Elucidation of Novel Factors Driving Inflammation and Pancreatic Beta-Cell Death in Type 2 Diabetes

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Copyright statement

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

5-HT1B	5-hydroxytriptamine 1B serotonin receptor
ADP	Adenosine diphosphate
AMPK	Adenosine monophosphate activated protein kinase
APO1	Apoptosis antigen 1
APS	Ammonium persulfate
AT	Adipose tissue
ATC	Anaplastic thyroid cancer
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C/EBP	CCAAT-enhancer binding protein
CAD	Coronary artery disease
CCL	Chemokine ligand
CHOP	CCAAT-enhancer binding protein homologous protein
CRC	Colorectal cancer
CRP	C reactive protein
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
DAG	Diacyl glycerol
DAMP	Damage associated molecular patterns
DDIT3	DNA damage inducible transcript 3
DFO	Deferoxamine

DFS	Diabetic foot syndrome
DM	Diabetes mellitus
DN	Diabetic nephropathy
DR	Diabetic retinopathy
DTT	Dithiothreitol
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EIF2	Eukaryotic initiation factor 2
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated protein degradation
ERK	Extracellular signalling related kinase
ERSE	Endoplasmic reticulum stress-response element
ESCC	Esophageal squamous cell carcinoma
FASR	FAS receptor
FADD	FAS-associated protein with death domain
FFA	Free fatty acids
FFAR	Free fatty acids receptor
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOXO1	Forkhead box O1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCK	Glucokinase
GDM	Gestational diabetes mellitus

GH	Growth hormone
GLP1	Glucagon like peptide 1
GLT	Glucolipotoxic
GLUT	Glucose transporter
GPCR	G-protein coupled receptor
GSIS	Glucose stimulated insulin secretion
GSK	Glycogen synthase kinase
GWAS	Genome wide associated studies
HCC	Hepatocellular carcinoma
HFD	High fat diet
HIF-1α	Hypoxia inducible factor 1α
HMGB1	High mobility group box 1
HRE	Hypoxia responsive elements
IAPP	Islet amyloid polypeptide
ICAM	Intracellular cell adhesion molecule
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IFNγ	Interferon gamma
IGF	Insulin growth factor
IκB	Inhibitor of Nuclear factor-kappa B
IKK	IκB Kinase
IL	Interleukin
IP3	Inositol triphosphate
IR	Insulin resistance

IRE1	Inositol requiring enzyme 1
IRS	Insulin receptor substrate
JAB1	c-Jun activation domain binding protein 1
JAK	Janus kinase
JNK	c-Jun N-terminal Kinase
KDR	Kinase domain receptors
KGF	Keratinocyte growth factor
KPNA	Karyopherin
LAR	Leukocyte common antigen-related
LKB1	Serine/Threonine kinase 1
LPS	Lipopolysaccharide
MAF	MAF transcription factor
MAPK	Mitogen activated protein kinase
MCL-1	Myeloid cell leukaemia sequence protein 1
MCP1	Monocyte chemoattractant protein 1
MIP1α	Macrophage inflammatory protein 1α
MMP	Matrix metalloproteinase
MODY	Maturity onset diabetes of the young
mTOR	Mammalian target of rapamycin
NAC	N-acetylcysteine
NDR	Nuclear dbf2-related
NEUROD	Neuronal differentiation factor
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor

NGN3	Neurogenin 3
NGS	Next generation sequencing
NHS	National Health Service
NIK	Nuclear factor-kappa B-inducing kinase
NKX	NK homeobox
NLRP3	Nucleotide binding domain & leucine-rich repeat containing protein 3
NLS	Nuclear localisation signal
NMIIA	Non-muscle myosin heavy chain IIA
NO	Nitric oxide
NSCLC	Non-small cell lung carcinoma
OA	Oleic acid
OSCC	Oral squamous cell carcinoma
PA	Palmitic acid
PAI1	Plasminogen activator inhibitor 1
PAX	Paired box
PCR	Polymerase chain reaction
PDK1	Protein kinase 3 phosphoinositide dependent protein kinase 1
PDX1	Pancreatic duodenal homeobox-1
PERK	Protein kinase Ribonucleic acid-like endoplasmic reticulum kinase
PHDs	Prolyl hydroxylases
PI3K	Phosphatidyl inositol 3 kinase
PIP2	Phosphatidyl inositol bisphosphate
PKB	Protein kinase B
PKC	Protein kinase C

PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
PRAME	Preferentially expressed antigen of melanoma
PRL	Prolactin
PUMP	Pathogen associated molecular pattern
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
REDD1	Regulated in development and DNA damage responses 1
RGD	REL homology domain
RHEB	RAS homologous enriched in brain
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute 1640
RYR	Ryanodine receptor
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SFA	Saturated fatty acid
SHH	Sonic hedgehog
SNV	Single nucleotide variant
SR	Sarcoplasmic reticulum
STAT	Signal transduction and activator of transcription
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TAD	Transactivation domain

TEMED	Tetramethylethylenediamine
TGFβ	Transforming growth factor β
TIMPs	Tissue inhibitors of metalloproteinases
TLR	Toll like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNFRSF	Tumour necrosis factor receptor super family
TSC1/2	Tuberous sclerosis complex 1/2
TXNIP	Thioredoxin-interacting protein
TZD	Thiazolidinediones
UFA	Unsaturated fatty acid
UPR	Unfolded protein response
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WAT	White adipose tissue
WHO	World Health Organisation
XBP1	X-box protein 1

*As a convention, throughout the thesis italics are used for designating gene names, while roman font is used for designating protein names.

Abstract

Type 2 diabetes (T2D) is a chronic metabolic disorder where failure to maintain normal glucose homeostasis is associated with, and exacerbated by, obesity and the concomitant elevated free fatty acid (FFA) concentrations typically found in these patients (Olokoba et al 2012). 463 million people are currently estimated to be living with diabetes, of which 229 million are undiagnosed. Importantly, around 90% of all cases are T2D cases.

Hyperglycaemia and hyperlipidaemia together contribute to a decline in pancreatic insulin-producing beta cell (β-cell) mass through activation of pro-inflammatory signalling pathways. Glucolipotoxicity (GLT) is the term given to the combined and damaging effect of increased glucose and FA levels on pancreatic β-cells (Poitout et al 2010). There are however a large number of molecules potentially able to modulate these pro-inflammatory pathways, and the mechanism(s) by which GLT induces inflammation remains poorly defined.

Utilising Illumina HiSeq next generation sequencing technology, I have analysed the β-cell transcriptome to identify those genes and proteins most sensitive to high glucose and FA environment. Data shows that of those molecules potentially able to activate inflammatory pathways, the S100 family of proteins are amongst the most highly upregulated by GLT. Independent PCR and immunoblot analysis have further confirmed upregulation of the three most highly expressed family members, namely S100A3, A4 and A5. Importantly, my data has established a link between S100A4 and NF-κB activation that is driven by glucose and FAs.

In order to determine wider mechanisms involved in the activation of NF-κB by S100A4, predictive pathway interaction maps have been generated for S100A4 based on the RNAseq data. This approach has uncovered a potentially novel interaction with a pro-inflammatory transcription factor not previously associated with T2D, HIF-1α, thereby establishing a link between inflammation and hypoxia, and by extent, between T2D and cancer. This provides a valuable strategy that can be further exploited to discover novel potential targets for therapeutic intervention in the treatment of T2D.

CHAPTER 1. Introduction

1.1 Diabetes

Diabetes mellitus (DM) describes a collection of metabolic disorders characterised by decreased insulin sensitivity and subsequent high blood glucose levels, known as hyperglycaemia. People with diabetes have an increased risk of developing a number of serious life-threatening health complications resulting in high medical care costs, decreased quality of life and increased mortality (Baena-Díez *et al.* 2016).

1.1.1 Epidemiology

For the past 20 years, the International Diabetes Federation (IDF) has been measuring the prevalence of diabetes in order to produce estimates of its global burden and its impact nowadays, and predict figures for 2045 (Cho *et al*. 2018). It was estimated that in 2019 there were 463 million people (aged 18-99 years) with diabetes worldwide. It is important to note that almost half of all people (49.7%) living with diabetes are undiagnosed. (Michelstown cohort, University College Cork). Alarmingly, these figures are expected to rise to 700 million by 2045 (IDF).

In 2017, approximately 5 million deaths of people in the 18-99 years age range worldwide were attributable to diabetes. Increased life expectancies have contributed significantly to this exponential rise, with diabetes now constituting the cause of almost 10% of global mortality (Cho *et al.* 2018). Diabetes has rapidly gained positions in the ranking of causes of death worldwide: in the year 2000 it was not included in the top 10 causes, whereas in 2017 it has ascended to the sixth position, although still behind heart and pulmonary diseases, cancers and dementias (World Health Organisation, WHO).

1.1.2 Health and economic consequences of diabetes

People with diabetes have an increased risk of developing a number of health problems. Consistent high blood glucose levels can lead to serious diseases, mostly affecting the

cardiovascular system and the peripheral and autonomic nervous systems. Importantly, around two thirds of people suffering from diabetes die from cardiovascular disease (CVD) (Low Wang *et al.* 2016); diabetes is the most frequent cause of severe chronic kidney disease, and is the leading cause of end-stage renal disease in western countries (Shahbazian *et al*. 2013). Furthermore, diabetes is the leading cause of blindness globally as a consequence of diabetic retinopathy (Lee *et al*. 2015), and the leading cause of lower limb amputation due to diabetic nephropathy and a combination of other conditions (Narres *et al*. 2015). The mechanisms leading to these diabetic comorbidities will be detailed in section 1.2.2.

The global healthcare expenditure on people with diabetes aged 18-99 was estimated to be US dollars 850 billion in 2017 and is expected to increase by 7% to US dollars 958 billion by 2045 (Cho *et al*. 2018). The diabetes prevalence estimates, together with the deaths attributable to diabetes and healthcare expenditure associated to it, present a large social, financial and health system burden worldwide, which needs to be addressed urgently.

1.1.3 Types of diabetes and current treatments

Diabetes is classified according to etiological type. There are four main groups:

a) Type 1 Diabetes (T1D)

T1D is an auto-immune condition, characterised by the destruction of the insulin producing pancreatic β-cells, due to the development of islet autoantibodies. It accounts for between 5-10% of all diabetes cases (IDF). T1D commonly originates in childhood or adolescence although it can appear at any age. Signs and symptoms of the severe insulin deficiency and hyperglycaemia characteristic of T1D include polydipsia (increased thirst), polyphagia (increased appetite), polyuria (increased urination), weight loss, and fatigue (Kahanovitz *et al.* 2017). Treatments for T1D include insulin therapy and pancreas or islet transplantation. In the case of insulin therapy, T1D patients need daily insulin injections in order to control their blood glucose levels. Failure to do so will lead to death (International Diabetes Federation).

b) Type 2 Diabetes (T2D)

T2D is the most common form of diabetes, accounting for 80% to over 95% of cases, and has an increasing prevalence worldwide (Dardano *et al.* 2014). It usually appears in people over the age of 40. In fact, T2D used to be called adult-onset diabetes; however, the number of children being diagnosed with T2D has rapidly increased in the last 20 years, an observation that correlates with a striking increase in both the prevalence and the degree of obesity in children and adolescents in many populations (Han *et al.* 2010, Pulgaron *et al.* 2014). T2D is a metabolic disease characterised by chronic hyperglycaemia secondary to insulin resistance (IR) (Rojas *et al*. 2018). Development of T2D is affected by genetic and/or environmental factors, including lifestyle habits such as high fat diet and lack of exercise; this leads to decreased insulin secretion or IR, resulting in insufficient insulin effect. Symptoms include frequent and abundant urination, tingling in limbs and tiredness or lack of energy (International Diabetes Federation). Risk factors for the development of T2D include weight, inactivity, family history and having been born to a mother with gestational diabetes (Mayo Clinic). T2D also used to be called non-insulin dependent diabetes, as treatment doesn't include insulin therapy. Instead, people with T2D can often initially manage their condition through exercise and diet. However, over time most people will require administration of drugs such as metformin, for its anti-hyperglycaemic properties but also for its ability to improve endothelial dysfunction, oxidative stress, insulin resistance, lipid profiles and fat redistribution (Rojas *et al*. 2013). If a treatment based on changes in the diet and oral medication is insufficient, insulin injections might be necessary.

c) Gestational Diabetes Mellitus (GDM)

Gestational diabetes is a form of diabetes consisting of high blood glucose levels during pregnancy, affecting 5-20% of pregnant women (Diabetes UK). GDM usually arises in the second or third trimester, and the reasons include an increase in hormones produced by the placenta, together with an increase in sexual hormones, in particular oestrogen, progesterone, and cortisol, that contribute to a disruption of the glucose-insulin balance, leading to IR (Alfadhli 2015). In addition, increased maternal adipose deposition, decreased exercise, and increased caloric intake contribute to this state of glucose intolerance. Glucose metabolism disorders often return to normal after delivery but there is an increased risk in developing T2D, metabolic syndrome and CVD in the future for the mother, and an increased risk of developing T2D in the child. Approximately half of the

women with a history of GDM develop T2D within five to ten years after delivery (Herath *et al.* 2017). Methods to decrease the risk of pregnancy-related health issues in women suffering from GDM includes controlling blood sugar levels through changes in the diet (for example by increasing the intake of low glycaemic index foods to avoid peaks of insulin) and increased physical activity, and if this can't be achieved, medication or insulin injections may be required (National Health Service, NHS).

d) Other types of diabetes include:

- Maturity Onset Diabetes of the Young (MODY) includes several disorders caused by monogenic defects in β-cell function (Schober *et al*. 2009). It is caused by a mutation in a single gene, which is inherited in an autosomal dominant pattern. Hyperglycaemia appears at an early age (generally before age 25 years). This single-gene mutation impacts the ability of the pancreatic β-cells to produce or secrete insulin, with minimal or no defects in insulin action (American Diabetes Association 2017).
- Neonatal Diabetes, a form of diabetes diagnosed under the age of 6 months. It differs from T1D in that it is not an autoimmune condition (Diabetes UK).

T2D, accounting for 80% to over 95% of all the diabetes cases, is the primary focus of this research.

1.2 Type 2 Diabetes

Global prevalence of T2D is rapidly growing as a consequence of lifestyle changes, urbanization and population aging (Chen *et al*. 2012). There is a strong social correlation between the incidence of T2D and a dramatic shift towards sedentary lifestyle in the last decades (Blas *et al.* 2010). This includes higher exposure to obesogenic environments, such as lower levels of physical activity and the consumption of excess calories, starting from young ages, which can lead to other complications including obesity and CVD. Consequently, recently more and more children are being diagnosed with T2D (Pulgaron *et al*. 2014).

1.2.1 Etiology of T2D

The prevailing theory for the pathogenesis of T2D describes it as a multifactorial disease, arising from a combination of the presence of T2D risk alleles in various genes, causing defective insulin secretion and insulin response, together with certain environmental factors including westernized food patterns, obesity, and sedentary lifestyle. This will be discussed in more detail below.

a) Heritability

Extensive evidence supports the principle of inherited genetic susceptibility as an important risk factor for the development of T2D. Importantly, different studies propose ranging contributions of heritability to the pathogenesis of T2D between 25% and 80%, evidencing that there is still a lot of research to be conducted regarding this aspect of the disease (Prasad *et al.* 2015). In any case, it has been seen that the method of genetic transmission of these risk alleles does not follow simple Mendelian patterns (Murea *et al*. 2012). Moreover, certain ethnic groups have a higher risk of developing T2D, independent of metabolic risk factors profiles. These include Hispanics, African Americans, Pacific Islanders, and American Indians. Possible reasons for ethnic differences in susceptibility to T2D could be either shared or unique cultural, environmental and genetic effects (Murea *et al*. 2012).

While the major environmental factors are well known, identification of the genetic factors has been a challenge. However, recent years have seen an explosion in the identification of genetic variants in risk and protection of T2D, thanks to the development of throughput technology that has allowed for genome-wide association studies (GWAS) and next-generation sequencing (NGS) (Prasad *et al.* 2015). While each identified variant explains only a very small proportion of the risk of T2D in the human population, they have greatly contributed to our understanding of disease pathogenesis (Prasad *et al.* 2015). Most of the T2D risk alleles identified correspond to genes that have an impact on glucose stimulated insulin secretion, including adenylate cyclase 5 (*ADCY5*), forkhead box O1 (*FOXO1*), glucokinase (*GCK*), insulin like growth factor 2 mRNA binding protein 2 (*IGF2BP2*) and serum/glucocorticoid regulated kinase 1 (*SGK1*), amongst others (Imamura *et al.* 2011). By 2011, GWAS had identified nearly 52 common risk variants that associate with T2D (Wheeler *et al.* 2011). Importantly, in the following years, advances in technology has allowed this number to increase to 153 risk variants, associated to more

than 120 loci (Prasad *et al.* 2015). Together, these genes comprise only 10% of the estimated heritability in T2D, suggesting that substantially larger association studies are needed to identify most T2D loci in the population. Consequently, the concept of "missing heritability" emerged along with novel hypotheses for the presence of other genetic determinants such as common copy number variations, or the interplay of different factors (epistasis, gene-environment interaction), to account for the unexplained heritability (Eichler *et al*. 2010).

Two trends have recently emerged from the study of T2D genetics. The first one evidences that T2D genetic architecture is likely polygenic and characterized by many loci detectable only in hundreds of thousands of samples, arguing that much larger collections of genetic data will be necessary to discover disease-relevant variants in the population. The second trend states that the increasing number of genes and/or processes linked to T2D will only increase the diversity of approaches necessary to translate these associations to mechanistic insight, although common resources and workflows have begun to emerge (Flannick *et al.* 2016).

More recently, GWAS were used to identify a genetic risk score that establishes the strongest association with T2D status in a population-based cohort, revealing that the genetic risk score had the potential to improve the accuracy of T2D risk prediction when added to alternative set of predictors (Lall *et al.* 2016).

b) Environment

Sedentary lifestyles and high-fat diets are behavioural factors that contribute to the high prevalence of T2D. The incidence of obesity among children has increased dramatically in recent decades, with about one-third of children in the USA currently being either overweight or obese (Pulgaron *et al.* 2014). Research has shown that youth who do not meet guidelines for dietary behaviour and physical activity have greater IR than those who do meet the guidelines (Huang *et al.* 2011). Moreover, it has been shown that lifestyle interventions (dietary modification, weight loss and exercise) in overweight adults with impaired glucose tolerance achieved 58% decline in the incidence of T2D (Tuomilehto *et al.* 2001).

1.2.2 Comorbidities of T2D

T2D is a leading cause of severe morbidities and disabilities worldwide, including blindness, chronic renal impairment, CVD and lower limb amputation. A study by Jelinek *et al.* reveals that at least one diabetic complication was present in over 80% of the investigated diabetic cohort, and that two or more complications were present in 50% of the studied population, with diabetic retinopathy being the most common single complication, followed by CVD (Jelinek *et al*. 2017).

a) Diabetic retinopathy (DR)

Diabetic retinopathy is one of the major microvascular complications of diabetes. It is currently the leading cause of blindness among adults in the western world, especially in patients who have had diabetes for a longer time (Chang *et al*. 2013). DR is characterised by the appearance of vascular and retinal lesions caused by chronic exposure to high blood glucose concentrations. Furthermore, it is well known that dyslipidemia is a major risk factor for the development of DR. For instance, van Leiden *et al.* reported that total levels of cholesterol and triglycerides, as well as body mass index (BMI), contribute to the development of DR regardless of the glycaemic levels (van Leiden *et al.* 2002).

b) Cardiovascular disease (CVD)

Another common complication associated with T2D is CVD, which constitutes the main cause of death and disability among T2D patients: about two-thirds of deaths in people with diabetes are due to CVD, of which approximately 40% are from ischemic heart disease, 15% from congestive heart failure, and about 10% from stroke (Low Wang *et al*. 2016). T2D patients typically have higher atherosclerotic plaque formation, higher atheroma volume and smaller coronary artery lumen diameter than non-diabetic individuals (Nicholls *et al*. 2008). There is also abundant epidemiologic data that supports an association between hyperglycaemia and increased cardiovascular risk (Elley *et al*. 2008, Eeg-Olofsson *et al*. 2010), by which hyperglycaemia attenuates endothelial function and lowers nitric oxide (NO) bioavailability (Williams *et al.* 1998) while increasing endothelial cell leukocyte adhesion (Perkins *et al.* 2015) mediated in part by increased oxidative stress and inflammation. IR has also been associated with increased cardiovascular risk (Ferrannini *et al.* 2007). People with IR have higher rates of hypertension, dyslipidemia and impaired glucose tolerance (Eddy *et al*. 2009), which contribute to development, progression, and complexity of atherosclerosis.

c) Diabetic nephropathy (DN)

DN, characterised by the presence of protein in urine (proteinuria), is one of the most serious long-term microvascular complications of T2D. It occurs in 20% to 40% of all diabetics (Gheith *et al.* 2016) and is responsible for a significant decline in their life expectancy (Ritz *et al.* 1999). DN is the leading cause of chronic kidney disease and end-stage renal disease, which constitutes the major workload of dialysis centres worldwide (Aldukhayel 2017).

d) Diabetic foot ulceration and limb amputation

Diabetic foot ulceration is a serious complication of T2D worldwide and the most common cause of hospitalization in diabetic patients (Volmer-Thole *et al*. 2016). The feet of diabetic patients are susceptible of developing a broad spectrum of clinical conditions, collectively known as diabetic foot syndrome (Frykberg *et al.* 2006), resulting from the combination of several diabetes-related complications. Feet deformity secondary to motor neuropathy, together with loss of protective sensation and impaired vision from diabetic retinopathy , increase the risk for minor feet trauma, leading to the appearance of diabetic foot ulcers (Reiber *et al*. 1999). Furthermore, impaired immunity related to chronic hyperglycaemia and subsequent infections will result in septic diabetic foot (Nather *et al.* 2008), which constitute over 90% of nontraumatic lower limb amputations (Tiwari *et al.* 2012).

e) Other complications

Other comorbidities of T2D also related to high blood glucose levels include slow wound healing (cuts and blisters can become serious infections if left untreated), hearing impairment, skin conditions (bacterial and fungal infections), sleep apnea (which can result in higher blood pressure), hypertension, Alzheimer's disease and diabetic neuropathy, which can also lead to impotence (Mayo Clinic).

1.2.3 Prevention of T2D

T2D incidence is increasing worldwide, driven by a rapidly changing environment and lifestyle, leading to increasing rates of overweight and obesity. In the fight against diabetes, prevention is key and must be a central focus for health policy and government

action, where programs based on lifestyle modification, mainly focusing on diet and physical activity, should provide a cost-effective opportunity to target overweight, high-risk individuals (Tuomilehto *et al.* 2001).

Prevention of weight gain and/or successful long-term weight loss maintenance seems the easiest way to prevent development of T2D (Liu *et al*. 2015). Three successful long-term intervention programs, the US Diabetes Prevention Program, the Finnish Diabetes Prevention Study, and the Chinese Da Qing Study, support the use of low-fat- and low-carbohydrate-based diets for successful weight loss maintenance and for T2D prevention (Liu *et al*. 2015). However, in recent years, some, mostly developed, countries are focusing on novel dietary approaches based on the recognition that macronutrient quality and source, together with avoidance of processed foods (particularly processed starches and sugars), are more important considerations (Franz *et al*. 2017).

The importance of nutrition in the management and prevention of T2D is clear. However, nutrition is also one of the most controversial and difficult aspects to manage. Firstly, diabetic patients' adherence is at stake, as it is a psychologic challenge to be "on a diet" for life. These patients might be consistent for the initial stages but struggle to maintain a healthy diet for longer periods. Secondly, most physicians are not trained in nutrition interventions, constituting a barrier to counselling patients (Kahan *et al.* 2017). Although progress has been made in understanding the best dietary advice for diabetes, broader problems exist. For instance, most dietary guidelines recommend increasing vegetable and fruit intake and decreasing consumption of highly processed foods, but the high cost of the former and the lower cost of the latter makes it hard for people to choose the healthy option in many situations.

The other point of focus in the prevention and management of T2D is physical activity. Abundant evidence shows that physical activity improves glycaemic control and decreases the risk of CVD and mortality in patients with T2D by increasing insulin sensitivity and glucose uptake in tissues like skeletal muscle (Hamasaki 2016, Short 2013). With regards to its prevention, the US Diabetes Prevention Program randomly assigned a cohort of 3234 individuals with impaired glucose tolerance to one of three interventions: standard lifestyle recommendations plus metformin treatment, standard lifestyle recommendations plus placebo, or an intensive program of lifestyle modification involving walking and moderate physical activity. Results indicated that the intensive

program of lifestyle intervention was the most effective program, decreasing the incidence of diabetes by 58% after a 2.8-year follow-up (Knowler *et al*. 2002).

1.2.4 Phases and diagnosis of T2D

T2D has a slow onset, progressing from an early asymptomatic stage with IR, to mild postprandial hyperglycaemia, to T2D requiring pharmacological intervention. Understanding the progression of diabetes is essential for effective and specific diagnosis and subsequent treatment for each stage of the disease.

a) Phases

Pre-diabetes, defined as a stage in which blood glucose concentrations are higher than normal but lower than the diabetes threshold, represents a high-risk state for diabetes and CVD development (American Diabetes Association 2017). It comprises three groups of individuals, constituting three different diagnostic criteria: Those with impaired fasting glucose (100–125 mg/dL), those with impaired glucose tolerance (140–199 mg/dL at 2 hours postprandial) and those with elevated levels of glycated haemoglobin (HbA1c) (39-46 mmol/mol) (Di Pino *et al*. 2016, Wu *et al.* 2017). Subjects with pre-diabetes have shown a high conversion rate to T2D (Knowler *et al*. 2002). Diagnosis of pre-diabetes and early intervention are therefore essential to prevent or delay the development of the disease and its complications.

Several studies support the observation that pre-diabetes is a risk factor for diabetes (Morris *et al*. 2013), CVD (Huang *et al.* 2016) and even cancer (Huang *et al.* 2014). However, little is known about the risk factors that trigger transition from pre-diabetes to T2D. A recent study by Wu *et al.* shows that critical factors determining this transition include poor metabolic health and increased BMI (Wu *et al.* 2017). There is extensive evidence, however, that shows that this transition comes accompanied by progressive loss of β-cell mass and function (Seino *et al.* 2010), and consequently impaired insulin secretion together with insulin deficiency with or without IR (Babu *et al.* 2006).

The presence of IR is considered to be an early primary defect in the progression towards T2D. It is characterised by an insufficient response of different tissues to a given concentration of insulin and by the consequent inability to induce glucose uptake and utilisation in these tissues (Kim *et al.* 2008).

b) Diagnosis

Historically, the diagnosis of diabetes was based on fasting blood glucose levels of higher than 7mmol/L, any blood glucose levels above 11.1mmol/L, or an abnormal glucose test. However, this has now been modified to include levels of glycated haemoglobin (HbA1c) (Seino *et al*. 2010). According to guidelines outlined by the WHO, levels of HbA1c below 42mmol/L indicate that a person is non-diabetic; levels between 42-47mmol/L indicate pre-diabetes, and levels higher than 48mmol/L indicate presence of T2D.

However, these traditional markers of glucose homeostasis may not be conclusive, and their use may be biased by several clinical and analytical factors. Moreover, not all subjects with pre-diabetes develop T2D, and conversely, a significant number of patients progress to T2D without going through prediabetes (Unwin *et al*. 2002). For these reasons, there is growing interest in new serum biomarkers of hyperglycemia to be used as alternatives or in conjunction with traditional measures to identifying subjects at risk for T2D and CVD (Di Pino *et al.* 2016). The most successful candidate so far is the monitoring of one-hour post-load glycaemia, which also provides physiopathological information, since it presents a strong correlation with markers of β-cell function, insulin secretion and insulin sensitivity (Bianchi *et al*. 2013).

1.2.5 Physiology of the pancreas and pancreatic β-cell homeostasis

The pancreas is a secretory organ, located at the rear of the abdomen, and is divided into head, body and tail (Röder *et al*. 2016). This organ is unique in that it possesses both endocrine and exocrine functions: it is made up of 95% exocrine tissue and is therefore considered to be an exocrine gland, but it is also made up of 5% endocrine tissue (Das *et al.* 2014).

Exocrine cells secrete pancreatic juice containing digestive enzymes (mainly amylase, pancreatic lipase and trypsinogen) into the pancreatic duct, which connects the pancreas to the duodenum. In contrast, pancreatic hormones (mainly insulin and glucagon) are released in an endocrine manner, directly into the bloodstream. The endocrine tissue component is made up of several types of cells, which are clustered together forming micro-organs known as islets of Langerhans. These islets are distributed throughout the pancreas, making up 2-3% of the total pancreatic mass (Striegel *et al*. 2015). They consist

of around 30% glucagon-producing α-cells and 60% insulin-producing β-cells. The remaining 10% is made up of δ-cells (somatostatin-producing), γ- or PP cells (pancreatic polypeptide-producing), and ε-cells (ghrelin-producing) (Ionescu-Tirgoviste *et al*. 2015).

Both insulin and glucagon play pivotal roles in maintaining glucose homeostasis. Together, these hormones tightly regulate blood glucose concentrations to avoid prolonged hyper- or hypoglycaemia. In order to do this, insulin is secreted by β-cells when blood glucose levels are high, in that way inhibiting glycogenolysis (the breakdown of glycogen into glucose), gluconeogenesis *(de novo* hepatic glucose synthesis), lipolysis (the breakdown of lipids into glycerol and fatty acids -FAs-) and ketogenesis (the breakdown of FAs and ketogenic amino acids like Leucine and Lysine into ketone bodies). On the other hand, when blood glucose levels are low, glucagon is secreted by α-cells, thereby stimulating those same processes (Robertson *et al*. 2007).

This tight control is regulated by the balance between glucose absorption from the intestine, production by the liver and uptake and metabolism by peripheral tissues such as muscle and adipose tissue (AT). A dysregulation of this tight mechanism leads to pathologic conditions, in particular to DM (Kahn *et al.* 2006)

In adults in normal physiological conditions, β-cells have an estimated life-span of approximately 60 days, after which they undergo programmed cell death (apoptosis) (Bonner-Weir 2000). These β-cells are replaced through 2 different processes: replication (proliferation) of existing β-cells, and neogenesis of new β-cells derived from progenitor cells that bud from the ducts of the exocrine pancreas. This balance is crucial for the maintenance of glucose homeostasis, as the ability of the pancreas to produce enough insulin to meet the body's requirements depends on the presence of the appropriate amount of β-cells (Sone *et al*. 2005).

β-cell replication and survival is regulated by a combination of factors, including nutrients (especially glucose and free fatty acids -FFAs-) (Oh 2015), insulin and other growth factors such as insulin growth factor 1 (IGF1), incretin hormones such as glucagon-like peptide 1 (GLP1) and transcription factors such as pancreatic duodenal homeobox1 (PDX1) (Prasadan *et al*. 2016). The role of each of these factors on the fate of β-cells is described below.

a) Nutrients

Glucose and FFAs are two main nutrients in energy metabolism and are of particular interest in β-cell equilibrium. Controlled increased levels of these nutrients have been seen to increase β-cell viability and function. However, chronic hyperglycaemia and hyperlipidemia will induce glucotoxicity and lipotoxicity respectively (detailed in section 1.3), impairing glucose and lipid metabolism and leading to β-cell damage (Halban *et al*. 2014).

i. Glucose

Both *in vivo* and *in vitro* studies have shown that controlled levels of glucose stimulate β-cell proliferation: mice and young rats subjected to glucose infusion for a short time (24-48 hours) exhibited increased β-cell mass (Alonso *et al*. 2007, Assefa *et al.* 2014) due mainly to the rapid activation of neogenesis of new endocrine cells and suppression of β-cell apoptosis (Bernard *et al.* 1999). The same effect was seen in the rat insulinoma pancreatic β-cell line INS-1 (Hügl *et al.* 1998), and in human islets (Maedler *et al.* 2006).

The mitogenic effect of glucose is associated with its ability to induce insulin secretion. Insulin is a strong proliferation inducer in some cell types, including pancreatic β-cells (Movassat *et al*. 1997). *In vitro*, glucose induces cell survival and proliferation through modulation of intracellular signalling molecules of the insulin signalling pathway, including insulin receptor substrate 2 (IRS2), phosphatidylinositol 3 kinase (PI3K), protein kinase B (PKB), glycogen synthase kinase (GSK3), extracellular signalling related kinase (ERK1/2), and mammalian target of rapamycin (mTOR) (Figure 1.1) (Srinivasan *et al.* 2002, Demozay *et al*. 2011, Oh 2015). Another mechanism responsible for the survival and proliferation of β-cells is the glucose dependent β-cell membrane depolarisation and subsequent Ca^{2+} influx: increased glucose uptake results in increased ATP production from glycolysis, shifting the ATP/ADP ratio. This causes the ATP-sensitive K^{+} channels to close, depolarising the membrane and causing the Ca^{2+} channels to open, resulting in increased Ca^{2+} influx from the cytosol. This increase in intracellular Ca^{2+} levels induces activation of calcineurine, a calcium-dependent phosphatase, which interacts with nuclear factor of activated T-cells (NFAT), promoting the expression of cell-cycle regulators and increasing β-cell proliferation (Figure 1.1). Accordingly, mice with a β-cell specific deletion of the calcineurine phosphatase regulatory subunit, calcineurin b1 (*Cnb1*) developed age-dependent diabetes, characterised by decreased β-cell proliferation and mass, diminished pancreatic insulin content and hypoinsulinemia (Heit *et al*. 2006).

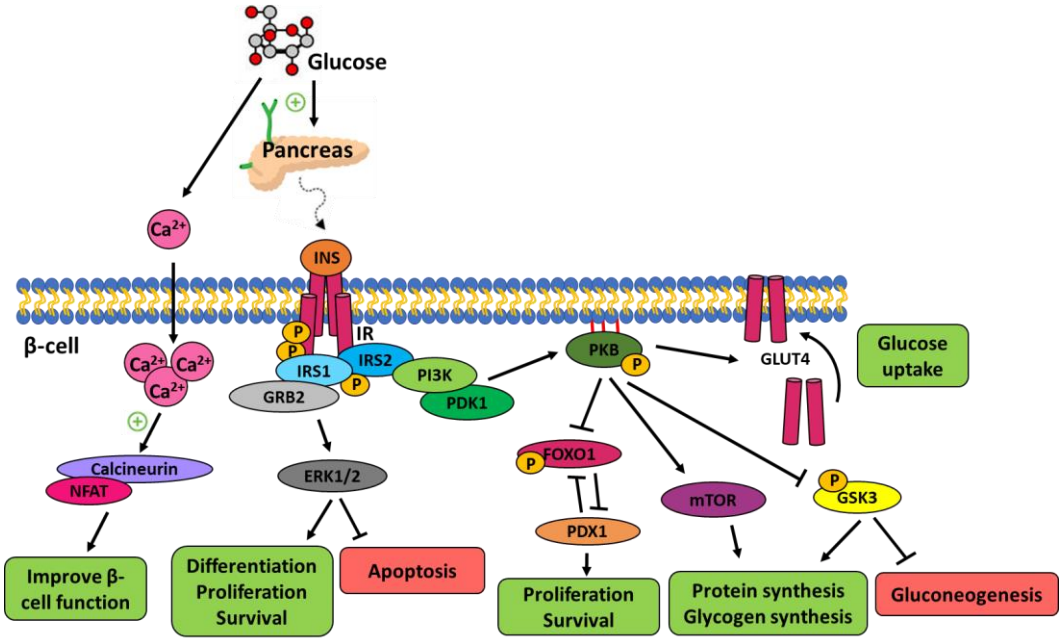


Figure 1.1: Mitogenic effect of glucose. Glucose-induced plasma membrane depolarisation and subsequent Ca^{2+} influx induces activation of calcineurin, which interacts with NFAT and promotes expression of cell cycle regulators. Glucose also induces insulin secretion from the pancreas, which acts on insulin receptors on target tissues, causing autophosphorylation of the insulin receptor, which serves as docking sites for IRS1 and IRS2, which get phosphorylated. These serve as docking sites for GRB2, which activates ERK1/2 cascade leading to cell differentiation, proliferation and survival. IRS1 and IRS2 also serve as docking site for PI3K and its adaptor, PDK1. They recruit PKB to the membrane and phosphorylate it. Activation of PKB induces phosphorylation and inactivation of FOXO1, thereby activating PDX1 and cell proliferation. PKB also induces mTOR-mediated protein synthesis, as well as phosphorylation and inactivation of GSK3, thereby promoting glycogen synthesis. Finally, activated PKB also induces GLUT4 transporter translocation to the plasma membrane, allowing glucose uptake (Boucher *et al.* 2014). Abbreviations: ERK1/2 extracellular signalling- related kinase 1/2; FOXO1 forkhead box protein O1; GLUT4 glucose transporter 4; GSK3 glycogen synthase 3; GRB2 growth factor receptor-bound protein 2; INS insulin; IR insulin receptor; IRS1, IRS2 insulin receptor substrate 1, 2; mTOR mammalian target of rapamycin; NFAT nuclear factor of activated T-cells; PDK1 protein kinase 3-phosphoinositide dependent protein kinase-1; PDX1 pancreatic duodenal homeobox 1; PI3K phosphatidylinositol 3-kinase; PKB protein kinase B.

ii. Free fatty acids

FFAs also play an important role on β-cell mass regulation. Acute exposure to FFAs stimulates insulin secretion and β-cell proliferation, while prolonged exposure decreases

glucose-stimulated insulin secretion and induces IR and β-cell dysfunction in both animal human models (Sharma *et al*. 2014).

Increased enteric nutrient supply, especially in the form of fats, induces β-cell mass proliferation through two processes. The first one is by modulating insulin release through FA metabolism (Röder *et al.* 2016), and the second one is through increased production of incretin hormone GLP1 (Perfetti *et al.* 2000).

The binding of long-chain FFAs such as oleic acid (18:1 cis-9, OA) to the G-protein-coupled free fatty acid receptor 1 (FFAR1) in β-cells leads to the activation of phospholipase C (PLC). PLC then hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). IP3 then docks on a calcium channel in the endoplasmic reticulum (ER), inducing the release and subsequent increase of Ca^{2+} concentration in the cytosol. This eventually triggers insulin secretion, which then acts as a mitogenic agent, increasing β-cell proliferation and survival, as well as β-cell function, ultimately increasing β-cell mass as described in Figure 1.1 (Itoh *et al*. 2003, Fujiwara *et al.* 2005) (Figure 1.2).

