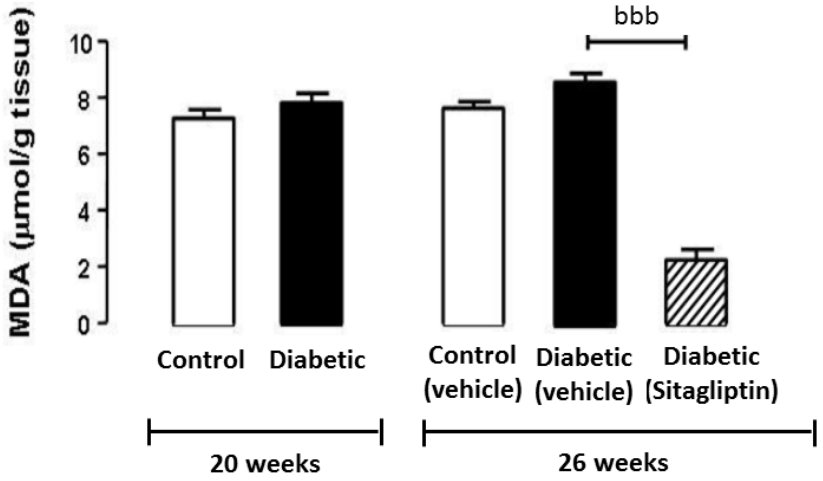


Figure 6.1 – Kidney lipidic peroxidation (MDA). MDA for the lean control and obese diabetic ZDF rats, in the initial and final times (6 weeks of vehicle or 10 mg/kg BW/day sitagliptin treatment). Data is expressed as mean ± sem of 8 rats/group. Comparisons between groups: b - diabetic untreated versus diabetic sitagliptin-treated rats. Three letters for $p < 0.001$. MDA – malondialdehyde**.**

6.3.3 Sitagliptin prevents aggravation of renal lesions

Glomerular lesions

Comparative analysis between lean control obese diabetic ZDF rats of 20 weeks of age revealed a significantly ($p \leq 0.001$) increased mesangial expansion, nodular sclerosis, glomerulosclerosis and glomerular atrophy in the obese diabetic animals, accompanied by a significant thickening of glomerular basement membrane and capsule of Bowman ($p < 0.01$) (Fig. 6.2 A and B). When aged 26 weeks, the obese diabetic rats showed aggravated glomerular basement membrane thickening and glomerular atrophy ($p < 0.001$), when compared with the lean control animals, accompanied by a significantly more intense expression of mesangial expansion and capsule of Bowman thickening ($p < 0.01$). Glomerulosclerosis was also significantly

more obvious in diabetic subjects ($p < 0.05$) (Fig. 6.2 C and D). Concerning ageing effects from 20 to 26 weeks in the lean rats, the most noted alterations were capsule of Bowman thickening ($P < 0.01$) and increase in nodular sclerosis ($p < 0.01$) (Fig. 6.2 A and C). In the obese diabetic rats, between 20 and 26 weeks, there was a statistically significant increase in glomerular basement membrane ($P < 0.05$) and capsule of Bowman thickening ($P < 0.05$). Hyalinosis of the vascular glomerular pole was absent in all lean rats but was present in the obese diabetic ZDF rats, as soon as 20 weeks of age, with a tendency for aggravation at 26 weeks.

Concerning the sitagliptin effects in the diabetic rats at 26 weeks old, there was a reduction of severity of fibrosis, demonstrated by the significant decrease of global glomerulosclerosis ($p < 0.01$), which is in agreement with the less severe nodular sclerosis ($p < 0.01$) (Fig. 6.3 A). Hyalinosis of the vascular glomerular pole was also significantly decreased (Table 6.2). Mesangial expansion and glomerular basement membrane thickening showed a trend for improvement in the sitagliptin-treated diabetic rats versus the untreated (Table 6.2; Fig. 6.3 B, C, and D). Therefore, mesangial expansion showed a 37.5% reduction in the most severe grade and glomerular basement membrane presented 12.5% reduction in grade 2 and 3 of lesion severity (Table 6.2). Glomerular atrophy showed a significant ($p < 0.01$) improvement in diabetic sitagliptin treated rats (Table 6.2; Fig. 6.4). When considering all the glomerular lesions, the diabetic rats presented a notorious pattern of lesion ($p < 0.001$), when compared with the lean animals, which was significantly ameliorated ($p < 0.05$) by chronic sitagliptin treatment (Fig. 6.6).

Table 6.2 – Scoring and distribution of glomerular lesions in lean control and obese diabetic ZDF rats kidneys at the final time, 26 weeks of age (6 weeks of vehicle or sitagliptin treatment)

Lesions	Group	Score			
		G0	G1	G2	G3
Mesangial expansion	Control	3	3	2	0
	Diabetic (Vehicle)	0	0	3	5^{aaa}
	Diabetic (Sitagliptin)	0	1	5	2
GBM thickening	Control	3	5	0	0
	Diabetic (Vehicle)	0	0	2^{aa}	6^{aa}
	Diabetic (Sitagliptin)	0	2	1	5
CB thickening	Control	1	6	1	0
	Diabetic (Vehicle)	0	0	4	4
	Diabetic (Sitagliptin)	0	4	0	4
Nodular sclerosis	Control	2	4	2	0
	Diabetic (Vehicle)	0	5	3	0
	Diabetic (Sitagliptin)	0	0	2^{bb}	6^{bb}
Glomerulosclerosis	Control	2	3	3	0
	Diabetic (Vehicle)	0	0^{a}	3	5^{aaa}
	Diabetic (Sitagliptin)	0	4^{bb}	4^{b}	0^{bbb}
Glomerular atrophy	Control	6	2	0	0
	Diabetic (Vehicle)	0	0	4^{aaa}	4^{aaa}
	Diabetic (Sitagliptin)	0	4^{bb}	2^{b}	2^{b}
Vascular Pole Hyalinosis	Control	8	0	0	0
	Diabetic (Vehicle)	2	1	2	3
	Diabetic (Sitagliptin)	0	7^{bbb}	1^{b}	0^{b}

Values are means ± SEM of n = 8 rats. Comparative analysis between groups a – Diabetic *versus* control rats at 26 weeks of age; b – Sitagliptin-treated diabetic diabetic *versus* untreated rats at 26 weeks of age. One, two or three letters for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. GBM, glomerular basement membrane; CB, Capsule of Bowman; G0, grade 0; G1, grade 1; G2, grade 2; G3, grade 3.

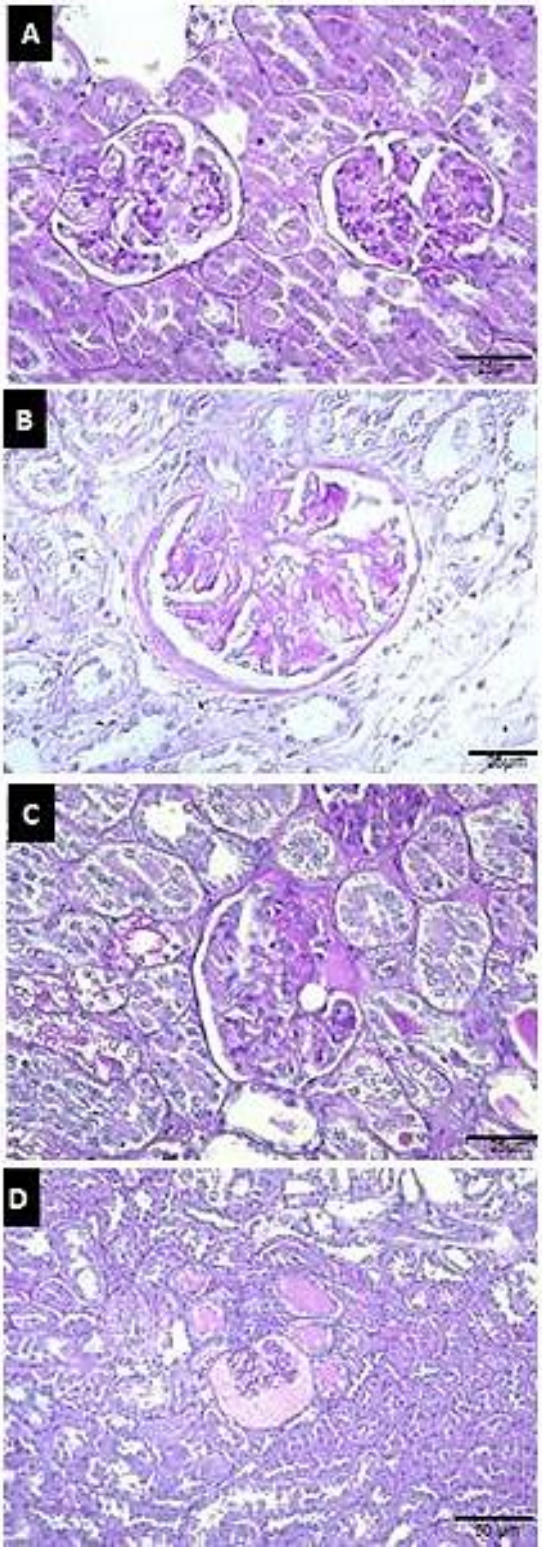


Figure 6.2 – Evolution of renal lesions with diabetes and age and in lean control and diabetic ZDF rats. (A) Normal renal histology in lean control rats at 20 weeks of age. (B) Glomerulus presenting grade 1 mesangial expansion and thickening of the capsule of Bowman in a lean control rat at 26 weeks of age, the tubulointerstitial pattern is normal. (C) Nodular sclerosis with *sinequia* attaching the tuft to Bowman's capsule, mesangial expansion and arteriolar sclerosis in a diabetic rat of 20 weeks. (D) Atrophic, sclerotic glomerulus, exhibiting filtrate fluid in Bowman's space. Note the presence of hyaline cylinders and the irregularity of tubular basement membranes, diabetic rat of 26 weeks, (PAS).

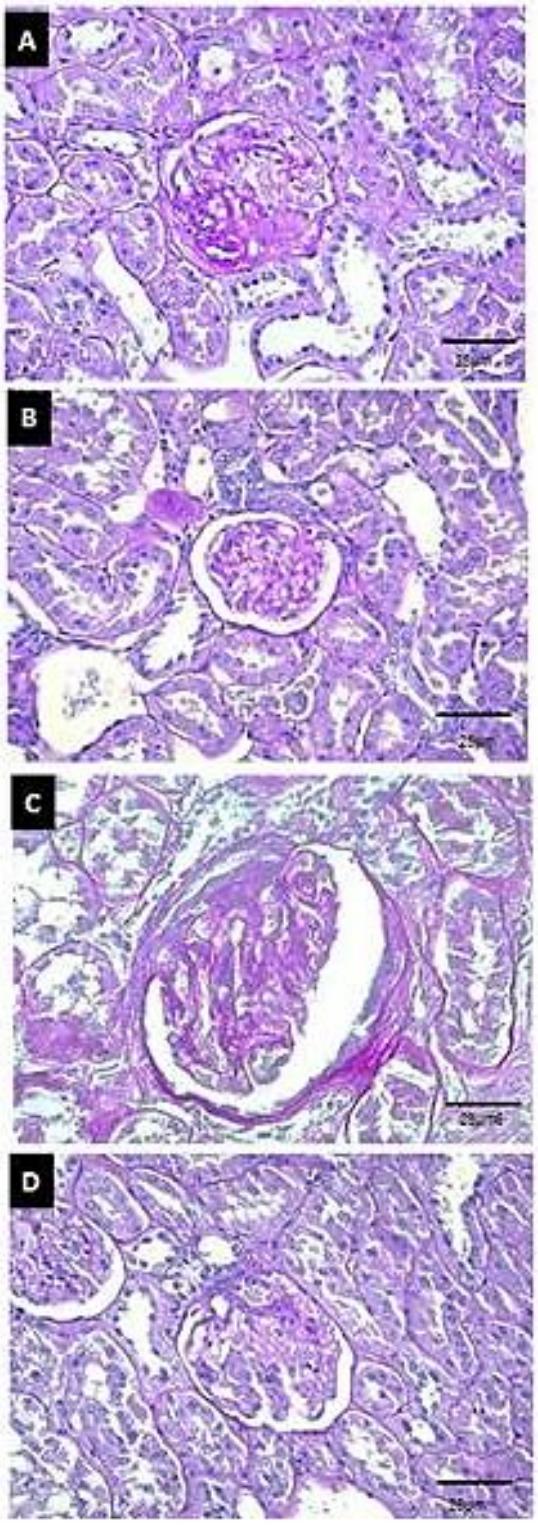


Figure 6.3 - Effects of chronic treatment with sitagliptin on glomerular lesions of diabetic ZDF rats. (A) Regression of glomerulosclerosis, with more glomeruli presenting the more benign nodular form of sclerosis. (B) Reduction in capsule of Bowman thickness and absence of sclerosis. (C) Although there is persistence of grade 2 capsular thickening, there is absence of sclerosis and only the presence of grade 1 mesangial expansion. (D) Presence of light mesangial expansion and hyalinosis of the vascular pole. Note in all figures the absence of hyaline cylinders and a more regular contour of the tubular basement membranes, (PAS).

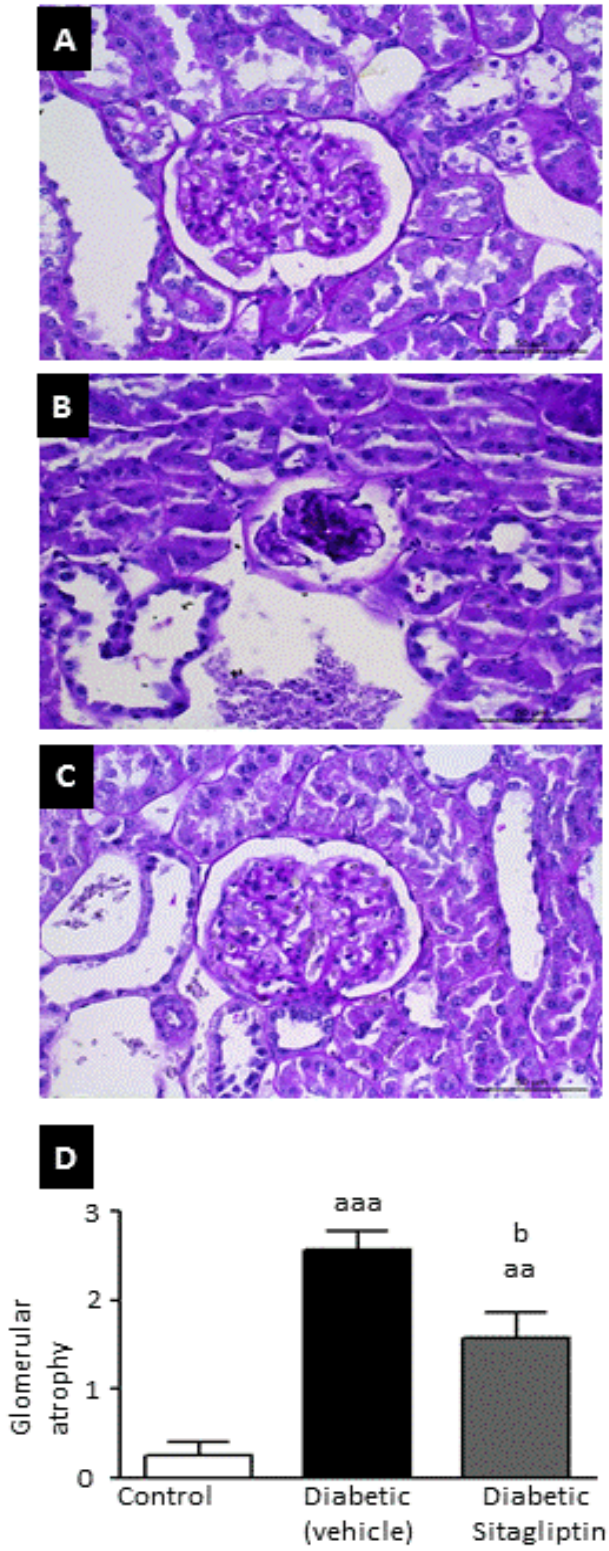


Figure 6.4 – Effects of sitagliptin treatment on glomerular atrophy in diabetic (ZDF) rats. Normal glomerulus (A). Glomerular atrophy in a diabetic untreated rat (B) versus a sitagliptin treated rat (C) showing a normal glomerulus; (PAS). (D) Data are expressed as mean ± SEM. Comparisons between groups (n=8 per group): a – significantly different from control and b – significantly different from diabetic; $p < 0.05$, $p < 0.01$ and $p \leq 0.001$ for one or three letters, respectively.

Tubulointerstitial lesions

When aged 20 weeks, the obese diabetic rats already presented a significant increase in tubular degeneration ($p < 0.01$), tubular basement membrane irregularity, and IFTA ($p < 0.01$), when compared with the lean controls animals. The differences between these groups were more pronounced when aged 26 weeks, in which the obese diabetic subjects showed marked aggravation of hyaline cylinders, tubular basement membrane irregularity and IFTA ($p \leq 0.001$), together with significant increase in tubular degeneration ($p < 0.01$) (Table 6.3). The most significant ageing alterations found in the lean rats were tubular basement membrane irregularity ($p < 0.01$) and IFTA ($p < 0.01$), while in the obese diabetic animals, these were mainly IFTA ($p \leq 0.001$) (Fig. 6.5 B) and hyaline cylinders ($p < 0.01$) aggravation.

Sitagliptin significantly prevented the appearance of hyaline cylinders ($p \leq 0.001$) and IFTA ($p < 0.05$) (Table 6.3; Fig. 6.5 C) in chronically treated diabetic rats, together with a trend to decrease basement membrane irregularity (by 50%) and tubular degeneration (37,5%) in grade 3 of lesion severity (Table 6.3; Fig. 6.5). Calcification of tubular epithelium was only present in diabetic rats, which did not suffer any mentionable recovery with sitagliptin treatment (Table 6.3). When considering all the tubulointerstetial lesions, the diabetic rats presented a pattern of lesion ($P < 0.001$), when compared with the lean animals, which was significantly ameliorated ($P < 0.001$) by chronic sitagliptin treatment (Figure 6.6).

Table 6.3 – Scoring and distribution of tubular lesions in lean control and obese diabetic ZDF rats kidneys at the final time, 26 weeks of age (6 weeks of vehicle or sitagliptin treatment)

Lesions	Group	Score			
		G0	G1	G2	G3
Hyaline cylinders	Control	6	2	0	0
	Diabetic (Vehicle)	0	0	7^{aaa}	1
	Diabetic (Sitagliptin)	0	8^{bb}	0	0
TBM irregularity	Control	2	5	1	0
	Diabetic (Vehicle)	0	0	1	7^{aaa}
	Diabetic (Sitagliptin)	0	3^{bb}	2	3^{bb}
Tubular calcification	Control	8	0	0	0
	Diabetic (Vehicle)	5	3	0	0
	Diabetic (Sitagliptin)	4	4	0	0
IFTA	Control	2	6	0	0
	Diabetic (Vehicle)	0	0	3^{aa}	5^{aaa}
	Diabetic (Sitagliptin)	1	2	3	2^{bb}
Tubular degeneration	Control	4	4	0	0
	Diabetic (Vehicle)	0	1^{aa}	4^{aaa}	3^{aaa}
	Diabetic (Sitagliptin)	0	3^{b}	5^{b}	0^{bb}

Values are means ± SEM of n = 8 rats. Comparative analysis between groups a – Diabetic *versus* control rats at 26 weeks of age; b – Sitagliptin-treated diabetic diabetic *versus* untreated rats at 26 weeks of age. One, two or three letters for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. TBM, tubular basement membrane; IFTA, interstitial fibrosis and tubular atrophy; G0, grade 0; G1, grade 1; G2, grade 2; G3, grade.