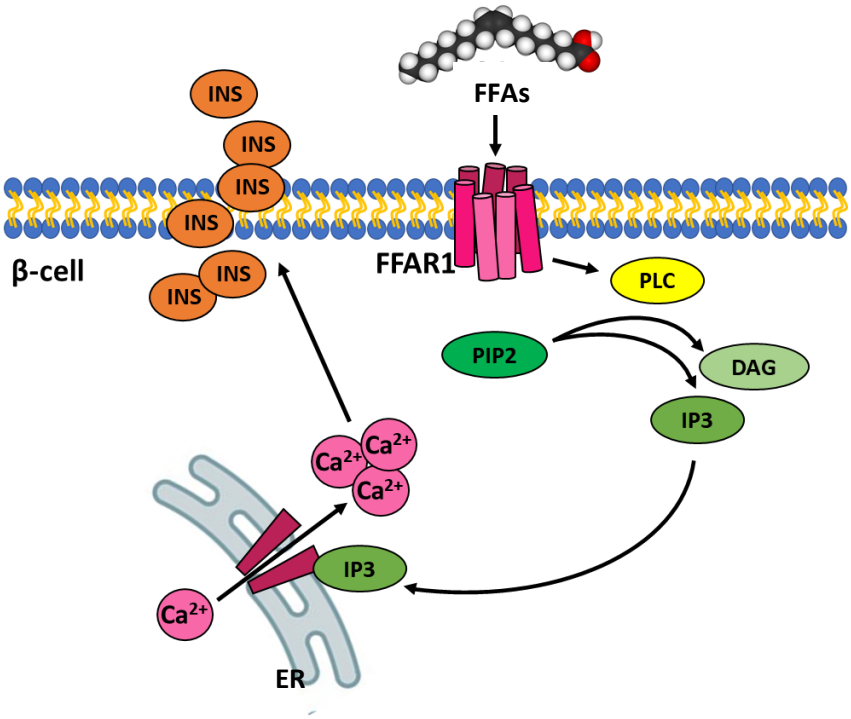


Figure 1.2: Effect of FFAs on β-cell mass regulation. OA binds to FFAR-1 in β-cells, leading to the activation of PLC, which hydrolyses PIP2 into DAG and IP3. IP3 then docks on a calcium channel in the ER and induces release and subsequent increase of Ca^{2+} concentration in the cytosol, which triggers insulin secretion from β-cells. Abbreviations: DAG diacylglycerol; ER endoplasmic

reticulum; FFAs free fatty acids; FFAR1 free fatty acid receptor 1; INS insulin; IP3 inositol-1,4,5-triphosphate; PIP2 phosphatidylinositol-4,5-bisphosphate; PLC phospholipase C.

b) Incretin hormones

GLP1 is an incretin hormone secreted by the intestine after glucose and FA ingestion, which activates several processes in the pancreas, including glucose-stimulated insulin secretion and β-cell growth and survival. It mediates activation of the pancreas-duodenum homeobox-1 *PDX1* gene, which encodes a transcription factor whose expression is essential for the regulation of genes associated with pancreatic cell differentiation and maturation (Li *et al.* 2005). A study reveals that *PDX1+/−* mice exhibit progressive glucose intolerance in association with defective glucose-stimulated insulin secretion (Brissova *et al.* 2002), whereas restoration of *PDX1* expression in experimental models of murine diabetes enhances β-cell function and expands β-cell mass (Kushner *et al*. 2002). Apart from inducing *PDX1* expression, GLP1 also mediates β-cell proliferation through transactivation of the epidermal growth factor receptor (EGFR) and subsequent activation of the PI3K signalling pathway (Buteau *et al*. 2003).

c) Insulin and growth factors

Insulin and insulin-like growth factors 1 and 2 (IGF1 and IGF2) also play an important role in the regulation of β-cell homeostasis. IGF1 binds to IGF1 receptor, stimulating its intrinsic tyrosine kinase activity, which in turn phosphorylates members of the IRS family. Upon phosphorylation, IRS1 and IRS2 activate the PI3K/PKB signalling pathway (Figure 1.1), subsequently activating expression of *PDX1* (Fujimoto *et al*. 2009). An *in vitro* study using mouse insulinoma MIN-6 cells shows that insulin receptor knockdown leads to decreased β-cell proliferation (Ohsugi *et al*. 2005). Furthermore, an *in vivo* study shows that mice with a specific deletion of the β-cell insulin receptor exhibit a progressively impaired glucose tolerance and diminished β-cell mass (Otani *et al.* 2003). Moreover, animal models deficient in *IRS2* develop T2D because of failed β-cell compensation (Withers *et al*. 1998). Conversely, it has been seen that increased *IRS2* expression promotes β-cell replication and prevents diabetes in mice (Hennige *et al*. 2003). These results suggest that IRS2 is essential for β-cell mass growth and β-cell compensation in order to meet the increased insulin demand that arises in the face of peripheral IR.

d) Transcription factors and other hormones

β-cell commitment involves expression of a relatively well-defined hierarchy of transcription factors. As mentioned above, PDX1 plays a crucial role for β-cell function, regulating the early development and commitment of certain progenitor cells in the foregut to form a pancreatic bud (Gu *et al.* 2002). Other transcription factors involved in maintaining β-cell mass and function include neurogenin 3 (NGN3), neuronal differentiation factor 1 (NEUROD), paired box 4 (PAX4), NK homeoboxes (NKX2.2, NKX6.1, NKX6.2) and MAF BZIP transcription factors (MAFB, MAFA), amongst others, each affecting downstream developmental and functional aspects of the endocrine pancreas (Prasadan *et al*. 2016). For instance, lack of *NGN3* in mice during development leads to the formation of a pancreas without an endocrine component, causing embryos to die soon after birth (Jenny *et al*. 2002). Disruption of *NEUROD* in mice caused perinatal lethality and impaired islet formation and maturation with a significant decline in β-cell mass (Naya *et al*. 1997). Loss of *PAX4* expression in mice results in a lack of pancreatic β- and δ-cell development, causing death soon after birth due to diabetes (Brink *et al.* 2001). Many other transcription factors regulate the expansion and maturation of β-cells specifically. Sussel *et al.* reported that *NKX2.2* mutant mice failed to develop β-cells and died soon after birth due to severe hyperglycaemia (Sussel *et al.* 1998), whereas it was also reported that lack of *NKX6.1* led to decreased β-cell neogenesis from progenitor cells in the embryo, as well as to poor expansion of mature β-cells is adults (Sander *et al.* 2000). The MAF family proteins MAFA and MAFB also play a central role in later development and maturation of endocrine cells (Abdellatif *et al*. 2015). In the embryonic pancreas, a significant number of insulin-positive cells express *MAFB*, and as part of the β-cell maturation process, these cells transition from *MAFB+/MAFA−/INS+* intermediate cells to *MAFB−/MAFA+/INS+* mature β-cells, correlating with a high level of *PDX1* expression (Nishimura *et al*. 2009).

Other growth factors have been shown to regulate β-cell growth, survival, differentiation, and insulin secretion, including growth hormone (GH), prolactin (PRL), and IGF1, together with their receptors (GHR, PRLR, and IGF1R respectively) (Vasavada *et al*. 2006, Huang *et al.* 2014). Binding of GH and PRL to their receptor triggers Janus kinase 2 (JAK2) activation, resulting in tyrosine phosphorylation of the receptor and subsequent activation of several intracellular signalling pathways, including signal transducers and activators of transcription (STATs), Ras/ERK, and PI3K/PKB (Waters *et al*. 2015). This leads to activation of cyclin 2, which is essential for β-cell proliferation (Friedrichsen *et al*. 2003).

1.2.6 Pancreatic β-cell compensation, dysfunction and death

Total plasma insulin levels depend on insulin production and secretion by β-cells, which in turn depends on the total number of β-cells (β-cell mass) and the output of each of these cells (β-cell function) (Chen *et al.* 2017).

Over the development of T2D, chronically elevated levels of blood glucose and FAs lead to IR. In order to overcome it, β-cells need to increase insulin secretion in a compensatory manner, and this is achieved by an increase of both β-cell mass and function (Kahn *et al*. 1993). Glucose is considered to be the main driving force of increased β-cell mass during β-cell compensation. It has been seen that hyperglycaemia leads to increased glycolysis in both isolated rat islets and pancreatic INS-1 cells, and subsequent upregulation of *Irs2* expression as a result of increased cytosolic Ca^{2+} levels (Lingohr *et al.* 2006). Increased IRS2 protein levels and activity triggers a signalling cascade that enhances β-cell replication and survival via PI3K/PKB activation (Weir *et al.* 2007).

Importantly, obesity plays a very significant role on the progression of the diabetic state: it has been shown that obese diabetic patients exhibit a higher degree of IR and compensatory hyperinsulinemia that lean diabetic patients (Kahn *et al*. 2006). In fact, several studies have reported that β-cell mass is generally increased in overweight or obese non-diabetic rodent (Flier *et al.* 2001) and human (Hanley *et al.* 2010) subjects. However, only a small fraction of obese individuals with IR progresses to T2D (LeRoith 2002), indicating that the determining factor for the development of T2D is not the presence of IR, but β-cell dysfunction (Ashcroft *et al.* 2012).

β-cell compensation is able to keep glucose levels normalised for a period of time (Kim *et al*. 2008). However, this increased workload puts a lot of stress on β-cells, which will start to deteriorate gradually, involving loss of both β-cell mass and function (Hanley *et al*. 2010, Inaishi *et al*. 2016) (Figure 1.3). β-cell dysfunction will lead to impaired glucose tolerance, which is reflected by the increase in fasting blood glucose in an asymptomatic but still potentially pathologic stage characterised by mild hyperglycaemia. The progression from this stage to early T2D is marked by a gradual decline in insulin secretion secondary to β-cell dysfunction (Fonseca 2009). It is only when β-cell function begins to fail, and insulin secretion decreases, that patients show levels of hyperglycaemia high enough for T2D diagnosis (Yagihashi 2012).

Currently, most clinical treatments of T2D are aimed to elevate insulin levels by increasing β-cell function. In the light of a potential exhaustive effect on β-cells, more basic research turns to study mechanisms to regenerate β-cell mass or to preserve β-cell function rather than increase it (Chen *et al.* 2017).

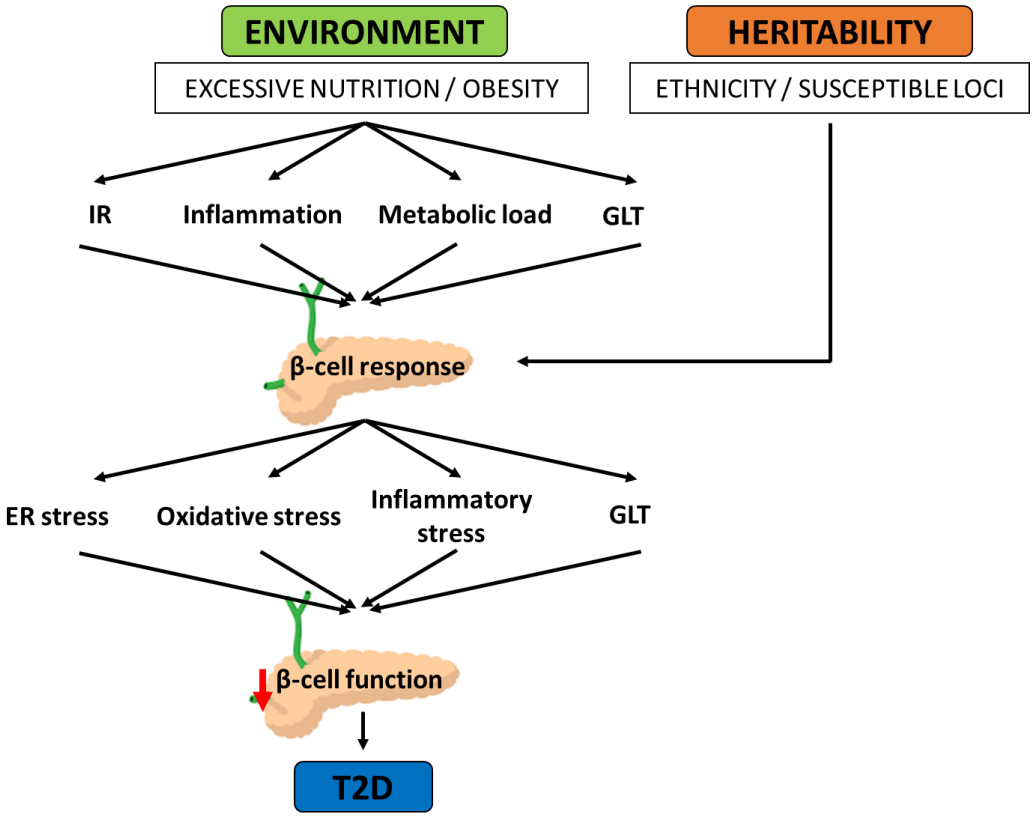


Figure 1.3: Stressors of the pancreatic β-cell. In the excessive nutritional state characteristic of obesity, hyperinsulinemia arises, followed by IR and subsequent hyperglycaemia and hyperlipidemia. This increases metabolic load and induces chronic inflammation. The pancreatic islet response includes ER stress, oxidative stress, inflammatory stress and metabolic stress (glucolipotoxicity), leading to loss of islet cell integrity. If untreated, these stressors increase with time, promoting β-cell dysfunction and ultimately loss of β-cell mass, which marks the onset of T2D. Abbreviations: ER endoplasmic reticulum; GLT glucolipotoxicity; IR insulin resistance; T2D type 2 diabetes.

β-cell compensation puts an excessive stress on β-cells, leading to failure in metabolism regulation and causing chronic exposure of β-cells to increased levels of glucose, FFAs and islet amyloid polypeptide (IAPP), as well as cytokines such as interleukin 1 beta (IL1β), as described below. This results in oxidative stress or ER stress induction in the β-cell, which

leads to the activation of signalling pathways of cell death, resulting in the eventual decline of β-cell mass (Ma *et al.* 2012) (Figure 1.3).

Accordantly, progressive decline of β-cell mass through apoptosis is a pathological hallmark of T2D. In fact, the extent of β-cell mass deficit can be used to determine the clinical staging of diabetes (Seino *et al*. 2010): even before the diagnosis of diabetes, more than 50% of β-cells have already disappeared in patients with impaired fasting glucose. In the case of overt diabetes, more than 70% of β-cells appear to be diminished (Butler *et al.* 2003).

There are many types of cell death. Some of the ones that that modulate survival of pancreatic β-cells are apoptosis, autophagy and pyroptosis (Scarlatti *et al.* 2009, Rojas *et al*. 2018). These are described below.

a) Apoptosis

The major cause of β-cell mass loss in models of diabetes is most likely a dramatically increased rate of β-cell apoptosis (Butler *et al*. 2007, Marchetti *et al*. 2007). As a consequence of β-cell dysfunction, diminished insulin signalling through the IGF pathway appears to be an important common mechanism leading to decreased *PDX1* expression, a common feature in states of β-cell failure (Chang-Chen *et al.* 2008). As described in the previous section (1.2.5), insulin/IGF signalling is an important regulator of β-cell proliferation through activation of *PDX1* expression via the IRS1/IRS2-PI3KPKB pathway (Fujimoto *et al.* 2009) (Figure 1.4A). Proof of this is that deletion of the β-cell insulin receptor in mouse insulinoma MIN6 cells causes a decline in β-cell mass (Ohsugi *et al.* 2005), while *in vivo* disruption of *Irs2* in mice leads to β-cell failure due to decreased proliferation and an increased rate of apoptosis (Kubota *et al*. 2000).

Chronic exposure to high glucose levels can also lead to β-cell apoptosis in a process termed glucotoxicity (Jonas *et al*. 2009), which induces oxidative stress, ER stress, and an inflammatory reaction in the β-cell (this will be described in detail in section 1.3.1). Chronically elevated glucose levels induce β-cell apoptosis through many mechanisms. One of them is through the upregulation of the FAS receptor (FASR), also known as apoptosis antigen 1 (APO1), or tumour necrosis factor receptor superfamily member 6 (TNFRSF6). This is a death receptor, which induces caspase-8 and -3 activation and subsequent β-cell apoptosis (Maedler *et al.* 2001) (Figure 1.4B). A study by Kim *et al.* in MIN6 mouse pancreatic β-cells shows that exposure to chronic high glucose also induces β-cell apoptosis through decreased glucokinase (GCK) expression and therefore

decreased interaction with the mitochondria, which leads to increased BAX oligomerization, cytochrome C release, and caspase-3 activation (Kim *et al.* 2005). Another study using rat pancreatic INS-1 cells reported an increase in glucose-induced thioredoxin-interacting protein (TXNIP), a proapoptotic β-cell factor, which came accompanied by an increase in caspase-3 expression and activity (Chen *et al*. 2008).

Another pathological hallmark of T2D is islet amyloid deposition, which has been linked to β-cell loss and apoptosis (Jurgens *et al*. 2011). IAPP is a regulatory peptide which is co-secreted with insulin by the β-cell (Kahn *et al*. 1990). It inhibits glucagon secretion and has a dual role on insulin, inhibiting excessive stimulated insulin secretion (negative feedback), as well as inducing basal insulin secretion (positive feedback). It has the ability to aggregate into pancreatic islet amyloid deposits, which are seen particularly in association with T2D patients (Westermark *et al.* 2011). Studies have demonstrated that β-cell death is due to formation of IAPP aggregates, which form toxic oligomers capable of altering the structural and functional stability of the cellular membranes, resulting in β-cell apoptosis (Lorenzo *et al.* 1994, Abedini *et al*. 2013). Furthermore, *in vivo* studies of IAPP deposition in transgenic rodent models have shown that this accumulation precedes fasting hyperglycaemia and is associated with decreased β-cell function and mass (Udayasankar *et al.* 2009). There is also evidence that IAPP induces β-cell death through ER stress. As mentioned, IAPP is co-secreted with insulin. Therefore, when β-cells are overcompensating for IR, hyperinsulinemia will come accompanied by increased levels of IAPP (Martel *et al.* 2017). Eventually, increased protein expression leads to the accumulation of unfolded proteins inside the ER, and consequently to ER stress (Huang *et al.* 2007). Chronic ER stress then activates three ER stress sensors, namely inositol requiring enzyme 1 (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). Activated IRE1 functions as an endogenous ribonuclease which splices the X-Box protein 1 (XBP1)-coding gene, resulting in a form of XBP1 that drives transcription of genes encoding proteins involved in ER-associated protein degradation (Lee *et al.* 2003). Activated PERK phosphorylates its downstream target protein, the eukaryotic initiation factor 2 (EIF2), resulting in the inhibition of global protein translation (Yoshida *et al.* 2003). Finally, under ER stress, ATF6 translocates to the Golgi where it undergoes cleavage, yielding an active transcription factor, NATF6, which is subsequently translocated to the nucleus and activates ER stress response element related genes such as the CCAAT-enhancer-binding protein homologous protein (CHOP)

transcription factor, which promotes DNA fragmentation, stopping the cell cycle and inducing apoptosis (Westermark *et al*. 2011) (Figure 1.4C).

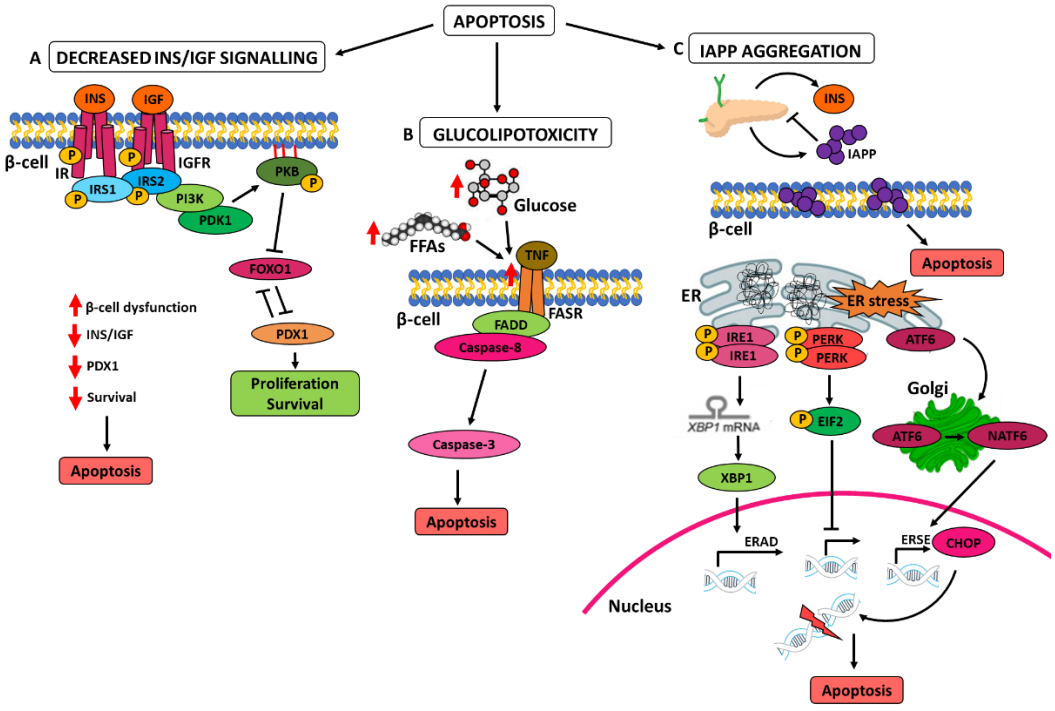


Figure 1.4: Activation of apoptosis in β-cells. A) Increased β-cell dysfunction leads to decreased INS/IGF signalling, resulting in decreased *PDX1* expression via attenuation of the IRS1/IRS2-PI3K-PKB pathway, consequently leading to increased apoptosis. **B**) Increased circulating levels of glucose and FFAs (glucolipotoxicity) upregulate expression of FASR, which upon activation by TNFs recruits FADD and activate caspase-8, which then activates caspase-3, leading to apoptosis. **C)** IAPP, co-secreted with insulin, forms aggregates in β-cells, disrupting the cell membrane and inducing apoptosis. Furthermore, increased protein synthesis leads to ER stress, which induces IRE1, PERK and ATF6 activation. IRE1 activation leads to trascription of a form of XBP1 which induces expression of ERAD; activation of PERK induces phosphorylation of EIF2, resulting in the inhibition of global protein translation; ER stress induces ATF6 translocation to the Golgi, where it is cleaved to NATF6, which translocates to the nucleus and activates transcription of CHOP, an ER stress response element, promoting DNA fragmentation and inducing apoptosis. Abbreviations: ATF6 activating transcription factor 6; CHOP CCAAT-enhancer-binding protein homologous protein EIF2 eukaryotic initiation factor 2; ER endoplasmic reticulum; ERAD endoplasmic reticulum associated protein degradation; ERSE endoplasmic reticulum stress response element; FADD FAS-associated protein with death domain; FASR FAS receptor; FFAs free fatty acids; FOXO1 forkhead box O1; IAPP islet amyloid polypeptide; IGF insulin-like growth factor; IGFR insulin-like growth factor receptor; INS insulin; IR insulin receptor; IRE1 inositol requiring enzyme 1; IRS1/2 insulin receptor substrate 1/2; PDK1 protein kinase 3-phosphoinositide dependent protein kinase-1; PDX1

pancreas-duodenum homeobox-1; PERK protein kinase RNA-like endoplasmic reticulum kinase; PI3K phosphatidylinositol 3 kinase; PKB protein kinase B; TNF tumour necrosis factor; XBP1 X-Box protein 1.

b) Autophagy

Autophagy is a key molecular mechanism for maintaining cellular physiology and promoting cell survival. It balances sources of energy in response to nutrient stress through sequestration of cytoplasmic material such as damaged organelles within a double membrane autophagosome, which then fuses with a lysosome that supplies acid hydrolases that degrade the contents (Parzych *et al.* 2013), while at the same time generating energy in the form of ATP (Glick *et al*. 2010).

Autophagy has generally been considered to be a catabolic, energy generating pathway that allows the cell to adapt to environmental stress (Levine *et al*. 2008). However, more recently this process has been described as a double-edged sword, being able to promote both cell survival and/ or cell death (Yang *et al.* 2017). It is now clear that autophagy is involved in the pathogenesis of several diseases such as cancer or infectious and autoimmune diseases (Levine *et al*. 2008), and its role in the pathogenesis of T2D and β-cell death has also been investigated (Yang *et al.* 2017).

Autophagy is triggered by low cytosolic ATP levels through induction of AMP-activated protein kinase (AMPK), and by hypoxia and ER stress through induction of regulated in development and DNA damage responses 1 (REDD1). Both signals inhibit mTOR through induction of tuberous sclerosis complex (TSC1/2) and subsequent inhibition of RAS homologous enriched in brain (RHEB) GTPase activity, ultimately inducing autophagy. Conversely, autophagy is inhibited by increased insulin/IGF signalling, as well as by other growth factor receptors that activate PI3K and PKB, leading to induction of mTOR activity and cell growth (Glick *et al.* 2010) (Figure 1.5).

mTOR is a key player in nutrient sensing and in regulating cell growth and autophagy. It constitutes a control point downstream of growth factor and insulin signalling, hypoxia and ATP levels. mTOR is activated downstream of growth factor receptor signalling and subsequent activation of PI3K and PKB when nutrients are available, to promote cell growth and inhibit autophagy (Sabatini 2006) (Figure 1.5).

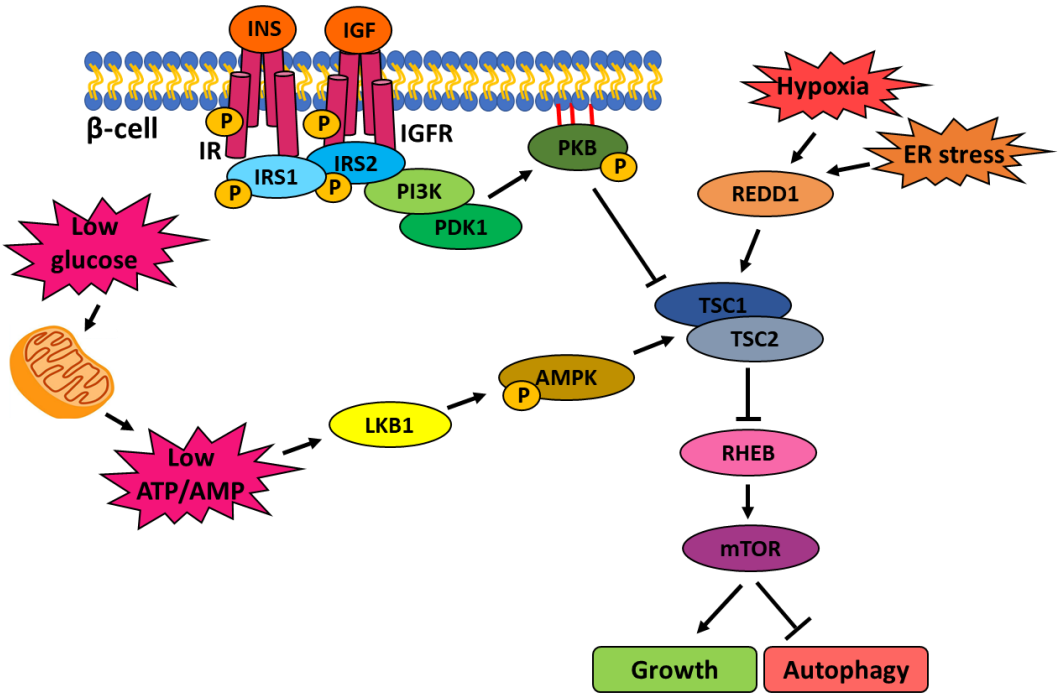


Figure 1.5: Regulation of autophagy in T2D. Low nutrient supply leads to low mitochondrial ATP generation. The low ATP/ADP ratio activates LKB1, which phosphorylates AMPK, inducing AMPK-mediated activation of TSC1/2. Similarly, increased levels of oxidative and ER stress, as well as hypoxia, induce REDD1-mediated activation of TSC1/2. This leads to inhibition of RHEB and consequently to decreased mTOR activation and induction of autophagy. Conversely, INS/IGF signalling activates the PI3K/PKB pathway, which inhibits TSC1/2, thereby inducing mTOR activity and promoting cell growth. Abbreviations: ADT adenosine diphosphate; AMPK adenosine monophosphate-activated protein kinase; ATP adenosine triphosphate; ER endoplasmic reticulum; IGF insulin-like growth factor; IGFR insulin-like growth factor receptor; INS insulin; IR insulin receptor; IRS1/2 insulin receptor substrate 1/2; LKB1 Serin/Threonin kinase 11; mTOR mammalian target of rapamycin; PDK1 protein kinase 3-phosphoinositide dependent protein kinase-1; PI3K phosphatidylinositol 3 kinase; PKB protein kinase B; REDD1 regulated in development and DNA damage responses 1; RHEB RAS homologous enriched in brain; T2D type 2 diabetes; TSC1/2 tuberous sclerosis complex.

T2D progression through impaired pancreatic β-cell function and development of IR has been associated with dysregulation of mTOR signalling and autophagy (Laplante 2012, Lee *et al.* 2014). Several studies suggest that enhanced autophagy plays a protective role against nutrient surplus-induced oxidative and ER stress in pancreatic β-cells (Hur *et al.* 2010, Bartolome *et al.* 2012). However, constitutively activated autophagy has

detrimental effects on pancreatic β-cells, causing autophagy-induced cell death (Demirtas *et al.* 2016).

Interestingly, Fujimoto *et al.* have claimed to have unpublished data showing that *PDX1+/-* β-cells, as well as *PDX1*-diminished mouse insulinoma MIN6 cells present increased autophagy (Fujimoto *et al*. 2009), indicating that *PDX1* also mediates β-cell survival through regulation of autophagy.

c) Pyroptosis

Pyroptosis is a pro-inflammatory response-mediated type of programmed cell death involving the formation of a macromolecular complex known as inflammasome, and subsequent caspase-1 activation (not related with apoptotic cell death) (Byrne *et al*. 2013). An inflammasome is a protein complex that acts as a sensor for pathogen or damage-associated molecular patterns (PAMPs or DAMPs) (Saïd-Sadier *et al.* 2012).

To date, several inflammasomes have been described: the NLRP3 inflammasome (made up of sensor molecule NLRP3, adaptor protein ASC and pro-caspase 1), the AIM2 inflammasome (made up of sensor molecule AIM2, adaptor protein ASC and pro-caspase 1), the NLRP1 inflammasome (constituted by sensor protein NLRP1 and pro-caspase 1), and the NLRP4 inflammasome (made up of sensor protein NLRP4 and pro-caspase 1). Among these, the NLRP3 inflammasome is the most extensively studied. Importantly, reactive oxygen species (ROS) generated by the mitochondria under situations of increased metabolic stress induce expression of IL1β, a pro-inflammatory cytokine related to IR and β-cell function, which activates the NLRP3 sensor protein (Gonzalez *et al*. 2018). When activated, NLRP3 interacts with the adaptor protein ASC and recruits and activates pro-caspase-1 to produce caspase-1, which mediates cell death through pyroptosis, a process involving fragmentation of DNA, pore formation in the plasma membrane and ultimately cellular lysis (Miao *et al.* 2011).

d) Other mechanisms of β-cell loss

Traditionally, it has been believed that loss of insulin-producing cells was solely caused by different mechanisms of cell death. However, there is growing evidence that β-cells can undergo de-differentiation (they display decreased expression of β-cell identity genes and increased expression of β-cell de-differentiation markers) and/or trans-differentiation to other islet cell types (especially to glucagon-producing α-cells) (Dor *et al.* 2013). An increase in the number of α-cells consequently leads to hyperglucagonemia, which is also

associated with T2D (Gromada *et al.* 2007). There is evidence showing that loss of β-cell identity (decreased expression of key β-cell markers such as genes encoding transcription factors involved in the β-cell maturation process like *MAFA*, and proteins implicated in glucose-stimulated insulin secretion like GLUT2) with the conversion of β-cells into α-cells occurs in murine models of T2D (Talchai *et al*. 2012) and in human pancreatic islets *ex vivo* (Spijker *et al*. 2013). More recently, new markers for β-cell de-differentiation have been identified, including two members of the fibroblast growth factor (FGF) family, FGF1 and FGF2, and several transcription factors, including SOX9, HES1, MYC, and tumour necrosis factor receptor superfamily member 11b (TNFRSF11B), as shown by their expression in the pancreas of T2D patients (Diedisheim *et al.* 2018).

It is still unknown why β-cells variably respond to stress by de-differentiating, trans-differentiating or dying. The differential response might depend on the type of stress, or might be associated with particular islet dysfunctions (Hudish *et al.* 2019).

1.3 Glucolipotoxicity

1.3.1 Glucotoxicity: adverse effect of glucose on pancreatic β-cells

The level and duration of the hyperglycaemic state are crucial in determining the fate of β-cells. As mentioned in sections 1.2.5 and 1.2.6, prolonged hyperglycaemia has a proapoptotic effect on β-cells, which is referred to as glucose toxicity, or glucotoxicity. This term refers to the progressive and irreversible detrimental effects of chronically elevated glucose levels on β-cell function, which lead to decreased insulin gene expression and synthesis (Poitout *et al*. 2008). A number of stress-related mechanisms have been proposed to explain how chronically elevated glucose levels impair β-cell function and increase β-cell apoptosis rates (Oh 2015).

a) Endoplasmic reticulum stress

The ER is responsible for the synthesis of all secreted proteins including insulin, the most abundant protein produced by β-cells. As a result of insulin resistance, hyperglycaemia arises, with subsequent increased workload for production of insulin on β-cells (Hanley *et al*. 2010, Inaishi *et al.* 2016). This causes ER stress, which leads to translational attenuation of proinsulin and degradation of insulin mRNA, which, together with the accumulation of

unfolded proteins inside of the ER, triggers the activation of a defence mechanism executed by the β-cell known as unfolded protein response (Rojas *et al*. 2018). This pathway is responsible for enhancing protein folding capacity via increased production of chaperones and enzymes for protein maturation (Scheuner *et al*. 2008). This phenomenon is accompanied by an upregulation of ER-associated degradation proteins and components of autophagy to promote elimination of unfolded and aggregated proteins (Fonseca *et al.* 2011). However, this adaptive response can induce molecular mechanisms that lead to ER stress-induced β-cell apoptosis (Oh *et al*. 2013).

Other ER stress responses include increased activation of IRE1 and ATF6 and subsequent expression of XBP1 and CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP) (Figure 1.4), which lead to β-cell death and subsequent transition from pre-diabetes to diabetes as it has been reported in β-cell lines and isolated islets from rat, mouse, and humans (Marchetti *et al*. 2007, Lipson *et al.* 2006).

b) Mitochondrial dysfunction and oxidative stress

In normal physiological conditions, pyruvate is generated as the final product of glycolysis in the cytosol. It then enters the mitochondria, where it is converted into acetyl-coenzyme-A, which is further oxidized within the tricarboxylic acid (TCA) cycle, yielding reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$). These products serve as electron donors for the mitochondrial electron transport chain, where ATP is produces, generating low levels of ROS (Liemburg-Apers *et al.* 2015). However, chronically elevated glucose levels increase pyruvate production and subsequent mitochondrial activity, and consequently, the generation of ROS in islet cells, inducing oxidative stress (Oh *et al.* 2015). Moreover, because of the high demand for insulin production, β-cells are among the most metabolically active tissues and highly rely on oxidative phosphorylation for ATP production, thereby further increasing ROS generation.

Importantly, pancreatic β-cells are susceptible to oxidative stress due to the lack or low expression levels of antioxidants such as catalase, glutathione peroxidase, and superoxide dismutase, enzymes that protect cells from ROS and subsequent downstream damage (Tiedge *et al.* 1997). The consequential imbalance from ROS overproduction and the low β-cell antioxidant capacity results in oxidative stress, which diminishes the activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Giacco *et al*. 2010), thus increasing the concentrations of all the intermediate glycolytic compounds

upstream of GAPDH, as well as of the first metabolite of the glycolytic pathway, glucose (Du *et al*. 2003). Given the already overt hyperglycemia, and the increase in glucose concentration as a result of decreased GAPDH activity, the normal route for glycolysis gets saturated and excess glucose is shunted towards alternative ROS-generating pathways, further increasing oxidative stress.

There are five pathways that can branch off the glycolytic pathway under chronic hyperglycaemic conditions (Robertson 2004). These pathways play a minor role in glucose metabolism under normoglycaemic conditions but can become major pathways to flux increased levels of glucose and other intermediates. These pathways are the polyol pathway (conversion of glucose to fructose) (Tang *et al.* 2012); the hexosamine pathway (conversion of fructose-6P to glucosamine-6P) (Lima *et al.* 2012); the protein kinase C (PKC) activation pathway (glyceraldehyde-3-phosphate from the breakdown of fructose 1:6-bisphosphate increases synthesis of diacylglycerol (DAG), which activates PKC) (Teshima *et al*. 2014); the receptor for advanced glycation end products (RAGE) pathway (methylglyoxal formation from glyceraldehyde 3-phosphate modifies proteins by adding a glycated haemoglobin (HbA1c)) (Wells-Knecht *et al.* 1995); and the glyceraldehyde autoxidation pathway (autoxidation of glyceraldehyde-3-phosphate generates hydrogen peroxide and α-ketoaldehydes) (Wolff *et al*. 1987). Ultimately, all five pathways have been linked to ROS production and subsequent oxidative stress, and consequently to the pathogenesis of diabetes and its complications (Robertson 2004).

Indeed, it has been reported that ROS can induce IR (Yang *et al.* 2014), impair insulin synthesis (Robertson *et al.* 1992), and impair insulin secretion (Karunakaran *et al.* 2013). Additionally, isolated islets from T2D patients display increased expression of markers of oxidative stress compared with healthy controls, including nitrotyrosine and 8-hydroxy-2-deoxyguanosine, correlating with the degree of IR or impaired insulin secretion (Del Guerra *et al*. 2005). Furthermore, it has been seen that treatment of islets, Zucker diabetic fatty (ZDF) rats and db/db mice with anti-oxidants such as N-acetylcysteine (NAC) results in decreased production of markers for oxidative stress, improved insulin gene expression and improved glycaemic control (Poitout *et al*. 2008).

Another molecular mechanism through which chronic hyperglycaemia can cause deteriorating β-cell function is via decreased expression of *PDX1* and *MAFA* (Robertson 2004, Rojas *et al*. 2018). The possibility that oxidative stress is responsible for the decreased expression of *PDX1* and *MAFA* was highlighted by early studies showing that

NAC treatment of β-cell lines and rodent models of T2D protected against diminished *PDX1* and *MAFA* gene expression and loss of insulin gene expression and secretion induced by exposure to high glucose concentrations (Tanaka *et al*. 1999, Tanaka *et al*. 2002).

c) Inflammation

It has been reported that prolonged exposure of human islets to hyperglycaemia triggers β-cell production of IL1β, a pro-inflammatory cytokine, and subsequent activation of nuclear factor kappa B (NF-κB), which triggers the activation of an inflammatory response that culminates in autocrine apoptosis (Maedler *et al.* 2002). It has been seen that anti-inflammatory therapies with the use of IL1β antagonists can preserve some β-cell functional mass in T2D (Donath 2014). This topic will be described in detail in section 1.4.2.