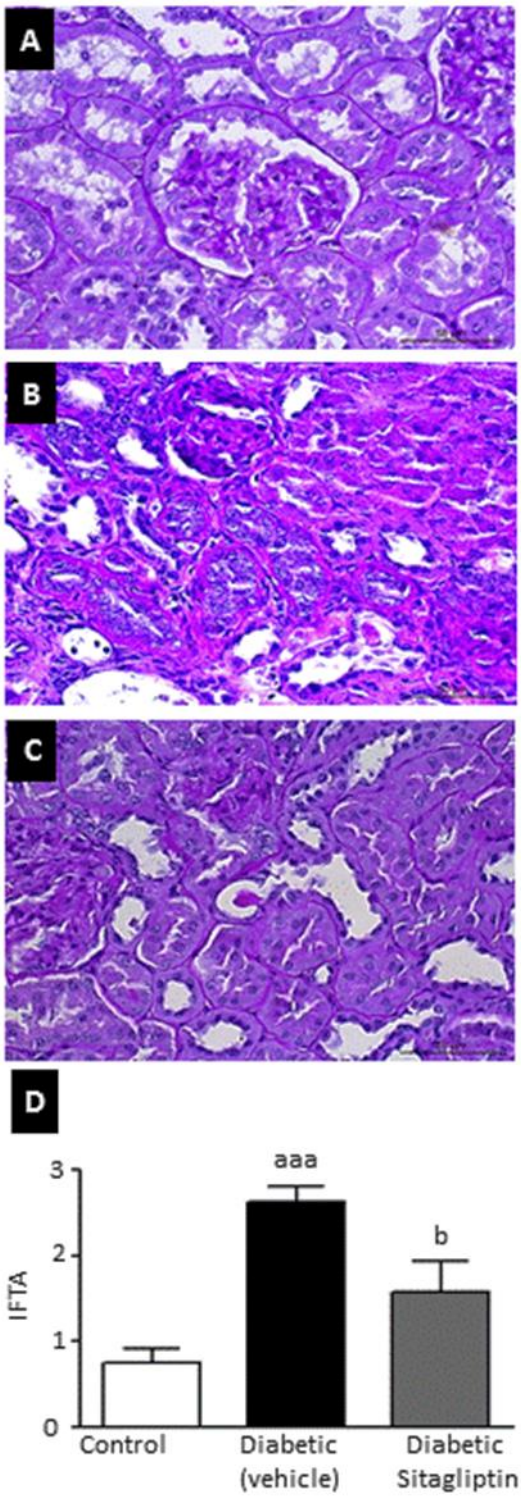


Figure 6.5 – Effects of sitagliptin treatment on IFTA in diabetic (ZDF) rats. Normal interstitial tissue (A) IFTA in a diabetic untreated rat (B) versus a sitagliptin treated rat (C) showing normal interstitial support tissue and regular normal tubules, (PAS). (D)Data are expressed as mean ± SEM. Comparisons between groups (n=8 per group): a – significantly different from control and b – significantly different from diabetic; $p < 0.05$, $p < 0.01$ and $p \leq 0.001$ for one or three letters, respectively.

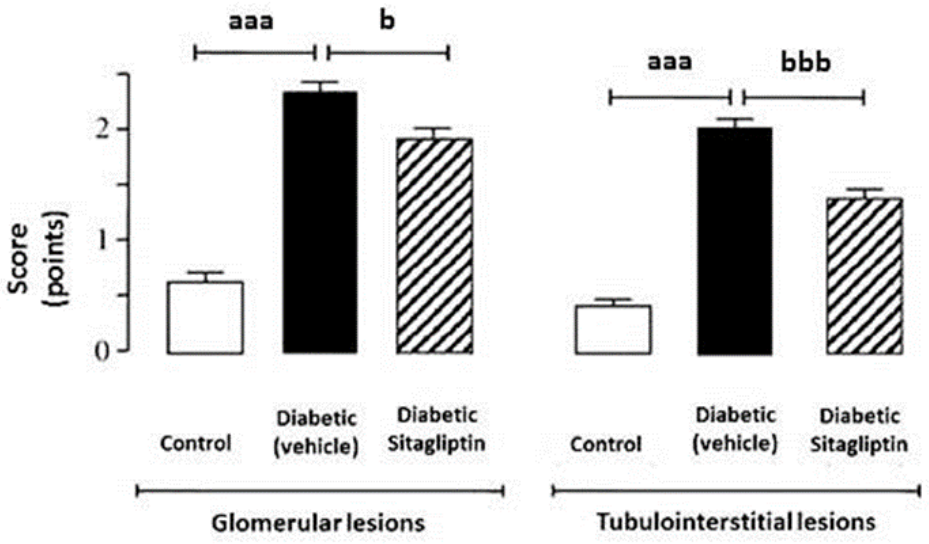


Figure 6.6 – Effects of chronic sitagliptin treatment on glomerular and tubulointerstitial lesions in obese diabetic ZDF rats, at the final time (26 weeks). Data is expressed as mean ± SEM of n=8 rats. Comparisons between groups (n=8 per group): a – lean control vs diabetic ZDF rats and b – Untreated diabetic vs sitagliptin treated diabetic rats; $p < 0.05$ and $p \leq 0.001$ for one or three letters, respectively.

Vascular lesions

Arteriolar hyalinosis was only found in the diabetic rats, which aggravated between 20 and 26 weeks ($p < 0.05$). Arteriosclerosis was only detected in lean animals when aged 26 weeks but was present in the diabetic rats at 20 weeks which also exhibited aggravation of sclerosis at the final time, with 62.5% of the animals exhibiting grade 1 and 25% grade 2 lesions, in comparison to its lean counterparts, which showed 50% of animals in grade 1 and none in grade 2 (Table 6.4). Sitagliptin promoted a 50% improvement in the most severe form of hyalinosis (grade 2) and reduced the incidence of arteriosclerosis in the treated diabetic rats by 12.5% (Table 6.4).

Table 6.4 – Scoring and distribution of vascular lesions in lean control and obese diabetic ZDF rats kidneys at the final time, 26 weeks of age, (6 weeks of vehicle or sitagliptin treatment)

Lesions	Group	Score		
		G0	G1	G2
Arteriolar hyalinosis	Control	8	0	0
	Diabetic (Vehicle)	1[aa]	1	6[aa]
	Diabetic (Sitagliptin)	3	3[b]	2[b]
Arteriosclerosis	Control	4	4	0
	Diabetic (Vehicle)	1	5	2
	Diabetic (Sitagliptin)	3	4	1

Values are means ± SEM of n = 8 rats. Comparative analysis between groups a – Diabetic *versus* control rats at 26 weeks of age; b – Sitagliptin-treated diabetic diabetic *versus* untreated rats at 26 weeks of age. One, two or three letters for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. G0, grade 0; G1, grade 1; G2, grade 2.

6.3.4 Sitagliptin decreases the inflammatory state in the diabetic kidney

The pro-inflammatory cytokines IL-1β and TNF-α are thought to contribute to an inflammatory response in the diabetic kidney. Therefore, their cellular distribution was evaluated in kidney frozen sections by immunohistochemistry and their mRNA levels by RT-qPCR (Fig. 6.7).

The relative expression of TNF-α mRNA in the diabetic kidney (437.1 ± 73.0%; $p < 0.05$) was significantly higher when compared to control animals (100.0 ± 51.0%); sitagliptin treatment prevented the effect in the diabetic rats (96.5 ± 24.4%; $p < 0.05$) (Fig. 6.7 A1). Although no significant differences were observed, the relative expression of IL-1β mRNA levels shows a trend to increase in diabetic animals (186.4 ± 25.1%) comparatively to non-diabetic animals (100.0 ± 11.9%); once again, sitagliptin administration presented a trend to reduce IL-1β mRNA levels in the diabetic animals (122.9 ± 30.6%) (Fig. 6.7 A2). Additionally, diabetes markedly increased the immunoreactivity of IL-1β and TNF-α in cells around the glomeruli that are probably tubular cells and/or recruitment and accumulation of interstitial inflammatory cells; sitagliptin treatment decreased the overexpression of IL-1β and TNF-α protein levels in the diabetic kidney (Fig. 6.7 B1 and B2).

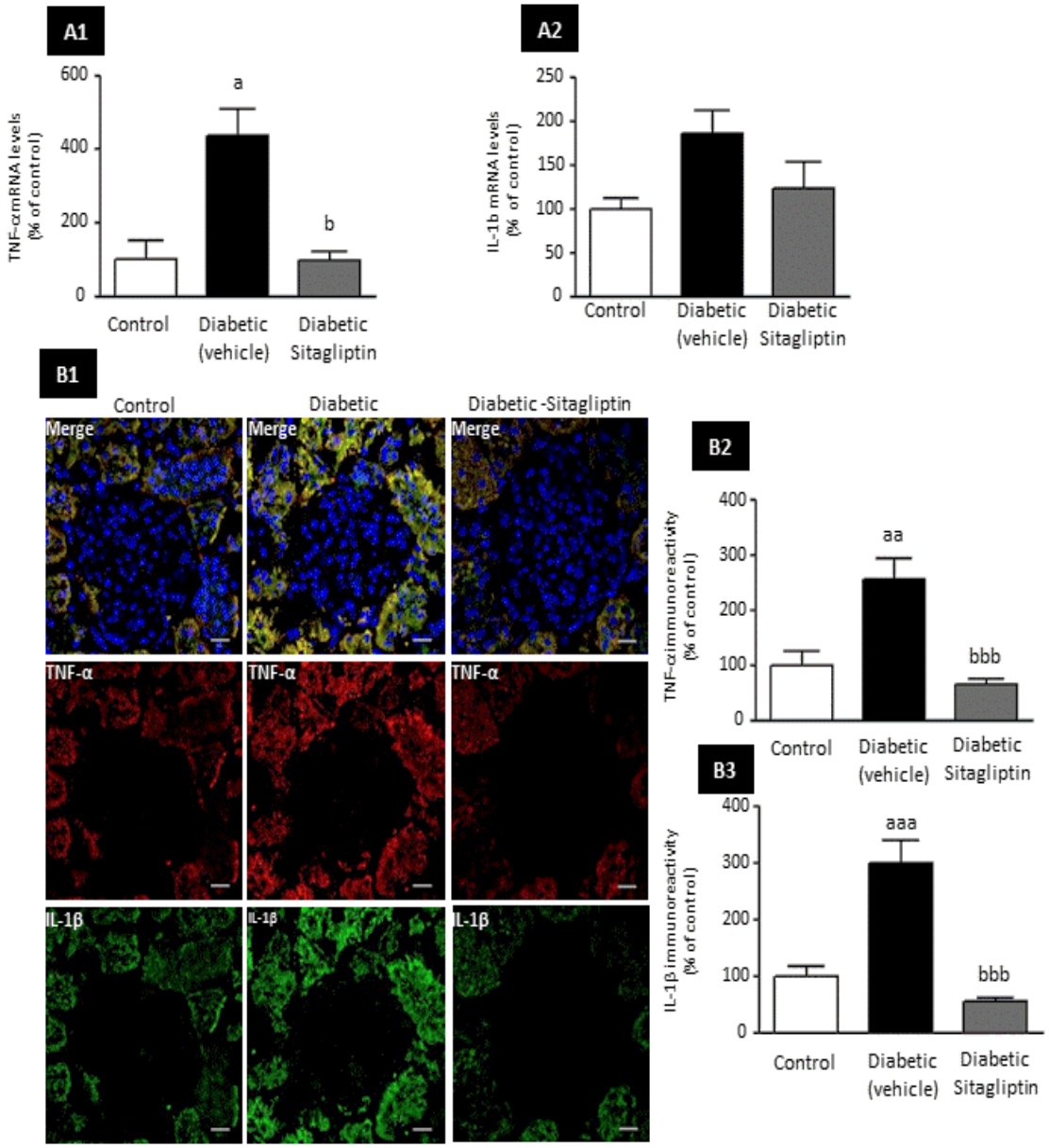


Figure 6.7 – Sitagliptin decreases the pro-inflammatory cytokines IL-1β and TNF-α in the diabetic kidney. (A) mRNA expression of mediators of inflammation, TNF-α (A1) and IL-1β(A2), in the kidney. (B) Representative confocal images showing TNF-α immunoreactivity (red), IL-1β immunoreactivity (green), and nuclear staining with DAPI (blue) in kidney sections (B1), as well as immunoreactivity quantitation for TNF-α (B2) and IL-1β (B3). Bars = 20 μm. Data is expressed as percentage of control and represent the mean ± SEM Comparisons between groups (n = 6 per group): a – lean control vs diabetic ZDF rats and b – untreated diabetic vs sitagliptin treated diabetic rats; $p < 0.05$, $p < 0.01$ and $p \leq 0.001$ for one, two or three letters, respectively.

6.3.5 Sitagliptin protects the kidney against apoptotic cell death induced by diabetes

It is well established that the ratio between Bax, a pro-apoptotic protein, and Bcl-2, an anti-apoptotic one, determines the response to a cell death signal, being considered an indicator for the activation of apoptosis (Xiang et al., 1996). The levels of Bax and Bcl-2 were determined by Western Blotting, in total kidney of lean and diabetic rats, as well as Bax and Bcl-2 mRNA levels. In addition, it has been described that Bid, a pro-apoptotic protein member of the Bcl-2 family, has an important role in mitochondrial cell death pathway (Kluck et al., 1999). In this context, we also evaluated Bid protein levels in the kidney by Western Blotting.

The diabetic animals presented significant increases in the kidney mRNA and protein of Bax/Bcl-2 ratio (Fig. 6.8 A and B, resp.), when compared to control rats. Sitagliptin treatment showed an anti-apoptotic effect since it was able to prevent the diabetes-induced increment of mRNA (Fig. 6.8 A and protein B) Bax/Bcl-2 ratio. Diabetes also induced a significant increase in Bid levels (173.57 ± 22.64% of control; $p < 0.01$) comparatively to non-diabetic ZDF rats, which was prevented in diabetic rats under sitagliptin treatment (113.02 ± 9.43%; $p< 0.05$) (Fig. 6.8 C).

As shown in Fig. 6.8 D, apoptosis increased in the kidney of diabetic animals, as assessed by the increase of TUNEL-positive cells, particularly in tubular cells. Treatment with sitagliptin decreased the number of TUNEL-positive cells in the kidney of diabetic animals (Fig. 6.8 D).

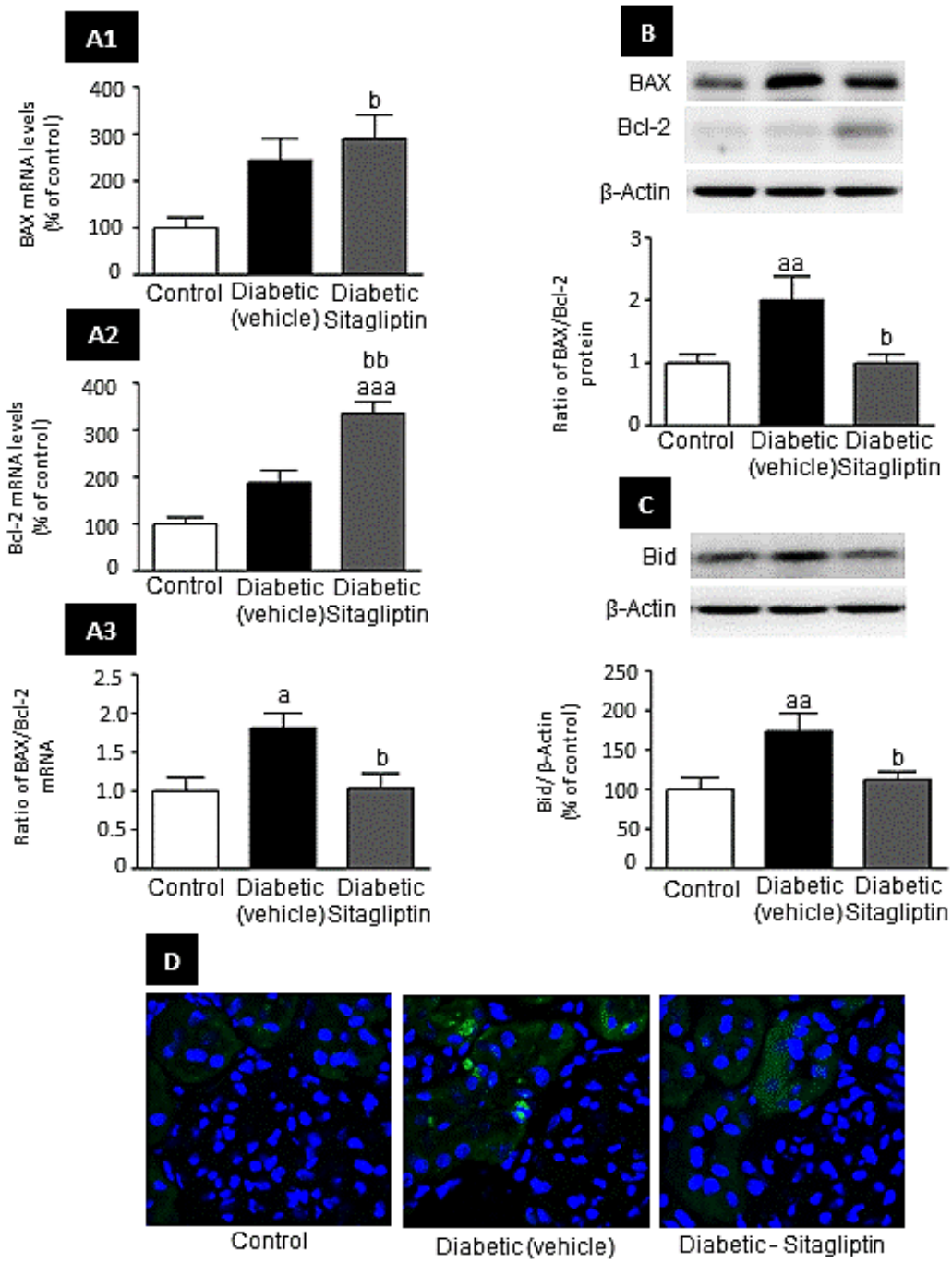


Figure 6.8 – Effect of sitagliptin treatment in Bax, Bcl-2 and Bid content and TUNEL-positive cells in the diabetic kidney. (A) mRNA expression of Bax (A1) and Bcl-2 (A2) in the kidney and Bax/Bcl-2 ratio (A3). (B and C) Protein levels assessed in total kidney cell lysates by Western Blotting in lean control, diabetic and sitagliptin treated diabetic rats: (B) protein levels of Bax and Bcl-2 and Bax/Bcl-2 ratio; (C) protein levels of Bid. The Western Blots shown are representative of each group of animals. Data are expressed as percentage of control and represent the mean $\pm$ SEM. Comparisons between groups (n = 6 per group): a – lean control vs diabetic ZDF rats and b – untreated diabetic vs sitagliptin treated diabetic rats; $p < 0.05$, $p < 0.01$ and $p \leq 0.001$ for one, two or three letters, respectively. (D) Representative confocal images for each group of animals showing TUNEL-positive (green) cells and nuclear counterstaining with DAPI (blue) in kidney sections (6 μm).

6.4 DISCUSSION

DN has emerged as the leading cause of ESRD, and thus, preventing or delaying its evolution, has been a major goal in biomedical research. The development of innovative therapeutic alternatives such as the incretin enhancers (including sitagliptin), able to target not only hyperglycaemia but also multiple pathophysiological processes involved in diabetes development and evolution, seems more likely to be beneficial, as has been shown in recent approaches (Abel et al., 2010; Ferreira et al., 2010). Our present study reports the progression of renal disease in ZDF rats and demonstrates that a daily chronic administration of low-dose sitagliptin noticeably reduces renal injury in this model.

It is well known that a commonly accepted animal model of DN has not been available. The ZDF rat is characterized by hyperglycaemia, hyperinsulinaemia, hyperlipidaemia, moderate hypertension, obesity and progressive renal injury (Peterson et al., 1990). These rats develop nephropathy by 12 weeks of age, earlier than in most of the other models of T2DM, characterized by focal segmental glomerulosclerosis (FSGS) associated with glomerulomegaly and mesangial expansion (Hoshi et al., 2002). Thus, this animal model is useful for preclinical evaluation of pharmacological strategies designed to target human DN.

In the present study, the animal's ages were selected according to the moment of initiation of relative insulinopaenia (20 weeks) and on the presence of significant diabetic complications (26 weeks). Although the literature describes in this animal model an earlier nephropathy, our animals were fed with normal rodent maintenance chow (with 2.9% of lipids) for developing all the different stages of T2DM in latter times than those described for this animal model. Therefore, if we intend to analyse renal lesions when rats presented lower insulin levels, those are the proper animal's ages. In order to achieve a better correlation between our animal observations and the human nephropathy process, we decided to adapt a recent human pathologic classification for DN (Tervaert et al., 2010). Despite the fact that our untreated diabetic ZDF rat presented lower BW than their lean counterparts, our data show that along with the metabolic changes occurring over time in these rats,

nephropathy resembles human DN in terms of morphology. The significant BW loss of diabetic ZDF rats corresponds to the time of notorious depletion of serum insulin levels compared with age-matched lean ZDF rats, which was an expected profile and is in agreement with disease aggravation.