1.3.2 Lipotoxicity: adverse effect of fatty acids on pancreatic β-cells

In normal physiological conditions, lipids play multiple and very important biological functions. They constitute the main energy reserve, serve as a signalling molecules, constitute the basis for steroid hormone biosynthesis, and are the major components of biological membranes (Jo *et al*. 2016). In β-cells, lipids are metabolised through the mitochondrial β-oxidation pathway to provide energy. However, in hyperglycaemic conditions, β-oxidation is diminished, decreasing the rate of lipid detoxification, which results in an accumulation of intracellular lipid metabolites such as long-chain acyl-CoA, which mediates the toxic effects of chronically elevated FFAs (Véret *et al.* 2014). Acute FA overload in β-cells amplifies insulin secretion, but, similarly to the effects of elevated levels of glucose for a prolonged period, chronically elevated levels of FAs cause β-cell dysfunction, a process that has been termed lipotoxicity (Giacca *et al.* 2011). It has been seen that prolonged exposure of isolated islets of non-diabetic rats or insulin-secreting cell lines to elevated levels of FAs is associated with impairment of insulin gene expression and inhibition of glucose-stimulated insulin secretion (Zhou *et al*. 1994), inhibition of expression of cell differentiation genes (Giacca *et al*. 2011), and induction of β-cell death through apoptosis (Kharroubi *et al.* 2004) as shown by increased DNA fragmentation, caspase-3 activity and expression of apoptotic genes (Oh 2015). A number of mechanisms have been proposed to explain how chronically elevated levels of FAs impair β-cell function and increase β-cell apoptosis rates.

a) Formation of ceramide and other lipid derivatives

Increased intracellular lipid storage due to elevated circulating levels of FAs increases *de novo* synthesis of ceramides as well as *de novo* synthesis of long chain Acyl-CoAs, such as palmitoyl-CoA. While ceramides are capable of decreasing insulin expression through the activation of c-Jun N-terminal kinase (JNK) and subsequent phosphorylation of IRS Serine/Threonine residues (Rojas *et al*. 2018), long chain Acyl-CoAs have been seen to inhibit several steps in the glycolytic pathway, including glucokinase activity, thereby attenuating insulin signalling (Sokolowska *et al.* 2019). Furthermore, it has been seen that treatment of INS-1 β-cells with palmitic acid (16:0, PA), the most common saturated fatty acid (SFA) found in animals and plants (Carta *et al*. 2015), induces ceramide synthesis and accumulation by a dual mechanism involving the enzymes serine palmitoyl-transferase and ceramide synthase 4, resulting in the formation of ceramides with specific N-acyl chain lengths (Véret *et al*. 2011), which have also been seen to induce β-cell death through apoptosis. Importantly, elevated levels of ceramide have been reported in the islets of ZDF rats, and early studies show that treatment with fumonisin B1, a ceramide synthase inhibitor, prevents β-cell apoptosis induced by increased ceramide biosynthesis (Shimabukuro *et al*. 1998). Furthermore, ceramides inhibit PKB by blocking its phosphorylation, allowing FOXO1 to repress PDX1 activity, thereby decreasing insulin synthesis, as well as β-cell proliferation and survival (Boslem *et al*. 2012) (Figure 1.4A).

b) Endoplasmic reticulum stress

ER stress has been linked to β-cell apoptosis in situations of chronic exposure to high levels of FAs (Biden *et al.* 2014), with a reported increase in the presence of ER stress markers in pancreatic islets of T2D patients (Marchetti *et al*. 2007). The mechanism underlying the generation of ER stress induced by SFAs such as PA in β-cells involves processes such as the loss of Ca^{2+} ions and protein overload.

The ER constitutes the main intracellular Ca^{2+} reservoir. Controlled release of Ca^{2+} ions into the cytosol is a critical step for insulin synthesis, making β-cells particularly sensitive to ER stress (Hara *et al.* 2014). It was recently reported that sorcin, a Ca^{2+} sensor protein in the ER, is downregulated under lipotoxic stress conditions (Marmugi *et al.* 2016). Additionally, lipotoxicity disrupts ER-to-Golgi protein trafficking due to protein overload, resulting in impaired proinsulin maturation and loss of insulin content (Preston *et al.* 2009), and consequently to ER stress-induced apoptosis. This is triggered through several

mechanisms, including perturbation of membrane lipid composition, which promotes IRE1 and PERK activation (Volmer *et al*. 2013), leading to apoptosis (Figure 1.4A).

c) Mitochondrial dysfunction and oxidative stress

Increased circulating FA levels leads to incomplete mitochondrial FA oxidation and increases ROS production, resulting in oxidative stress and lipotoxicity (Tumova *et al*. 2016). Increased ROS levels are the determinant triggering factor for β-cell dysfunction. MIN-6 cells and rat islets exposed to FAs for 48 hours exhibit diminished glucose-stimulated insulin secretion, an effect that can be reversed with the use of antioxidants such as taurine. It has also been seen that the use of NAC and tempol as antioxidants prevents the impairment in β-cell function induced by FAs *in vivo* during hyperglycaemic clamping, and *in vitro* in isolated rat islets treated with OA (Oprescu *et al.* 2007). The molecular mechanisms of ROS-mediated lipotoxicity have been analysed using RINm5F and INS-1 β-cells, as well as primary islets; only long-chain (>C14) saturated, non-esterified FAs were described to be toxic to β-cells (Elsner *et al*. 2011). In fact, a study by Busch *et al.* demonstrated that expression levels of the stearoyl-CoA desaturase enzyme correlates with β-cell resistance to the proapoptotic effect of PA, indicating that the capacity to desaturate FA might have a protective effect against lipotoxicity (Busch *et al*. 2005).

d) Inflammation

It has been reported that FAs can induce inflammatory toxicity by directly activating inflammatory pathways in pancreatic islets, demonstrating that lipotoxicity may interact with inflammatory factors that initiate, sustain, and cause β-cell loss (Donath *et al*. 2013). This topic will be described in detail in section 1.4.2.

e) Autophagy

Under normal conditions, autophagy is inhibited by mTOR, a modulator activated by INS/IGF signalling or in states of sufficient nutrients. On the other hand, upon induction of ER stress or increased ROS exposure, autophagy is stimulated to protect the cell by clearing accumulated damaged components (Oh *et al.* 2018), as it has been reported in cultured β-cells after prolonged PA exposure (Martino *et al.* 2012). However, FAs have been shown to interfere with autophagic flux under diabetic conditions, suggesting that when there is excessive autophagy, the cellular defence mechanism may turn to the apoptotic pathway (Pugazhenthi 2014).

f) Other mechanisms

FA signalling can affect transcriptional or translational levels of anti-/pro-apoptotic or survival genes. It has been observed that PA is able to directly inhibit *PDX1* and *MAFA* expression, repressing β-cell survival (Poitout *et al.* 2008), as well as to induce translational repression of the myeloid cell leukaemia sequence 1 protein (MCL1), an anti-apoptotic factor of the BCL2 family (Allagnat *et al.* 2011). Furthermore, it has been seen that FFA signalling can block glucose-induced β-cell proliferation *in vivo* in mice and *in vitro* in rat INS-1 pancreatic β-cells through induction of cell cycle inhibitors (Pascoe *et al*. 2012).

1.3.3 Glucolipotoxicity: toxic effect of chronic exposure to high glucose and fatty acids

We have seen the adverse effects that both chronically increased levels of glucose alone (glucotoxicity), and FAs alone (lipotoxicity) have on pancreatic β-cells. The term glucolipotoxicity refers to the combination of both high blood glucose and FA levels, and the harmful effects that this combination has on pancreatic β-cells regarding both function and survival (Poitout *et al*. 2010). Importantly, both *in vitro* (Briaud *et al*. 2001) and *in vivo* (Briaud *et al.* 2002) studies have shown that the effect of lipotoxicity is synergistically increased in the presence of concomitantly elevated glucose levels. Reports also indicate that treatment with a lipid lowering drug does not protect animals from islet dysfunction and diabetes, whereas the use of a glucose lowering drug does (Tanaka *et al*. 2002), suggesting that lipotoxicity requires concomitant hyperglycaemia to damage islet function, whereas glucotoxicity can exert harmful effects on islets in the absence of elevated circulating FAs (Fujimoto *et al.* 2009, Somesh *et al.* 2013). In any case, the combined effect of glucotoxicity and lipotoxicity is greater than any of them alone (Bagnati *et al*. 2016).

1.4 Obesity, Inflammation and T2D

Obesity is a worldwide pandemic that continues to grow at an alarming rate. It is a serious health problem that increases morbidity and mortality of a variety of acute and chronic diseases, most notably T2D and CVD (Vachharajani *et al.* 2009). Some of the detrimental consequences of obesity have been attributed to the induction of a low-grade chronic inflammatory state that results in the production and secretion of inflammatory mediators from the enlarged pool of activated adipocytes, the main cell type found in AT, thus contributing to the pathogenesis of several obesity-related diseases including T2D (Dandona *et al*. 2004).

1.4.1 Obesity as a chronic inflammatory disease: dysregulation of adipose tissue

The obesity-induced low-grade inflammatory response is triggered by an excess of nutrients, which cause AT and pancreatic β-cells to secrete pro-inflammatory cytokines, (Alexandraki *et al*. 2006), as it has been reported in obese humans and linked to IR (Alexandraki *et al.* 2006). Furthermore, T2D-associated complications in kidneys, arteries, and eyes are also characterised by inflammatory processes (Guarner *et al.* 2014).

AT is made up of a wide variety of cell types, including adipocytes, pre-adipocytes, tissue matrix, nerve tissue, stromal-vascular cells, macrophages, endothelial cells and fibroblasts (Frayn *et al*. 2003). As obesity develops and AT depositions grow, a variety of cell populations begin to exhibit an inflamed or stressed state, becoming activated (Johnson *et al.* 2012). This becomes apparent though various mechanisms: induction of hypoxia due to lack of oxygen availability as a consequence of rapid tissue expansion (Johnson *et al.* 2012), mitochondrial uncoupling and subsequent oxidative stress through increased production of ROS due to excess nutrient processing (Wojtczak *et al.* 1993), and production of a variety of anti- and pro-inflammatory adipokines and cytokines by the activated pool of adipocytes (Johnson *et al.* 2012).

a) Hypoxia

Rapid AT expansion causes a decrease in oxygen availability, exposing cells to hypoxia (Johnson *et al.* 2012). This will result in activation of hypoxia inducible factor 1α (HIF-1α), a transcription factor that activates transcription of several apoptosis-related genes

including p53, p21 and BCL2 (Carmeliet *et al.* 1998), as well as other factors and proteins involved in several other pathologies. For instance, HIF-1α activates transcription of vascular endothelial growth factor (VEGF), GLUT and plasminogen activator inhibitor 1 (PAI1), contributing to the development and progression of several types of cancer (Liu *et al.* 2012). HIF-1α also blocks adipocyte secretion of anti-inflammatory adipokines such as adiponectin, thereby contributing to the establishment of a pro-inflammatory milieu (Fakhruddin *et al.* 2017). This topic is described in detail in section 1.6.

b) Mitochondrial dysfunction and oxidative stress

In section 1.3 we saw how hyperglycaemia and hyperlipidemia can induce mitochondrial dysfunction and oxidative stress in the β-cell through increased ROS production. This excess of nutrients, as well as presence of an obesogenic environment, can also cause mitochondrial uncoupling in hypertrophied adipocytes with the subsequent increase in ROS production both from increased mitochondrial function and from increased NADPH oxidase activity (Wojtczak *et al.* 1993). This triggers abnormal signalling pathways involving NF-κB activation and subsequent induction of inflammatory cytokines, chemokines, adhesion molecules and growth factors, causing numerous complications including vascular dysfunction, atherosclerosis and inflammation.

1.4.2 Inflammation and T2D: activation of the NF-κB transcription factor

Exposure of tissues to harmful stimuli such as microbial pathogens, irritants, or toxic cellular components, including toxic levels of glucose and FAs, trigger an inflammatory response. These events are controlled by a variety of extracellular molecular regulators, mainly cytokines and chemokines, that mediate recruitment of immune cells to the site of inflammation, which in the case of T2D could be AT or the pancreas (Turner *et al.* 2014).

a) Adipose tissue

Rapid growth of AT causes the release of AT-specific inflammatory cytokines, referred to as adipokines. This process is primarily mediated by the activation of NF-κB, the central pro-inflammatory transcription factor, and results in low-grade inflammation throughout AT (Illán-Gómez *et al.* 2012).

Adipokines, together with AT-secreted hormones, are the link between obesity and obesity-related complications, including T2D (Gnacińska *et al*. 2009). Amongst the AT-secreted hormones we can find leptin, which stimulates secretion of tumour necrosis factor α (TNFα) from circulating monocytes, and resistin, which is associated with an increased production of pro-inflammatory cytokines from macrophages and pro-inflammatory adipokines from adipocytes through NF-κB activation (Vachharajani *et al*. 2009) (Figure 1.6). AT-secreted adipokines include TNFα, IL6, and C reactive protein (CRP), with its production being in turn enhanced in response to TNFα and IL6, hence amplifying the pro-inflammatory cascade (Vachharajani *et al.* 2009). Under stimulation of TNFα, adipocytes are also able to secrete chemokines, which induce macrophage activation (switch from anti-inflammatory (M2) to pro-inflammatory (M1) type macrophages) and infiltration into AT (Figure 1.6). In fact, despite the ability of the adipocytes to secrete pro-inflammatory cytokines (adipokines), it is the activated macrophages infiltrated in the AT the ones responsible for the main release of these mediators (Weisberg *et al.* 2003). These include TNFα, IL1, IL6 and monocyte chemoattractant protein 1 (MCP1) (Jung *et al.* 2014), which impair adipocyte insulin sensitivity and stimulate further activation and infiltration of peripheral monocytes and macrophages into AT (Amrani *et al*. 1996) (Figure 1.6).

NF-κB is a redox-sensitive transcription factor that can be activated by a wide variety of stimuli, including oxidative stress (Sanz *et al*. 2010). ROS-mediated activation of NF-κB can activate transcription of a wide range of pro-inflammatory and profibrotic genes, causing vascular dysfunction, atherosclerosis, and inflammation. These include, as mentioned above, cytokines such as TNFα, IL1β, IL2, IL6 and IL12; adhesion molecules including E-selectin and vascular and intracellular cell adhesion molecules (VCAM1, ICAM1); growth factors including transforming growth factor β (TGFβ); and chemokines, including MCP1 (Pedruzzi *et al.* 2012) (Figure 1.6).

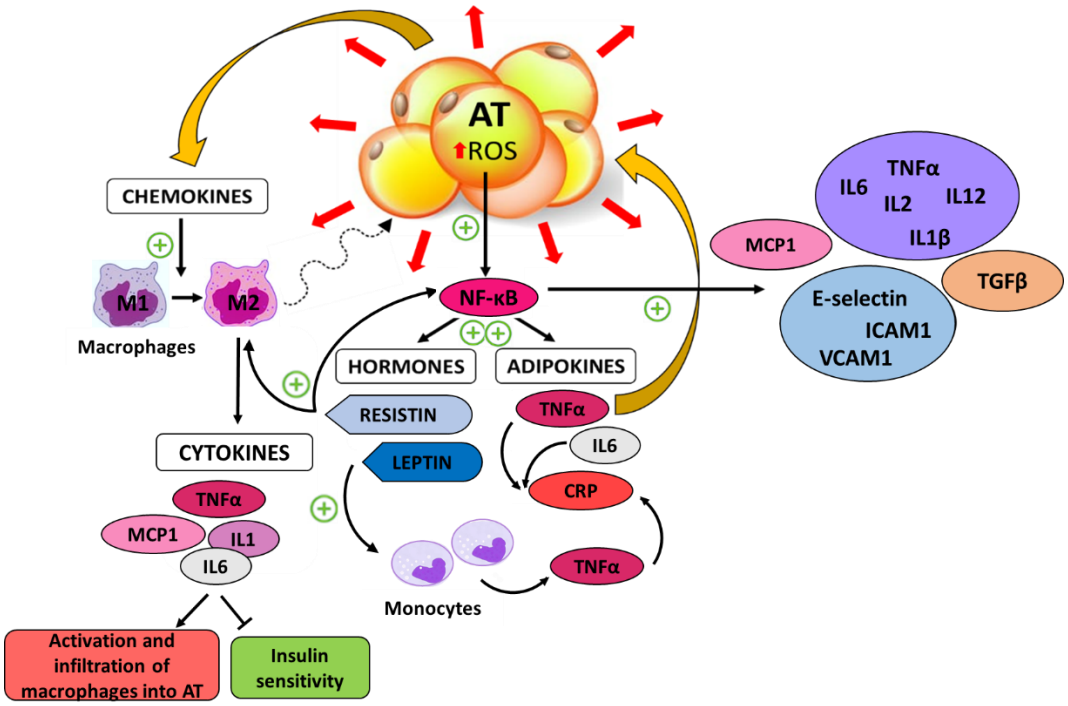


Figure 1.6: ROS-mediated activation of inflammation in AT. Rapid growth of AT causes mitochondrial dysfunction and subsequent induction of ROS production. This activates the NF-κB transcription factor, which induces AT production and secretion of several hormones including leptin and resistin, and adipokines such as TNFα, IL6 and CRP. Amongst them resistin induces pro-inflammatory cytokine production from macrophages and pro-inflammatory adipokine production from adipocytes through NF-κB activation, while leptin induces TNFα secretion from monocytes. TNFα produced by AT induces AT production of chemokines, which will attract and activate macrophages, which switch from anti-inflammatory (M2) to pro-inflammatory (M1), leading to macrophage infiltration into AT and further production of pro-inflammatory cytokines such as TNFα, IL6 and IL1, and chemokines such as MCP1, all of which impair AT insulin sensitivity. ROS-mediated activation of NF-κB directly activates transcription of cytokines TNFα, IL1β, IL2, IL6 and IL12, adhesion molecules E-selectin and VCAM1, ICAM1, TGFβ, and MCP1. Abbreviations: AT adipose tissue; CRP C reactive protein; IL6 interleukin 6; MCP1 monocyte chemoattractant protein 1; NF-κB nuclear factor kappa κB; ROS reactive oxygen species; TGFβ transforming growth factor β; TNFα tumour necrosis factor α; VCAM1, ICAM1 vascular and intracellular cell adhesion molecules.

While some of the mechanisms involving the roles of pro- and anti-inflammatory cytokines still remain to be fully resolved, it is clear that dysregulated production of adipokines caused by excess AT, together with AT dysfunction, contributes to the development of obesity-related metabolic diseases such as T2D (Villarroya *et al.* 2018).

b) Pancreas

Growing evidence from recent studies suggests that an important determining factor for β-cell dysfunction and failure in T2D is chronic pancreatic islet inflammation (Westwell-Roper *et al*. 2014). Interestingly, several reports have indicated that FAs potentiate inflammatory toxicity by directly activating inflammatory pathways in pancreatic islets, demonstrating that lipotoxicity may interact with inflammatory factors that initiate, sustain, and cause β-cell loss (Donath *et al.* 2013). Different types of FA have different effects on the lipotoxicity of β-cells (Acosta-Montaño *et al.* 2018). As mentioned before, only SFAs have been associated with adverse health effects. In particular, in section 1.3.2 we described how PA has a major role in β-cell failure through mechanisms including the induction of ceramide formation, the build-up of proinsulin in the ER lumen and subsequent generation of ER stress, the induction of mitochondrial dysfunction and subsequent increase in oxidative stress, the attenuation of β-cell proliferation capacity, and the induction of autophagy and β-cell apoptosis. However, PA has also been seen to induce chemokine (CXCL1 and CCL3) and cytokine (IL6 and IL8) expression within pancreatic islets *in vitro* through NF-κB activation and induction of ER stress (Igoillo-Esteve *et al.* 2010), promoting an inflammatory response. By contrast, unsaturated fatty acids (UFAs), such as OA, are generally associated with protective effects (Acosta-Montaño *et al.* 2018). Interestingly, treatment of monocytes with docosahexaenoic acid (DHA), a polyunsaturated fatty acid (PUFA), has been described to inhibit PA-induced monocyte secretion of pro-inflammatory cytokines such as IL1β (Snodgrass *et al.* 2016). However, the effects of DHA on IL1β release form β-cells remains to be determined.

Interestingly, an elevated number of islet-associated macrophages and an increased expression of IL1β have been reported in pancreatic islets from T2D subjects (Richardson *et al*. 2009, Eguchi *et al*. 2017). Moreover, recent evidence shows that enhanced macrophage infiltration results in an evident increase in pro-inflammatory cytokine production through NF-κB activation, resulting in β-cell release of chemokines, which drive recruitment of neutrophils, monocytes, and lymphocytes to the pancreas in obesity (Lackey *et al.* 2016, Collier *et al.* 2017). Accordingly, it has been seen that depletion of resident islet macrophages in HFD-fed mice lowers IL1β expression and improves β-cell insulin secretion (Westwell-Roper *et al.* 2014), while inhibition of IL1β signalling with the use of IL1β antibodies or antagonists for the IL1Ra receptor, decreases immune cell infiltration and improves β-cell function and glucose homeostasis in rats with T2D (Ehses *et al.* 2009, Böni-Schnetzler *et al.* 2019).

As mentioned in section 1.2.6, β-cell dedifferentiation has been proposed as a mechanism underlying β-cell mass loss in T2D. A recent study shows that this mechanism might be triggered by the action of pro-inflammatory cytokines including IL1β, IL6 and TNFα. In this study, exposure of cultured human and mouse islets to IL1β induced downregulation of the identity maintaining transcription factor FOXO1. Furthermore, treatment with anti-IL1β, anti-TNFα or the NF-κB inhibitor sodium salicylate was shown to improve insulin secretion of isolated islets *in vivo* (Nordmann *et al.* 2017).

1.4.3 The NF-κB transcription factor

NF-κB is a critical transcription factor involved in a broad range of biological processes, including immune and inflammatory responses, cell survival, stress responses and maturation of various cell types. While NF-κB activation is required to protect organisms from harmful environmental effects, defects in its regulation can lead to various diseases including chronic inflammation and cancer (Shih *et al.* 2011).

NF-κB is a family of ubiquitously expressed transcription factors, consisting of hetero- or homodimers of 5 Rel homology domain (RHD)-containing polypeptides (RelA or p65, RelB, c-Rel, p50 and p52) and their stoichiometric inhibitor proteins, IκBs. The RHD contained in the 5 subunits mediates dimerization, DNA binding, interaction with IκBs and nuclear translocation. The five monomers can form at least 15 different dimers which are cell type- and stimulus-specific, of which the most common is the one formed by p50 or p52 with RelA (p65), also known as NF-κB1 (Christian *et al.* 2016). The RelA, RelB and c-Rel subunits also contain a transactivation domain (TAD) which is responsible for the transcriptional activity of dimers containing these subunits (Hoffmann *et al.* 2006). On the other hand, the p50 and p52 subunits are regulated by processing of precursor proteins p105 and p100 respectively, and they do not contain a TAD. In fact, when they homodimerize they act mainly as transcriptional repressors (Christian *et al*. 2016).

The classical inhibitor proteins of NF-κB consist of the single polypeptide IκBs: IκBα, IκBβ and IκBε. In resting cells, IκB binds and sequesters NF-κB dimer in the cytoplasm, thereby preventing nuclear translocation and subsequent DNA binding and transcriptional activation (Shih *et al*. 2011).

NF-κB activation can be mediated through two mechanisms, giving rise to different cell responses. Firstly, the rapid and reversible inflammatory and immune response that leads to transcriptional activation of proinflammatory factors typically occurs through the activation of the canonical pathway. Secondly, the slower and irreversible developmental response including immune cell differentiation and maturation typically occurs through activation of the non-canonical pathway (Shih *et al*. 2011). However, this pathway has recently been described to also regulate several aspects of innate and adaptive immune responses, as evidenced by the fact that dysregulated non-canonical NF-κB activation can also contribute to the pathogenesis of inflammatory diseases (Sun 2017).

In most cell types, NF-κB is activated through the canonical pathway, where stimuli from diverse immune receptors such as TNFR, IL1R or toll-like receptor (TLR) leads to activation of a trimeric inhibitor of κB kinase (IKK) complex, composed of catalytic (IKKα and IKKβ) and regulatory (IKKγ) subunits. This complex phosphorylates IκB, which gets ubiquitinated and subsequently undergoes proteasomal degradation. NF-κB1 is released, allowing its translocation to the nucleus, thereby activating expression of various inflammatory mediators including TNFα, IL6 and MCP1 (Lee *et al.* 2014) (Figure 1.7).

Importantly, NF-κB activity is increased in obese subjects, and it has been seen that its inhibition improves IR (Yuan *et al.* 2001). In non-obese individuals, NF-κB signalling is mainly activated through lipopolysaccharide (LPS)-mediated activation of TLR4. However, in obesity, increased levels of circulating FFAs also signal through TLR4 (Shi *et al*. 2006), further activating NF-κB (Novotny *et al*. 2012) (Figure 1.7). Additionally, TNFα and IL1β, both increased in AT of obese and diabetic rodents, signal through TNFR and IL1R respectively to activate NF-κB, mediating the proinflammatory response (Lackey *et al*. 2016).

Activation of the non-canonical NF-κB pathway is initiated by ligands of a subset of the TNFR superfamily members including CD40 (Sun 2017). This ligand-receptor interaction will activate the NF-κB-inducing kinase (NIK), which phosphorylates and activates the IKK catalytic subunit IKKα. IKKα then phosphorylates p100, which gets ubiquitinated and subsequently undergoes selective proteasomal degradation of its C-terminal, leading to the generation of p52. This allows the formation of the RelB:p52 dimer, also known as NF-κB2, which is insensitive to inhibition by IκB, and thus localizes to the nucleus (Sun 2011) (Figure 1.7), mediating processes such as survival of B cells (Gardam *et al.* 2008), generation and function of T helper (T_H) cells (Yu *et al.* 2014), differentiation of monocytes

(Dejardin 2006), production of chemokines (Xia *et al.* 1997), and glucagon responses in hepatocytes (Sheng *et al.* 2012). However, when this pathway is overactivated due to the elevated production of its inducing agents, pathological conditions arise: Anomalous B cell survival renders resistant self-reactive B cells, contributing to the accumulation of autoantibodies associated with inflammatory diseases (Mackay 2004); chronic production of chemokines promotes recruitment of inflammatory cells (Maijer *et al.* 2015); and atypical glucagon responses are associated with metabolic diseases (Sheng *et al.* 2012).

It is also worth mentioning that signalling through TNFR receptors can stimulate both the canonical and the non-canonical NF-κB pathway, and mediate biological processes that involve the functional cooperation between both pathways (Sun 2017).

Importantly, it has been seen that TNFR5 (also known as CD40) expression and subsequent downstream NF-κB activation is increased in pancreatic β-cells under conditions of glucolipotoxicity, but also in isolated mouse islets from HFD-fed mice, as well as in isolated human islets (Bagnati *et al.* 2016). CD40-CD40L interactions are required for many immune processes including cytokine and chemokine production, and CD40-CD40L signalling is involved in the pathophysiology of many inflammatory diseases, including atherosclerosis, inflammatory bowel disease, rheumatoid arthritis and diabetes (Peters *et al.* 2009). CD40 and its ligand, CD40L are expressed in immune cells (B cells, T cells, dendritic cells and monocytes) but also in non-immune cells such as platelets, endothelial cells, fibroblasts and pancreatic β-cells. CD40 expression in β-cells is increased upon exposure to proinflammatory cytokines including TNFα, IL1β and IFNγ, all abundantly present in the diabetic pancreas (Barbé-Tuana *et al.* 2006). CD40L can be found soluble in plasma (derived from activated platelets) or bound to the membrane of immune cells. In diabetic conditions, these immune cells infiltrate pancreatic β-cells, allowing CD40-CD40L interaction and activating the NF-κB signalling pathway (Seijkens *et al.* 2013).

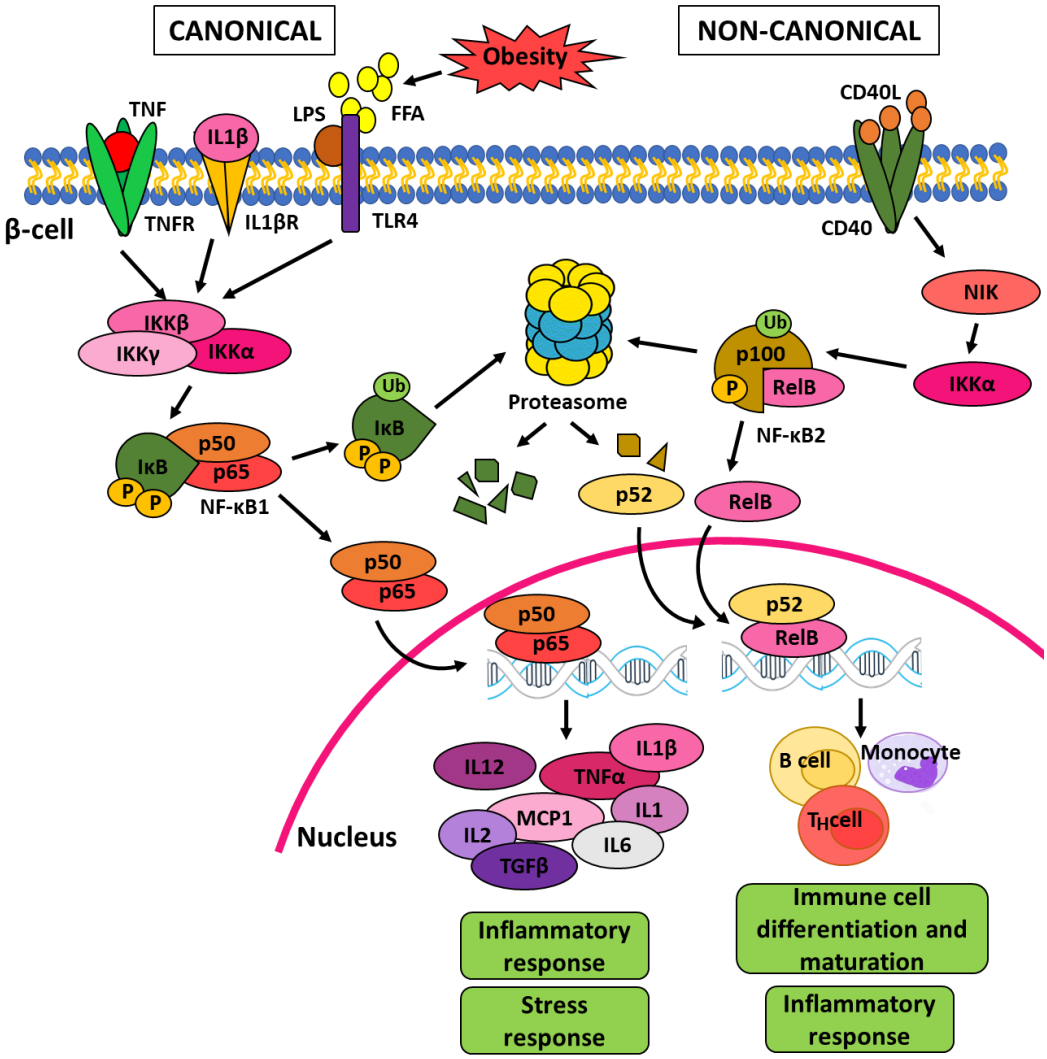


Figure 1.7: Canonical and non-canonical NF-κB signalling. Activation of the canonical NF-κB pathway is initiated through TNFR, IL1R or TLR signalling, leading to activation of the IKK complex. IKK phosphorylates IκB, which gets ubiquitinated and undergoes proteasomal degradation. NF-κB1 is released, allowing its translocation to the nucleus, thereby activating expression of TNFα, IL6 and MCP1 amongst other inflammatory mediators. Activation of the non-canonical NF-κB pathway is initiated by ligands of a subset of the TNFR superfamily members including CD40. Ligand binding will activate NIK, which phosphorylates and activates IKKα. IKKα then phosphorylates p100, which gets ubiquitinated and undergoes selective proteasomal degradation of its C-terminal, leading to the generation of p52. This allows the formation of NF-κB2, which is insensitive to inhibition by IκB, and thus localizes to the nucleus, mediating processes involving immune cell maturation. Abbreviations: FFA free fatty acids; IL1β/1/2/6/12 interleukin 1β/1/2/6/12; IL1R interleukin 1 receptor; IκB inhibitor of kappa B; IKK inhibitor of kappa B kinase; LPS lipopolysaccharide; MCP1 monocyte chemoattractant protein 1; NF-κB nuclear factor kappa B; NIK nuclear factor kappa B inducing kinase; TGFβ transforming growth factor β; T_H T-helper; TLR4 toll-like receptor 4; TNF/α tumour necrosis factor/α; TNFR tumour necrosis factor receptor.

1.5 S100 Proteins

1.5.1 Structure, expression, regulation and function

The S100s are a multigenic family encoding a set of non-ubiquitous Ca^{2+}-modulated proteins, which are implicated in multiple intracellular and/or extracellular regulatory activities (Donato 2001). To date, at least 25 such proteins have been identified, with varying expression and distribution amongst different tissues and cell types (Marenholz *et al.* 2004).

a) Molecular structure

Each S100 protein is encoded by an individual gene (Marenholz *et al*. 2004). Of the 25 human *S100* genes, 19 (group A S100 proteins) are located within chromosome 1q21 (Figure 1.8A). Other members map to different regions, including 7q22-q3 (*S100A11P*), 21q22 (*S100B*), Xp22 (*S100G*), 4p16 (*S100P*), and 5q13 (*S100Z*) (Marenholz *et al.* 2004). Every member of the S100 protein family has a similar molecular mass of 10–12 KDa, and they each share 25-65% similarity in their amino acid sequence. They exist as anti-parallel homo and heterodimers, with each monomer consisting of two helix-loop-helix EF-hands (EF-1 and EF-2) connected by a hinge region and flanked by conserved hydrophobic residues at the C- and N-terminal ends (Schäfer *et al.* 1996) (Figure 1.8B).

In the last 15 years, three-dimensional structures of S100 proteins have been determined in the Ca^{2+}-free (apo), Ca^{2+}-bound, and target-bound states (Réty *et al.* 1999, Otterbein *et al.* 2002). These studies have revealed that upon Ca^{2+} binding, the S100 proteins undergo a conformational change that allows interaction with a target protein. As mentioned above, the S100 protein exists in a dimeric form, with each monomer containing two EF-hand motifs. The N-terminal EF-hand comprises helix I, Ca^{2+}-binding site I and helix II, separated by a flexible hinge region from the C-terminal EF-hand that includes helix III, Ca^{2+}-binding site II and helix IV (Santamaria-Kisiel *et al.* 2006). Ca^{2+} binding to site I results in alterations of its backbone conformation, adopting a 'Ca^{2+}-ready' state. This involves a ~40° rotation of helix III, resulting in a more open structure and allowing the exposure of a broad hydrophobic region including residues from helices III and IV in the C-terminal EF-hand and linker region. This regulates protein activity by enabling the S100 proteins to interact with a variety of target proteins including receptors and other S100 members, as well as other molecules (Chazin 2011, Zimmer *et al.* 2013).

Some S100 members are proposed to interact with the same target molecules. For instance, S100A1, S100A6 and S100B bind annexin A6 (Arcuri *et al.* 2002), while S100A1, S100A2, S100A4 and S100B interact with the tumour suppressor p53 (Mueller *et al.* 2005, Fernandez-Fernandez *et al.* 2005). This might not be surprising given the significant sequence similarities between most of the S100 proteins. However, there is a fine level of discrimination that avoids random interaction of targets with all S100 proteins. Structural studies of S100-target complexes have shown that S100 family members use different mechanisms for target recognition despite the similar conformational change induced by Ca^{2+} binding in all S100 family members (Bhattacharya *et al.* 2003, Lee *et al.* 2008, Kiss *et al.* 2012, Ozorowski *et al.* 2013). Moreover, the region exposed upon Ca^{2+} binding comprises the most variable portions of the S100 sequences (hinge and C-terminal regions), which is enough to discriminate against different target proteins (Santamaria-Kisiel *et al.* 2006). The distribution of hydrophobic and charged residues, together with differences in surface geometries, contribute to the variety of target binding patterns observed amongst S100 family members (Ozorowski *et al*. 2013, Wafer *et al.* 2013).