Nephropathy in this model has previously been described as FSGS associated with glomerulomegaly and mesangial expansion, findings characteristically seen in patients with obesity and metabolic syndrome associated with T2DM (Kambham et al., 2001; Kato et al., 2009). In the literature, we found that the descriptions of tubulointerstitial lesions are mentioned only in passing and as secondary pathological features (Hoshi et al., 2002; Gassler et al., 2001). Renal vascular pathology has not been described. The data presented herein provides morphologic characterization of progressive nephropathy, including the glomerular, tubulointerstitial and vascular lesions in the kidney of ZDF rats.

The lean ZDF rats demonstrated, when aged 20 weeks, thickening of GBM, mesangial expansion, nodular sclerosis and IFTA, which further aggravates with age. These observations are in accordance with Vora et al. (1996) and could be classified as non-diabetic renal lesions attributed to ageing in this strain. All the obese diabetic ZDF rats presented significant worsening of glomerular, tubulointerstitial and vascular lesions compared with lean ZDF controls in both ages analysed (20 and 26 weeks). In the obese diabetic ZDF rats, the severity of the lesions aggravates with diabetes progression, confirming a link between diabetes (hyperglycaemia and hyperlipidaemia) and progressive renal injury.

In patients with DN, the initial physiological change is glomerular hyperfiltration, while the initial morphological change is glomerular hypertrophy. At 26 weeks of age, the obese ZDF rats exhibit an aggravation of the lesions described for 20 weeks rats, including mesangial expansion, glomerular basement membrane thickening, and glomerular hypertrophy. We observed that tubulointerstitial lesions are dependent on glomerulosclerosis, which is suggested by the aggravation of both glomeruli and interstitium. Vascular pole hyalinization and arteriosclerosis also suffer aggravation with age. All of these histological alterations were accompanied by an augmentation of kidney weight. In the obese diabetic ZDF rats, glomerular hypertrophy, expansion in the mesangial area related to the mesangial matrix, and

renal hypertrophy were noted. In the present study, we did not evaluate the progression of proteinuria, which was well documented by others (Schöfer et al., 2004; Janiak et al., 2006), but serum BUN levels were significantly increased in the obese diabetic ZDF rats when compared to the lean controls, suggesting a deficient kidney function. Nevertheless, serum creatinine levels were unchanged between groups, which are in accordance with others (Schrijvers et al., 2006).

Chronic sitagliptin (low-dose) treatment ameliorated all lesions (glomerular, tubulointerstitial and vascular), except the tubular epithelium calcification, in the diabetic-treated rats. Chronic sitagliptin administration was able to decrease BUN to levels analogous to those observed in lean controls, suggesting an amelioration of kidney function. The mechanism by which a low-dose of sitagliptin, which was unable to completely normalize the hyperglycaemic profile of the diabetic rats, is able to positively modulate kidney function is unknown. We may hypothesize that significant improvement of circulating levels of TGs (Chapter 5) may result in the attenuation of renal injury in sitagliptin-treated diabetic ZDF rats. One explanation for this is that the augment of insulin levels by sitagliptin inhibits adipose tissue hormone-sensitive lipase (HSL) activity and, thus, adipose tissue fatty acid release. In addition, insulin (together with the increased availability of GIP as the results of DPP-4 inhibition) may enhance adipose tissue fatty acid re-esterification and, thus, increase adipose tissue triacylglycerol (TAG) deposition. In the present work, we did not measure fat pads and lipid depots in the kidney, and, thus, we cannot confirm our hypothesis. Nevertheless, we intend to further perform red oil staining in the kidney in order to assess lipotoxicity and the putative effects of sitagliptin. However, some previous data from our studies should be mentioned. We have demonstrated that this low-dose chronic sitagliptin treatment is able to promote a favourable impact on chronic inflammation and oxidative stress, which are key players of diabetes pathophysiology and may precede and further potentiate tissue damage (Ferreira et al., 2010). Despite the lower dose used, we have previously demonstrated beneficial effects of sitagliptin on metabolic profile and reduction in inflammatory markers, as well as an amelioration of endocrine and exocrine pancreatic lesions (Ferreira et al., 2010). The histomorphological observations were in accordance with the improvement in pancreatic β-cell function, as suggested by the sitagliptin-evoked augment in HOMA-

beta (Chapter 5). The effects of chronic DPP-4 inhibition in increasing β-cell mass and function over time may occur, at least in part, by the augmentation of glucose-stimulated insulin secretion. This effect is believed to be primarily mediated via stabilization of the incretin hormones contents, including of GLP-1 (Mu et al., 2006). We also observed a weight gain (Chapter 5) of sitagliptin-treated diabetic ZDF animals that could be attributed to amelioration in dysmetabolism. This metabolic improvement was accompanied by a reduction in inflammatory markers (CRP and IL-1β) and in pancreatic oxidative stress, as previously documented by our group (Ferreira et al., 2010). Our results also agree with studies performed by other groups, which have been suggesting an antioxidant and anti-inflammatory effect of incretin modulators, due to attenuation of the deleterious effects of the AGEs-RAGE-oxidative stress axis and to protection against the cytokine-induced apoptosis and necrosis (Matsui et al., 2011; Li et al., 2005; Zhang et al., 2011).

Although large body of evidence indicates that oxidative stress is involved in the progression of fibrosis to ESRD in experimental and human DN (Chang et al.,2005), we cannot fully corroborate these facet in our study, at least when comparing kidney lipid peroxidation between untreated diabetic ZDF rats with their lean controls. Further studies should better address this aspect, namely by assessing other relevant kidney markers of oxidative stress, including AGEs, as well as relevant antioxidant measures. However, our work suggests a favourable impact of sitagliptin treatment on kidney oxidative stress profile, expressed by reduced amount of lipid peroxidation, which might be further confirmed with additional parameters, but that is in agreement with recent studies from Vaghasiya et al. (2011) which have reported a significant decrease in renal lipidic peroxidation by sitagliptin in diabetic rats with renal damage.

Experimental evidence linking hyperlipidaemia (Chapter 5) to renal injury and progression of renal fibrogenesis has been well documented; lipids can modulate the progression of chronic renal diseases and may even be primary factors in renal injury pathogenesis (Rosario et al., 2005). Additionally, the synergistic effects of hyperlipidaemia and diabetes on the development of renal injury have been recently observed in several animal models (Lassila et al., 2004; Dominguez et al., 2000). In ZDF rats, Chander et al. (2004) and Suzaki et al. (2006) suggested that hyperlipidaemia, in concert with hyperglycaemia, may be responsible for the increased oxidative stress

and initiation and aggravation of injury in the kidneys of these animals. Thus, we may hypothesize that the ability of sitagliptin to lower plasma lipids (Chapter 5), as well as to promote a more favourable redox status in the kidney, as confirmed in the present study by the reduction of lipid peroxidation products, may have contributed to its renoprotective effects. Furthermore, the positive effects demonstrated in peripheral insulin resistance and pancreas lesions, together with the antihypertensive effect (Ferreira et al., 2010), might be viewed as relevant contributors to the renoprotection afforded by sitagliptin in this study. On the other hand, we could not exclude possible effects due to sitagliptin-evoked GLP-1 increment. In fact, previous studies from other groups have linked GLP-1 with protection of mesangial cells, amelioration of Na^+, acid-base and fluid homeostasis, overall contributing to renoprotection (Girardi et al., 2008; Ishibashi et al., 2011).

Accumulating evidences point to a critical role of inflammation and pro-inflammatory cytokines in the development and progression of DN (Dalla Vestra et al., 2005; Saraheimo et al., 2003). Our results clearly indicate that diabetes leads to increased IL-1β and TNF-α immunoreactivity in the kidney. These results are corroborated by other authors that described an increased expression of those pro-inflammatory cytokines in the diabetic kidney (Navarro et al., 2006), leading to enhanced vascular endothelial permeability, oxidative stress, renal hypertrophy, and tubulointerstitial lesions (Lim et al., 2012). Recently, it has been reported that in the kidney of diabetic ZDF (fa/fa) rats, the expression of vascular cell adhesion molecule-1 (VCAM-1) increases with concomitant infiltration of white blood cells, as well as enhanced production of inflammatory cytokines, such as TNF-α and IL-1β, leading to renal cell injury (Wang et al, 2012). In addition, previous works have shown that a decrease in inflammation promotes an amelioration of DN (Wu et al., 2006; Yozai et al., 2005; Tone et al., 2005). In the present study we found that sitagliptin was able to prevent the increase in both mRNA and protein levels of the pro-inflammatory cytokines IL-1β and TNF-α in the diabetic kidneys. These results, obtained by immunohistochemistry and RT-qPCR, seem to be in agreement with previous studies from our group, which reported decreased IL-1β and TNF-α levels in the serum (Ferreira et al., 2010) and in the retina (Gonçalves et al., 2012) of ZDF (fa/fa) rats treated with sitagliptin. Together, these findings seem to indicate that chronic

sitagliptin treatment corrected the inflammatory state in diabetic microvascular complications, particularly in DN. In addition, it was reported that sitagliptin decreases local inflammation in other tissues, such as the adipose tissue and pancreatic islets of obese mice (Dobrian et al., 2011).

Moreover, our immunohistochemistry studies also revealed that sitagliptin was able to prevent the diabetes-induced increase in IL-1β and TNF-α, mainly in the cells around the glomeruli, which are probably tubular cells and/or accumulation of interstitial inflammatory cells. It has been shown that inflammatory cells, such as macrophages, lymphocytes, and monocytes, are often found in tubular compartment (Furuta et al., 1993; Mezzano et al., 2003), showing that interstitial inflammatory cells infiltrates are associated with progression of renal injuries in DN (Chow et al, 2004; Ninichuk et al., 2007). Monocyte chemotactic protein-1 (MCP-1) played a key role in promoting recruitment and infiltration of macrophage in the diabetic kidney (Chow et al., 2006). It has been described that hyperglycaemia increases expression of MCP-1 in tubular cells of the diabetic kidney (Chow et al., 2006; Mezzano et al., 2003). The pro-inflammatory transcription factor NF-κB was also detected mainly in tubular cells of human and rat kidney, with T2DM and overt nephropathy (Morcos et al., 2002). Furthermore, NF-κB regulates gene expression of several molecules involved in inflammation, which includes MCP-1, IL-1β and TNF-α (Guijarro et al., 2001). Based on these evidences, the increased expression of NF-κB and MCP-1 in tubular cells of diabetic kidneys can be a plausible explanation for our results, which should be further clarified.

The activation of signalling pathways linked to cell death resulting from chronic hyperglycaemia and to a state of low-grade chronic inflammation contributes to an increase in apoptosis. It is well established that members of the Bcl-2 family are key regulators of cell death. In the present study, a pro-apoptotic state seems to be favoured in the kidney of diabetic ZDF rats, which can lead to loss of renal cells and consequent renal dysfunction (Wagener et al., 2009; Sanchez et al., 2010. This increase in cell death by apoptosis appears to be mediated by Bax and Bid. Additionally, it has been demonstrated that glucose-induced ROS production initiates podocyte apoptosis and its depletion both, *in vitro* and *in vivo*, leading to DN (Susztak et al., 2006). Therefore, a good glycaemic control could reduce ROS production and the consequent

risk of cell death. In addition, sitagliptin was able to ameliorate serum TGs contents, thus reducing lipotoxicity-evoked apoptosis in the kidney tissue (Lee et al., 2011).

Accordingly to what was previously reported in the retina of ZDF rats, treatment with sitagliptin reduced this pro-apoptotic state and cell death by apoptosis in the kidney (Gonçalves et al., 2012). It has been previously described that activation of incretin receptors in pancreatic β-cells promotes resistance to apoptosis through the activation of several pathways leading to inhibition of caspase-3, by increasing expression of Bcl-2 and decreasing expression of Bax (Kim et al., 2005; Wang et al., 2002). Moreover, GLP-1 has anti-apoptotic actions via alteration of the Bcl-2 family proteins in several cell types. In fact, it was found that GLP-1 upregulates Bcl-2 and inhibits Bax expression in cholangiocytes (Marzioni et al., 2010), neuronal cells (Liu et al., 2009; Qin et al., 2008)], and endothelial cells (Zhan et al., 2012). In addition, it was shown that GLP-1 enhances Bcl-2 upregulation (Natalicchio et al., 2010), Bcl-2-associated death promoter (BAD) inactivation (Quoyer et al., 1989), and caspase-3 activity reduction (Tews et al., 2009) in pancreatic β-cells. Here, we confirmed and expanded these data by demonstrating that increased levels of GLP-1 through DPP-4 inhibition prevents the Bax/Bcl-2 mRNA and protein increase and reverses the increase in Bid and TUNEL-positive cells induced by chronic hyperglycaemia in the ZDF kidney rats.

In conclusion, our findings have shown amelioration of DN, and specifically of glomerulosclerosis, tubulointerstitial and vascular kidney lesions, by a chronic administration of a low dose of sitagliptin that does not reduce hyperglycaemia below a rather high level (partial, but significant, correction), indicative of non-compensated diabetes. The present study demonstrated that sitagliptin delays the development of nephropathy in ZDF rats, concomitantly with hypoglycaemic, hypolipidaemic and antioxidant effects, as well as, to anti-inflammatory and anti-apoptotic properties. Sitagliptin might be viewed as a promising preventive renoprotective therapeutic strategy against the development and/or progression of DN.

Chapter 7

General discussion

Prevalence and incidence of T2DM is increasing rapidly worldwide and comprises the majority (around 90%) of DM patients (IDF, 2016; IDF, 2015; WHO 2013; Lyssenko, 2013). In Portugal the numbers are equally alarming, as they follow the international trends in prevalence (Observatório Nacional da Diabetes, 2015). Statistically, DN, a major complication of diabetes, is now the single most common cause of ESRD worldwide and one of the main causes of death in diabetic patients, being also acknowledged as an independent risk factor for CVD (Narres et al., 2016; Van den Brand, 2016).

The central key features of T2DM are a defect in insulin resistance and/or insulin secretion, which lead to hyperglycaemia and disrupt the normal relationship between insulin sensitivity and pancreatic β-cell function (Virally et al., 2007). Degeneration of Langerhans islets with β-cell loss is secondary to insulin resistance and is regarded as the most important lesion for disease progression (Kahn et al., 2000; Marchetti et al., 2010; Cerf, 2013; Yagihashi et al., 2016). Apoptosis, low-grade inflammation and oxidative stress, which are mainly fuelled by hyperglycaemia and hyperlipidaemia, are key mediators of insulin resistance, β-cell degradation, and development of T2DM complications (Marchetti et al., 2010; Piya et al, 2013; Guo, 2014; Jung et al., 2014; Le Lay et al., 2014; Chen et al., 2015; Tangvarasittichai et al., 2015; Keane et al., 2015). Major long-term macrovascular (ischaemic heart disease, stroke and peripheral vascular disease) and microvascular (nephropathy, retinopathy and neuropathy) complications of T2DM arise from the persistent dysfunction in these multiple metabolic pathways (Filla et al., 2016).

Treatment regimens that reduce the levels of HbA1c to near or below 7% result in a significant reduction of risk in microvascular complications and diabetes-related death (IDF, 2012; Nathan et al., 2014; NICE, 2015; ADA; 2016; Ayadurai et al., 2016; Rayman, 2016). Current recommendations by the Consensus of ADA and EASD justify the selection of appropriate treatment based on its capability to achieve and maintain desired glycaemic goals (Rayman, 2016; Kahn et al., 2014; Nathan et al., 2014; Inzucchi et al., 2012). Despite all recomendations, many patients spend a long time well outside the recommended glycaemic range and, therefore, have an increased risk for developing micro and macrovascular complications (De Fronzo, 2009; Mata-Cases et al., 2017). Traditional treatments for T2DM, although complying

variably to standard glycaemic goals, do not seem to address the progressive decline in β-cell function and patients continue to advance in their disease state (Raz et al., 2006; De Fronzo, 2009; Karaca et al., 2009). Effective glycaemic control will likely require the use of medications that target both β-cell dysfunction and insulin resistance (Russell-Jones et al., 2012; Wajchenberg, 2013).

Currently it is becoming clearer that T2DM management must envision not only glycaemic control, but also and particularly, the mechanisms behind progression of pancreatic deterioration. Therefore, preservation of β-cell mass is regarded as the mainstay of disease control and prevention of evolutional complications, such as DN. In this sense, the improvement of strategies able to prevent or delay the evolution of diabetes and of DN, as one of the major complications, remains a major goal in biomedical research.

DPP-4 inhibitors, such as sitagliptin, have shown potential to target not only hyperglycaemia but also multiple pathophysiological processes and tissues involved in the progression of diabetes and of its complications (Abu-Amara et al, 2012; Aston-Mourney et al., 2013; Lee et al., 2013; Picatoste et al., 2013; Satoh-Asahara et al., 2013). Nonetheless, the precise protective mechanisms sitagliptin might exert in the pancreas and other tissues targeted by T2DM, specifically the kidney, have not yet been fully elucidated and need further research.

Thus, the main objective of our study was to evaluate the effects (and possible underlying mechanisms) of sitagliptin treatment, during 6 weeks, in ZDF rats, as a model of obese T2DM. In the first part of the study we evaluated the effects of sitagliptin treatment on glycaemic, lipidic and insulinaemic profiles and investigated some of the possible protective underlying pathways in the pancreas of ZDF rats, focusing on histopathological lesions and mediators of apoptosis, inflammation, angiogenesis and proliferation. In the second part, we assessed the effects of sitagliptin on renal lesions evolution and evaluated oxidative stress, apoptosis and inflammatory pathways as possible mechanisms underlying the putative renoprotective effects of this drug.

Concerning the ZDF rat model of T2DM, our results have demonstrated the key features encountered in diabetic patients. Hence, at the beginning of the study (20 weeks of age) the diabetic rats presented hyperglycaemia, hypercholesterolaemia,

hypertriglyceridaemia, increased HbA1c and hyperinsulinaemia, accompanied by insulin resistance (HOMA-IR). Furthermore, the diabetic rats lost weight, evident at 20 weeks of age, which was considered a sign of diabetic pathophysiological progression. At the end of the study protocol, when animals were 26 weeks old, the diabetic rats aggravated their disease state, displaying higher hyperglycaemia, accompanied by increased HbA1c, insulin resistance and reduced plasma concentration of insulin, suggesting a state of relative insulinopaenia. Moreover, the diabetic rats continued to lose weight and showed aggravated hypercholesterolaemia and hypertriglyceridaemia. During the course of the study, diabetic rats treated once a day with sitagliptin showed a remarkable beneficial effect on several important parameters, such as a reduction of glucose and HbA1c levels, jointly with a partial correction of insulin reduction and an improvement in insulin resistance (HOMA-IR), which is in agreement with other reports (Mu et al., 2009; Verspohl et al., 2009; Ahrén, 2010; Ji et al., 2016; Kondo et al., 2016). Furthermore, reduction of BW was prevented and hypertriglyceridaemia corrected, in agreement with others studies (Moritoh et al., 2009; Verspohl et al., 2009; Van Genugten et al., 2012; Gallwitz, 2014; Ji et al., 2016). The amelioration of serum TGs levels are in accordance with a recent meta-analysis of randomized clinical trials, which reveal that sitagliptin alone, or added to other antihyperglycaemic agents, significantly improve serum TGs concentration (Fan et al., 2016).

In order to establish a baseline for the study of the histopathological lesions in the diabetic pancreas, we relied mostly on prior descriptions of animal models of diabetes (Butler et al., 2003; Nugent et al., 2008), as detailed descriptions for the human pancreas (Bonner-Weir et al., 2008) were (and still are) lacking in T2DM bibliography, probably due to the scarcity of human diabetic pancreas for research. Moreover, we could not find a pre-existing semiquantitive histopathological classification for any pancreatic lesions, which we required for our comparative evaluation. Therefore, we adopted the semiquantitive scoring of the Banff classification for renal allograft histopathology (Soleza et al., 2008) and adapted it to diabetic pancreas pathology.