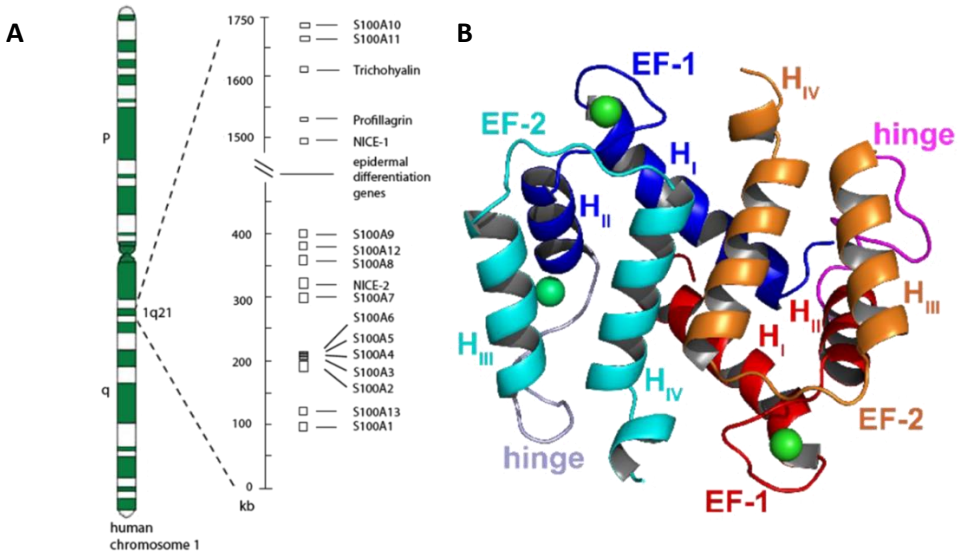


Figure 1.8: A) S100 gene cluster on chromosome 1q21. Most human S100 genes are located on chromosome 1q21. Genes located in the cluster region are indicated. p and q indicate the short and the long arm of the chromosome, respectively. Human S100B, S100P, S100Z and S100G are located on chromosomes 21q22, 4p16, 5q14 and Xp22 respectively (not shown). **B) Dimer molecular structure of S100 proteins**. All members of the S100 protein family contain a pair of EF-hand domains (EF-1 and EF-2), involved in intracellular Ca^{2+} binding. The binding of metal ions (Ca^{2+}, Zn^{2+} and Cu^{2+}) results in a conformational change that exposes a hydrophobic region that interacts with other proteins including receptors, other S100 members and several other components (Srikrishna *et al.* 2011).

b) Expression pattern of S100 proteins

Members of the *S100* gene family show different patterns of both cell and tissue-specific expression (Appendix 1). Expression of S100 proteins is strictly regulated in order to maintain immune homeostasis (Donato 2003). Calprotectin (S100A8/A9), for example, is constitutively expressed in monocytes, neutrophils, and dendritic cells (Averill *et al.* 2011). However, upon activation, it is also expressed in fibroblasts (Rahimi *et al.* 2005), mature macrophages (Ingersoll *et al.* 2009), vascular endothelial cells (Yao *et al.* 2010) and keratinocytes (Grimbaldeston *et al.* 2003). In addition, epigenetic mechanisms also play a key role in regulating their gene expression (Lindsey *et al.* 2007), with methylation of DNA CpG islands being a common method of gene transcription repression. Accordingly, DNA hypomethylation has been reported to significantly induce expression of several members of the *S100* members in prostate and gastric cancer (Wang *et al.* 2007, Wang *et al.* 2010).

c) Function of the S100 proteins

The S100 proteins have been implicated in the control of a broad range of intracellular and/or extracellular functions including regulation of cell apoptosis, proliferation, differentiation, migration/invasion, energy metabolism, Ca^{2+} homeostasis, protein phosphorylation and inflammation in different cell types (Donato *et al.* 2013). Some of the S100 protein functions are outlined below.

i) S100 proteins as damage associated molecular pattern (DAMP) molecules

After cell damage/stress or activation of neutrophils and macrophages, S100 proteins are released to the extracellular space, where they play a key role in the regulation of immune homeostasis, post-traumatic injury and inflammation (Xia *et al.* 2018). They can act as DAMP molecules to activate immune cells and endothelial cells by binding to TLRs and RAGE (Figure 1.9). For example, binding of S100A8/A9 to TLR4 initiates a signalling cascade that regulates inflammation, cell proliferation, differentiation and tumour development in an NF-κB-dependent manner (Vogl *et al*. 2007). Furthermore, S100A12 binding to RAGE has been described to induce expression of vascular and intercellular adhesion molecule 1 (VCAM1 and ICAM1) on endothelium, while also increasing NF-κB-induced expression of proinflammatory cytokines such as TNFα by other inflammatory cells (Hofmann *et al.* 1999).

DAMPs play a key role in the pathogenesis of many inflammatory diseases such as rheumatoid arthritis, osteoarthritis or atherosclerosis. Importantly, S100A4, S100A8/9,

S100A11 and S100A12 have been found to be upregulated in the synovial tissue, synovial fluid, or serum of rheumatoid arthritis patients (Klingelhöfer *et al*. 2007, Baillet *et al.* 2010, Cerezo *et al.* 2017). S100A8/A9 expression was also found to be increased in the synovium of a collagenase-induced osteoarthritis mouse model (Cremers *et al*. 2017), while S100A12 levels were significantly increased in the synovial fluid of osteoarthritis patients compared to healthy controls (Wang *et al.* 2013). Lastly, S100 proteins are also involved in the pathogenesis of atherosclerosis. S100A8, S100A9, and S100A12 have an important role in the mediation of inflammation and have been reported to increase atherosclerosis in human and rodent models through interactions with RAGE, which plays an important role in endothelial dysfunction and inflammation (Harja *et al.* 2008, Oesterle *et al.* 2015).

DAMPs also play a role in the pathogenesis of neurodegenerative diseases. S100B serum levels have been found to be intimately related to the severity of diseases such as Alzheimer's disease (Chaves *et al.* 2010) and Parkinson's disease (Schaf *et al.* 2005). Interestingly, it has also been reported that S100A10, also known as p11, increases expression of the 5-hydroxytryptamine 1B serotonin receptor (5-HT1B) in HeLa cells and brain tissue and that its expression is decreased in rodent models of depression (Svenningsson *et al.* 2006).

ii) S100 proteins in macrophage migration, invasion and differentiation

Macrophages play a crucial role in tumour growth and metastasis through modulation of local inflammation, inhibition of anti-tumour immunity and stimulation of angiogenesis (Sica *et al*. 2007). They are recruited to tumour sites by chemoattractants such as chemokine ligands (CCL) 3-8, VEGF and macrophage inflammatory protein 1 alpha (MIP1α) (Dandekar *et al.* 2011). An increasing number of findings show that many S100 proteins contribute to leukocyte adhesion and migration. For instance, as well as inducing pro-inflammatory cytokine production in macrophages through the activation of the NF-κB and p38 mitogen activated protein kinase (MAPK) pathways (Sunahori *et al.* 2006), the release of S100A8/A9 has been suggested to facilitate monocyte and neutrophil migration (Eue *et al*. 2000); S100A12 has been shown to induce the production of pro-inflammatory cytokines IL6 and IL8 through RAGE-dependent NF-κB activation, resulting in the recruitment of monocytes (Sorci *et al.* 2013, Yang *et al.* 2007); S100A10 has been reported to mediate macrophage migration to tumour sites, as shown by decreased plasmin generation and matrix metalloproteinase (MMP) 9 activation in macrophages in *S100A10*-deficient mice (O'Connell *et al.* 2010); Finally, S100A9 is capable of inhibiting myeloid cell

differentiation through generation of ROS (Cheng *et al.* 2008), while S100A8 and S100A9 have been shown to mediate the cell arresting effect of TNFα on the differentiation of myeloid-derived suppressor cells into dendritic cells and macrophages in a RAGE-dependent manner (Sade-Feldman *et al*. 2013).

d) S100 proteins in disease

As we have seen, S100 proteins play a major role in the development of numerous inflammatory conditions such as rheumatoid arthritis or osteoarthritis. However, the greatest contribution of S100 proteins to pathology is in the cancer field. There is abundant evidence showing that a dysregulated expression of a variety of S100 proteins is a common feature in many types of cancer (Bresnick *et al.* 2015). Some of these will be described below.

i) S100 proteins as biomarkers for specific diseases

Since S100 proteins can be detected in body fluids, they may be used as biomarkers where there is altered expression level associated with a specific disease (Foell *et al.* 2003). For instance, S100A4 has recently been reported as a novel biomarker and a critical regulator of glioma stem cells, with its enhanced expression contributing to the presentation of a metastatic phenotype (Chow *et al.* 2017); increased serum levels of S100A6 have been reported in gastric cancer patients (Zhang *et al.* 2014); S100A7 levels have been found to be increased in cerebrospinal fluid and brain of Alzheimer's disease subjects (Qin *et al*. 2009); S100A12 serum levels have been seen to correlate with extensive coronary atherosclerosis in patients with coronary artery disease, diabetes mellitus and chronic kidney disease. Increased S100A12 plasma levels have also been associated with diabetic retinopathy and macrovascular events in T2D patients (Dong *et al.* 2015); increased serum concentrations of S100A8/A9 have been detected in obese individuals (Mortensen *et al.* 2009) and in patients with coronary artery diseases (Ionita *et al.* 2009); Importantly, S100A8/A9 has also proven to be a useful biomarker as its faecal detection can be used to differentiate inflammatory bowel disease from irritable bowel syndrome (Konikoff *et al.* 2006); finally, S100B has been considered as a prognostic marker of the acute phase of neurologic damage after traumatic brain injury and large volume cerebral infarction (Thelin *et al*. 2016), and it has been associated with some genetic disorders as it was found to be overexpressed in patients with Down syndrome (Lu *et al.* 2011), an even to certain mood disorders as a consequence of glial pathology (Schroeter *et al.* 2013).

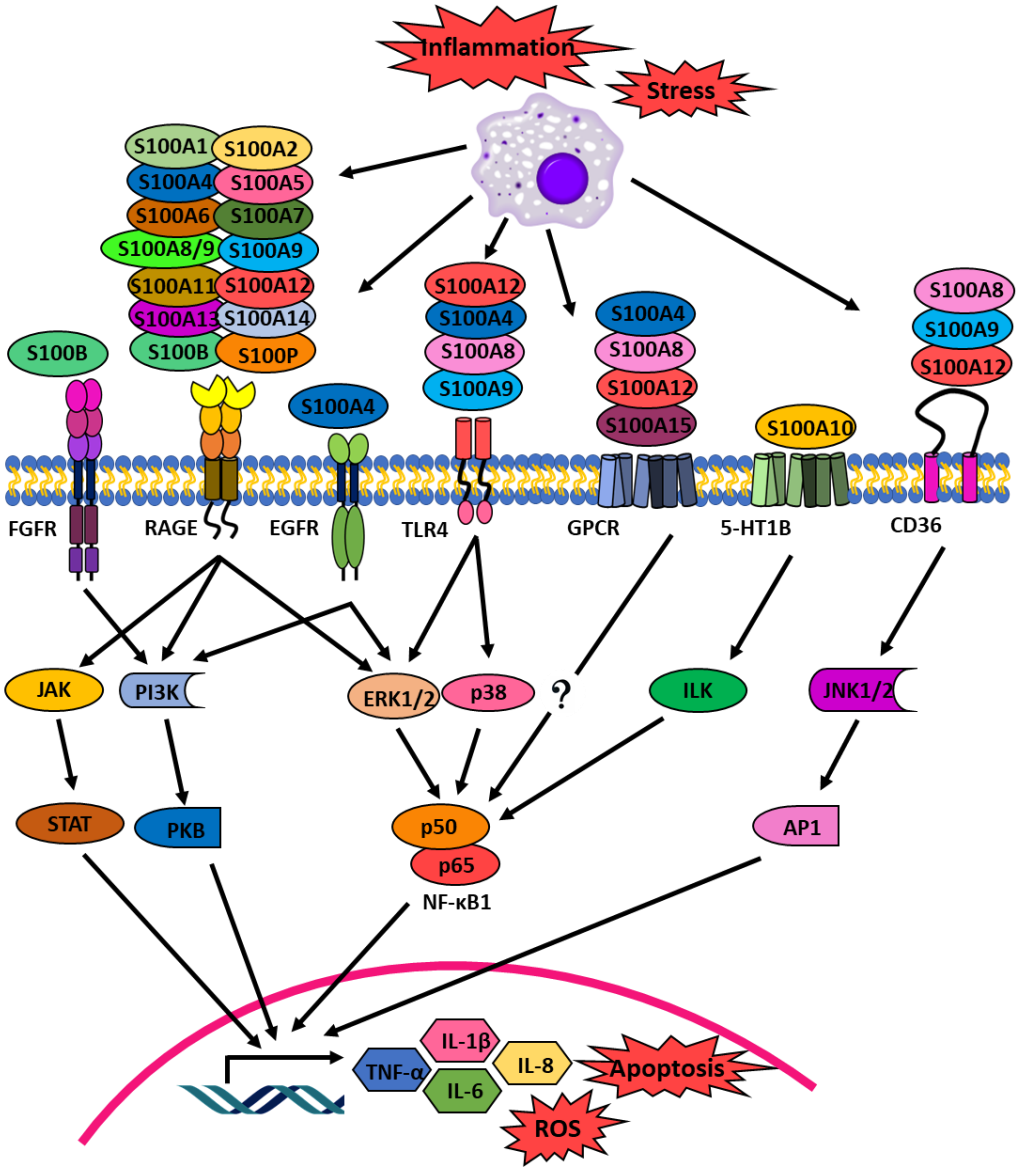


Figure 1.9: Extracellular S100s signalling. S100 proteins are released from inflammatory cells including fibroblasts, macrophages, lymphocytes and neutrophils in response to inflammation and stress. They signal through a range of cell surface receptors to activate several inflammatory signalling pathways which ultimately activate transcription of proinflammatory factors including TNF-α, IL-1β, IL-6 and IL-8, as well as other mechanisms that lead to ROS formation and apoptosis. S100B signals through FGFR to activate the PI3K/PKB pathway. Most S100s signal through RAGE to activate the JAK/STAT, PI3K/PKB and ERK/NF-κB pathways. EGFR-mediated signalling can also activate the PI3K/PKB and ERK/NF-κB pathways. GPCR-mediated signalling can also activate NF-κB although the mechanism involved in unknown. S100A10 signals through the 5-HT1B receptor to activate ILK and NF-κB, while CD36-mediated signalling activates the JNK/AP1 pathway. Abbreviations: 5-HT1B 5-hydroxytriptamine 1B serotonin receptor; AP1 activator protein 1; CD36 cluster of differentiation 36; EGFR epidermal growth factor receptor; ERK extracellular signalling-

related kinase; FGFR fibroblast growth factor receptor; GPCR G protein coupled receptor; IL interleukin; ILK integrin-linked protein kinase; JAK Janus kinase; JNK c-Jun N-terminal kinase; NF-κB nuclear factor κB; PI3K phosphatidyl inositol 3 phosphate; PKB protein kinase B; RAGE receptor for advanced glycation end products; STAT signal transducer and activator of transcription; TLR4 toll-like receptor 4; TNF-α tumour necrosis factor alpha.

ii) S100 proteins as therapeutic targets in disease

As described above, the S100 proteins interact with an extensive range of protein targets and contribute to a great variety of intracellular and extracellular functions (Donato *et al.* 2013), thereby regulating multiple cellular processes such as proliferation, differentiation and migration and/or invasion. Consequently, the S100 proteins play key roles in a wide range of pathologies including cancers, autoimmune diseases and chronic inflammatory disorders.

The identification of an increasing number of S100-target molecules has provided key insights into the chemical and physical factors regulating target selectivity, which can be used for the development of specific S100 therapeutic strategies. Accordingly, an increasing number of studies indicate that S100 proteins may serve as therapeutic targets for certain disease conditions, as indicated below.

A great number of S100 proteins bind to TLR4 (Vogl *et al.* 2007, Foell *et al.* 2013, Cerezo *et al.* 2014) and RAGE (Leclerc *et al.* 2009) (Figure 1.9). Among them, S100A8/S100A9, whose levels are found to be elevated in the serum of patients suffering from rheumatoid arthritis and other inflammatory conditions (Austermann *et al.* 2018), elicits most of its effects via these receptors (Vogl *et al.* 2007, Björk *et al.* 2009). While the S100A8/S100A9 heterodimer can bind TLR4, high extracellular Ca^{2+} concentrations induce the formation of S100A8/S100A9 tetramers (Brini *et al.* 2013), which blocks its interaction with TLR4, providing an autoinhibitory mechanism for modulating S100A8/9 biological activity (Vogl *et al.* 2018).

Substantial evidence shows that tissue and serum levels of many S100 proteins correlate with disease severity during tissue or local inflammation (Donato *et al.* 2013, Kessel *et al.* 2013). In addition, we have seen how extracellular S100 proteins can function as DAMPs, triggering proinflammatory responses and inducing autoimmune conditions and inflammatory disorders (Foell *et al.* 2007, Donato *et al.* 2013, Xia *et al.* 2018). Function-blocking antibodies targeting cell surface receptors and ligands have been widely used as

therapeutics for the treatment of numerous pathologies including cancers and immune disorders (Brufsky 2010, Saif 2013, König *et al.* 2016, Hofmann *et al.* 2018). Given the extensive literature showing that extracellular S100 proteins mediate inflammatory responses in many pathological conditions, mostly through cell receptor signalling (Bresnick *et al.* 2015, Austermann *et al.* 2018), the use of S100 neutralizing antibodies may provide a novel and effective therapeutic strategy to treat these conditions. To date, antibodies targeting S100A8/A9, S100A4, S100A7, and S100P have demonstrated efficacy for several pathological conditions (Björk *et al.* 2009, Grum-Schwensen *et al.* 2015, Padilla *et al.* 2017, Dakhel *et al.* 2014). Some of these are described in further detail below.

S100A8/A9 is the best characterized S100 family dimer with respect to extracellular functions. Elevated extracellular S100A8/S100A9 levels are strongly associated with inflammatory and autoimmune diseases, including rheumatoid arthritis, systemic sclerosis (Austermann *et al.* 2018) and diabetic nephropathy (Burkhardt *et al.* 2009), amongst others. Furthermore, it has been seen that elevated S100A8/A9 expression in the tumour microenvironment or in plasma correlates with aggressive disease (Tidehag *et al.* 2014, Miller *et al.* 2017). In particular, as described earlier, extracellular S100A8/A9 plays a central role in the recruitment of myeloid cells and myeloid-derived suppressor cells, thereby promoting the establishment of a pre-metastatic niche, as well as tumour growth (Cheng *et al.* 2008, Ichikawa *et al.* 2011). It also induces the expression of serum amyloid 3, which recruits CD11b+ myeloid cells to pre-metastatic sites (Hiratsuka *et al.* 2008), enabling the formation of a proinflammatory environment that recruits circulating tumour cells; It has been seen that S100A8 and S100A9 neutralizing antibodies block the recruitment of both myeloid cells and circulating tumour cells (Hiratsuka *et al*. 2006, Hiratsuka *et al.* 2008), as well as blocking their interaction with TLR4 or RAGE, thereby inhibiting TNFα release (Björk *et al.* 2009). It has also been reported that peptibodies (peptide-Fc fusion proteins) directed towards S100A8 and S100A9 decrease tumour-related complications in multiple cancer models (Qin *et al.* 2014). Moreover, S100A4 and S100B have been shown to participate in neoplastic disorders by binding to p53 and suppressing its phosphorylation, thereby leading to its downregulation (Fernandez-Fernandez *et al.* 2005). Therefore, targeting these proteins and suppressing their interaction would restore p53 tumour suppression function. Together, these findings highlight the potential use of function blocking or neutralising antibodies as both therapeutic and diagnostic reagents.

Moreover, early studies reported that several anti-allergic drugs are able to bind to S100A12 and S100A13 and block downstream RAGE signalling and subsequent NF-κB activation (Shishibori *et al.* 1999).

In addition to direct interaction strategies to modulate S100 proteins in disease, covalent modification may also regulate the extracellular functions of S100 proteins. For instance, transglutaminase 2 (TG2)-mediated crosslinking of S100A11 dimers is necessary for activation of p38 MAPK signalling in chondrocytes (Cecil *et al.* 2008). In addition, several S100 proteins have been seen to be S-nitrosylated, including S100B (Bajor *et al.* 2016), S100A1 (Lenarčič *et al.* 2012) and S100A8/A9 (Lim *et al.* 2010). Finally, sumoylation and phosphorylation of S100 proteins have also been reported (Miranda *et al.* 2010, Schenten *et al.* 2018). Therefore, targetting these modifications may also constitute an indirect way to modulate S100 structure or function, thus impacting upon disease pathology and progression.

Although growing evidence shows details of many S100 proteins regulation, further studies are required to fully reveal the underlying mechanisms by which S100 proteins participate in a variety of disease conditions. Therefore, future directions in this area should focus on the development of therapeutic approaches targeting S100 proteins, verification of their therapeutic potential in both preclinical and clinical settings, and elucidation of their underlying mechanisms of action.

1.5.2 S100A4

Of all the S100 family members, S100A4 is one of the the most extensively studied, and constitutes the focus of this thesis. S100A4 is also known as metastasin (MTS1), PEL98, 18A2, 42A, P9KA, CAPL, calvasculin and fibroblast-specific protein (FSP1) (Watanabe *et al.* 1992). The human *S100A4* gene is located on chromosome 1q21, and consists of four exons that encode a protein with 101 amino acid residues (Ravasi *et al.* 2004). It is ubiquitously expressed, and is present both intra- and extracellularly, with intracellular levels being high both in the cytoplasm and in the nucleus (Malashkevich *et al.* 2008).

S100A4 plays an important role in many physiological functions including cell motility, adhesion and proliferation, but also in various pathological processes such as invasion and metastasis (Roh *et al.* 2014). Intracellular S100A4 binds to cytoskeletal proteins including

F-actin and non-muscle myosin heavy chains (Ford *et al.* 1997), both of which are involved in cellular stability and/or migration (Tarabykina *et al.* 2007). By contrast, extracellular S100A4 regulates the expression of extracellular matrix (ECM)-remodelling enzymes such as MMPs, which are involved in facilitating cellular migration in different tissues (Schmidt-Hansen *et al*. 2004), and most importantly, it can also signal through membrane receptors to activate proinflammatory pathways (Cerezo *et al.* 2011).

1.5.2.1 S100A4 signalling

a) Extracellular S100A4

Under various pathological stimuli, numerous inflammatory cells including lymphocytes, macrophages and neutrophils upregulate their own expression and release of S100A4 into the extracellular space in the form of plasma membrane-derived macrovesicles (Ambartsumian *et al.* 2016). It is not clear how extracellular S100A4 exerts its effects, but it has been shown to bind several different cell-membrane receptors including RAGE, EGFR, TLR4 and IL10 receptor (IL10R) (Donato *et al.* 2013, Grotterød *et al*. 2010) (Figures 1.9 and 1.10).

RAGE is a membrane spanning protein of the immunoglobulin superfamily. Although its basal expression is low in most tissue types, it can be upregulated as a cellular response to pathogenic environments such as inflammatory conditions (Yan *et al*. 2009). Increased expression of RAGE and some of its ligands has been found in atherosclerotic lesions from diabetic subjects who died suddenly from cardiovascular complications (Burke *et al*. 2004). Furthermore, multiple studies indicate that RAGE is expressed at low levels in the human kidney in physiological conditions, but that its expression is increased in kidney failure-related diseases, including diabetes (Suzuki *et al*. 2006).

RAGE are well-established interaction partners of S100A4 (Chaabane *et al.* 2015). In addition to inducing smooth muscle proliferation in atherosclerosis (Chaabane *et al.* 2015), it has been reported that binding of extracellular S100A4 to these receptors increases the migratory and invasive capabilities of colorectal cancer cells via activation of MAPK/ERK and NF-κB, and via hypoxia signalling through upregulation of hypoxia-inducible factor 1α (HIF-1α) (Dahlmann *et al*. 2014) (Figure 1.10).

Extracellular S100A4 signalling activates several major proinflammatory pathways, including the MAPKs p38 and ERK1/2 (Novitskaya *et al.* 2000, Cerezo *et al.* 2014). This

triggers leukocyte migration and recruitment during immune responses, inducing a self-amplifying pro-inflammatory cycle through the upregulation of several pro-inflammatory cytokines (IL1β, IL6, and TNFα), acute phase reactants, granulocyte colony-stimulating factors, and well-known inflammation-associated S100 family members including S100A8 and S100A9, thereby establishing an inflammatory milieu (Cerezo *et al.* 2011).

Extracellular S100A4 also triggers the activation of another major proinflammatory transcription factor, namely NF-κB (Grotterød *et al.* 2010, Yammani *et al.* 2006). Interestingly, both NF-κB (Bond *et al.* 2001) and MAPKs ERK1/2 (Boyd *et al.* 2005) are key transcriptional regulators of several MMPs (Borghaei *et al*. 2009), and consequently possible mediators of S100A4-induced stimulation of cell migration and metastasis (Figure 1.10).

The underlying mechanisms of S100A4-mediated activation of MAPKs and NF-κB are not completely understood. In chondrocytes, these signalling events depend on interaction with RAGE (Yammani *et al.* 2006), whereas S100A4-induced signalling in primary neurons and endothelial cells seems RAGE-independent (Kiryushko *et al*. 2006). It has been widely demonstrated that extracellular S100A4 specifically activates NF-κB in human cancer cell lines through the classical NF-κB activation pathway (Boye *et al.* 2008, Kim *et al.* 2017) to promote cell migration and metastasis. Although little is known about the role of S100A4 in the activation of the inflammatory processes mediated by NF-κB in many autoimmune diseases, fibrosis, and other disorders, it has been recently proposed that S100A4 constitutes a link between cancer-related metastasis and inflammation (Ambartsumian *et al.* 2016). Accordingly, a link between T2D and cancer, mediated through inflammation, is proposed in the final chapter of this thesis (chapter 6).

b) Intracellular S100A4

Intracellular S100A4 was firstly identified in tumour cells, and accordingly, extensive evidence shows that an upregulation in S100A4 intracellular levels correlates with increased tumour cell motility (Takenaga *et al.* 2006, Tsukamoto *et al.* 2013). Besides tumour cells, intracellular S100A4 is expressed in normal cells and tissues, including fibroblasts and cells of the immune system (Li *et al.* 2010). For instance, it has been seen to be expressed in astrocytes, where its levels increase after injury, inducing astrocyte migration and repair responses (Takenaga *et al.* 2006). Importantly, a model of S100A4 (-/-) mice shows impaired recruitment of macrophages to inflammation sites in vivo,

whereas macrophages derived from these mice showed defective chemotaxis in vitro (Li *et al.* 2010). Overall, these findings indicate that intracellular S100A4 plays a major role in conferring migratory capacity to cells, mainly to non-metastatic tumour cells during an epithelial to mesenchymal transition, as well as to cells of the immune system including lymphocytes, neutrophils and macrophages during the progress of the immune response (Garrett *et al.* 2006).

One of the mechanisms through which this intracellular S100A4 upregulation is thought to take place is through TGF-β-induced expression (Matsuura *et al.* 2010). Secretion of proinflammatory cytokines and other factors such as TGF-β by activated immune cells and fibroblasts signal through the SMAD2/SMAD3 pathway to induce expression of intracellular S100A4, which is then able to interact with cytoskeleton–associated target molecules such as acto-myosin filaments, tropomyosin or non-muscle myosin heavy chain IIA (NMIIA) (Figure 2). This interaction destabilises the myosin II assembly, promoting its dissociation and remodelling, ultimately resulting in enhanced migration (Dulyaninova *et al.* 2005). In the case of fibroblasts and immune cells, this enhanced migration allows subsequent infiltration into the affected regions, inducing the release of inflammatory factors and thereby contributing to the aggravation of pathological processes (Helfman *et al.* 2005). Additionally, intracellular S100A4 is also able to bind to p53. This interaction inhibits p53 phosphorylation and subsequent activation, thereby modulating transcription of cell cycle-regulating genes, and consequently stimulating apoptosis (Garrett *et al.* 2006). Importantly, intracellular interactions of S100A4 with the mentioned cytoskeleton target molecules as well as with p53 are Ca^{2+}-dependent, thus linking the cellular functions of these proteins with changes in intracellular Ca^{2+} concentrations and consequently with the energetic status of the cells (Garrett *et al.* 2006).

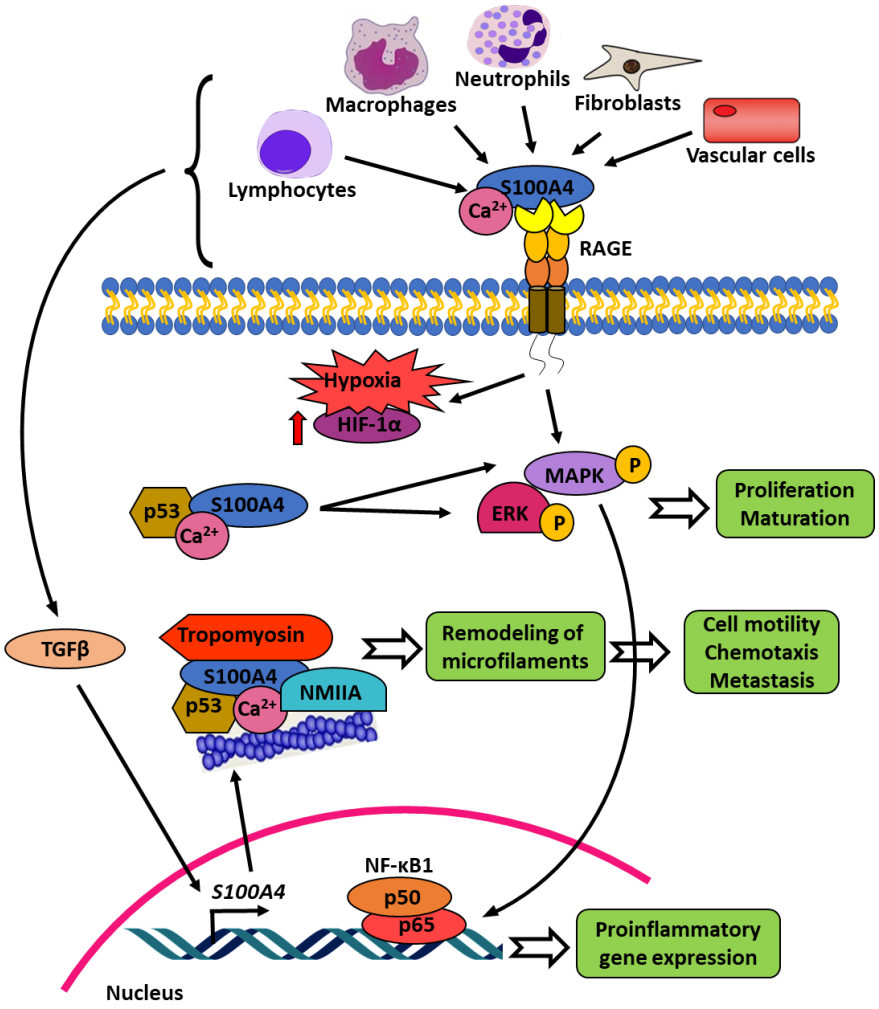


Figure 1.10: Intracellular and RAGE-mediated extracellular S100A4 signalling. Extracellular S100A4, released from fibroblasts, macrophages, lymphocytes, neutrophils, vascular cells, and other bone marrow derived cells, signals through RAGE, leading to increased phosphorylation of MAPKs and subsequent activation of NF-κB, inducing expression of pro-inflammatory genes. On the other hand, TGFβ secreted from immune cells induces intracellular expression of *S100A4*, which can combine with numerous target molecules, such as NMIIA, tropomyosin, p53, and actin, to form complexes that facilitate the remodelling of microtubes and microfilaments to enhance cell motility and chemotaxis, contributing to the infiltration of fibroblasts, immune cells and vascular cells into the affected region. In addition, binding of intracellular S100A4 to p53 promotes cell proliferation and collagen expression via MAPK activation and phosphorylation of ERK. Finally, extracellular S100A4 signalling through RAGE can also activate hypoxia signalling through upregulation of HIF-1α. Abbreviations: ERK extracellular signalling-related kinase; HIF-1α hypoxia inducible factor 1α; MAPK mitogen-activated protein kinases; NF-κB nuclear factor κB; NMIIA non-muscle myosin heavy chain IIA; RAGE receptor for advanced glycation end products; TGFβ transforming growth factor β.

1.5.2.2 S100A4 in disease

a) S100A4 in cancer

Cancer is a significant worldwide health problem in both economically developing and developed countries (Torre *et al*. 2015), and its burden is expected to further increase due to growing and aging global populations, especially in developing countries. Environmental factors such as increased pollution, together with unhealthy lifestyles such as tobacco smoking, alcohol consumption, unhealthy diet and lack of exercise amongst others further increase the risk of developing cancer. There were 14.1 million new cancer cases and 8.2 million cancer deaths worldwide in 2012 (Torre *et al.* 2015). In the last years, the number of cancer cases and deaths have increased worldwide, making it the second leading cause of death behind cardiovascular disease. To date, strategies for cancer prevention have been effective against only a relatively small proportion of human cancers through vaccination, early intervention, or changes in the lifestyle. However, most human cancers continue to develop, progress, and metastasize due to the absence of effective management strategies (Torre *et al.* 2015).

Cancer metastasis is a multi-step process in which a number of proteins have been identified to be involved at the molecular level (Jin *et al.* 2017), including S100A4 (Garrett *et al.* 2006). S100A4 has gained increasing attention for its role in the development of different types of cancers (Hou *et al.* 2018). It has been well established that S100A4, secreted from both tumour and non-malignant cells, plays an important role in the regulation of angiogenesis, cell migration and inflammation (O'Connell *et al.* 2011, Dahlmann *et al*. 2016). It was first shown to be associated with tumour metastasis in 1989 (Ebralidze *et al.* 1989), and later, it was found that transfection of *S100A4* could enhance the tumorigenic potential and induce the metastatic phenotype *in vivo* (Davies *et al.* 1994).

Overexpression of S100A4 as an indicator of poor prognosis and high metastatic potential was first proposed in human breast cancer in the year 2000 (Rudland *et al*. 2000). Since then, S100A4 has been found to be overexpressed in many cancers, which has been strongly associated with poor prognosis of many tumour types including brain, medullar thyroid, breast, colorectal, ovarian, liver, prostate, pancreatic, bladder, lung, oesophageal, gallbladder, and gastric cancers as well as in osteosarcoma, leukaemia and malignant melanoma (Gongoll *et al.* 2002, Taylor *et al.* 2002, Gross *et al*. 2014, Chen *et al*. 2014, Bresnick *et al*. 2015, Zakaria *et al*. 2016). Detection of S100A4 expression becomes

therefore a promising candidate biomarker in cancer early diagnosis and prediction of cancer metastasis (Fei *et al*. 2017).

As described earlier, intracellular S100A4 expressed by cancer cells, fibroblasts and immune cells, forms covalent interactions with target molecules including actin, NMIIA and tropomyosin (Fei *et al.* 2017), all of which are associated with cell migration, metastasis and tumour cell spread (Tarabykina *et al*. 2007, Li *et al.* 2010). Other S100A4-binding target proteins include p53, methionine aminopeptidase 2, and liprin-β1, although only a few of them have been confirmed *in vivo* (Bresnick *et al*. 2015). Amongst them, it has been reported that S100A4 binds to p53 both *in vitro* and *in vivo,* resulting in inhibition of p53 phosphorylation and consequently, in decreased p53 activation, and that this leads to apoptosis induction, loss of p53 function in tumours and a more aggressive phenotype during tumour progression (Grigorian *et al.* 2001).

As an extracellular protein, S100A4 released from tumour and/or stromal cells can alter the tumour microenvironment by stimulating angiogenesis and attracting immune cells to the growing tumour lesions (Ambartsumian *et al*. 2001), as well as by promoting the secretion of various cytokines and growth factors. Importantly, studies using breast adenocarcinoma and cervical carcinoma cell lines (Mueller *et al*. 1999) have shown that extracellular S100A4 can signal through RAGE to induce nuclear translocation of intracellular S100A4, which can also act as a transcription factor for various genes, including those encoding adherence junction proteins, and thereby regulating cell motility (Hsieh *et al.* 2004).

S100A4 has been described as a metastasis-inducing but not a tumour-initiating oncogene, as it was observed that it did not influence tumorigenesis in *S100A4*-transgenic mice, but could promote metastasis when overexpressed in the primary tumour (Ambartsumian *et al*. 1996). After carrying out experiments with transgenic mice, it was also suggested that *S100A4* needs to couple with an oncogene in order to induce cancer (Davies *et al*. 1996). The proposed mechanism by which S100A4 promotes metastasis in many cancer types is via epithelial-to-mesenchymal transition (EMT), a complex molecular process involving a change in cell morphology and function in which cells acquire fibroblastic phenotype and stem cell features (Smith *et al*. 2016). TGFβ, a key triggering factor of the EMT process, induces upregulation of *S100A4* through the activation of the SMAD pathway (Matsuura *et al.* 2010), decreasing expression of

epithelial cell markers and increasing expression of mesenchymal cell markers (Ning *et al.* 2018).

S100A4 was initially described to promote EMT through downregulation of the cell-adhesion molecule E-cadherin (Keirsebilck *et al*. 1998). Since then, other mechanisms of S100A4-induced activation of EMT have been described in different types of cancer: for example, in colorectal cancer, S100A4-induced EMT is mediated by TGFβ-induced activation of PI3K/PKB/mTOR/ribosomal protein S6 kinase beta 1 (p70S6K) signalling pathway (Wang *et al*. 2014); in pancreatic cancer, it is mediated by the sonic hedgehog-gli1 (SHH-GLI1) signalling pathway (Xu *et al.* 2014); in gallbladder cancer, overexpression of c-Myc and MMP14 induces loss of E-cadherin expression followed by an increase in S100A4 expression (Kohya *et al*. 2003); in prostate cancer, NF-κB-dependent transcriptional activation of MMP9 induces S100A4-mediated cell invasion and malignant phenotypes (Saleem *et al*. 2006); In osteosarcoma, S100A4-induced tumour invasion and metastasis is also mediated via the dysregulation of MMPs and the expression of tissue inhibitors of metalloproteinases (TIMPs) (Bjørnland *et al.* 1999); finally, in leukaemia, preferentially expressed antigen of melanoma (PRAME) suppresses heat shock protein HSP27 and S100A4 expression, inducing cell apoptosis and inhibiting cell proliferation and tumorigenicity (Tajeddine *et al.* 2008).

b) S100A4 in non-tumour pathophysiologies

Even though S100A4 is best known for its significant role in promoting cancer progression and metastasis, upregulation of *S100A4* expression has also been associated with various non-tumour pathophysiological processes such as tissue fibrosis, inflammation, immune reaction, neuroprotection and cardiovascular events (Fei *et al*. 2017), although the underlying mechanisms remain unclear.

Numerous studies indicate that the S100A4-mediated EMT plays a vital role in the occurrence and development of both tumour and non-tumour pathophysiologies. The EMT process can be categorized into three subtypes depending on the phenotype of the output cells (Kalluri *et al*. 2009). Type I EMT (non-pathological tissue development) involves the transition of primordial epithelial cells into motile mesenchymal cells that eventually form the basic body of gastrulation, and which are then re-induced as secondary epithelial cells; type II EMT (pathological conditions) involves the transition of secondary epithelial or endothelial cells to resident or inflammation-induced fibroblasts in response to persistent inflammation and fibrosis; finally, type III EMT is part of the

metastatic process by which epithelial carcinoma cells in their primary nodule form migrate to a distant site via blood circulation to reform as a secondary tumour nodule (Zeisberg *et al*. 2009).

S100A4 regulates tissue fibrosis associated with type II EMT via various mechanisms. The initiation of *S100A4* expression in epithelial cells that have undergone EMT promotes the generation of ECM components such as collagen, elastin, and other proteins, providing a basis for the onset of tissue fibrosis (Okada *et al*. 2017). Furthermore, TGFβ-induced *S100A4* expression stimulates fibroblasts to secrete fibronectin, contributing to the formation of a pro-inflammatory niche (Tomcik *et al*. 2015). S100A4 is therefore considered to be a specific fibroblast maker, and thus is frequently used to monitor or predict the mechanism of tissue fibrosis (Smith *et al.* 2016). Simultaneously, extracellular S100A4 secreted in response to inflammatory cytokines signals through RAGE, promoting the recruitment and chemotaxis of macrophages, neutrophils, and leukocytes via the activation of the MAPK and NF-κB pathways (Grotterød *et al*. 2010), thereby activating a self-amplifying pro-inflammatory cycle by upregulating several pro-inflammatory cytokines, including IL1β, IL6, and TNFα, and thus regulating inflammation and immune functions (Li *et al*. 2010, Zhou *et al*. 2015). S100A4 also participates in angiogenesis, thus inducing its metastasis-promoting mechanisms via interaction with annexin 2 and stimulation of MMP production (Schmidt-Hansen *et al.* 2004, Semov *et al*. 2005).

Interestingly, it has been reported that certain members of the S100 protein family have obesity-facilitating properties. For instance, S100B promotes obesity by impairing insulin sensitivity (Fujiya *et al.* 2014), while overexpression of S100A16 can enhance adipogenesis in 3T3-L1 preadipocytes (Liu *et al.* 2011). S100A4 expression is also known to be involved in the pathogenesis of several autoimmune diseases and other inflammatory conditions such as rheumatoid arthritis, systemic sclerosis, psoriasis (Klingelhöfer *et al*. 2007), diabetic retinopathy (Abu El-Asrar *et al*. 2014) and inflammatory myopathies (Cerezo *et al*. 2011). Given that S100A4 white adipose tissue (WAT) expression has been reported to associate positively with expression of genes involved in inflammation and immune cell activation, as well as with those involved in ECM formation, organization and migration (Arner *et al*. 2018), and given that obesity has been described to be a state of low-grade chronic inflammation (Castro *et al.* 2017), it is reasonable to hypothesise that S100A4 may also have obesity-facilitating properties.

Adipokine secretion from WAT has been linked to WAT dysfunction and metabolic complications of obesity. Fat cell size is potentially an important factor linking dysfunctional WAT to metabolic disease (Laforest *et al.* 2015). Irrespective of body fat level, WAT can be composed of many small fat cells (hyperplasia) or few but large fat cells (hypertrophy), with the latter being closely associated with IR, risk of T2D and other metabolic abnormalities (Arner *et al*. 2010). The correlation between S100A4 expression and fat cell size suggests that S100A4 may be a marker of WAT hypertrophy, which could in turn explain its association with IR (Arner *et al.* 2018). S100A4 could therefore be classified as a novel adipokine that associates with a pernicious adipose phenotype, including adipocyte hypertrophy and increased expression and secretion of proinflammatory factors (Arner *et al.* 2018).

The known association between S100A4 and cancer, together with the proposed association between S100A4 and obesity, raises the possibility that it may at least in part be involved in linking obesity/IR with cancer. It is known that in cancer cells, WNT/β-catenin signalling increases *S100A4* gene transcription, leading to an increase in tumour progression and invasiveness (Stein *et al.* 2006a). Conversely, the inhibition of this pathway attenuates S100A4 mRNA levels, and hence cell migration and invasion (Stein *et al.* 2011). Other factors known to be involved in regulating S100A4 expression in different cancer cell types are C/EBPβ (Aguilar-Morante *et al.* 2015), c-Myb (Liu *et al.* 2014) and SHH (Xu *et al.* 2014).

Whilst the pathways involved in the transcriptional regulation of S100A4 in different AT cells are yet to be fully elucidated, given the commonality between obesity/IR and WAT dysfunction to both T2D and certain types of cancer, it is tempting to speculate involvement of S100A4 in both. Indeed, the auto-inflammatory component of T2D is, in part, associated with the excessive AT proliferation that causes hypoxia in AT (Gonzalez *et al.* 2018). Rapid AT expansion causes a decrease in oxygen availability, exposing cells to hypoxia. This will result in activation of HIF-1α, a transcription factor that activates transcription of several apoptosis-related genes, as well as other factors including S100A4. Given that HIF-1α can also participate in the ROS response that results from hyperglycaemia in diabetes, this may therefore represent a unifying molecular mechanism in diabetes (this is described in more detail in section 1.6 and Figure 1.11).

1.5.2.3 S100A4 therapeutics

S100A4 has proven to be a valuable biomarker and therapeutic target for many types of cancer. As a biomarker, the detection of S100A4 levels in tumour tissues or in body fluids could predict prognosis and metastasis of cancer patients in the early stages, whereas as a target, the inhibition of *S100A4* expression can decrease metastasis *in vivo* (Hernández *et al.* 2013). Several molecular targetting strategies for S100A4 have been developed. The use of these new techniques has made it possible to discover, for instance, the exact atomic structure of the interaction between intracellular S100A4 and NMIIA (Kiss *et al.* 2012). However, there is a currently unmet clinical need to develop new therapeutic agents that function to modulate S100A4 expression and activity.

S100A4 expression is intimately associated with the proliferation, aggressive phenotype and metastatic behaviour of numerous types of human cancers, and is an indicator of poor outcome of cancer patients. Therefore, targetting S100A4 expression or biological function may provide a novel approach to fight metastatic cancer, improve prognosis and increase survival rates of cancer patients, as well as to combat non-tumour pathophysiological processes such as tissue fibrosis, inflammation, immune reaction, neuroprotection and cardiovascular disease.

Applicable therapies to decrease the S100A4-mediated metastatic potential include inhibition of S100A4 expression using miRNA-, siRNA- or shRNA-based knockdown of S100A4, the use of neutralizing antibodies, or the use of specific small molecule inhibitors. Furthermore, it was reported in 1996 that ribozyme-based knockdown of S100A4 successfully decreased the S100A4-mediated osteosarcoma metastatic phenotype (Maelandsmo *et al.* 1996). More recently, it has been seen that shRNA-mediated knockdown of *S100A4* decreases metastasis formation in colorectal cancer *in vivo* (Dahlmann *et al.* 2012), and that siRNA-mediated S100A4 knockdown significantly decreased proliferation, induced apoptosis and inhibited the invasive potential of anaplastic thyroid cancer cells *in vitro*, and abdominal cavity metastasis and tumour growth *in vivo* (Zhang *et al*. 2016).

Moreover, miR-3189-3p mimics have been seen to intensify the effects of S100A4 siRNA on the inhibition of proliferation and migration of gastric cancer cells (Bian *et al.* 2018). S100A4 neutralising antibodies have been shown to decrease tumour metastasis and to inhibit T-cell migration in pre-metastatic lungs, (Grum-Schwensen *et al.* 2015) and in

mouse models of breast cancer (Klingelhöfer *et al.* 2012), as well as to block pancreatic tumour growth in immunocompromised mice *vivo* (Hernández *et al.* 2013).

As mentioned earlier, transcription of *S100A4* is controlled by the WNT/β-catenin pathway (Dahlmann *et al*. 2016), therefore, compounds that block the formation of the β-catenin complex or promote its degradation such as calcimycin (Sack *et al.* 2011), niclosamide (Sack *et al.* 2011), or sulindac (Dahlmann *et al*. 2016) will be able to inhibit *S100A4* transcription. In fact, it has been seen that *in vitro* treatment of colorectal cancer cells with niclosamide, an antihelminthic agent, lowers WNT-dependent *S100A4* expression and subsequently inhibits tumour cell migration, invasion, proliferation and colony formation (Sack *et al.* 2011). Importantly, the use of niclosamide as a therapeutic agent against S100A4 is not only restricted to anti-cancer therapies, as it has also been seen to decrease NADPH oxidase, mTOR and NF-κB activity in murine activated primary microglia, a model or neuroinflammation (Serrano *et al.* 2019). Sorafenib, an inhibitor of numerous kinases, have also been reported to downregulate expression of *S100A4* and block osteosarcoma progression and metastasis *in vitro* by targeting the Raf/MEK/ERK pathway (Walter *et al.* 2014). Finally, treatment with interferon-gamma (IFNγ) has also been seen to downregulate *S100A4* mRNA in osteosarcoma, breast, and colon carcinoma cells (Andersen *et al.* 2003).

1.6 Hypoxia and T2D

The term hypoxia is defined as the failure of the tissues, for any reason, to receive an adequate supply of oxygen (Cafaro 1960). Oxygen concentration is closely associated with cellular proliferation, division and survival, and is generally maintained by homeostatic mechanisms operating at the cellular and tissue levels (Taylor *et al*. 2017, Fratantonio *et al.* 2018).

Hypoxia is a characteristic of both physiological and pathological immunological niches. Some examples of the former are the bone marrow, lymphoid tissue, placenta and intestinal mucosa, where physiological hypoxia controls innate and adaptive immunity by modulating immune cell proliferation, development, and effector function, largely via transcriptional changes driven by hypoxia-inducible factors (HIFs) (Taylor *et al.* 2017). Conversely, in pathological immunological niches such as tumours and chronically

inflamed, infected or ischaemic tissues, pathological hypoxia can drive tissue dysfunction and disease development through immune cell dysregulation (Taylor *et al*. 2017).

a) Hypoxia-induced adipose tissue dysfunction

As mentioned above, hypoxia is the deprivation of oxygen from tissues. This is a common issue found in obese patients, particularly in AT, and is thought to be one of the mechanisms by which pro-inflammatory adipose signalling is initiated and maintained (Hodson *et al*. 2013). The association between hypoxia and obesity involves an hypertrophic process by which adipose cell size increases up to 140–180μm, which is more than the 100μm diffusion distance of oxygen, resulting in a diminished oxygen supply to this tissue (Jang *et al*. 2013).

The activation of the hypoxic response in AT also involves the build-up of ECM, which brings inflammatory cells and overall dysregulation to AT (Sun *et al.* 2012). Importantly, one of the key indicators of obesity-related hypoxia in AT is the release of adipokines that cause widespread systemic inflammation (Tkacova *et al*. 2013). A study shows that inhibition of HIF-1 signalling in AT of HFD-fed mice resulted in improvements in obesity and IR, and that these improvements were associated with the induction of adiponectin, an anti-inflammatory adipokine secreted by AT, and with the subsequent increase in insulin sensitivity (Jiang *et al.* 2011).

b) Hypoxia-induced pancreatic dysfunction

Cellular oxygen consumption is a determinant factor of intracellular oxygen levels. Because of the increased mitochondrial respiration under high glucose conditions, pancreatic β-cells consume large amounts of oxygen in a short time, inducing a hypoxic state (Sato *et al.* 2011). Accordingly, pancreatic islets of diabetic mice, but not those of control mice, showed moderate levels of hypoxia through higher expression of HIF-1α transcription factor and its target genes, which was accompanied by a selective downregulation of *MAFA* and *PDX1*, amongst others, all of which play important roles in β-cell function (Sato *et al*. 2014). Consistent with the altered expression of these genes, abnormal insulin secretion and increased apoptosis was detected in hypoxic mouse pancreatic MIN6 cells. In addition, hypoxia also inhibits the adaptive unfolded protein response in β-cells through activation of JNK and DNA-damage inducible transcript 3 (DDIT3), a process associated with impaired ER-to-Golgi protein trafficking and subsequent increased apoptosis (Bensellam *et al.* 2016). Importantly, hypoxia has recently been seen to activate the NLRP3 inflammasome and NF-κB signalling in LPS-

primed mouse MIN6 cells, a process mediated through upregulation of ROS and thioredoxin-interacting protein (TXNIP) (Chen *et al.* 2018). These results suggest that hypoxia is a novel stressor of β-cells and that hypoxic stress plays a key role in the deterioration of β-cell function.

1.6.1 HIF-1α and T2D

Hypoxia is a hallmark of inflamed, infected or damaged tissue. The adaptation to insufficient tissue oxygenation is regulated by HIFs, which are the key mediators of the cellular response to hypoxia. However, these factors are also associated with pathological conditions such as inflammation, bacteriological infection or cancer. In addition, HIFs are central regulators of many innate and adaptive immunological responses, including migration, antigen presentation, production of cytokines and antimicrobial peptides, phagocytosis, and cellular metabolic reprogramming (Krzywinska *et al.* 2018).

HIF is a heterodimeric transcription factor composed of two subunits, an oxygen sensitive HIF-α subunit and a constitutively expressed HIF-β subunit. H1F1 (histone cluster 1, H1a) is part of the HIF family, and is a key controlling element in many hypoxic responses. In normoxic conditions, the oxygen-dependent prolyl hydroxylases (PHDs) hydroxylate HIF-1α, priming it for poly-ubiquitination by the von Hippel-Lindau (VHL) tumour suppressor complex, which leads to its proteasomal degradation (Franke *et al.* 2013). In situations of hypoxia however, PHDs are inactivated, therefore HIF-1α is stabilized. It is then able to translocate to the nucleus, where it binds to its heterodimerization partner HIF-1β and other co-factors, and together they induce transcription of target genes involved in a broad range of physiological functions including angiogenesis, erythropoiesis, metabolism, autophagy and apoptosis (Biddlestone *et al.* 2015), such as erythropoietin (*EPO*), *VEGF*, *GLUT* and *PAI1* (Fakhruddin *et al*. 2017) by binding to hypoxia responsive elements (HRE) in their promoter region (Figure 1.11). Increased levels of these factors are considered to aggravate ECM deposition and induce recruitment of inflammatory cells, bringing overall dysregulation to AT (Sun *et al*. 2012). In AT, HIF-1α also blocks adipocyte secretion of anti-inflammatory adipokines such as adiponectin, contributing to the formation of a hypoxia-induced inflammatory milieu as well as contributing to insulin resistance. Furthermore, since HIF-1α is also capable of increasing transcription of profibrotic genes, it can greatly contribute to the pathogenesis of some diabetic-

associated complications such as renal fibrosis (Haase 2012). Importantly, in addition to hypoxia, other factors found to be upregulated in diabetes such as angiotensin II, TGFβ, PKC, and ROS, can also activate HIF-1α even in nonhypoxic conditions (Jarad *et al.* 2009) (Figure 1.11).

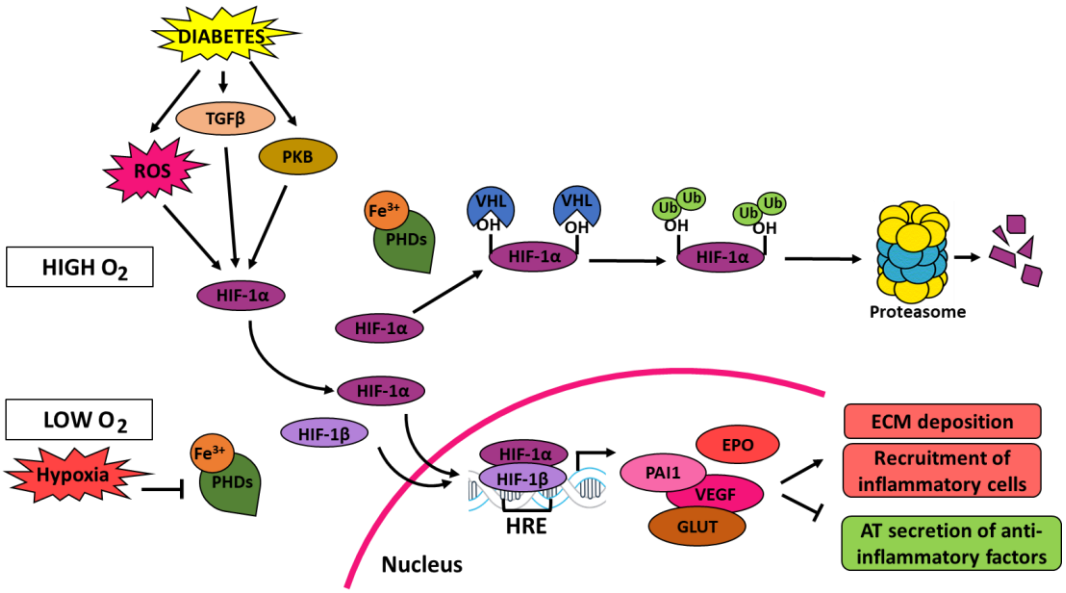


Figure 1.11: Oxygen-dependent and inflammatory factors-mediated regulation of HIF-1α. In normoxic conditions, PHDs hydroxylate HIF-1α, priming it for poly-ubiquitination by VHL, which leads to its proteasomal degradation. In situations of hypoxia however, PHDs are inactivated, therefore HIF-1α is stabilized and translocates to the nucleus, where it binds to HIF-1β and other co-factors. Together, they bind to HRE in the promoter region of target genes including EPO, PAI1, VEGF and GLUT. Increased levels of these factors aggravate ECM deposition and induce recruitment of inflammatory cells. In AT, HIF-1α also blocks adipocyte secretion of anti-inflammatory factors. In addition to hypoxia, other factors found to be upregulated in diabetes such as TGFβ, PKC, and ROS, can also activate HIF-1α even in nonhypoxic conditions. Abbreviations: EPO erythropoietin; GLUT glucose transporter; HIF-1α hypoxia inducible factor 1α; HRE hypoxia responsive elements; PAI1 plasminogen activator inhibitor 1; PHDs prolyl hydroxylases; PKB protein kinase B; ROS reactive oxygen species; TGFβ transforming growth factor β; VEGF vascular endothelial growth factor; VHL von Hippel-Lindau ubiquitin-ligase.

1.6.2 HIF-1α and S100A4

As mentioned in section 1.5.2.2, available knowledge on the role of S100A4 in cancer is much more extensive than on its role in diabetes. Interestingly, S100A4 has been found to be upregulated in gastric cancer cells after being exposed to hypoxia (Zhang *et al.*

2010). Moreover, recent evidence shows that hypoxia-induced *S100A4* expression promotes hepatocellular carcinoma metastasis and EMT formation (Dou *et al*. 2016). It has been seen that exposure of several different types of tumour cells to hypoxia increases hypomethylation of the first intron of the *S100A4* gene, facilitating the binding of HIF-1α to HRE in the *S100A4* promoter region, thereby increasing *S100A4* expression and consequently inducing tumour cell invasiveness and metastasis (Liu *et al.* 2010, Horiuchi *et al.* 2012, Fei *et al.* 2017), showing that *S100A4* is a target gene for HIF-1α regulation.

Even though the effect of hypoxia and HIF-1α on *S100A4* expression in the diabetic panorama has not been fully explored yet, all the evidence linking inflammation and cancer with diabetes suggests that HIF-1α-induced *S100A4* expression might also play an important role in the pathogenesis of diabetes.

1.7 Transcriptome profiling and bioinformatic analysis

The transcriptome is the collection of transcripts in a given living organism and represents the link between the information encoded in the DNA and the phenotype. mRNA regulation depends on the action of a combination of cis-acting proteins that bind to gene flanking regions. (Licatalosi *et al*. 2010).

The transcriptome has for long been considered to primarily consist of ribosomal RNA (rRNA, 80-90%), transfer RNA (tRNA, 5-15%), messenger RNA (mRNA 2-4%) and a small fraction of intra- and intergenic noncoding RNA (ncRNA, 1%) with undefined regulatory functions (Lindberg *et al.* 2010). However, recent evidence has shown that the amount of noncoding genetic material increases with organism complexity, ranging from 0.25% in prokaryotic species to 98.8% in humans (Taft *et al.* 2007). Additional complexity is added by the fact that most of the transcripts identified so far are cell- and tissue-specific and can vary depending on various environmental factors such as in response to growth factors or other stimuli. Transcriptome analysis will therefore open the doors towards a more complete knowledge of many biological issues such as the onset and progression of disease.

The main goal of transcriptome analysis is to identify, characterise and register all the transcripts expressed within a specific cell/tissue and in a given condition, but more

importantly, it can also allow the quantification of the differential expression of transcripts in both physio- and pathological conditions, which can constitute the molecular basis of a disease (Costa *et al.* 2010).

Tools for analysing RNA such as Northern Blot or reverse transcription PCR (RT-PCR) have been available for years. However, rapid and high throughput analysis of the transcriptome became possible only after the development of more advanced techniques such as gene expression microarrays (detailed in section 1.7.1). These techniques offer the possibility to perform a complete transcriptional characterisation of all organisms. Moreover, they allow transcriptome profiling of model of diseases both *in vitro* and *in vivo* in an unbiased and highly sensitive manner, without previous knowledge of the genes involved, offering crucial information for the study of the pathogenesis of a wide variety of diseases.

1.7.1 Microarrays vs RNA sequencing (RNAseq)

a) Microarrays

Expression microarrays are high throughput techniques that allow simultaneous measurement of the expression of a set of genes. The starting point is an array of short oligonucleotide probes complementary to the transcripts whose presence is to be investigated, and immobilised on a solid substrate. Probe design is based on genome sequence and usually multiple probes are designed per gene. Transcripts are extracted from cells or tissues, labelled with fluorescent dyes, hybridised to the arrays, washed and scanned with a laser. Probes that correspond to transcribed RNA hybridise to their complementary target, and measurement of light intensity is used to quantify gene expression (Malone *et al*. 2011).

However, this technique has some limitations. Firstly, it needs to rely on existing knowledge of genome sequence, so it cannot be used to identify novel genes. Secondly, it shows lack of reproducibility within microarrays designed by different companies, and it has been seen that the use of different laser scanners introduces variation in the fluorescent readout of the same samples (Tan *et al*. 2003). More importantly, because of background noise and cross-hybridization, microarrays have difficulty detecting genes

with low expression levels, and thus cannot distinguish ‘‘no’’ from ‘‘low’’ expression (Zhao *et al*. 2014).

Nonetheless, microarrays have been successfully and extensively used for the identification of differentially expressed genes in different conditions. Accordingly, several studies have investigated the effect of β-cell exposure to high concentrations of glucose and have identified clusters of genes differentially expressed at specific glucose concentrations (Bensellam *et al.* 2009).

b) RNA sequencing

The development of high throughput next-generation sequencing has revolutionized transcriptomics by enabling RNA analysis through the sequencing of complementary DNA (cDNA) (Wang *et al*. 2009). This method, termed RNA sequencing (RNAseq), has important advantages over previous approaches and has revolutionized our understanding of the complex and dynamic nature of the transcriptome. Rather than using molecular hybridisation to detect transcript molecules of interest, RNAseq analyses transcripts present in the starting material by direct sequencing.

After sequencing, the resulting reads are either aligned to a reference genome or assembled *de novo* without the genomic sequence to produce a genome-scale transcription map including both the transcriptional structure and expression level for each gene (Wang *et al*. 2009).

c) RNAseq vs microarray

The use of RNAseq presents some advantages over the use of microarrays. Because RNAseq provides direct access to the sequence, this technique can be used to study species for which a full genome sequence is not available. Moreover, it allows detection of expressed regions of the genome that correspond to novel genes, not currently identified. Another strength of RNAseq is the possibility of quantifying individual transcript isoforms and single nucleotide variants (SNV) which is highly important as genetic polymorphism has been shown to be vital in the identification of defective genes associated with inherited diseases (Zhao *et al*. 2014). Moreover, RNAseq has very low, if any, background signal because cDNA sequences can be uniquely mapped to single regions of the genome (Wang *et al*. 2009). Finally, RNAseq shows a higher sensitivity in direct measurement of low abundant transcripts, as well as in detection of changes in expression of these transcripts under different conditions (Zhao *et al*. 2014).

However, RNAseq also presents several disadvantages compared to microarrays, including their higher cost. Another important concern about this technique is the depth to which sequencing needs to be performed (how many times a single sample must be sequenced) in order to effectively sample the transcriptome. For highly expressed genes, small amounts of sequencing are sufficient, but for genes with medium and low expression levels, many reads are necessary. Another consideration about arrays and sequencing is the quantity and size of the data. In expression arrays, raw data consists of image files that may be around 30MB per array. These are then transformed into text files containing the fluorescence intensities for each gene. For RNAseq however, the Illumina instrument generates more than 600GB of data files, so far more complex bioinformatic tools are necessary to analyse the data output (Zhao *et al.* 2014).

Importantly, RNAseq has been used to characterise the β-cell transcriptome, allowing the identification of β-cell specific genes, splicing events and intergenic RNA that could play an important role in the regulation of β-cell function (Ku *et al*. 2012). In addition, this technique has also allowed identification of long noncoding RNAs in human pancreatic islets, some of which were found to be dysregulated in diabetes (Morán *et al*. 2012). Recently, several single-cell RNAseq studies have also been used to generate transcriptional profiles of β-cells from human pancreas of healthy and T2D individuals, identifying alterations in gene expression in T2D, as well as identifying novel genes that had not been previously associated with T2D (Segerstolpe *et al.* 2016, Xin *et al.* 2016). Furthermore, another study investigated the effects of FA exposure on the β-cell, revealing that PA changed the expression of 1325 genes in human islets, (in particular linked to ER stress, ubiquitin and proteasome function, autophagy and apoptosis); inhibited transcription factors involved in the regulation of the β-cell phenotype (including PAX4 and GATA6); and shifted alternative splicing of 3525 transcripts (Cnop *et al*. 2014).

1.8 Aims

1.8.1 Analyse the RNAseq data to identify key factors upregulated in glucolipotoxicity

The main aim of this project is to use the RNAseq data obtained previously in our laboratory (Bagnati *et al.* 2016) to identify differentially expressed genes resulting from the combined effect of high glucose and high fatty acid (FAs) exposure of rat INS-1 pancreatic β-cells. Furthermore, Metacore and other bioinformatic software will be used to construct specific pathway interaction maps between the most differentially expressed genes in glucolipotoxic (GLT) conditions compared to control. It is hypothesised that significant links between these upregulated genes will arise, which will give us insight into the underlying mechanisms involved in the pathogenesis of T2D.

1.8.2 Validate the RNAseq data for differential expression of selected genes

In order to validate the differential expression of several selected genes from the RNAseq data, INS-1 cells will be incubated in control and GLT conditions for 72 hours, and subsequently, changes in mRNA and protein expression levels of selected genes will be observed through qPCR and western blot. Together with the bioinformatic analysis results, it is hypothesised that this will allow identification of key factors driving GLT-induced damage to β-pancreatic cells, including S100A4, making it the main focus of this project.

1.8.3 Investigate a potential link between S100A4 and inflammation through NF-κB activation

After validating the increased expression of selected genes, including S100A4, in GLT conditions compared to control, the next step will be to establish a link between S100A4 upregulation and NF-κB activity, and consequently associate it with the activation of inflammatory pathways. To this end, the GLT-induced increase in NF-κB protein expression, nuclear localisation and concomitant transcriptional activity will be measured

through western blot, indirect immunofluorescence and transcription factor activity assay respectively. After this, siRNA transient knockdown technology will be used on INS-1 cells to determine the effect that S100A4 has on NF-κB protein expression, nuclear localisation, and transcriptional activity. It is hypothesised that S100A4 upregulation will ultimately drive an increase in NF-κB activity, and that S100A4 knockdown will reverse this effect.

1.8.4 Identify interaction partners of S100A4

With the use of the pathway interaction map obtained through RNAseq data bioinformatic analysis, potential interacting partners of S100A4 will be identified. siRNA technology will be used on INS-1 cells to transiently knockdown one of these interacting partners, namely HIF-1α, and its effect on S100A4 and NF-κB will be assessed. Further analysis of the pathway interaction map will allow the identification of additional factors linked to HIF-1α transcriptional activity. Their role in the GLT-induced NF-κB activation process will also be tested with the use of siRNA technology and subsequent measurement of its effects on HIF-1α, S100A4 and NF-κB. It is hypothesised that suppression of this newly identified factor, namely the KPNA2 importin, which is potentially responsible for HIF-1α nuclear translocation, will decrease HIF-1α transcriptional activity, and consequently S100A4 expression and NF-κB activation.

1.8.5 Establish a link between T2D and cancer through S100A4 and inflammation

The PC3 prostate cancer cell line will be used throughout the project parallelly to INS-1 cells and subjected to the same experiments and treatments (DFO-induced hypoxia). The aim of this last part of the thesis is to observe and compare the effects of hypoxia and subsequent activation of HIF-1α transcriptional activity, S100A4 expression and NF-κB-induced inflammatory pathways activation in cancer cells to its effects on pancreatic β-cells. Given the well-established role of S100A4 on cancer initiation and progression, concordant results on INS-1 cells will strengthen our main hypothesis, by which S100A4 will be in part responsible for the initiation of the inflammatory process characteristic of T2D.

CHAPTER 2: Materials and Methods

2.1 Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and all plasticware purchased from Thermo Fisher (Waltham, MA, USA) unless otherwise stated.

The INS-1 cell line was obtained from Philippe Halban's group, who made the initial characterisation of the cell line in 1993 (Neerman-Arbez *et al.* 1993).

The PC3 cell line was obtained from the John Van Geest Cancer Research Centre (Nottingham Trent University), who originally obtained it from ATCC.

2.2 Solutions

Table 2.1: Name and composition of solutions used throughout the project.

SOLUTION	COMPOSITION
Roswell Park Memorial Institute complete media (RPMI complete media)	RPMI-1640 (#R7755) plus 26mM sodium bicarbonate ($NaHCO_3$, #S5761), 10mM Hepes (#H7006), 50µM β-mercaptoethanol (#M6250), 1% v/v sodium pyruvate ($C_3H_3NaO_3$, Thermo Fisher #11360039), 1% v/v penicillin/streptomycin (Thermo Fisher #10378016), 10% v/v fetal bovine serum (FBS, Thermo Fisher #16000044)
100mM oleic acid stock solution	0.0304g oleic acid (OA, #O7501), 0.5ml ethanol, 0.5ml dH_2O (final ethanol concentration in media: 0,001% v/v)*
100mM palmitic acid stock solution	0.0255g palmitic acid (PA, #P0500), 1ml ethanol (final ethanol concentration in media: 0,002% v/v)*
Glucolipotoxic media (GLT media)	RPMI complete media (composition detailed above) plus 16mM glucose (#G7021), 200µM OA (from stock detailed above), 200µM PA (from stock detailed above), 2% w/v bovine serum albumin (BSA, #A4303)
Dulbecco's Modified Eagle Medium (DMEM)	DMEM (Thermo Fisher #12491-015) plus 15mM Hepes, 10% v/v FBS, 1% v/v penicillin/streptomycin/L-glutamine (Thermo Fisher #10378016)
100mM deferoxamine stock solution	0.066g deferoxamine (DFO, #D9533), 1ml dH_2O
10mM MG132 stock solution	0.0048g MG132 (#M8699), 1ml dH_2O
Radioimmunoprecipitation assay buffer (RIPA buffer)	150mM sodium chloride (NaCl, #S5886), 0.1% Triton X-100 (#X100), 0.5% sodium deoxycholate ($C_{24}H_{39}NaO_4$, #D6750), 0.1% sodium dodecyl sulfate (SDS, #L3771), 50mM Tris-base (#RDD008), pH 8.0 + protease inhibitor tablet (Roche, #5892970001)

Protein loading buffer	950µl 4X Laemmli buffer (Bio-Rad #1610747), 50µl β-mercaptoethanol
Lower buffer	1.5M Tris-base, pH 8.8
Upper buffer	0.5M Tris-base, pH 6.8
Running Buffer	250mM Tris-base, 1.92M glycine (#G8898), 35mM SDS, pH 8.3
Transfer buffer	60% 5X Trans Blot Turbo transfer solution (Bio-Rad #1704270), 20% dH_2O, 20% ethanol
Phosphate buffered saline (PBS) 10X	137mM NaCl, 2.7mM potassium chloride (KCl, #P9333), 10mM sodium phosphate dibasic (Na_2HPO_4, #S3264), 2mM potassium phosphate monobasic (KH_2PO_4, #P9791), pH 7.2
Tris-buffered saline (TBST) 10X	1.5M NaCl, 0.5M Tris-base, 0.05% Tween-20 (#P1379), pH 7.6
TAE buffer 10X	400mM Tris-acetate (#T1258), 10mM Ethylenediamine tetra-acetic acid (EDTA, Thermo Fisher #AM9912), pH 8.2
Paraformaldehyde solution, (PFA)	4% w/v PFA (#441244), 1% v/v sodium hydroxide (NaOH, #71687)

*Final ethanol concentration in the culture media are practically negligible so its presence will have no effect on cell viability.

2.3 Cell culture

2.3.1 Cell lines

a) INS-1

The main cell line used in this project is the pancreatic β-cell line INS-1, derived from a rat insulinoma by x-ray irradiation. INS-1 cells display important characteristics of the pancreatic β-cell, including high insulin content and responsiveness to glucose within a physiological range. However, despite being responsive to glucose, the total amount of insulin content in the proliferative cells is only about 20% of that of primary β-cells (Skelin *et al*. 2010). The reason for using this cells line is based on its display of β-cell characteristics. Other insulinoma cell line commonly used in β-cell research is RINm5F. However, this cell line has been reported to display abnormal glucose transport and glucose sensitivity (Halban *et al*. 1983), making it unsuitable for our research.

b) PC3

The second cell line used in this project is the cell line known as PC3. It is an androgen-independent human prostate cancer cell line derived from bone metastasis (Kaighn *et al*. 1979). It is widely used in prostate cancer research, and in this project, it is used for its

high expression of S100A4, and as a positive control for the role of S100A4 in the activation of NF-κB-mediated inflammation.

2.3.2 Growth conditions and cell passaging

a) INS-1

INS-1 cells were cultured in RPMI-1640 media containing 11mM D-glucose and supplemented with 10mM HEPES, 26mM sodium bicarbonate and 50µM β-mercaptoethanol, and adjusted to pH 7.4. The complete media was then supplemented with 10% v/v FBS, 1% v/v sodium pyruvate and 1% v/v Penicillin/Streptomycin. Cells were cultured in T75 flasks and incubated at 37°C and 5% CO_2.

b) PC3

PC3 cells were cultured in DMEM media containing 25mM D-glucose and supplemented with 15mM HEPES, 10% v/v FBS and 1% v/v Penicillin/Streptomycin/L-Glutamine. Cells were cultured in T75 flasks and incubated at 37°C and 5% CO_2.

Passaging of both cell lines was conducted when 80-85% confluent. The media was first aspirated, and the adherent cells were washed twice with 5ml of sterile phosphate buffered saline (PBS). The cells were detached by adding 2ml of trypsin-EDTA 0.05% (Thermo Fisher, MA, USA, #25300062) in the case of INS-1 cells, or 2ml of diluted in PBS (1:10) 10X trypsin (#T4549) in the case of PC3 cells, and incubated at 37°C for 5 minutes. Cells were harvested in 10 ml media (complete RPMI for INS-1 cells or DMEM for PC3 cells) and centrifuged at 400 x g for 5 minutes. The supernatant was discarded, and the pellet resuspended in 10ml media. A fraction of the suspension containing 1,000,000 cells was added to a new T75 flask containing 15ml of fresh media for cell maintenance, and either 200,000 cells were added to each well of a 6-well plate containing 2ml of fresh media, or 100,000 cells were added to each well of a 12-well plate containing 1ml of fresh media for experiments set-up. Cell counting methodology is described below.

It is worth mentioning that, even though the glucose concentrations used here are much higher than the normal physiological glucose concentrations (4.0-6.0mM for humans), they are the established glucose concentrations used in rodent models of diabetes, for which 5.0 to 11.0mM is physiological, while 20.0-25.0mM is considered pathological

(Martino *et al.* 2012). Regarding FFA concentrations, 200µM would not be considered pathological in humans (physiological levels range from 200µM to 4mM). However, in rodents, anything above 100µM is considered pathological (Martino *et al.* 2012).

2.3.3 Cell counting

In order to seed the cells in flasks or in 6 or 12 well plates, cells were counted manually. After centrifugation and resuspension in fresh media (described above), 10µl of cell suspension was added to a Neubauer chamber (Figure 2.1). Cells in the four 1mm x 1mm squares were counted, and the average calculated, resulting in the number of cells present in 0.1mm^3, or 0.1µl. This number was then multiplied by 10,000 to obtain the number of cells per ml.

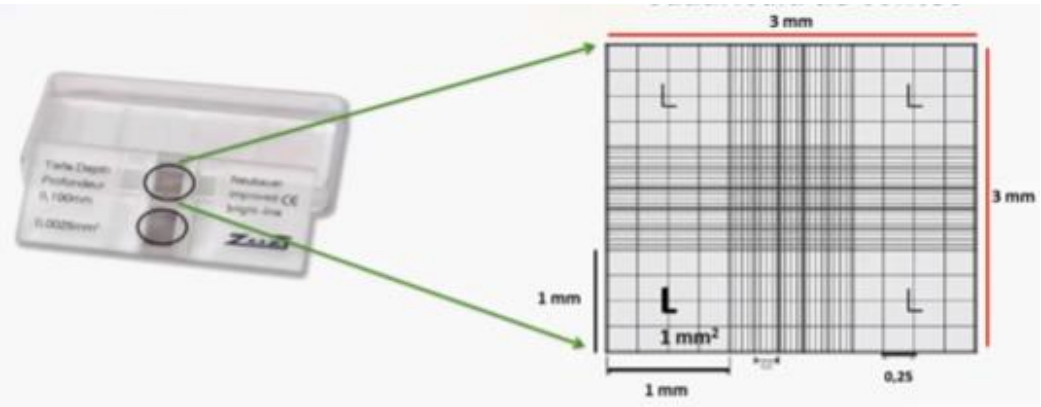


Figure 2.1: Schematic representation of a Neubauer chamber for cell counting. Cells in the four 1mm x 1mm squares were counted, the average calculated, and the result multiplied by 10,000 to obtain the number of cells per ml.

2.3.4 Glucolipotoxic treatment of INS-1 cells

In order to replicate the extracellular glucolipotoxic (GLT) milieu characteristic of T2D, the RPMI-1640 media was supplemented with 16mM D-glucose (to achieve a final concentration of 27mM), 200µM sodium oleate (oleic acid) and 200µM palmitic acid (Bagnati M *et al.* 2016). To do this, a 100mM stock solution of each fatty acid was prepared as described in Table 2.1. 200µM of each fatty acid were first conjugated to 2% BSA, which was then used to supplement the RPMI-1640 media together with the additional glucose. This media was then incubated in a water bath at 37°C for 45 minutes to allow the fatty acids to conjugate to the BSA. Finally, the media was filtered before adding it to the INS-1

cells 24 hours after being seeded, which were then incubated in GLT media for a further 72 hours before harvesting them for subsequent experiments.

2.3.5 Hypoxic treatment of INS-1 and PC3 cells

In order to mimic the hypoxic state characteristic of the proinflammatory environment during obesity-induced T2D (in the case of INS-1 cells) and the hypoxic state characteristic of growing tumours (in the case of PC3 cells), DFO (final concentration 100µM) was added to the cells 24 hours after being seeded (a 100mM stock was prepared by dissolving it in dH_2O, aliquoted in 1-time use aliquots and frozen at -20ºC). Cells were then incubated for 72 hours to match the GLT treatment timeframe.