In our study, the ZDF rat presented altered morphological islet architecture, the presence of inflammation, fibrosis, and congestion in the endocrine and exocrine pancreas, as well as islet cell vacuolization. These lesions exhibited significant

aggravation from the initial to the final observational time. Sitagliptin treatment of diabetic rats was able to prevent the aggravation of both endocrine and exocrine pancreatic histopathological lesions. Diabetic lesion improvement was also documented by Yeom et al. (2011), using the TUNNEL assay and Ki-67 staining in the Akita mice. The authors reported beneficial effects of sitagliptin on the islets via reduced apoptosis and increased proliferation. In other two studies using des-fluoro-sitagliptin, DPP-4 inhibition protected islet morphology, reduced the number of apoptotic cells increased β-cell mass in the streptozotocin (STZ)-induced and in the db/db diabetic mouse models (Shirakawa et al. 2016; Takeda et al., 2012). These results were further corroborated by other authors (Shirakawa et al. 2011a; Takeda et al. 2012; Pappachan et al., 2015). In fact, our studies on pancreatic apoptosis confirmed the previously mentioned studies by revealing, in the diabetic rats under sitagliptin treatment, a net reduction in the Bax/Bcl-2 ratio. Others studies have also described that activation of incretin receptors in pancreatic β-cells promotes resistance to apoptosis through activation of several pathways leading to an increase in the expression of Bcl-2 and to a decrease in the expression of Bax (Wang et al., 2002; Kim et al., 2005; Natalicchio et al., 2010; Hou et al., 2015; Chang et al., 2016; Chon et al., 2016).

In our work, sitagliptin promoted an increased expression of PCNA (a marker of proliferation) in the pancreas, which is in line with the β-cell mass expansion. In addition, sitagliptin-treated rats presented overexpression of VEGF, which appears to be accountable for the needed vascular support for tissue regeneration. The overexpression of pro-proliferative and pro-angiogenic mediators have also been alluded in other studies (Parnaud et al., 2011; Pascoe et al., 2012; Sharma et al., 2015). A study with sitagliptin-treated Wistar rats reported significantly increased immunostaining of glucagon, GLP-1 and GLP-1R, when compared to the untreated diabetic animals (Karabulut et al., 2015). These beneficial effects seem to be GLP-1-dependent (Van Genugten et al., 2012; Gallwitz et al., 2014) and mediated by the transcriptional activation of anti-apoptotic and pro-survival genes, as well as, by the suppression of pro-apoptotic genes in the β-cells (Mu et al. 2006; Duttaroy et al. 2011; Han et al. 2011; Kim et al. 2016). Other DPP-4 inhibitors, like linagliptin, were found to protect isolated human islets from gluco-, lipo-, and cytokine toxicity, which are crucial

diabetogenic mechanisms, via promotion of oxidative stress and inflammation (Shah et al. 2013). Vildagliptin, another DPP-4 inhibitor, also preserved β-cell mass in diabetic HFD-induced obese mice through induction of β-cell proliferation and inhibition of apoptosis, as well as, by reduction of islet inflammation (Omar et al. 2013). Our results show a significant decrease in IL-1β, one of the main inflammatory cytokines, which have been corroborated by others that have suggested anti-inflammatory properties of gliptins in distinct animal models and tissues, as well as, in T2DM patients (Gonçalves et al., 2012; Joo et al., 2013; Lee et al., 2013; Satoh-Asahara et al., 2013; Derosa et al., 2013; Dai et al., 2014; Röhrborn et al., 2015).

Insulin resistance is recognized as a key factor in the pathogenesis of T2DM (Prudente et al., 2009; Beguinot et al., 2010; Yagihashi et al., 2016), resulting from anomalies in the insulin cascade, which is regulated by many biochemical mechanisms (Prudente et al., 2012; De Lorenzo et al., 2013; Marinho et al., 2015). In our study, sitagliptin treatment was able to completely reduce pancreas TRIB3 expression. TRIB3 gene has been implicated in coding inhibitors of insulin signalling cascade. Reduction of TRIB3 expression might be an important mechanism for the decline of insulin resistance and improvement of insulin secretion found in the diabetic rats under sitagliptin treatment (Yagihashi et al., 2016; Beguinot et al., 2010; Prudente et al., 2009). In humans, TRIB3 has also been associated with insulin resistance and is found to be overexpressed in T2DM patients (Prudente et al., 2009; Prudente et al., 2012; Gong et al., 2009). Another aspect by which TRIB3 seems to be associated with the evolution of insulin resistance and pancreas degradation is its role as inductor of β-cell apoptosis and inhibitor of proliferation. Therefore, it is hypothesized that, by downregulating the expression of TRIB3, sitagliptin sponsored further anti-apoptotic effects and enhanced β-cell proliferation, thus contributing to the beneficial effects afforded by this DPP-4 inhibitor in this animal model (Humphrey et al., 2014).

Although gliptins have been suggested has having the ability to preserve both β-cell function and mass in animal models of diabetes, little is still known regarding their cytoprotective effects on the human pancreas (Shirakawa et al., 2016; Yeom et al., 2011; Ferreira et al., 2010; Matveyenko et al., 2009; Maida et al., 2009). DPP-4 inhibition in humans over an extended time period does not seem to confirm long-term cytoprotective properties in β-cells, as shortly after cessation of therapy the

improvement in β-cell function is not maintained, deeming this improvement as functional rather than structural (Van Genugten et al. 2012; Chon et al., 2016). This discrepancy might be due to variation of dosage and other protocol variants in pre-clinical studies, as well as, to the differing expression and localization of DPP-4 in different animal species. The pancreatic islets of rodents show an exclusive expression of DPP-4 in the β-cells, with little expression in the α-cells, while human and pig islets mostly express DPP-4 in the α-cells (Liu et al. 2014; Omar et al. 2014). The physiological implication of the inter-species differences in the localization of DPP-4 is still unclear.

Our histomorphological evaluation of the exocrine pancreas showed a notable improvement between untreated and sitagliptin-treated diabetic animals. However, our results are in contradiction with the data reported by Matveyenko et al. (2009) that used a DPP-4 inhibitor in human IAPP transgenic (HIP) rats and with the results of Nachnani et al. (2010) that used an injection of a GLP-1 agonist. Both studies have suggested that the enhancement of endogenous GLP-1 levels could induce undetected low grade asymptomatic chronic pancreatitis. The FDA, in 2009, emitted safety concerns regarding incretin-induced pancreatitis. These safety concerns have been recently deflated by research and cohort studies (Yabe et al., 2015; Egan et al., 2014; Aston-Mourney et al., 2013). The strongest evidence currently available came from three large cardiovascular safety studies with DPP-4 inhibitors and from meta-analysis recently published of non-randomized and randomized clinical trials: Trial Evaluating Cardiovascular Outcomes with Sitagliptin (TECOS), Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus (SAVOR-TIMI 53) and Examination of Cardiovascular Outcomes with Alogliptin versus Standard of Care in Patients with Type 2 Diabetes Mellitus and Acute Coronary Syndrome (EXAMINE). It was concluded that there was no significant difference between the placebo and the DPP-4 inhibitor treated groups with regard to pancreatitis or pancreatic cancer (Holman, 2015; Yabe et al., 2015; Egan et al., 2014; Aston-Mourney et al., 2013). In addition, FDA and European Medicines Agency (EMA) concluded that a causal association between incretin-based drugs and pancreatitis or pancreatic cancer cannot be established with the current data. However, both agencies have not reached a final conclusion so far regarding such a causal relationship and will continue to investigate this safety signal (Egan et al., 2014).

DN is the single most common cause of ESRD and is acknowledged as an independent risk factor for CVD, being one of the main causes of incapacity and of mortality in diabetic patients (Narres et al., 2016; Van den Brand, 2016). DN is primed by diabetic metabolic dysregulations and haemodynamic variations, through activation of the RAAS, which triggers a number of cell signalling cascades that mediate cellular responses by activation of key transcription factors. In reaction to such signals, renal cells (such as tubular epithelial cells, podocytes and mesangial cells) will produce chemokines, growth factors, and pro-fibrotic cytokines. These responses contribute to a cycle of inflammation, oxidative stress, cellular injury, progressive fibrosis, and loss of glomerular filtration. Podocyte loss, endothelial dysfunction, alterations in the glomerular basal membrane and tubular injury, all contribute to the increase in proteinuria during the development and progression of DN (Górriz et al., 2015, Kawasaki et al., 2015).

Our study reports the progression of renal disease in ZDF rats and demonstrates that a daily chronic administration of a low-dose of sitagliptin noticeably reduces renal function and injury in this model. In fact, we found significantly increased levels of serum BUN in the diabetic rats when compared to the lean control rats, suggesting a deficient kidney function. Sitagliptin treatment was able to decrease BUN levels to values identical to those observed in lean controls, suggesting an amelioration of renal function. Nevertheless, serum creatinine levels were unchanged between groups, which are in accordance with others using the ZDF rat as animal model (Schrijvers et al., 2006).

In order to achieve a better correlation between the lesions observed in our animal model with the human nephropathy profile, we adopted in our study the international histopathologic classification, currently approved for human DN (Taevert et al., 2010). It is noteworthy that until we published our work, previous research had only briefly alluded to tubulointerstitial lesions, as a secondary lesion in DN, such as Hoshi et al. (2002) and Gassler et al. (2001). The description of lesions in the major vessels of the kidney was absent in animal model studies and could only be found in scarce human DN reports. From 2010, the histopathological observation of these vessels has become mandatory for DN classification (Taevert et al., 2010).

Nephropathy in this model has previously been described as FSGS, now called nodular sclerosis that is associated with glomerulomegaly and mesangial expansion, which are features seen in T2DM patients (Chander et al., 2004; Kim et al., 2016). In our obese diabetic ZDF rats, the severity of lesions aggravated with diabetes progression, confirming a link between diabetes and the progressive renal injury. In patients with DN, the initial physiological change is glomerular hyperfiltration, while the initial morphological change is glomerular hypertrophy. At the final time of our experiment, the diabetic rats exhibited aggravation of the lesions described in our starting point, including mesangial expansion, glomerular basement membrane thickening, glomerular hypertrophy and glomerulosclerosis. We observed that tubulointerstitial lesions were linked to glomerulosclerosis, which was suggested by the aggravation of both the glomeruli and the interstitium. The diabetic metabolic environment and the prolonged interaction of albuminuria (and other substrates in the glomerular filtrate) with the tubular system, seems to incite renal oxidative stress and cortical interstitial inflammation, with subsequent hypoxia and tubulointerstitial fibrosis, contributing to DN progression (Tonolo et al., 2014). The pro-inflammatory transcription factor NF-κB regulates the gene expression of several molecules involved in inflammation, including IL-1β and TNF-α (Guijarro et al., 2001; Hojs et al., 2015; Kumar et al., 2015; Ozkok et al., 2016). This factor has been mainly detected in tubular cells of diabetic rats and humans with overt DN (Kumar et al., 2015; Morcos et al., 2002). Vascular pole hyalinization and arteriosclerosis also suffered aggravation with age in diabetic rats. All of these histological alterations were accompanied by an increment of kidney weight. In our study, the obese diabetic ZDF rats presented glomerular hypertrophy, expansion in the mesangial area related to the mesangial matrix, and renal hypertrophy. Although we were unable to evaluate the progression of proteinuria, which was well documented by others, we consider that the increase of hyaline cylinders in 26 weeks diabetic rats may be related to this phenomenon (Janiak et al., 2006; Espinel et al., 2015; Kawasaki et al., 2015; Garsen et al., 2016).