2.3.6 Cryo-conservation and cell recovery

Both INS-1 and PC3 cells were collected and cyro-conserved when they reached 80-85% confluency. The media was aspirated, and cells were washed twice with sterile PBS, detached by adding trypsin-EDTA and incubated at 37°C for 5 minutes. The cells were harvested in their respective media and centrifuged at 400 x g for 5 minutes. The supernatant was aspirated, and the pellet was resuspended in 10ml of their respective media. Cells were counted as described above and then centrifuged again at 400 x g for 5 minutes, and finally resuspended in the required volume of synth-a-freeze (Thermo Fisher, MA, USA, #A1254201) in order to obtain a concentration of 1,000,000 cells per ml. 1ml of cell suspension was then transferred to each cryovial, which were stored in a Mr. Frosty container (Thermo Fisher, MA, USA, #5100-0001) at -80°C for 48 hours and then transferred into liquid nitrogen for long term storage.

When needed, cryo-vials were taken out of the liquid nitrogen tank and rapidly defrosted in a water bath at 37ºC. The suspension of cells was then added drop by drop to a T75 flask containing 15ml of fresh pre-warmed media and the flask was incubated at 37ºC and 5% CO_2 until ready to passage again. A few passages were allowed after defrosting before setting up any experiments with the cells.

2.3.7 Mycoplasma detection

INS-1 and PC3 cells were regularly checked for mycoplasma infection using an EZ-PCR mycoplasma test kit (Biological Industries, CT, USA, #20-700-20) following manufacturer's instructions. Briefly, a sample of cell culture supernatant was used to sediment possible mycoplasma, after which a PCR was carried out to amplify any possible mycoplasma present in the sample. The PCR product was then analysed through agarose gel DNA electrophoresis, and the gel visualized using GeneSnap software (Syngene, Bangalore, India).

No positive mycoplasma results were obtained in the cell cultures used for the experiments described in this thesis.

2.4 Protein analysis

2.4.1 Cell lysis

As mentioned before, 200,000 cells were seeded into 6-well plates and incubated in the appropriate conditions (control, glucolipotoxic or hypoxic). Following the incubation period (72 hours), cells were washed in cold PBS and lysed in 200µl RIPA buffer (Table 2.1) containing a 1x protease inhibitor tablet per 10ml RIPA buffer (Roche Applied Sciences, Basel, Switzerland, #5892970001) over ice. Cell scrapers were used to harvest cells from the surface of the plates and disrupt cellular integrity. Lysates were transferred to 1.5ml Eppendorf tubes, kept on ice for 40 minutes and vortexed for 1 minute every 5 minutes. The tubes were then centrifuged at 16,000 x g for 10 minutes at 4ºC to pellet cell debris and the supernatant containing the protein lysate was transferred to a clean 1.5ml Eppendorf tube and stored at -20ºC until used for subsequent analysis, or at -80ºC for long term storage.

2.4.2 Bicinchoninic acid (BCA) assay

Protein concentration of cell lysates was measured using Pierce™ BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher, MA, USA, #23225). This is a colorimetric assay technique that involves two steps: first, a biuret reaction results in faint blue colour from the reduction of cupric ion to cuprous ion. Second, the chelation of BCA with the cuprous ion results in an intense purple colour. The BCA/copper complex is water-soluble and exhibits

a strong linear absorbance at 562nm with increasing protein concentrations. The purple colour can be measured at any wavelength between 540nm and 590nm. The BCA assay has a broad dynamic range, being capable of measuring protein concentrations of 0.5µg/ml to 1.5mg/ml in a linear manner. A BSA stock (2mg/ml) from the kit was used to make serial dilutions for protein standards preparation according to manufacturer's instructions, with concentrations of 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.025 and 0mg/ml, in order to construct a standard calibration curve for protein quantification.

To conduct the assay, a 10µl aliquot of the protein sample (diluted in RIPA buffer if necessary if protein concentration is too high) or BSA standard was added to a 96-well plate, to which 200µl of combined BCA assay kit reagent was added (49 parts reagent A + 1 part reagent B). The plate was incubated at 37ºC for 30 minutes, after which absorbance at 595nm was determined using an iMark™ Microplate Absorbance Reader (Bio-Rad, CA, USA), and the sample's protein concentration calculated from the standard calibration curve.

2.4.3 Bradford assay

In order to determine nuclear protein concentration for subsequent NF-κB activity assay (detailed in section 2.8), the Pierce™ BCA Protein Assay Kit could not be used, as the nuclear extraction buffer (NEB) from the Nuclear/Cytosol Fractionation kit (Biovision, CA, USA, #K266) used to dissolve the nuclear extract contains Dithiothreitol (DTT), a reducing agent which binds to the Cu^{2+} ions present in the BCA mixture, therefore artificially contributing to the signal. Instead, Bradford reagent was used.

The Bradford method is a dye-based assay in which the dye, Coomassie blue, binds to the protein present in the sample. The interaction causes the dye to shift from its reddish/brown form (absorbance maximum at 465nm) to its blue form (absorbance maximum at 610nm). The difference between the two forms of the dye is greatest at 595nm, so that is the optimal wavelength to measure the dye-protein complex. The Bradford assay is linear over a short range, typically from 0mg/mL to 2mg/mL, often making dilutions of a sample necessary before analysis. A 0.15M solution of NaCl was used to make serial dilutions of a 10mg/ml BSA stock in order to create a standard curve for protein determination, with concentrations of 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.025 and 0mg/ml.

To conduct the assay, a 20µl aliquot of the nuclear protein extract (diluted in NEB if necessary if protein concentration is too high) or BSA standard is added to a 96-well plate, to which 200µl of Bradford reagent is added. The plate incubated at room temperature for 2 minutes, time in which a blue-coloured product is formed. Absorbance at 595nm was measured within 5 minutes using an iMark™ Microplate Absorbance Reader (Bio-Rad, CA, USA), and the sample's protein concentration calculated from the standard calibration curve.

2.4.4 SDS-PAGE

Acrylamide/bisacrylamide (#A3574) gels were hand poured using the Bio-Rad system. Different resolving gel percentages were used depending on the size of the protein of interest: higher percentage gels for detection of low molecular weight protein (10-30KDa) and lower percentage gels for detection of high molecular weight proteins (90-130KDa). Firstly, a resolving gel was prepared as shown in Table 2.2 and poured between two glass plates 1mm apart, with a layer of 70% ethanol poured on top to displace any air bubbles. The gel was allowed to polymerise for 30 minutes at room temperature. After polymerising, the ethanol was discarded by tilting the clamp stand holding the glass plates and a stacking gel was prepared as shown in Table 2.2. The mix was then poured on top of the resolving gel with a ten teeth comb in place to form the wells and the gel was allowed to polymerise for another 30 minutes.

Table 2.2: Recipes for different percentages of acrylamide/bisacrylamide gels used in western blotting. Volumes are calculated for 2 gels.

	Resolving				**Stacking**
Components	**7.5%**	**10%**	**15%**	**20%**	**4%**
H_2O	4.85 ml	4.0 ml	2.35 ml	720 µl	3.1 ml
30% Acrylamide/ Bisacrylamide (29:1)	2.5 ml	3.33 ml	5.0 ml	6.68 ml	650 µl
Tris-Base 1.5M	2.5 ml	2.5 ml	2.5 ml	2.5 ml	-
Tris-Base 0.5M	-	-	-	-	1.25 ml
APS (10%) (#A7460)	50 µl	50 µl	50 µl	50 µl	25 µl
SDS (20%)	50 µl	50 µl	50 µl	50 µl	25 µl
TEMED (#T9281)	7.5 µl	7.5 µl	7.5 µl	7.5 µl	5 µl

The prepared gels were placed into electrophoretic tanks and these were submerged in running buffer (Table 2.1) and allowed to equilibrate for 15 minutes at room temperature. Between 20-30µg protein was denatured by adding 4x Laemmli loading buffer containing β-

mercaptoethanol and being incubated at 95°C for 5 minutes. The combs were removed from the stacking gel and the denatured protein was loaded on the gel alongside 5µl of a molecular weight marker (Bio-Rad, CA, USA, #1610374). The gel was electrophoresed at a constant voltage of 90V for 120 minutes. Subsequently, proteins were transferred to a 0.2µm pore nitrocellulose membrane (Bio-Rad, CA, USA, #1620112) using Bio-Rad transfer buffer (Table 2.1) and a Bio-Rad Trans-Blot Turbo Transfer unit, using a semi-dry transfer program either for mixed (7 minutes), high (10 minutes) or low (5 minutes) molecular weight proteins. Successful transfer was confirmed using Ponceau S solution (#P7170) before immunoblotting. Briefly, the membrane was incubated in 5 ml of Ponceau solution for one minute, removed and gently washed three times with TBST until bands were visible.

2.4.5 Immunoblotting

Nitrocellulose membranes were blocked in 5% BSA/TBST for 1 hour at room temperature in a rocking platform to prevent non-specific binding. The nitrocellulose membrane was incubated with the primary antibody diluted at the required concentration (Table 2.3) in 5% BSA/TBST overnight at 4°C on a tube roller. Membranes were washed in TBST three times for 10 minutes before being incubated in the appropriate secondary antibody (anti-mouse IgG, anti-rabbit IgG or anti-chicken IgG) diluted at the required concentration (see Table 2.3) in 5% BSA/TBST for 1 hour at room temperature and gentle rocking. The membrane was then washed three times in TBST for 10 minutes prior to visualization. The membrane was removed from the incubating tray and 2ml of ECL solution (Amersham, GE Healthcare, IL, USA, #RPN2235) was added to the membrane, which was then incubated for 2 minutes in the dark. Specific protein bands were detected by chemiluminescence using the Image Reader LAS 4000 (Fujifilm).

Table 2.3: Details and concentrations for the different primary and secondary antibodies used in western blotting.

Type	Antibody	Company	Catalogue number	Source	Concen-tration	Speci-ficity
1ary	β-Actin	Cell Signaling	8H10D10	Mouse	1:1000	h, m, r
	α-Tubulin	Cell Signaling	3873	Mouse	1:4000	h, m, r
	S100A3	Thermo Fisher	PA5-39372	Rabbit	1:1000	h, m, r
	S100A4	Sigma	HPA007973	Rabbit	1:250	h, m, r
	S100A5	Biorbyt	orb128695	Rabbit	1:1000	h, m, r
	NF-κB	Thermo Fisher	PA5-16545	Rabbit	1:200	h, m, r
	HIF-1α	Thermo Fisher	PA1-16601	Rabbit	1:1000	h, m, r
	HIF-1α	Thermo Fisher	H1alpha67	Mouse	1:500	h, m, r
	KPNA2	Abcam	ab37628	Chicken	1:2000	h, m, r
	H2A	Abcam	ab18975	Rabbit	1:1000	h, m, r
2ary	α-mouse IgG	Bio-Rad	172-1011	Goat	1:2000	-
	α-rabbit IgG	Bio-Rad	170-6515	Goat	1:5000	-
	α-chicken IgG	Abcam	ab6753	Rabbit	1:5000	-

2.4.6 Band densitometry

In order to measure and quantify the levels of the protein of interest present in the sample, bands densitometry was quantified using Aida Image Analyzer (Elysia-Raytest) and normalized to actin levels. In brief, the program was used to draw circles around the bands corresponding to the protein of interest, which were assigned a densitometry value proportional to the intensity of the band. The same procedure was carried out for the actin bands, and the value obtained was used to normalise the levels of the protein of interest.

2.4.7 Indirect immunofluorescence

Another technique for measuring protein levels and changes in expression, as well as changes in subcellular localisation is indirect immunofluorescence. 200,000 or 100,000 cells were seeded on sterile glass coverslips in 6 or 12-well plates respectively, and 24 hours later, the appropriate conditions were applied for 72 hours. After the incubation period, cells were washed with cold PBS and fixed with 4% paraformaldehyde in PBS (Table 2.1) for 10 minutes at room temperature. Cells were washed three times for 10 minutes with PBS (all the washes from this point were done in these same conditions) and

permeabilized with 0.2% Triton X-100 in PBS for 2-5 minutes at room temperature. Cells were washed and then blocked with 5% BSA/PBS for 1 hour at room temperature in a rocking platform to prevent non-specific binding. The primary antibody was then added to the cells in blocking solution at the required concentration (Table 2.4) and cells were incubated for 1 hour at room temperature in a rocking platform. Cells were then washed before being incubated with the appropriate Alexa fluor 488 conjugated secondary antibody: anti-mouse IgG (Thermo Fisher, MA, USA), anti-rabbit IgG (Thermo Fisher, MA, USA) or anti-chicken IgG (Abcam, Cambridge, UK) (Table 2.4) in the dark for 1 hour at room temperature in a rocking platform. Cells were then washed again before being incubated in Hoechst 33258 solution (Thermo Fisher, MA, USA, #H1398) at 0.5µg/ml for 5 minutes at room temperature in the dark and in a rocking platform. Cells were then washed one last time and coverslips were placed facing down on slides with a drop of mounting solution (90% glycerol in PBS). Slides were allowed to dry at room temperature for 1 hour in the dark before being stored at 4ºC.

Table 2.4: Details and concentrations for the different primary and secondary antibodies used in immunofluorescence.

Type	Antibody	Company	Catalogue number	Source	Concen-tration	Specificity
1ary	S100A4	Sigma	HPA007973	Rabbit	1:250	h, m, r
	NF-κB	Thermo Fisher	PA5-16545	Rabbit	1:100	h, m, r
	HIF-1α	Thermo Fisher	H1alpha67	Mouse	1:50	h, m, r
	KPNA2	Abcam	ab37628	Chicken	1:250	h, m, r
	488 α-mouse IgG	Thermo Fisher	A32766	Donkey	1:1000	h, m, r
Fluorescent 2ary	488 α-rabbit IgG	Thermo Fisher	A21206	Donkey	1:1000	-
	488 α-chicken IgG	Abcam	ab150169	Goat	1:1000	-

2.5 RNA analysis

2.5.1 Primer design and sequence information

Primers for real time qPCR were designed using a combination of NCBI gene search, Primer Quest Tool (Integrated DNA Technologies, IA, USA) and NCBI primer blast. First, NCBI gene search was used to obtain specific mRNA sequences for the genes and species of interest. Then, this mRNA sequence was input into Primer Quest Tool, which was used to obtain potential primer pairs for each gene. Finally, each potential pair was input into NCBI primer blast to test the specificity of each pair. For each gene, a pair of primers was chosen which did not amplify any other unwanted transcript. Specific forward and reverse primers were designed 18-25bp long, with a GC content no higher than 55% and a melting temperature of 57-62°C, to obtain a 100-140bp amplicon. Primers were purchased from Sigma-Aldrich and tested using PCR (detailed in section 2.5.2). Sequences of all the primers used can be found in Table 2.5.

Table 2.5: Details of primers used for quantitative PCR.

Species	Target gene	Sequence (F)	Tm (F)	Sequence (R)	Tm (R)	Product size
Rat	*GAPDH*	CATCTCCCTCAC AATTCCATCC	58.5	GAGGGTGCAGCGAAC TTTAT	58.3	100
	S100A3	CCGGGAGTGTG ACTACAATAAA	57.8	TTTGAAGTACTCGTG GCAGTAG	58.1	124
	S100A4	AACCTCTCTGTT CAGCACTTC	57.9	GTGGAAGGTGGACAC TATTACAT	57.9	102
	S100A5	GTCCTCTCTCTT CCTTCAGAGTA	58.2	GCTACCCTCTCTCCCT GAATA	57.8	107
	HIF-1α	GGTGGATATGT CTGGGTTGAG	57.8	AGGGAGAAAATCAAG TCGTGC	58.6	128
	RELA	CAGATACCACTA AGACGCACC	58.2	TCCTTCCCCACAAGTT CATG	57.4	127
Human	*GAPDH*	CGGTGACTAAC CCTGCGCT	62.3	GCCCAATACGACCAA ATCAGAGAAT	61.4	140
	S100A4	CTCTACAACCCT CTCTCCTCA	57.6	AAGGTGGACACCATC ACATC	57.5	105

2.5.2 Primer validation

A Taq-polymerase master mix (Qiagen, Hilden, Germany, #201203) was prepared containing both forward and reverse primers and the sample cDNA. This mix was put into the thermo cycler (see Tables 2.8 and 2.9 for reaction set up and amplification conditions respectively) and a PCR was conducted to check for single product amplification. PCR products were then electrophoresed in a 2% agarose gel (method described in section 2.3.7) at 70V for 30 minutes. The gel was then visualized using GeneSnap software (Syngene, Bangalore, India).

Table 2.6: PCR reactions setup for primer check.

Reagent	Volume per reaction (µl)
Taq PCR Master Mix	10
FP 10µM	0.8
RP 10µM	0.8
cDNA (25ng)	0.33
dH_2O up to 20µl	8.07

Table 2.7: Cycling conditions for primer check PCR.

Phase	Temperature (ºC)	Time	Cycles
Initial denaturation	94	3 min	1
Denaturation	94	45 secs	35
Annealing	61.5	45 secs	
Extension	72	30 secs	
Final extension	72	10 min	1

2.5.3 Sample preparation

INS-1 and PC3 cells were seeded and treated in control, glucolipotoxic or hypoxic conditions in either 6-well plates or T75 flasks. Following the treatment incubation period, cells were detached using trypsin-EDTA. The cells were then centrifuged at 400 x g for 5 minutes to form a pellet. 1ml of sterile PBS was used to resuspend the pellet and a maximum of 5 x 10^5 cells were transferred to a new 1.5ml tube, which was centrifuged at 400 x g for 5 minutes again to collect cells for RNA extraction.

2.5.4 RNA extraction

To extract RNA from INS-1 and PC3 cells, a RNeasy® Micro Kit (Qiagen, Hilden, Germany, #74004) was used. Cells were harvested, centrifuged and counted as described before, and then a volume corresponding to 500,000 cells was collected in a 1.5ml Eppendorf tube and centrifuged again. The pellet was then homogenised in 350µl of RLT buffer (cell lysis buffer). 1 volume of 70% ethanol (350µl) was then added and mixed well by pipetting. This step promotes selective binding of RNA to the RNeasy membrane. The resulting cell suspension was transferred to a RNeasy® Mini spin column in a 2ml collection tube and centrifuged for 15 seconds at 8000 x g. The flow through was discarded. In order to remove contaminant genomic DNA from the RNA solution, a DNase incubation mix (10µl DNase I in 70µl RDD buffer) (Qiagen, Hilden, Germany, #79254) was added drop by drop directly to the spin column membrane, which was incubated at room temperature for 15 minutes. After the incubation, 350µl of RW1 buffer was added to wash the membrane-bound RNA, and the spin column was centrifuged for 15 seconds at 8000 x g. The collection tube containing the flow through was discarded. The column was placed in a new 2ml collection tube and 500µl RPE buffer was added to the column to remove traces of salts left on the column due to buffers used earlier. The column was then centrifuged at 8000 x g for 15 seconds and the flow through was discarded. 500µl of 80% ethanol was then added to the spin column to help wash away the salts, and then the column was centrifuged for 2 minutes at 8000 x g. The collection tube containing the flow through was discarded and replaced. The spin column was then centrifuged with the lid open for 5 minutes at 16,000 x g to dry the membrane. The collection tube was discarded and replaced with a 1.5ml new collection tube. 20µl RNase free water was then added to the centre of the membrane and the column was centrifuged for 1 minute at 16,000 x g to elute the RNA. This was then stored at -80ºC until used for subsequent analysis.

2.5.5 RNA quantification

RNA concentration and quality were analysed by adding 1µl of the sample to a NanoDrop spectrophotometer (Thermo Fisher, MA, USA). A conversion factor based on the extinction coefficient for RNA (A_{260} of 1.0 = 40µg/ml) was used to calculate RNA concentration, which correlates with absorbance at 260nm in a linear manner according to the Beer-Lambert law. To assess the quality and purity of the sample, measurements at 280nm (wavelength at which aromatic amino acids absorb) and 230nm (wavelength at

which other contaminants such as guanidine thiocyanate, common in nucleic acid purification kits, absorb) were also taken. The ratios A_{260}/ A_{280} and A_{260}/ A_{230} can be used to assess RNA quality and purity, where a ratio of 2 or above is indicative of a pure RNA sample.

2.5.6 cDNA synthesis

In order to carry out quantitative PCR, single stranded complementary DNA (cDNA) is needed. cDNA synthesis was carried out using the extracted RNA as starting material, using High Capacity cDNA Reverse Transcription kit (Thermo Fisher, MA, USA, #4368814). In each reaction 1.5µg of total RNA was used for reverse transcription. See Tables 2.10 and 2.11 for reaction set up and amplification conditions respectively.

Table 2.8: Reagents and volumes used for reverse transcription PCR for cDNA synthesis.

Reagent	Volume (µl)
Reverse Transcription buffer	2
dNTPs	0.8
Random Primers	2
Reverse Transcription enzyme	1
RNA	(1.5µg)
RNase free H_2O	(up to 20µl)

Table 2.9: Cycling conditions for reverse transcription PCR for cDNA synthesis.

Phase	Temperature (ºC)	Time
Primer annealing	25	10 min
DNA polymerization	37	120 min
Enzyme deactivation	85	5 min
Maintenance	4	∞

2.5.7 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) measures the amount of PCR product generated per cycle using a fluorescent label. QuantiNova SYBR® Green PCR kit (Qiagen, Hilden, Germany, #208054) was used for qPCR. During the amplification step (40 cycles long), a fluorescent dye binds to the DNA molecules and fluorescence values are recorded for each cycle. The fluorescence signal is directly proportional to the DNA concentration over a broad range, and the point at which fluorescence is first detected as statistically significant above the background is called the cycle threshold or CT value. The higher the initial amount of

sample DNA, the sooner the accumulated product is detected and therefore the lower the CT value. A negative control containing no cDNA was examined alongside each sample, therefore any signal detected would be sign of contamination. *GAPDH* was used as reference gene for target gene expression normalization. See Tables 2.12 and 2.13 for qPCR reaction set up and cycling conditions respectively.

Table 2.10: Reagents and volumes for master-mix used in RT-qPCR.

Reagent	Volume (µl)
2X SYBR green mix	10
Forward Primer (10µM)	1
Reverse Primer (10µM)	1
cDNA	0.33 (25ng)
RNase free H_2O	7.67 (up to 20µl)

Table 2.11: Cycling conditions for RT-qPCR.

Temperature (ºC)	Time	Cycles
95	5 secs	40
61.5	12 secs	
72	20 secs	

2.5.8 qPCR data analysis

The qPCR data obtained was analyzed using the $2^{-\Delta\Delta CT}$ method, a relative quantification strategy that calculates a ratio between the target and reference gene. The reference gene used throughout the qPCR experiments was *GAPDH*. A CT value obtained from computer software Rotor Gene Q series (Qiagen, Hilden, Germany) was converted into Δ_{CT} to calculate the relative fold change of a treated sample against a control sample, known as calibrator. The calibrators for the qPCR reactions carried out in this project were the control (untreated samples).

The fold change relative to the calibrator sample was calculated as follows:

1. Δ_{CT} = CT target gene – CT reference gene (normalization to housekeeping gene, *GAPDH*, to minimize sample to sample variation).
2. $\Delta\Delta_{CT}$ = Δ_{CT} sample (treated) - Δ_{CT} calibrator (untreated).
3. $2^{-\Delta\Delta CT}$ = gene fold change of treated sample relative to the calibrator sample.

2.6 RNA sequencing

RNA sequencing technology is providing new biological insight into the transcriptome. It is a high throughput sequencing method that can be used to determine gene structure (including the identification of exons and introns) and gene-splicing patterns, as well as other post transcriptional modifications. It also allows the detection of rare and novel transcripts and the quantification of any changing expression transcript levels, which means that this technique can be used to study the effect of a particular treatment on transcriptome regulation.

2.6.1 Sample and library preparation

RNA sequencing was conducted using INS-1 cells. In order to do this, cells were left untreated or were treated using GLT conditions for 72 hours. After this time, RNA was extracted and RNA quality and integrity were assessed using an Agilent Bioanalyser (Agilent, CA, USA). This device determines abundance of 18S and 28S ribosomal RNA (rRNA) to assess the integrity of RNA. 28S and 18S exist in equal concentrations in the cell, but 28S is double fluorescent. As RNA degradation occurs, there is a gradual decrease in the 18S to 28S ribosomal band ratio. Consequently, a 28S/18S ratio of 2 is considered to be good quality RNA. Only RNA meeting the above criteria was sent to Sarah Lamble laboratory in Oxford for the subsequent stages of library preparation and sequencing, using an Illumina mRNAseq Sample preparation kit (Illumina, CA, USA). To create the library, mRNA was fragmented, and fragments were converted to cDNA using reverse transcriptase and random primers.

Adapters were then ligated to the end of the cDNA fragments, allowing them to be hybridized to a single read flow cell. A quality control analysis of the library was then performed, based on the quantification of DNA concentration, using Agilent Bioanalyzer (Agilent, CA, USA).

The library was then used to perform paired-end sequencing over one lane of a flow cell on an Illumina-HiSeq 2000 instrument (Illumina, CA, USA) in Oxford. This technology relies on the attachment of randomly fragmented genomic DNA to a flat and transparent surface. Attached DNA fragments were amplified to create an ultra-high-density sequencing flow cell with millions of clusters, each containing ~1000 copies of the same

template. Finally, these templates were sequenced using four-colour DNA SBS (Ssequencing By Synthesis) technology that uses reversible terminators with removable fluorescent dyes.

2.6.2 RNAseq data analysis

The resulting raw RNAseq data was analysed by Dr. Rob Lowe (Blizard Institute, Barts and The London School of Medicine and Dentistry, London, UK). The Illumina instrument produced quality-scored base calls. The sequencing output files (compressed FASTQ files) were then used for the secondary analysis.

Reads were aligned to a reference genome using Top Hat v 2.0.9:
http://tophat.cbcb.umd.edu.

Reads aligned to exons, genes and splice junctions were quantified using the reference genome "rn4", extracted from UCSC:
http://genome.ucsc.edu/goldenPath/credits.html#rat_credits.

Data visualisation and interpretation as well as gene and transcript expression quantification were conducted using the HTseq-count program:
http://www-huber.embl.de/users/anders/HTSeq/doc/count.html.

In order to correct for in-sample distributional differences within the read counts (such as differences in total counts), and within sample gene-specific effects (such as gene length or GC-content effects), a normalization process was performed using the program DEseq:
http://www.bioconductor.org/packages/devel/bioc/html/DESeq.html

Finally, the differential expression statistical significance was calculated by comparing the experimental read values to the control samples, and p-values were subsequently adjusted using the Bonferroni formula.

2.6.3 RNAseq pathway analysis

In order to identify enriched pathways and functions between the differentially expressed genes, the RNAseq data was loaded in several pathway analysis programs, namely Panther (http://www.pantherdb.org/) and Metacore (https://clarivate.com/cortellis/).

Panther and Metacore allow the identification of enriched pathways, molecular functions, biological processes, cellular components, protein classes and associated diseases amongst a given list of genes. The programs calculate the number of genes that are

enriched within a specific pathway and give also the statistical significance of any enrichment. Enrichment is considered statistically significant when there are more genes in the list associated with a particular pathway than it would be expected by chance based on the total number of genes associated with that pathway.

2.7 Small interfering RNA (siRNA) transfection

Small interference RNA (siRNA) technology was used to transiently knockdown cellular proteins. A pool of 4 different siRNAs (Dharmacon, GE Healthcare, CO, USA) alongside a transfection reagent (chosen after an optimisation process testing three different transfection reagents, listed below) were used for each target molecule. Briefly, 100,000 INS-1 and PC3 cells were seeded into separate 12-well plates 24 hours before transfection in order to be 40-50% confluent at the time of transfection. siRNA-transfection reagent complexes were prepared for each sample to be transfected (one complex per well of the 12-well plate) as described below for each transfection reagent. Parallelly, negative controls were carried out preparing scrambled siRNA (ssRNA)-transfection reagent complexes which were added to the negative control wells to ensure that the effect of a specific siRNA knockdown is only due to its specificity and not to the addition of an exogenous nucleic acid.

2.7.1 Lipofectamine RNAiMAX

siRNA-Lipofectamine RNAiMAX (Thermo Fisher, MA, USA, #13778030) complexes were prepared as follows: In a 1.5ml sterile tube, 2µl of siRNA (10µM) were diluted in 50µl of serum-free Optimem media (Invitrogen, CA, USA, #51985-026) and mixed gently. In a separate 1.5ml sterile tube, 3µl of Lipofectamine RNAiMAX were diluted in 50µl of serum-free Optimem media and mixed gently. The two solutions were combined, and the complex was incubated at room temperature for 5 minutes. Media was removed from the cells and replaced with 900µl of complete RPMI-1640 media. Subsequently, the 100µl of the transfection solution prepared earlier was added. Following a 24 hours incubation period, the transfection solution was removed and 1ml of either complete RPMI-1640 (control conditions), GLT media (glucolipotoxic conditions) or complete media + DFO (hypoxic conditions) was added to the cells and was incubated for 72 hours. Subsequent validation of knockdown was carried out using western blot.

2.7.2 TransIT-X2

siRNA-TransIT-X2 (Mirus Bio, WI, USA, #MIR6003) complexes were prepared as follows: In a 1.5ml sterile tube, 2.5µl of siRNA (10µM) and 3µl of TransIT-X2 were diluted in 100µl of serum-free Optimem media and mixed gently. The complex was incubated at room temperature for 20 minutes. Media was removed from the cells and replaced with 900µl of complete RPMI-1640 media. Subsequently, the 100µl of the transfection solution prepared earlier was added. Following a 24 hours incubation period, the transfection solution was removed and 1ml of either complete RPMI-1640 (control conditions), GLT media (glucolipotoxic conditions) or complete media + DFO (hypoxic conditions) was added to the cells and incubated for 72 hours. Subsequent validation of knockdown was carried out using western blot.

2.7.3 INTERFERin

siRNA-INTERFERin (Polyplus, Illkirch-Graffenstaden, France) complexes were prepared as follows: In a 1.5ml sterile tube, 2µl of siRNA (10µM) and 4µl of INTERFERin were diluted in 200µl of serum-free Optimem media (Invitrogen, CA, USA) and mixed gently. The complex was incubated at room temperature for 10 minutes. Media was removed from the cells and replaced with 1ml of complete RPMI-1640 media. Subsequently, the 200µl of the transfection solution prepared earlier was added. Following a 24 hours incubation period, the transfection solution was removed and 1ml of either complete RPMI-1640 (control conditions), GLT media (glucolipotoxic conditions) or complete media + DFO (hypoxic conditions) was added to the cells and was incubated for 72 hours. Subsequent validation of knockdown was carried out using western blot.

2.8 NF-κB activity

NF-κB activity is measured in this project as it is responsible for the regulation and transcription of multiple genes involved in the mediation of immune and inflammatory responses. The kit used, the Trans-AM NF-κB p65 Transcription Factor Assay kit (Active Motif, CA, USA, #40596), detects and quantifies transcription factor activation via an ELISA assay. This assay was carried out according to manufacturer's instructions, which are described in detail below (see section 1.8.2). In essence, the active form of the

transcription factor present in our samples binds to a consensus sequence immobilized in an oligonucleotide-coated plate. A primary antibody specific for the epitope on the active form of the NF-κB transcription factor is added, followed by subsequent incubation with adequate secondary antibody. Finally, addition of developing solution triggers a colorimetric reaction which can be easily quantified.

2.8.1 Nuclear extraction

Nuclear extracts are required for the NF-κB transcription factor assay, as they contain the activated NF-κB form. Nuclear extracts were obtained using the Nuclear/Cytosol Fractionation kit according to manufacturer's instructions, as described in detail below. All buffers required for the extraction were prepared in advance according to manufacturer's instructions.

200,000 INS-1 and PC3 cells were cultured separately in 6-well plates and 24 hours later, either complete RPMI-1640 (control conditions), GLT media (glucolipotoxic conditions) or complete media + DFO (hypoxic conditions) was added for 72 hours. After the incubation period, the cells were detached from the plate surface using trypsin-EDTA and centrifuged at 400 x g for 5 minutes to form a cell pellet. This pellet was resuspended and washed in PBS and a maximum of 2 x 10^6 cells were transferred to a new tube. The suspension was centrifuged again, the supernatant discarded and 200µl of cytosol extraction buffer-A (CEB-A) was added to the pellet, which was fully resuspended by vortexing vigorously for 15 seconds. The suspension of cells was then incubated on ice for 10 minutes. Following this incubation, 11µl of ice-cold cytosol extraction buffer-B (CEB-B) was added, the tube was vortexed for 5 seconds, incubated on ice for 1 minute and then vortexed again for another 5 seconds. The tube was then centrifuged for 5 minutes at 16,000 x g at 4ºC and the supernatant (cytosolic fraction) was immediately transferred to a clean 1.5ml tube and placed on ice. The pellet containing the nuclei was resuspended in 80µl of ice-cold nuclear extraction buffer (NEB), vortexed vigorously for 15 seconds and returned to ice. This was repeated every 10 minutes for 40 minutes, after which the tube was centrifuged at 17,000 x g for 10 minutes at 4ºC. The supernatant (nuclear extract) was transferred to a clean 1.5ml tube and immediately used for nuclear protein concentration determination through Bradford assay and for NF-κB activity assay.

2.8.2 NF-κB activity assay

To measure NF-κB activity, the Trans-AM NF-κB p65 Transcription Factor Assay kit was used. All buffers (complete lysis buffer, complete binding buffer and washing buffer) and reagents were prepared according the manufacturer's instructions, either prior to the assay or prior to the step in which they were needed. In brief, 30µl of complete binding buffer was added to each well of the oligonucleotide-coated plate included in the kit. 20µl of sample containing 20µg of nuclear protein (diluted in lysis buffer if necessary) was added to the sample wells. A nuclear extract from Jurkat cells stimulated with tissue plasminogen activator (TPA) and calcium ionophore (CI) is included in the kit and is used as a positive control: 20µl of diluted Jurkat nuclear extract (1µl extract + 19µl lysis buffer) was added to the positive control wells. 20µl complete lysis buffer was added to the negative control (blank) wells. The plate was incubated at room temperature for 1 hour with gentle agitation on a plate rocker. The wells were then washed 3 times with diluted wash buffer and the plate tapped on paper towel to remove excess liquid. Subsequently, 100µl of diluted NF-κB antibody (1:1000) (included in the kit) was added to each well. The plate was incubated for 1 hour at room temperature without agitation. The wells were then washed 3 times with diluted wash buffer. 100µl of diluted HPR-conjugated secondary antibody (1:1000) (included in the kit) was then added to each well and the plate was incubated at room temperature for 1 hour without agitation. After this incubation, the wells were washed 4 times with diluted wash buffer, followed by the addition of 100µl of developing solution to each well. This was incubated for 3 minutes at room temperature in the dark before adding 100µl of stop solution to each well. Absorbance was measured at 450nm within 5 minutes using the iMark™ Microplate Absorbance Reader (Bio-Rad, CA, USA).

2.9 Statistical analysis

To determine statistical significance of results obtained in this thesis, statistical analysis using a two-tailed, unpaired T-test for analysis of two independent conditions was carried out using Microsoft Excel. A p-value of <0.05 was considered to be significant. Results are expressed as mean ± standard error of the mean (SEM). All results presented are derived from at least three independent experiments unless stated otherwise.

CHAPTER 3: Bioinformatic analysis and validation of RNAseq data

3.1 Effect of glucolipotoxicity on β-cell function and integrity

As detailed in chapter 1, T2D is a metabolic disorder with an increasing prevalence worldwide, mainly caused by the combination of chronic exposure to glucolipotoxicity and a sedentary lifestyle (Golson *et al.* 2010, Oh *et al.* 2013). Under chronically elevated concentrations of glucose and Fas, IR arises and β-cells initially respond by increasing secretion of insulin in a compensatory manner, a process which is accompanied by an increase in β-cell mass (Butler *et al.* 2003). However, this eventually leads to β-cell dysfunction, impairment of insulin secretion and production, and ultimately β-cell death, which leads to T2D (Mason *et al*. 1999, Ježek *et al*. 2018).

T2D is the most common type of diabetes, accounting for approximately 90% of all diabetic cases (Ortega *et al*. 2017). Over 80% of T2D patients are obese, with increased circulating glucose and FFA levels. The underlying molecular mechanisms for the development of T2D include ER stress, oxidative stress, lipotoxicity, and glucotoxicity, which ultimately trigger an inflammatory response (Oh 2015). Inflammation is a characteristic feature of many disease conditions that has initial beneficial effects such as encouraging tissue regeneration or preventing spread of infection, however, prolonged or chronic inflammation may aggravate a disease condition via tissue destruction. This is likely to be the case in the pathogenesis of T2D (Donath *et al*. 2009). Importantly, elevation of inflammatory markers such as cytokines and C-reactive protein (CRP) (Pereira *et al*. 2006) and other inflammatory mediators such as TNFα (Peraldi *et al*. 1996) and IL6 (Nieto-Vazquez *et al.* 2008) have been shown to be a feature of diabetes pathogenesis.

Although some of the underlying molecular mechanisms involved in β-cell dysfunction have been described, additional studies are required to discover new molecules involved in the process of activation of inflammatory pathways. These new molecules could potentially constitute novel targets for specific therapeutic strategies.

The aim of this study was to characterise the effects that chronic exposure of pancreatic β-cells to glucolipotoxicity has on inflammation-induced β-cell dysfunction. In order to do so, I focussed my research on identifying inflammatory gene expression changes caused by glucolipotoxic conditions.

Several studies associate dietary FAs with prevention or progression of non-transmissible chronic conditions such as T2D and cardiovascular diseases (Anderson *et al.* 2009). For instance, saturated FAs (SFAs) such as palmitic acid (16:0, PA), myristic acid (14:0, MA) and stearic acid (18:0, SA) have been associated with adverse health effects (Carta *et al*. 2017), while unsaturated FAs (UFAs) such as oleic acid (18:1, OA) and palmitoleic acid (16:1, PAO) are generally related to protective effects, like prevention of β-cell apoptosis, regulation of plasma glucose concentrations and enhancement of insulin sensitivity (Acosta-Montaño *et al*. 2018).

To replicate glucolipotoxic conditions, INS-1 rat pancreatic β-cells were exposed to elevated glucose and FA levels. The two FA used were palmitic acid and oleic acid (PA and OA), which are the most abundant FAs in human nutrition and therefore in blood circulation (Donath *et al*. 2009). PA is most abundant in meat and dairy products, and it can also be sourced from plants and microorganisms. It is one of the main FA involved in the lipotoxicity during T2D progression: it has been seen that prolonged exposure of β-cells to PA impairs insulin gene expression, inhibits β-cell secretory capacity and increases apoptosis (Hagman *et al*. 2005). On the other hand, OA is found mainly in animal and vegetable fats and oils. Despite there being evidence that OA has anti-apoptotic and anti-inflammatory protective properties, here it is used as a damaging agent given the increased concentration at which it is used and the chronic exposure to which cells are subjected.

More specifically, INS-1 cells were incubated for 72 hours in glucolipotoxic conditions (RPMI-1640 media supplemented to 27mM glucose, 200µM OA and 200µM PA), and in control conditions (complete RPMI-1640 media containing 11mM glucose). This compares to human models of glucolipotoxicity in which 11-20mM glucose is normally used as high glucose, and 4-6mM glucose for control conditions (Tsonkova *et al.* 2018).

Cell viability was initially assessed though observation of morphological changes. Although this is not an experimentally accepted method to do so, previous experiments carried out in our laboratory demonstrated that a 72 hour incubation of INS-1 cells in these conditions did not affect cell cycle, did not activate caspase 3 and did not induce apoptosis (data not shown). Accordingly, results in Figure 3.1 show that INS-1 cells cultured in control conditions for 72 hours display a stretched and pointed shape and grow in a single monolayer (Figure 3.1A), while a 3-day treatment with GLT induces morphological changes: cells turn more rounded in shape and tend to aggregate and grow

upwards rather than in the characteristic monolayer (Figure 3.1C). Although a 72 hour treatment with GLT did not visibly affect cell viability (cells were still attached and growing) (Figure 3.1C), a prolonged 168 hours (7 days) exposure to GLT results in β-cell death as it can be observed from the increasing number of floating cells (Figure 3.1D), while control cells remained healthy after the same incubation period (Figure 3.1B).

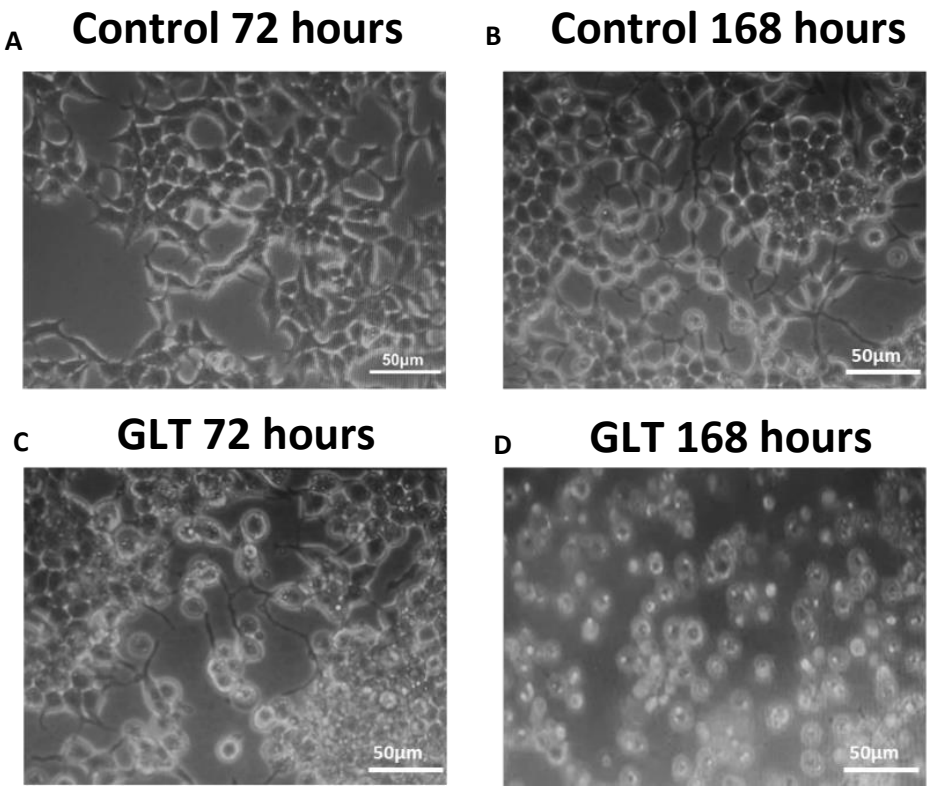


Figure 3.1: INS-1 cell morphology in control and GLT conditions for different incubation periods. Control or GLT treatment is added to the cells 24 hours after passage and experimental procedures carried out 72 hours later (A, C). Cells were left for 168 hours in both conditions to show changes in cell viability after a prolonged exposure to GLT (B, D). Scalebar 1cm:50µm.

3.2 Effect of glucolipotoxicity on β-cell gene expression

In order to gain further insight into the complex nature of diabetogenesis and to discover novel mechanisms that can affect activation of inflammatory pathways leading to β-cell dysfunction, a transcriptome profiling of the β-cell in conditions of glucolipotoxicity compared to control was performed using RNA sequencing.

3.2.1 RNA sequencing and pathway analysis

Previously in our laboratory, INS-1 cells were incubated for 72 hours in RPMI-1640 media supplemented to 27mM glucose alone (high glucose), in 200µM OA and 200µM PA alone (high fatty acids), or in the combination of both high glucose and high FAs (glucolipotoxicity), as well as in complete RPMI media for control conditions (Bagnati *et al.* 2016). Subsequently, RNA was isolated, and the samples converted to a library of cDNA fragments. High-throughput technology was then used to sequence each fragment. After sequencing, the resulting reads were aligned to a reference genome to produce a genome-scale transcription map including both the transcriptional structure and expression level for each gene (Wang *et al*. 2009), described in detail in section 2.6. Data analysis was carried out comparing three independent experiments for each condition.

Statistically significant data ($p<0.05$) was analysed for gene expression change using the Panther Classification System (http://pantherdb.org/). When genes were classified according to pathways, it was found that expression of a substantial number of genes linked to apoptosis and related processes such as oxidative stress and most notably inflammatory and immune response was affected by glucotoxic, lipotoxic and glucolipotoxic conditions (Figures 3.2, 3.3 and 3.4 respectively). As mentioned before, evidence shows that there must be increased glucose levels in order to observe adverse lipotoxic effects (Somesh *et al.* 2013). Importantly, both *in vitro* (Briaud *et al*. 2001) and *in vivo* (Briaud *et al*. 2002) studies have shown that the effect of lipotoxicity is synergistically increased in the presence of concomitantly elevated glucose levels. Accordingly, our data shows that the effect on the activation of inflammatory pathways was greater when both high glucose and high FAs were combined, inducing glucolipotoxicity (Figure 3.4). This can be most clearly observed in the figures by the increased expression of genes linked to “inflammation mediated by chemokines and cytokines” (represented in light green) shown in glucolipotoxic conditions (17.5%) compared to their expression in glucotoxic (8%) and lipotoxic (9,6%) conditions.

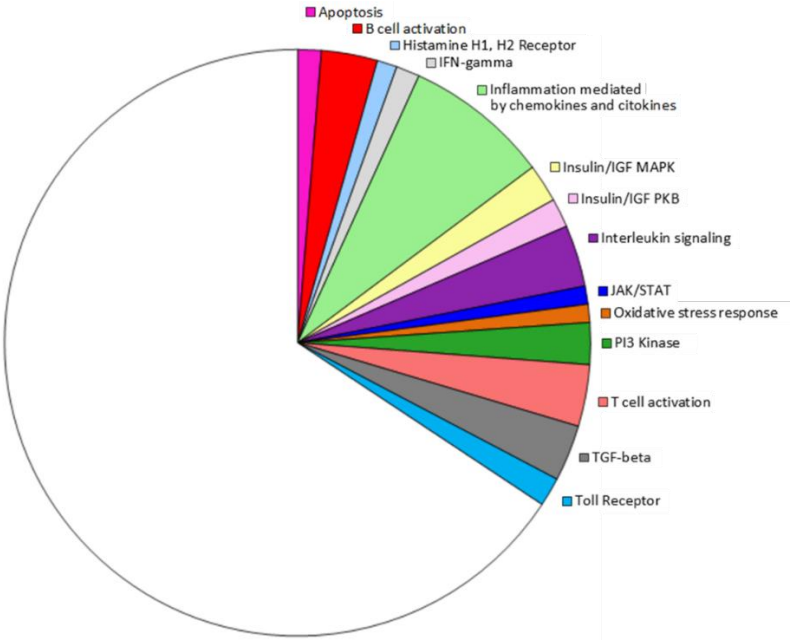


Figure 3.2: Inflammation markers differentially expressed in glucotoxicity compared to control conditions. Panther pathway analysis was used to construct a chart showing the significantly differentially expressed genes involved in inflammation-related processes in high glucose conditions compared to control. White space represents non-inflammation related genes which are also differentially expressed in these conditions.

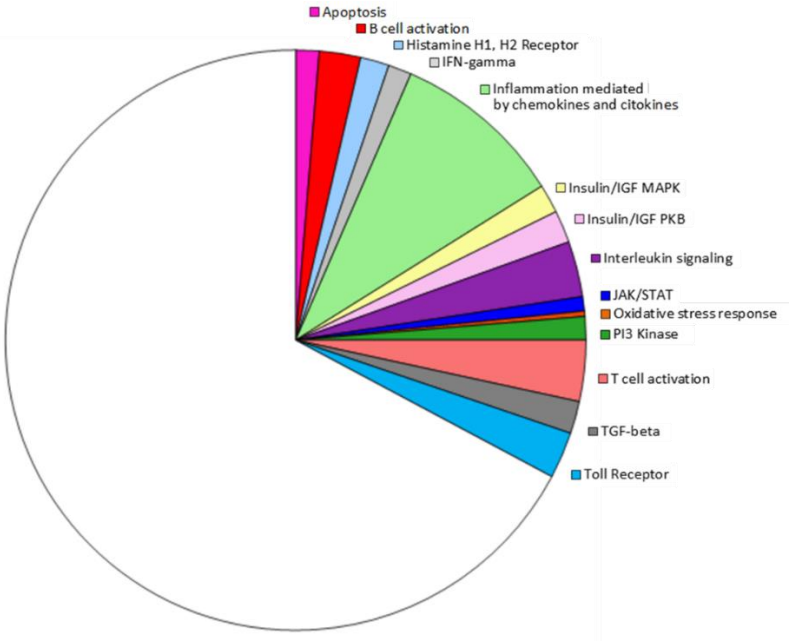


Figure 3.3: Inflammation markers differentially expressed in lipotoxicity compared to control conditions. Panther pathway analysis was used to construct a chart showing the significantly differentially expressed genes involved in inflammation-related processes in high fatty acids conditions compared to control. White space represents non-inflammation related genes which are also differentially expressed in these conditions.

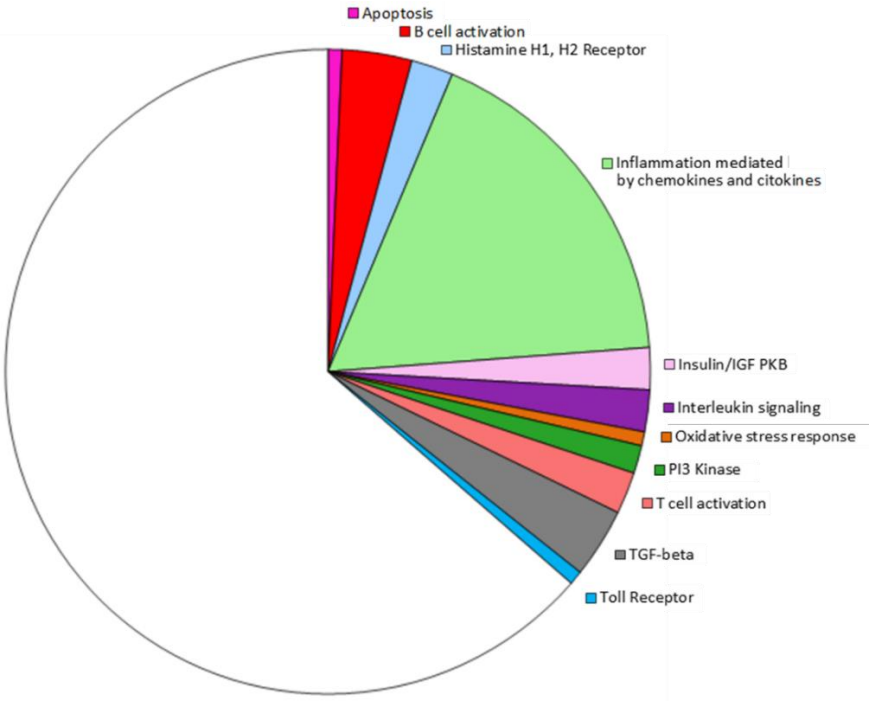


Figure 3.4: Inflammation markers differentially expressed in glucolipotoxicity compared to control conditions. Panther pathway analysis was used to construct a chart showing the significantly differentially expressed genes involved in inflammation-related processes in glucolipotoxic conditions compared to control. White space represents non-inflammation related genes which are also differentially expressed in these conditions.

3.2.2 S100s are overexpressed in glucolipotoxicity according to RNAseq data

Amongst the most significantly differentially expressed genes in the whole β-transcriptome, several members of the S100 family were identified to be upregulated in GLT conditions compared to control. The most differentially expressed S00 members were *S100A3, S100A4* and *S100A5*, with a 2.1-, 9.3- and 6.2-fold increased expression respectively (Figure 3.5, Table 3.1).

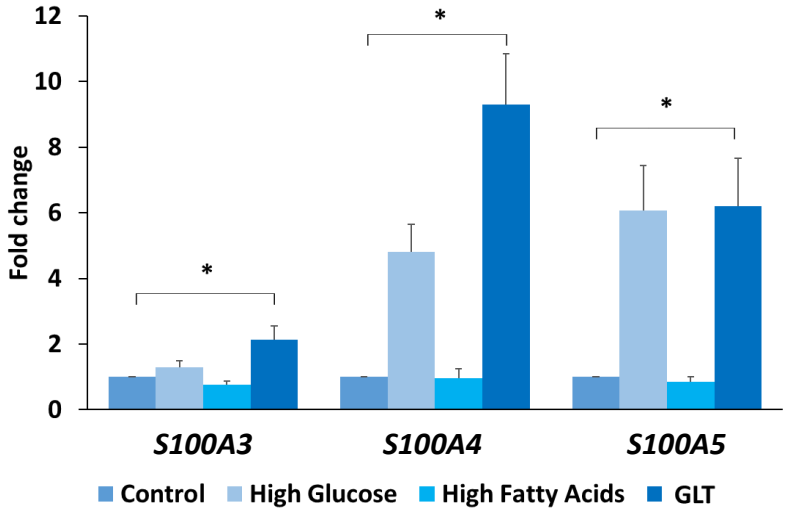


Figure 3.5: *S100A3, S100A4* and *S100A5* differential gene expression in high glucose, high fatty acids and glucolipotoxic conditions compared to control obtained from the RNAseq data. Previously in our laboratory, cells were incubated for 72 hours in RPMI (control), RPMI + 27mM glucose (high glucose), RPMI + 200µM OA and 200µM PA (high FAs), or RPMI + 27mM glucose, 200µM OA and 200µM PA (GLT). RNA was isolated, the sample converted to a library of cDNA frabgments which were then sequenced in a high-throughput manner to obtain short sequences. Resulting reads were aligned to a reference genome to produce a genome-scale transcription map including expression levels for each gene in the different conditions (Wang *et al*. 2009). Data was analysed and expressed as means + SEM of 3 independent experiments, *p<0.05. Abbreviations: FAs fatty acids; GLT glucolipotoxicity; OA oleic acid; PA palmitic acid.

Table 3.1: Fold change of *S100A3, S100A4* and *S100A5* gene expression in high glucose, high fatty acids and glucolipotoxicity compared to control obtained from the RNAseq data. Data analysis was done comparing three independent experiments. *p<0.05.

	Fold change		
Gene	**High Glucose**	**High Fatty Acids**	**Glucolipotoxicity**
S100A3	1.29	0.77	2.12*
S100A4	4.8	0.96	9.3*
S100A5	6.08	0.86	6.21*

3.2.3 Validation of RNAseq data: mRNA levels of S100A3, S100A4 and S100A5 are increased in GLT

In order to validate the RNAseq data, INS-1 cells were incubated for 72 hours in control RPMI media containing 11mM glucose, or RPMI media supplemented to 27mM glucose, 200µM OA and 200µM PA. Only GLT conditions were used compared to control as it were the conditions in which a higher differential expression was observed, as well as being more representative of the diabetic environment. Cells were subsequently lysed and total RNA was extracted and quantified as described in chapter 2. RNA was transcribed to cDNA and qPCR was performed using primers specific for S100A3, S100A4 and S100A5. Figure 3.6 shows the increased expression of the 3 members in conditions of glucolipotoxicity. I detected a 2-fold increase ($p<0.05$) in S100A3, a 4.2-fold increase ($p<0.0005$) in S100A4, and a 4.9-fold increase ($p<0.05$) in S100A5 mRNA levels in glucolipotoxicity compared to control.

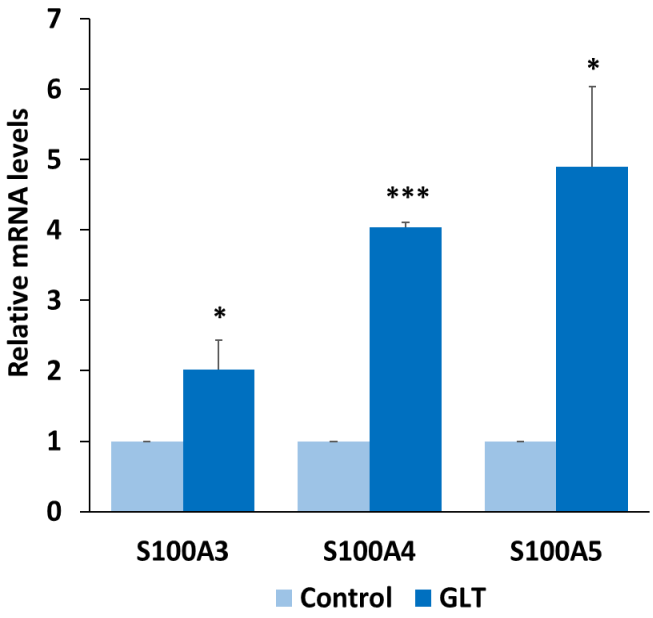


Figure 3.6: Differential expression of S100A3, S100A4 and S100A5 mRNA levels in glucolipotoxicity compared to control. INS-1 cells were incubated for 72 hours in RPMI (control) or RPMI + 27mM glucose, 200µM OA and 200µM PA (GLT), subsequently lysed and total RNA extracted. qPCR was performed using primers specific for each gene. Data represent ΔΔCt values expressed as a fold change compared to cells grown in control conditions and normalised to GAPDH internal control. Data is expressed as means + SEM of 3 independent experiments (*$p<0.05$, ***$p<0.0005$). Abbreviations: OA oleic acid; PA palmitic acid.

3.2.4 Validation of RNAseq data: Protein levels of S100A3, S100A4 and S100A5 are increased in GLT

In order to validate the RNAseq data, and the qPCR results for mRNA expression levels, INS-1 cells were incubated in control RPMI media and in RPMI media supplemented to 27mM glucose, 200µM OA and 200µM PA for 72 hours, lysed and total protein extracted and quantified as described in chapter 2. Lysates were electrophoresed on 15% polyacrylamide gels, and proteins were transferred to a nitrocellulose membrane. Specific antibodies for S100A3, S100A4 and S100A5 were used to incubate the membranes overnight. Figure 3.7 shows the increased protein expression of the three S100 members in conditions of glucolipotoxicity. Bands were observed at the correspondent molecular weights (around 12KDa for S100A3 and S100A4, and around 30KDa for S100A5). Bands were quantified and normalised to internal actin control using Image J, and a 2.2-fold increase ($p<0.05$) for S100A3, a 3.1-fold increase ($p<0.005$) for S100A4, and a 1.7-fold increase ($p<0.05$) for S100A5 protein levels were observed.

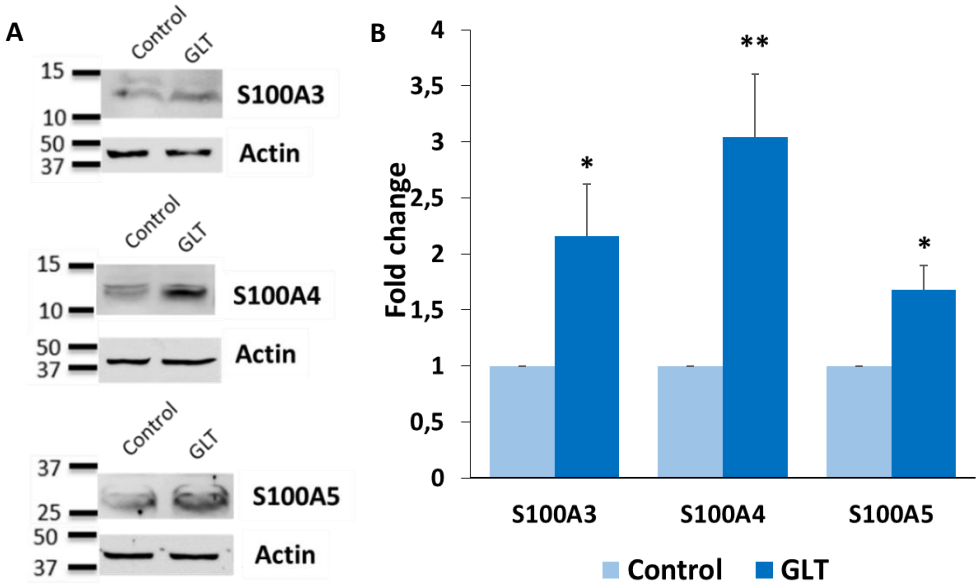


Figure 3.7: Differential expression of S100A3, S100A4 and S100A5 protein levels in glucolipotoxicity compared to control. INS-1 cells were incubated for 72 hours in RPMI (control) or RPMI + 27mM glucose, 200µM OA and 200µM PA (GLT), subsequently lysed. Lysates were separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with S100A3, S100A4 or S100A5 specific antibodies. **A)** Western blot of each S100 member, representative of 3 independent experiments; **B)** Quantification of anti-S100 western blot bands, normalised to internal actin control. Data is expressed as means + SEM of 3 independent experiments (*$p<0.05$, **$p<0.005$). Abbreviations: OA oleic acid; PA palmitic acid.

3.3 Discussion

The term glucolipotoxicity refers to the combined and deleterious effects of elevated glucose and FA levels on pancreatic β-cell function and viability (Poitout *et al.* 2010). Prolonged exposure of β-cells to high concentrations of glucose and FAs leads to a progressive impairment of β-cell function, more precisely to defects in insulin expression and secretion, and ultimately to β-cell death (Mason *et al*. 1999).

In order to mimic conditions of glucolipotoxicity, rat pancreatic INS-1 β-cells were initially cultured in four different conditions: control, high glucose (27mM), high FAs (200µM OA and 200µM PA) and high glucose and high FAs combined (27mM glucose, 200µM OA and 200µM PA), for 72 hours. Previous work in our laboratory demonstrated that a 72-hour incubation in these conditions did not affect negatively the cell cycle, and did not cause activation of caspase 3 or subsequent induction of apoptosis either (data not shown). However, a study by Zhou *et al.* reported an increase in apoptosis with progressive activation of caspase 3 in INS-1 cells after incubation with 30mM glucose and 200µM PA for 72 hours (Zhou *et al*. 2014). In contrast with our experimental procedures, this research group did not use OA as part of the GLT treatment, which is considered to have protective effects against inflammation and apoptosis (Acosta-Montaño *et al.* 2018). Also, considering that this is the most abundant FA in the human diet, our experimental conditions are more representative of physiological situations. Furthermore, the increased glucose concentration may also play a role in the induction of apoptosis. Similarly, El-Assaad *et al.* showed that incubation of INS-832/13 rat insulinoma β-cells as well as human islet β-cells with different combinations of glucose and FAs induced apoptosis (El-Assaad *et al*. 2003). However, this was seen using 300-400µM PA, which is double the concentration that was used in the present study, higher than what you would observe in a pathological situation, and therefore not completely representative of the diseased state.

Furthermore, previous data from our laboratory showed that incubation of INS-1 cells for 72 hours in GLT conditions induces a decrease in insulin mRNA levels and insulin content and secretion (data not shown). Importantly, data indicated that high glucose was the main driver of the attenuation of insulin levels and secretion, whereas FAs alone exerted little or no effects. On the other hand, the combination of glucose and FAs together potentiated the effect of glucose in impairing insulin secretion and production. These results are in line with previous investigations showing that FAs exert a negative effect on

β-cell function only in the presence of concomitant elevated glucose concentrations (Jacqueminet *et al*. 2000, Briaud *et al*. 2002, Somesh *et al*. 2013).

In brief, our experimental glucolipotoxic conditions do not affect cell viability, but impair β-cell function, especially insulin secretion and production (previous data obtained in our laboratory). This indicates that GLT induces an early stage of β-cell dysfunction, in which apoptosis is not induced, but in which some other pathological mechanism is initiated.

As these experimental conditions induce an early stage of β-cell dysfunction and failure, studying the differential gene expression induced by glucolipotoxicity may lead to the discovery of novel molecules involved in this process. RNA sequencing performed previously in our group identified thousands of genes differentially expressed in conditions of 27mM glucose alone, 200µM OA and 200µM PA alone, and the combination of high glucose and high FAS compared to control. When significantly differentially expressed genes were classified according to pathways, it was found that expression of a substantial number of genes linked to apoptosis and inflammatory and immune response was affected by glucolipotoxicity (Figures 3.2, 3.3 and 3.4). Again, previous data from our group shows that the effect on the activation of inflammatory pathways was greater when high glucose and high FAs were combined.

Among all the differentially expressed genes in the RNAseq data, I focussed my attention on S100A3, S100A4 and S100A5, three members of the S100 family of non-ubiquitous Ca^{2+}-modulated proteins. S100A3 has been linked to tumorigenesis and tumour aggressiveness in several types of cancer (Liu *et al.* 2013, Tao *et al*. 2017); S100A4 has been shown to play an important role in many physiological and pathological functions including cell motility, adhesion, proliferation, invasion, metastasis and inflammation (Cerezo *et al.* 2011, Roh *et al.* 2014); On the other hand, little is known about the biological roles of S100A5, but it is known that it might also be involved in inflammation and olfactory signalling (Wheeler *et al*. 2017).

Out of the three members, S100A4 resulted to be the most highly upregulated in the RNAseq data, in which it was upregulated almost 5 times in high glucose and more than 9 times in GLT conditions (Table 3.1), but did not change when cells were treated only in high FAs conditions, again in accordance with the observation that in order to exert its lipotoxic effects, FAs need concomitant high glucose concentrations.

Given that our results, as well as the evidence mentioned above, indicate a greater toxic effect when high glucose and high FA concentrations are combined, and that these

conditions are more representative of the diabetic environment than high glucose alone or high FAs alone, all the experiments from now on will be carried out only in conditions of glucolipotoxic compared to control.

The next step in our investigation was to validate the RNAseq data by demonstrating that both mRNA and protein levels of the three studied S100 members are increased in glucolipotoxicity conditions.

With regards to mRNA levels, our data confirms a statistically significant increase of all three members of the S100 family, albeit to a slightly lesser extent than the increase observed in the previously published RNAseq data (Bagnati *et al.* 2016), especially in the case of S100A4 and S100A5 (4.1 versus 9.3-fold increase and 4.9 versus 6.2-fold increase respectively) (Figure 3.6).

With regards to protein levels, again, our data shows a statistically significant increase of all three members of the S100 family, although to a lower degree than the increase observed in the previously published RNAseq data (Bagnati *et al.* 2016), especially for S100A4 and S100A5 (3.1 versus 9.3-fold increase and 1.7 versus 6.2-fold increase respectively). However, limited studies have explored the mRNA to protein expression correlation and the results have been relatively inconsistent (Guo *et al*. 2008). There are mainly three reasons for the poor correlations generally reported between the level of mRNA and the level of protein. Firstly, there are many complicated and varied post-transcriptional mechanisms involved in the process of mRNA translation to protein that are not yet sufficiently well-defined to be able to extrapolate protein levels from mRNA; secondly, some protein might be damaged or degraded in the extraction process; and/or thirdly, there is a significant amount of error and noise in both protein and mRNA experiments that limit our ability to get a clear picture (Baldi *et al*. 2001). Furthermore, the technologies used to quantify protein abundance still lag behind the more sensitive, high-throughput experimental techniques used to determine mRNA expression levels (Greenbaum *et al*. 2003). In spite of this observation, fold increases in our results are statistically significant, and more relevant than mRNA levels as functional proteins are the ones which exert biological functions.

Taken together, these results indicate that chronic exposure of INS-1 cells to elevated levels of glucose and FAs leads to the upregulation of S100A3, S100A4 and S100A5 at both mRNA and protein level. Given that out of the three members, S100A4 was the most highly upregulated member in the RNAseq data and in our protein expression results, and

that it has a very well established role in many other pathologies, especially in cancer and other inflammatory diseases (Fei *et al.* 2017), from now on the focus of this thesis will be this S100 member. In addition, the potential role of S100A4 in diabetes and glucolipotoxicity has not been described before, which makes it an interesting subject for further investigations and a candidate for potential therapeutic intervention.

In conclusion, these preliminary results open the way to the investigations that are going to be described in the next chapters, in order to characterise the function of S100A4 in the pancreatic β-cell in relation to glucolipotoxicity-induced inflammation and its role in the development of T2D.

CHAPTER 4: Investigation of a potential link between S100A4 and inflammation through activation of NF-κB

4.1 NF-κB

NF-κB is a key transcription factor involved in a broad range of biological processes, including immune and inflammatory responses, cell survival, stress responses and maturation of various cell types (Shih *et al.* 2011).

The NF-κB family consists of 5 members of ubiquitously expressed transcription factors, divided in two groups: Class I members NF-κB1 (p50/p105) and NF-κB2 (p52/p100), and Class II members RelA (p65), RelB, cRel. These subunits are able to associate with each other to form at least 15 different homo- or heterodimers, which are cell type- and stimulus-specific (Oeckinghaus *et al.* 2009). The most common dimer is composed of either p50 or p52 and p65 (Christian *et al*. 2016) (Figure 4.1B). Each subunit contains a Rel homology region (RHR) consisting of two folded domains, the amino-terminal domain (NTD) and the dimerization domain (DimD), and a carboxy-terminal region containing the nuclear localisation signal (L) (Figure 4.1A). The RelA, RelB and cRel subunits also contain a transcription activation domain (TAD), a region whose protein structure is incompletely understood, and which is responsible for the increase in NF-κB target gene expression resulting from NF-κB induction. Dimers containing at least one of these subunits will therefore function as transcriptional activators (Hoffmann *et al.* 2006, Huxford *et al.* 2009), activating the expression of various inflammatory mediators including TNFα, IL1β, IL6, and MCP1, amongst others (Lorenzo *et al.* 2011), giving place to the initiation of a broad inflammatory response (Figure 4.1B). The p50 and p52 subunits do not contain a TAD. As a result, NF-κB complexes consisting of p50 and/or p52 homo or heterodimers are able to translocate to the nucleus and bind to DNA but fail to activate target gene expression. In fact, they have been seen to function as transcriptional repressors (Christian *et al*. 2016).

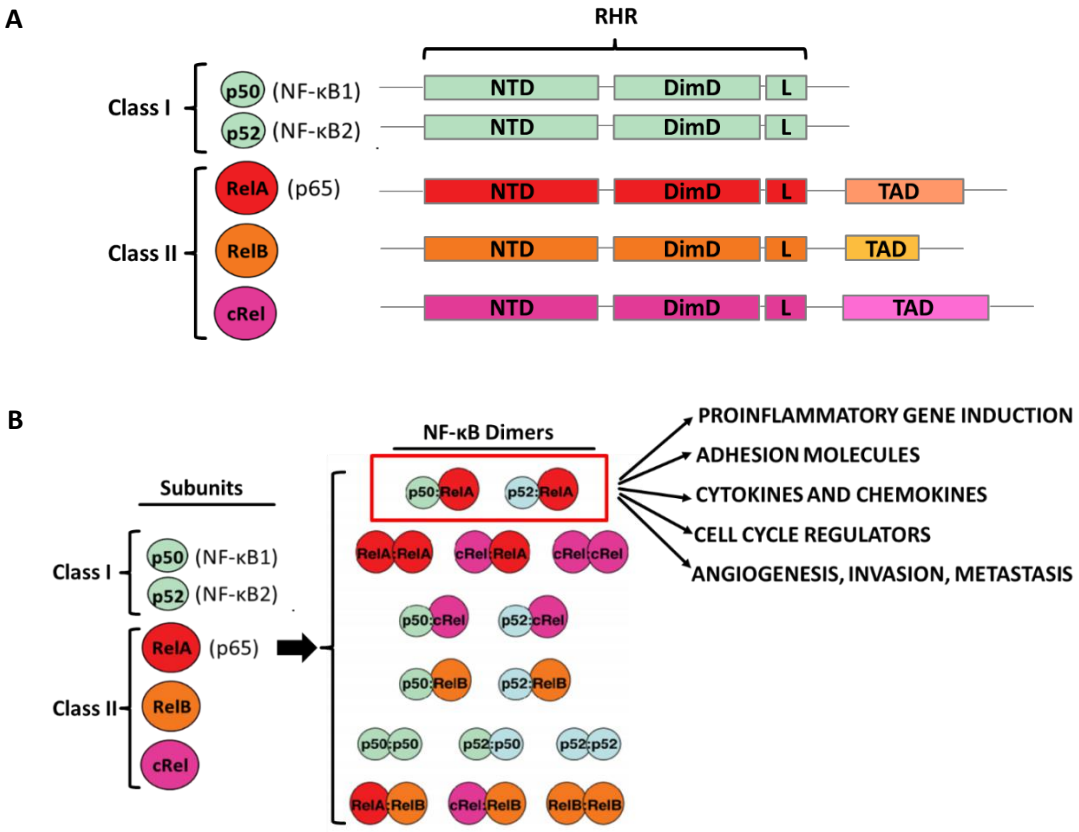


Figure 4.1: The NF-κB family. A) Structural domains. Each subunit contains a Rel homology region (RHR) consisting of two folded domains, the amino-terminal domain (NTD) and the dimerization domain (DimD), and a carboxy-terminal region containing the nuclear localisation signal (L). The RelA, RelB and cRel subunits also contain a transcription activation domain (TAD) responsible for the transcriptional activity of dimers containing these subunits **B)** Possible dimerization combinations. The most common dimer is the one formed by either p50 or p52 together with RelA (p65). This form of NF-κB is able to activate transcription of a wide range of genes involved in the regulation of inflammation, including inflammatory cytokines, chemokines and adhesion molecules. It is also able to mediate inflammation through the regulation of cell proliferation, apoptosis and differentiation.

In most cell types, NF-κB is activated through the canonical pathway, where stimuli from diverse immune receptors lead to induced phosphorylation and degradation of the NF-κB inhibitors IκBs, allowing the release of NF-κB, and its translocation to the nucleus (Lee *et al*. 2014).

In obesity and T2D, NF-κB activity is increased due to elevated levels of circulating FAs, which signal through TLR4 to activate the canonical NF-κB pathway (Shi *et al*. 2006, Novotny *et al*. 2012). Other cytokines and proinflammatory factors such as TNFα and IL1β

are also increased in AT of obese and diabetic subjects, and signal through TNFR and IL1R respectively to activate NF-κB, also through the canonical pathway (Lackey *et al*. 2016). This was described in further detail in chapter 1, section 1.4.3.

Although there is extensive knowledge on a wide variety of factors that activate NF-κB signalling to initiate the inflammatory response, there are still many other factors whose role on NF-κB activity has not yet been revealed. The aim of this section is to identify S100A4 as a novel factor able to activate or increase NF-κB activity in a glucolipotoxic model of T2D.

4.2 S100A4 and NF-κB

S100A4 has recently gained increasing attention for its role in the development of several pathologies through regulation of angiogenesis (Semov *et al*. 2005), cell migration (Garrett *et al.* 2006) and inflammation (O'Connell *et al.* 2011, Dahlmann *et al*. 2016). Upregulation of S100A4 expression has mainly been associated with many tumour-related processes (Fei *et al*. 2017), but also to various non-tumour pathophysiological processes such as tissue fibrosis, inflammation, immune reaction, neuroprotection and cardiovascular events (Fei *et al.* 2017), and consequently to several autoimmune diseases and other inflammatory conditions including rheumatoid arthritis (Klingelhöfer *et al.* 2007), diabetes retinopathy (Abu El-Asrar *et al*. 2014) and inflammatory myopathies (Cerezo *et al*. 2011). However, its role in obesity-related inflammation has not been well described yet.

Importantly, S100A4 binds to several cell-membrane receptors including RAGE, EGFR, TLR4 and IL10R (Donato *et al.* 2013, Grotterød *et al*. 2010) to activate various proinflammatory factors, including NF-κB (Yammani *et al*. 2006, Grotterød *et al.* 2010). This has been reported to take place in many cancer cell lines through induction of IKK-mediated phosphorylation and subsequent degradation of the NF-κB inhibitor IκBα (Boye *et al.* 2008), resulting in the activation of a self-amplifying pro-inflammatory cycle through the upregulation of NF-κB mediated pro-inflammatory cytokines expression (Li *et al.* 2010, Zhou *et al.* 2015).

RAGE, a membrane spanning protein of the immunoglobulin superfamily, presents a low expression in most tissue types. However, it has been seen to be upregulated in inflammatory conditions (Yan *et al*. 2009). Consequently, the combination of elevated

S100A4 levels in GLT conditions together with the presumed upregulated expression of RAGE in pathological situations suggests that S100A4 signalling may have an important role in the process of NF-κB activation and inflammation in glucolipotoxicity-induced diabetes.

4.3 Results

4.3.1 NF-κB expression and activity are increased in glucolipotoxicity

NF-κB is the main proinflammatory factor whose activation leads to the onset of the immune response. In order to determine whether NF-κB levels and activity were induced by glucolipotoxicity, I first measured NF-κB protein expression through western blot, as well as its subcellular localisation through indirect immunofluorescence. Finally, its transcriptional activity was measured through an NF-κB specific activity assay.

To do so, INS-1 cells were incubated for 72 hours in RPMI (control) or RPMI + 27mM glucose, 200μM OA and 200μM PA (GLT), lysed and total protein extracted and quantified as described in chapter 2. Figures 4.2A and 4.2B show the increased NF-κB protein expression in GLT compared to control. Bands were quantified and normalised to internal actin control, and a small but significant 1.3-fold increase ($p<0.05$) in NF-κB protein levels was observed. However, increased protein expression levels do not necessarily correlate with increased activity. INS-1 cells were therefore also cultured on coverslips in control or GLT conditions for 72 hours and subsequently incubated with specific NF-κB antibody followed by incubation with fluorescent secondary antibody as described in chapter 2 in order to determine its subcellular localisation through immunofluorescence. Figure 4.2C shows increased expression and increased nuclear localisation of NF-κB in GLT conditions, which indicates that GLT induces translocation of NF-κB, suggesting an induction of its transcriptional activity.