On histopathological evaluation, sitagliptin treatment ameliorated glomerular, tubulointerstitial and vascular lesions in diabetic rats. Other authors have shown similar results, with suppression of DPP-4 activity and/or protein expression, in which amelioration of kidney fibrosis was associated with the inhibition of endothelial-to-

mesenchymal transition (EndMT) and reduced levels of inflammatory and fibrotic markers (Panchapakesan et al.; 2013; Min et al., 2014; Panchapakesan et al.; 2015). These histopathological improvements have been disclosed by other studies (Tonolo et al., 2014; Vavrinec et al., 2014; Shi et al., 2016). Considering that sitagliptin was not able to completely normalize hyperglycaemia in the diabetic rats in our study, another key mechanism by which it can positively modulate kidney function and lesions might be through direct renal DPP-4 inhibition, via both GLP-1-dependent and GLP-1-independent pathways. GLP-1-dependent activity is sanctioned by the expression of GLP1-receptors in the kidney. GLP-1 has been associated to the protection of mesangial cells and to an amelioration of Na^+, acid-base, and fluid homeostasis, thus lowering BP, which collectively contributes to renoprotection (Ishibashi et al., 2012; Tanaka et al., 2014; Górriz et al., 2015). For the GLP-1-independent effects of DPP-4 inhibitors, in the kidney, several pathways have been suggested, including the other known substrates of DPP-4, such as high mobility group box 1 protein (HMGB1), Meprin β, neuropeptide Y (NPY), and peptide YY (PYY) (Von Websky et al., 2014; Panchapakesan et al.; 2015).

Furthermore, it is known that DPP-4 exhibits its enzymatic activity in both membrane-anchored cell-surface peptidase and as a smaller soluble form in blood plasma (Silva Júnior et al., 2015; Shirakawa et al., 2016; Shi et al., 2016). In fact, there are some studies suggesting that microvascular endothelial cells are the main sources of endogenous DPP-4 (Matheeussen et al., 2011; Romacho et al., 2016). In addition, in vitro studies showed that both DPP-4 mRNA expression and enzyme activity were enhanced by exposure of human glomerular endothelial cells to high glucose concentrations (Yu et al., 2016; Ahmed et al., 2015; Röhrborn et al., 2015). In agreement, our group has recently demonstrated that diabetic rats present an increased protein expression of DPP-4 in the kidney, when compared to nondiabetic animals (Marques et al., 2014).

Our results clearly indicate that diabetes leads to increased inflammation and pro-inflammatory cytokines, specifically IL-1β and TNF-α, in the kidney of diabetic ZDF rats. These results are corroborated by other authors that described an increased expression of those pro-inflammatory cytokines in the diabetic kidney (Navarro et al., 2006; Shi et al., 2016; Donate-Correa et al., 2015), leading to enhanced vascular

endothelial permeability, oxidative stress, renal hypertrophy, and tubulointerstitial lesions.

Using ZDF as animal model, we found that DPP-4 inhibition prevented the inflammatory profile and the pro-apoptotic state observed in the diabetic kidney, which might explain the improvement in renal dysfunction and injury (glomerular, tubulointerstitial and vascular damage). In fact, sitagliptin was able to prevent the increase in both mRNA and protein levels of the pro-inflammatory cytokines IL-1β and TNF-α in the diabetic kidneys of ZDF rats. Our current results, and studies by others, have shown that a decrease in inflammation promotes an amelioration of DN (Shi et al., 2016; Donate-Correa et al., 2015).

The activation of signalling pathways linked to cell death resulting from chronic hyperglycaemia and to a state of low-grade chronic inflammation contributes to an increase in apoptosis. In the present study, a pro-apoptotic state seems to be favoured in the kidney of the diabetic ZDF rats, which appears to be mediated by Bax and Bid. Sitagliptin prevented the Bax/Bcl-2 (mRNA and protein) ratio increase and reversed the increase in Bid and TUNEL-positive cells induced by chronic hyperglycaemia in the kidneys of our animal model. In addition, sitagliptin was able to ameliorate serum TGs contents, thus reducing lipotoxicity-evoked apoptosis in the kidney (Lee et al., 2011; Li et al., 2014; Martins et al., 2015; Glastras et al., 2016). Additionally, it has been demonstrated that glucose-induced ROS production initiates podocyte apoptosis and its depletion in *vitro* and *in vivo*, leading to DN (Susztak et al., 2006; Wagener et al., 2009; Sanchez et al., 2010). Therefore, the reduction of oxidative stress afforded by sitagliptin in this study could eventually reduce ROS production and the consequent risk of cell death. Other studies have demonstrated that GLP-1 receptor activation has also attenuated diabetic renal injury, including by reduction of kidney oxidative stress, inflammation and apoptosis (Hendarto et al., 2012; Kodera et al., 2014; Nakashima et al., 2014; Matsui et al; 2015; Mima, 2016).

Our findings clearly support that in diabetic ZDF rats the amelioration of hiperglycaemia with a therapeutic dose of sitagliptin confer independent renoprotective effects. These findings might contribute to the explanation of safety and efficacy profile of DPP-4 inhibitors in patients with T2DM and CKD and might aid to confirm the position of these new therapy classes in patients with CKD.

Supporting our results in both the pancreas and kidney, several reports have suggested that sitagliptin also confers cytoprotective effects on several tissues. Besides the present results, our group has also shown beneficial effects of sitagliptin in animal models of T2DM and T1DM in various organs. In fact, in the heart, a decrease in hypertension and in cardiometabolic disease parameters was observed, together with a decline in heart oxidative stress (Ferreira et al., 2010) and in the retina, an amelioration in the pro-apoptotic and inflammatory state was noted (Gonçalves et al., 2012; Gonçalves et al., 2014). Furthermore, during the recent years other authors also disclosed putative cytoprotective properties of sitagliptin and other DPP-4 inhibitors in the pancreas (Shirakawa et al. 2016; Pappachan et al., 2015; Chang et al., 2016; Chon et al., 2016; Hou et al., 2016) and in extrapancreatic tissues, including studies in the diabetic heart (Liu et al, 2015; McCormick et al., 2014; Apaijai et al., 2013; Picatoste et al., 2013; Read et al., 2010), related with vascular complications (Mima, 2016; Mita et al., 2016) and with endothelial dysfunction (Matsubara et al., 2013; Zhan et al., 2012), as well as, experiments performed in the diabetic liver (Ideta et al. 2015; Jung et al., 2014; Klein et al. 2014), adipose tissue (Shirakawa et al., 2016 ; Shirakawa et al., 2011a ; Shirakawa et al., 2011b) and kidney (Panchapakesan et al., 2015; Liu et al., 2012; Vaghasiya et al., 2011). Collectively, these parallel investigations have been reinforcing our own findings in the pancreas and kidney.

Nonetheless, further research is required to establish the exact key mechanisms by which these putative cytoprotective effects are stimulated in different tissues, if by an indirect action via insulin secretion increment or through direct tissular DPP-4 inhibition. If a direct action in tissues confirmed, then further research is needed to address whether protection is conferred via GLP-1-dependent (which is sustained by expression of DPP-4 and GLP-1R in tissues) and/or GLP-1-independent pathways (reinforced by the existence of multiple DPP-4 substrates).

To summarize, our study using an animal model of obese T2DM (the ZDF rat) has revealed that sitagliptin is able to ameliorate the glycaemic, insulinaemic and lipidic profiles and prevent aggravation of pancreatic and renal lesions, which were accompanied by cytoprotective properties related with anti-apoptotic, antioxidant and anti-inflammatory actions.

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Chapter 8

Main conclusions

The results obtained throughout this work allow for the delineation of the following main conclusions:

- During the experimental period, the ZDF diabetic rat model displayed similar features to human T2DM, with aggravation of dysmetabolism (hyperglycaemia, hyperlipidaemia and insulin resistance), oxidative stress, inflammation and development of lesions in the pancreas and in the kidney.

- Sitagliptin treatment during 6 weeks was able to partially ameliorate the evolution of T2DM dysmetabolism in the obese diabetic ZDF rats, viewed by the reduction of hyperglycaemia, hypertriglyceridemia and insulin resistance.

- In addition, sitagliptin treatment prevented the evolution of all histopathological diabetic lesions in the endocrine pancreas, with special incidence in inflammation, as well as, exocrine pancreatic damage.

- Sitagliptin showed pancreatic cytoprotective properties, suggested by the anti-apoptotic (reduced Bax/Bcl2 ratio) and anti-inflammatory (reduced IL-1β expression) effects, as well as, by decreased insulin resistance (reduced TRIB3 expression) and pro-proliferative (increased PCNA expression) and angiogenic (increased VEGF expression) actions.

- Regarding the kidney, sitagliptin treatment during 6 weeks was able to prevent evolution of histopathological lesions (glomerular, tubulointerstitial and vascular) in the obese diabetic ZDF rats, thus delaying aggravation of DN.

- Concomitantly, sitagliptin showed ability to promote renoprotection, involving antioxidant (decreased MDA), anti-inflammatory (reduced IL-1β expression) and anti-apoptotic (reduced Bax/Bcl-2 ratio, Bid expression and TUNEL positive cells) properties.

These pleiotropic effects reinforce the status of sitagliptin as a promising antidiabetic drug to sustain natural disease progress. Sitagliptin showed potential to

avert the decline of insulin secreting capacity in pancreatic islets through tissue-cytoprotective properties, thus suggesting a role in the prevention of T2DM evolution. Furthermore, the protective actions on the diabetic kidney open-up the possibility of using sitagliptin as a renoprotective therapeutic strategy against the development and/or delay of DN.

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