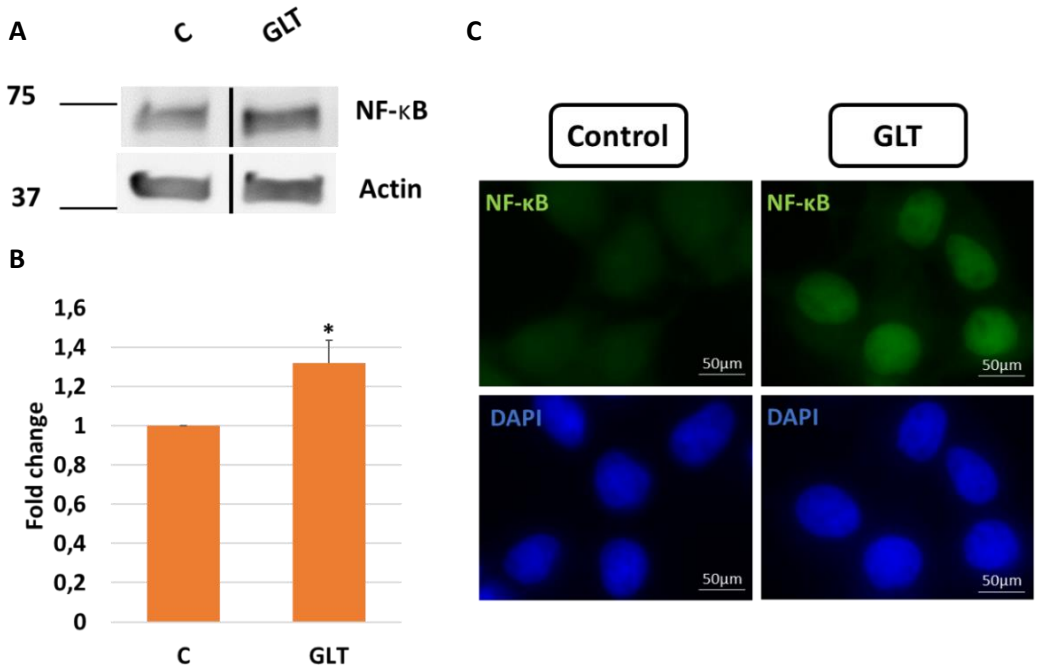


Figure 4.2: Effect of GLT on NF-κB protein levels and nuclear localisation. INS-1 cells were incubated for 72 hours in control or GLT conditions, subsequently lysed and total protein extracted. Lysates were electrophoresed on 12% polyacrylamide gels, and proteins were transferred to a nitrocellulose membrane. A specific antibody for NF-κB was used to incubate the membrane overnight. INS-1 cells were also cultured on coverslips in control or GLT conditions for 72 hours, after which they were fixed and incubated with specific NF-κB antibody, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken **A)** Representative western blot of 3 independent experiments; **B)** Quantification of anti-NF-κB western blot bands, normalised to internal actin control. **C)** Representative image from 3 independent experiments. Scalebar 1cm:50µm. Data is expressed as means + SEM of 3 independent experiments (*$p<0.05$)

In order to measure the increase in NF-κB activity induced by GLT quantitatively instead of qualitatively as shown in Figure 4.2C, an NF-κB transcription factor activity assay was performed. In brief, INS-1 cells were cultured in control and GLT conditions for 72 hours, after which their nuclear compartment was extracted using a nuclear/cytosol fractionation kit as described in chapter 2. Nuclear fractions are used instead of the total protein lysate as the active form of NF-κB would be contained in the nuclear fraction. Furthermore, this particular assay is specific for the p65/p50 dimer, which specifically activates transcription of proinflammatory cytokines.

The efficiency of the nuclear extraction was tested by measuring nuclear protein content in the nuclear extracts through western blot. of the After nuclear/cytosol fractionation, nuclear extracts were electrophoresed, and proteins were transferred to a nitrocellulose membrane. The membrane was then incubated in anti-histone H2A antibody to measure H2A presence in both nuclear and cytosolic fractions. Results in Figure 4.3 show low detection of H2A in the cytosolic fraction, while its presence in the nuclear fraction is substantial. Being an epigenetic marker, it should only be present in the nuclear fraction, however histones are synthesised in the cytosol so some may be present there. Furthermore, nuclei are damaged during extraction so some nuclear material may escape to the cytosolic fraction. These results indicate that the cytosolic/nuclear fractionation was successful, and therefore experiments using the nuclear fraction are reliable.

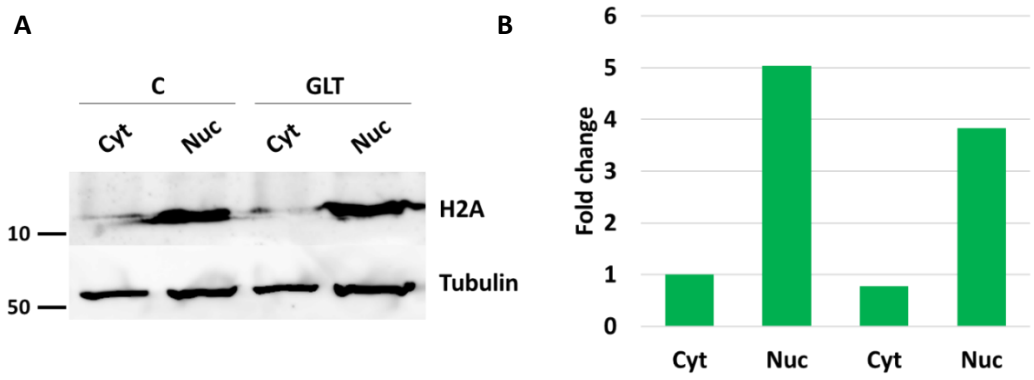


Figure 4.3: Histone H2A expression in cytosolic and nuclear fractions. INS-1 cells were incubated for 72 hours in control or GLT conditions, after which cells were lysed using a nuclear/cytosolic fractionation kit. Lysates were electrophoresed on 12% polyacrylamide gels, and proteins were transferred to a nitrocellulose membrane. A specific antibody for H2A was used to incubate the membrane overnight. **A)** Representative western blot; **B)** Quantification of anti-H2A western blot bands, normalised to internal tubulin control.

Nuclear extracts were then used for the transcription factor assay, which was performed according to manufacturer's instructions as detailed in chapter 2. Results in Figure 4.4 show that there is a significant 52% increase in NF-κB activity resulting from exposure of INS-1 cells to GLT conditions, confirming our results in Figure 4.2.

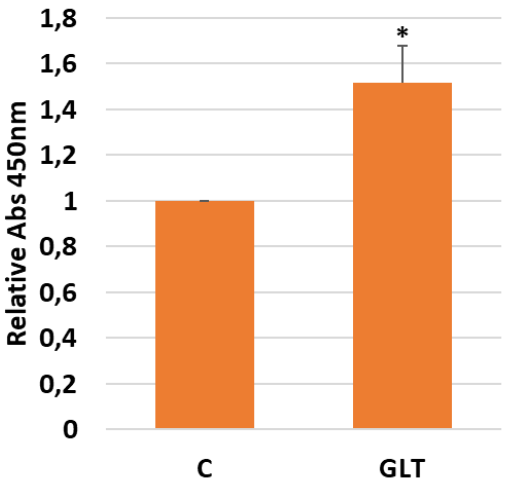


Figure 4.4: Effect of GLT on NF-κB activity. INS-1 cells were cultured in control or GLT conditions for 72 hours, after which cells were lysed using a nuclear/cytosolic fractionation kit. The nuclear compartment was added to the oligonucleotide coated wells of the activity assay plate, which was then incubated with specific NF-κB antibody and subsequent secondary antibody. Absorbance was read at 450nm to quantify NF-κB activity. Data is expressed as means + SEM of 3 independent experiments (*p<0.05).

4.3.2 S100A4 activates NF-κB

As observed in Figures 4.2 and 4.4, the replication of chronic glucolipotoxic conditions in INS-1 cells induces an increase in NF-κB protein expression, nuclear localisation and transcriptional activity. The next step was to investigate whether these changes were being induced by S100A4. To test this hypothesis, a transient knockdown of S100A4 was carried out using siRNA as described in chapter 2. Briefly, ssRNA/siRNA conjugated with transfection reagent was added to the INS-1 cells 24 hours after seeding, for a period of 24 hours. Media was then removed and control or GLT conditions were applied for another 72 hours, after which either total protein or nuclear fractions were obtained for subsequent western blot analysis or transcription factor activity assay respectively.

An optimisation process for S100A4 knockdown was initially carried out using 3 different transfection reagents (RNAiMAX, Transit-X2 and INTERFERin), and 3 different incubation periods (24, 48 and 72 hours), only in control conditions. The level of knockdown was checked through western blot (Figure 4.5).

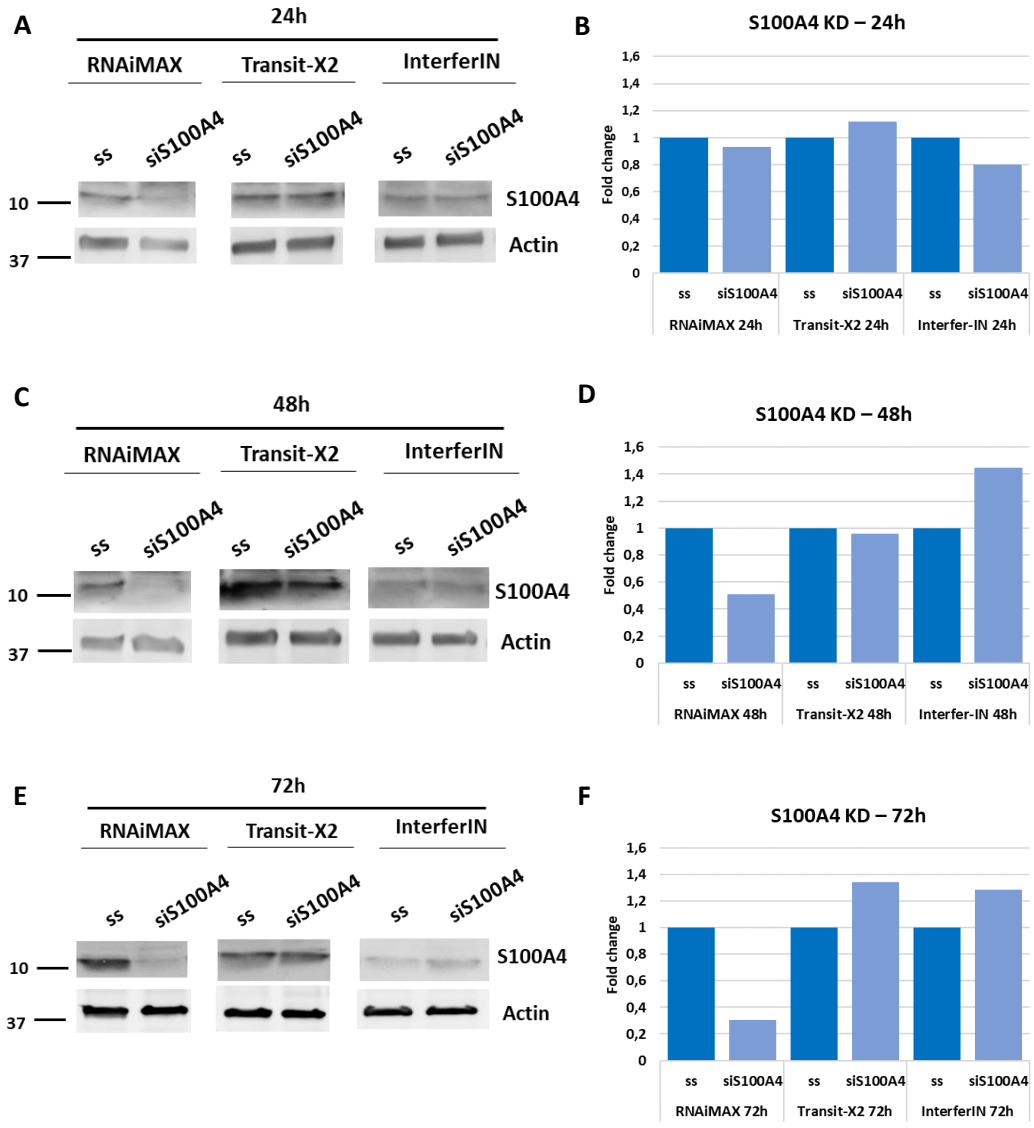


Figure 4.5: Optimisation process for S100A4 knockdown. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and replaced with control RPMI media for 24, 48 or 72 hours. Total protein was extracted, and lysates were separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with S100A4 specific antibody. **A, C, E)** Western blot of S100A4 after treatments for 24, 48 and 72 hours respectively, showing level of knockdown with the 3 different transfection reagents; **B, D, F)** Quantification of anti-S100A4 western blot bands, normalised to internal actin control, for 24, 48 and 72 hours treatments respectively.

Results in Figure 4.5 show that a 24-hour incubation period did not result in effective knockdown with any of the three transfection reagents (7% knockdown with RNAiMAX, no knockdown with Transit-X2 and 20% knockdown with INTERFERin) (Figures 4.5A and B). A 48-hour incubation period rendered a higher degree of knockdown with RNAiMAX (49%) but proved ineffective with both Transit-X2 and INTERFERin (Figures 4.5C and D). Finally, a 72-hour incubation period resulted in a 70% knockdown with RNAiMAX, while no knockdown was achieved with the other two transfection reagents (Figures 4.5E and F). In conclusion, the most effective transfection reagent for S100A4 knockdown was RNAiMAX, and the optimal incubation period was 72 hours, rendering a 70% S100A4 knockdown, as shown in Figure 4.5F. These were therefore the conditions selected to carry out all S100A4 knockdown experiments in INS-1 cells.

After the optimisation process, S100A4 was knocked down using RNAiMAX and an incubation period of 72 hours, following the manufacturer's instructions as described in chapter 2, and subsequently, total protein was obtained in order to measure NF-κB protein expression through western blot, again only in control conditions (Figures 4.6A and 4.6B). S100A4 knockdown was also conducted on INS-1 cells cultured on coverslips for 72 hours, after which cells were subjected to incubation with fluorescent antibody for immunofluorescence imaging (Figure 4.6C).

Results show that, following S100A4 knockdown (70%, **$p<0.005$) there was no significant decrease in NF-κB protein expression in control conditions (Figures 4.6A and 4.6B), as well as no changes in NF-κB subcellular localisation (Figure 4.6C).

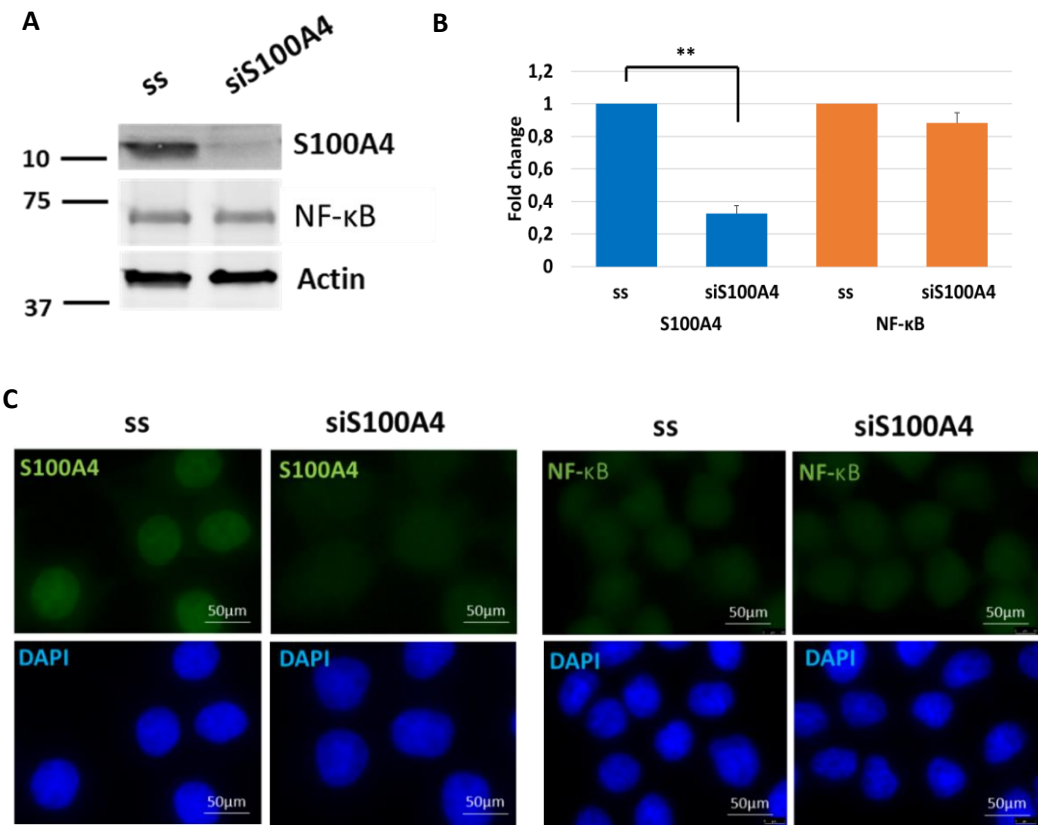


Figure 4.6: Effect of S100A4 knockdown on NF-κB protein levels and subcellular localisation in control conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and replaced with control RPMI media for 72 hours. Total protein was then extracted, and lysates were separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes which were then incubated with S100A4 or NF-κB specific antibodies. INS-1 cells were also cultured on coverslips, 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells and media replaced after 24 hours with control RPMI media for 72 hours. After fixing, cells incubated with specific S100A4 or NF-κB antibodies, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. **A)** Representative western blot of 3 independent experiments; **B)** Quantification of anti-S100A4 and anti-NF-κB western blot bands, normalised to internal actin control. **C)** Representative images from 3 independent experiments. Scalebar 1cm:50µm. Data is expressed as means + SEM of 3 independent experiments (**p<0.005).

As there were no apparent changes on NF-κB protein expression caused by S100A4 knockdown in control conditions, the same experiment was carried out, but this time using GLT conditions compared to control, as the absence of any effect could be due to the low basal expression and inactivity of NF-κB in control conditions. Again, S100A4 was knocked down as described above, this time applying control or GLT conditions after the

24-hour incubation with RNAiMAX-ss/siRNA complex, and subsequently, total protein was obtained to measure NF-κB protein expression through western blot (Figures 4.7A and 4.7B). INS-1 cells were also cultured on coverslips in control and GLT conditions for 72 hours and subjected to incubation with fluorescent antibody for indirect immunofluorescence (Figures 4.7C and 4.7D).

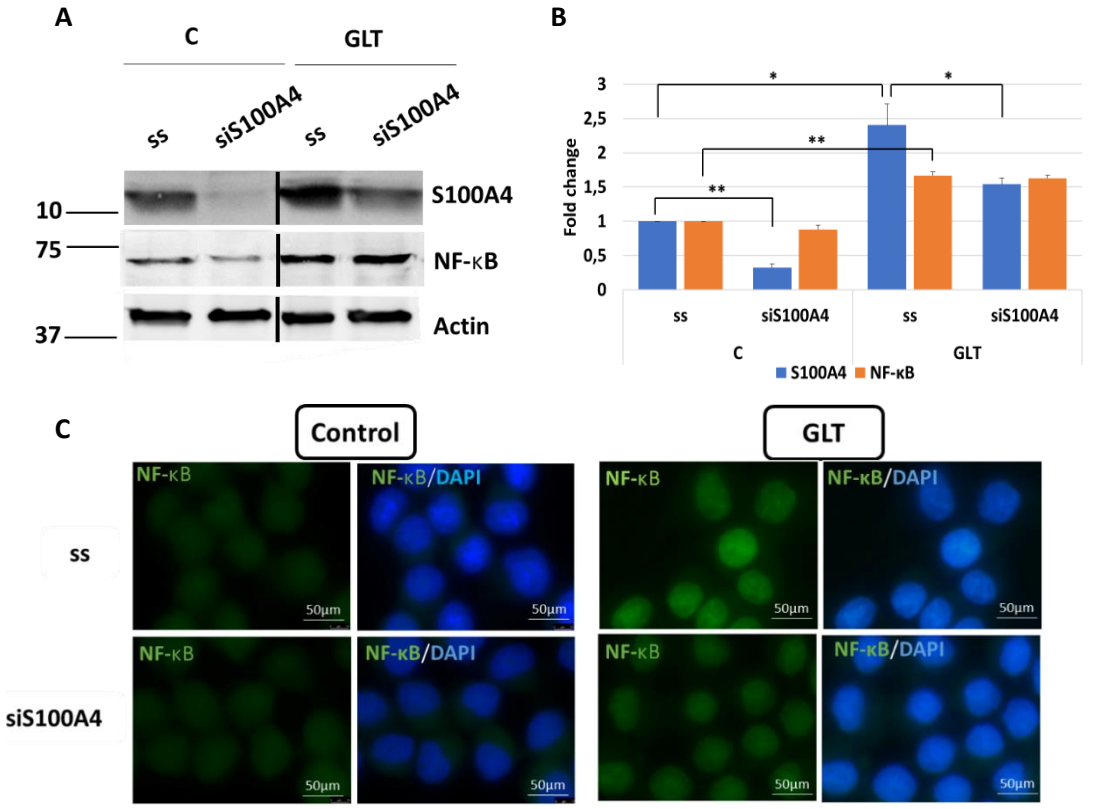


Figure 4.7: Effect of S100A4 knockdown on NF-κB protein levels and subcellular localisation in GLT conditions compared to control. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control or GLT conditions were applied for 72 hours. Total protein was extracted, and lysates were separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes which were then incubated with S100A4 or NF-κB specific antibodies. INS-1 cells were also cultured on coverslips, 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells and 24 hours later media was removed and control or GLT conditions were applied for 72 hours. After fixing, cells were incubated with specific S100A4 or NF-κB antibodies, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. **A)** Representative western blot of 3 independent experiments; **B)** Quantification of anti-S100A4 and anti-NF-κB western blot bands, normalised to internal actin control. **C)** Representative images from 3 independent experiments. Scalebar 1cm:50µm. Data is expressed as means + SEM of 3 independent experiments (*p<0.05, **p<0.005).

Results in Figures 4.7A and 4.7B show that NF-κB protein expression increases in GLT as we saw before, but is not affected by S100A4 knockdown. However, when we look at NF-κB subcellular localisation in control conditions (Figure 4.7C), NF-κB expression or nuclear localisation does not change in S100A4-knocked down cells, but we can observe a decrease in NF-κB nuclear expression and localisation in GLT treated cells in which S100A4 has been knocked down.

Together, these results suggest that S100A4 might have a role in NF-κB activation in GLT conditions without affecting its protein expression levels. This could include regulation of the inhibitor of NF-κB (IκBα) through its phosphorylation by the IκBα kinase (IKK) as it has been described before (Boye *et al.* 2008). In order to test this hypothesis, protein levels of IKK were measured in control and GLT conditions +/- siS100A4, as well as the subsequent degradation of IκBα in the same conditions (Figure 4.8).

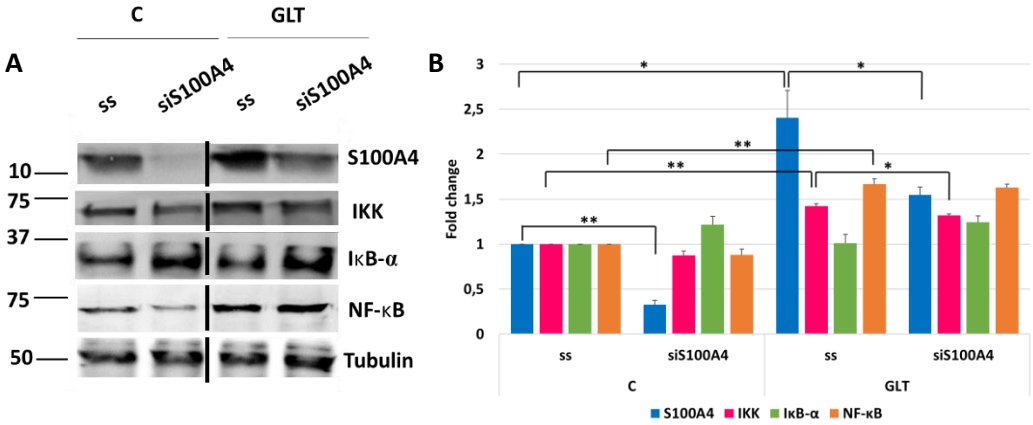


Figure 4.8: Effect of S100A4 knockdown on IKK, IκBα and NF-κB protein levels in GLT conditions compared to control. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control or GLT conditions were applied for 72 hours. Total protein was extracted, and lysates were separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes which were then incubated with S100A4, IKK, IκBα or NF-κB specific antibodies. **A)** Representative western blot of 3 independent experiments; **B)** Quantification of anti-S100A4, anti-IKK, anti-IκBα and anti-NF-κB western blot bands, normalised to internal tubulin control. Data is expressed as means + SEM of 3 independent experiments (*p<0.05, **p<0.005).

As mentioned before, even though NF-κB protein levels are not affected by S100A4 knockdown in GLT, the decrease in its nuclear localisation observed by immunofluorescence indicates that its activity is decreased. There is evidence that

S100A4 activates NF-κB in many cancer cell lines through induction of IKK-mediated phosphorylation and subsequent degradation of the NF-κB inhibitor IκBα (Boye *et al.* 2008). Here I wanted to check whether S100A4 knockdown affected the levels of IKK and IκBα. Results in Figure 4.8 show that IKK expression increases in GLT and decreases in S100A4-knocked down cells in GLT, suggesting that the mechanism by which S100A4 activates NF-κB in a glucolipotoxic environment is through activation of IKK. Simultaneously, protein levels of IκBα were measured, which should increase in S100A4-knocked down cells due to the decrease in IKK levels. As Figure 4.8 shows, this is also the case for IκBα, although the change is not statistically significant. Altogether, these results indicate that S100A4 is able to increase NF-κB activation through induction of IKK activity and subsequent degradation of IκBα.

A more direct method of measuring NF-κB activity is with the use of the NF-κB activity assay. To this end, nuclear extracts of S100A4-knocked down cells in control and GLT conditions were obtained and used to carry out the assay (Figure 4.9).

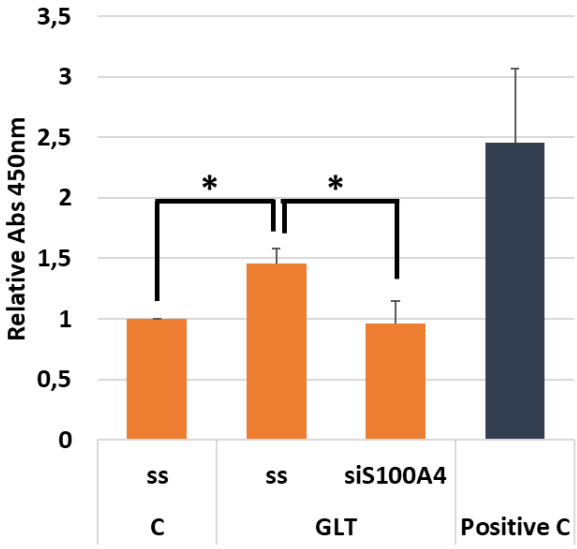


Figure 4.9: Effect of S100A4 knockdown on NF-κB activity in GLT conditions compared to control. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control or GLT conditions were applied for 72 hours, after which cells were lysed using a nuclear/cytosolic fractionation kit. The nuclear compartment was added to the oligonucleotide coated wells of the activity assay plate, and then incubated with specific NF-κB antibody and subsequent secondary antibody. Developing solution and subsequent stop solution was added to the wells, after which absorbance was read to measure NF-κB activity. Data is expressed as means + SEM of 3 independent experiments (*$p<0.05$).

Figure 4.9 shows that NF-κB activity increases in GLT conditions compared to control by 46% in cells treated with an ssRNA. This shows that the use of an ssRNA is not producing any effect per se, as it is the same result that we observed when measuring NF-κB activity in GLT compared to control (Figure 4.4). Interestingly, this effect is reversed by 109% when S100A4 is knocked down in GLT, indicating that S100A4 is indeed responsible for the activation of NF-κB.

4.4 Discussion and future directions

In this section, I wanted to explore a possible direct link between S100A4 and NF-κB. As mentioned before, S100A4 expression is known to be involved in the pathogenesis of several autoimmune diseases and other inflammatory conditions (Klingelhöfer *et al.* 2007, Cerezo *et al.* 2014). Existing knowledge on the well-established role of S100A4 in inflammatory diseases could suggest that S100A4 may also be involved in the pathogenesis of obesity-related diabetes, given that obesity has been described to be a state of low-grade chronic inflammation (Castro *et al*. 2017). Binding of S100A4 to RAGE has been seen to induce activation of NF-κB in human cancer cell lines through the classical NF-κB activation pathway (Boye *et al.* 2008, Kim *et al*. 2017) (detailed in chapter 1, section 1.4.3). However, very little is known about the role of S100A4 in the activation of the inflammatory processes mediated by NF-κB in many autoimmune diseases, fibrosis, and other disorders, which makes this a very exciting area to study and potentially exploit in future therapeutic intervention.

The results obtained in this section demonstrate that NF-κB protein expression and nuclear localisation increase in response to elevated glucose and FAs, indicating that glucolipotoxicity might be involved in the activation of NF-κB. Functional studies were carried out in order to confirm this hypothesis, in which NF-κB activity was measured using an NF-κB activity assay. Results showed that NF-κB activity increases by around 50% in conditions of glucolipotoxicity, confirming recently published work in our laboratory (Bagnati *et al*. 2016).

In order to determine whether S100A4 plays a role in this GLT-induced expression and activation of NF-κB, siRNA was used to transiently knockdown S100A4 and observe the effects that this had on NF-κB protein expression, subcellular localisation and activation. After a knockdown optimisation process, results showed that a 70% knockdown of

S100A4 did not have any effect on NF-κB protein expression or subcellular localisation in control conditions, which could be explained by the fact that NF-κB displays low basal expression and activity in control conditions, therefore knocking S100A4 down will not have any effect. However, when incubated in glucolipotoxic conditions, even though S100A4 knockdown did not affect NF-κB protein expression, it reversed the increased nuclear localisation induced by GLT. These results suggest that S100A4 might have a role in NF-κB activation in GLT conditions without affecting its protein expression, maybe through the regulation of IKK activity. In order to test this hypothesis, protein levels of IKK in control and GLT conditions +/- siS100A4 were measured, as well as the subsequent degradation of IκBα. Results show that IKK levels decreased in S100A4-knocked down cells in GLT, and that consequently, IκBα levels increased in these conditions. Experiments involving the measurement of IκB phosphorylation by IKK in S100A4-knocked down cells in control and GLT conditions could be carried out in order to confirm this hypothesis, as it would be a more direct way of determining the inhibitory activity of IκB rather than measuring its protein levels.

Finally, in the last section of this chapter, the role of S100A4 on NF-κB activation was assessed by performing an NF-κB activity assay +/- S100A4. Results show that S100A4 knockdown completely reverses the GLT-induced increase in NF-κB activity, indicating that S100A4 is responsible for this event.

In conclusion, results shown in this chapter indicate that S100A4 is likely involved in the glucolipotoxic-induced inflammatory process characteristic of T2D through an increase in NF-κB activation.

Additional experiments to further confirm the validation of our hypothesis include the measurement of IκB phosphorylation by IKK in S100A4-knocked down cells, as mentioned above, to confirm whether the activation of NF-κB by S100A4 involves induction of IKK activity.

Another more direct way to measure the effect of S100A4 knockdown on NF-κB activity could be to carry out a reporter assay based on NF-κB responsive promoter elements driving expression of a secreted luciferase, such as the one carried out by Badr *et al.* (Badr *et al*. 2009). A fluorescent reader would then be used to measure expression levels of the luciferase, with fluorescence signal positively correlating with NF-κB activity. Similarly, the measurement of NF-κB target gene expression through qPCR would also constitute another direct way of measuring NF-κB activity. Target genes that could be looked at

include Tumour Necrosis Factor Receptor Associated Factor 2 (TRAF2), Growth Arrest and DNA Damage-Inducible β (GADD45B) and Inhibitor of NF-κB kinase subunit β (IKKβ), all of them involved in stress or apoptosis-related pathways. Another direct way of measuring NF-κB transcriptional activity would be chromatin immunoprecipitation (ChIP), or performing western blot analysis against NF-κB p65 subunit.

Finally, measurement of RAGE mRNA and protein expression levels in INS-1 cells in control and GLT conditions, followed by knockdown or inhibition of RAGE signalling could also be carried out in order to block S100A4-mediated activation of NF-κB. This however, poses the problem that S100A4 is also able to signal through other receptors such as EGFR, TLR4 and IL10R, to activate NF-κB (Grotterød *et al*. 2010), therefore no change in NF-κB activation would be observed after RAGE knockdown.

RAGE is known to be involved in a variety of pathophysiological processes including immune/inflammatory disorders, tumours, and abnormalities associated with diabetes as arteriosclerosis or abnormal wound healing (Schlueter *et al*. 2003). RAGE expression is dependent on cell type and developmental stage. It is constitutively expressed during embryonic development, but then its expression decreases in adult life (Brett *et al*. 1993). RAGE is only expressed at low levels in a wide range of adult cells in physiological conditions, including endothelial cells, cardiomyocytes, neutrophils, macrophages, lymphocytes and dendritic cells (Ott *et al.* 2014), as well as in the adult central nervous system (CNS), glia and neurons (Huttunen *et al*. 2000). However, RAGE has been seen to be upregulated in inflammatory conditions (Yan *et al*. 2009).

Unfortunately, its expression is not included in the RNAseq data, probably because it is not one of the most differentially expressed genes. However, it would be interesting to see if its expression changes in glucolipotoxicity. Furthermore, knockdown or inhibition of RAGE signalling and subsequent measurement of NF-κB activity would indicate whether S100A4 signals through RAGE to activate NF-κB, although, as mentioned before, S100A4 could signal through other receptors to activate NF-κB (Grotterød *et al.* 2010).

CHAPTER 5: Identification of interaction partners of S100A4

5.1 S100A4 interaction network construction

As detailed in chapter 3, in order to gain further insight into the complex nature of diabetogenesis and to discover novel mechanisms that can affect activation of inflammatory pathways that lead to β-cell dysfunction, a transcriptome profiling of the pancreatic β-cell in conditions of glucolipotoxicity compared to control was performed using RNA sequencing. Analysis of the transcriptome allowed the identification of differentially expressed genes in conditions of glucolipotoxicity, including *S100A4*.

Whilst it has been established that S100A4 is linked to NF-κB, I sought to identify additional molecules that interact with the *S100A4* gene in the β-cell environment in order to potentially elucidate key pathways by which *S100A4* expression could be regulated in INS-1 pancreatic β-cells.

Together with our collaborators in Barts and The London School of Medicine and Dentistry, we used MetaCore™ (Clarivate Analytics) to generate a pathway interaction map for *S100A4* (Figure 5.1). MetaCore™ is a bioinformatic analysis programme which combines gene expression data input, in our case the RNAseq data produced previously in our laboratory, with data available in other databases to produce relevant pathways and networks associated with the specific genes of interest, and providing information about their pathological relevance.

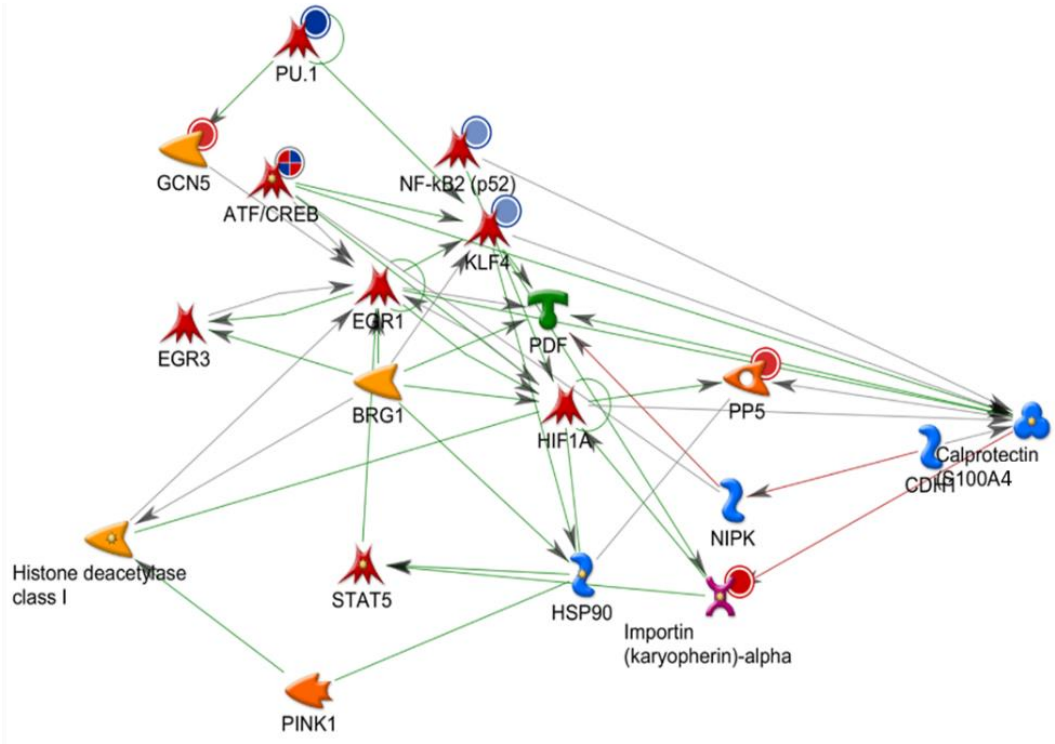


Figure 5.1: MetaCore™ interaction network for *S100A4*. RNAseq data was input into MetaCore™ analysis software, and *S100A4* was selected as the target gene upon which the interaction analysis was to be made. The interaction network shows the most relevant interactions between *S100A4* and other genes included in the RNAseq data, as well as the directionality of the interaction (depicted by the direction of the arrows), the effect of the interaction (red arrow indicates negative interaction, green arrow indicates positive interaction), and whether a gene is significantly differentially expressed in GLT conditions compared to control (depicted by the blue -downregulation- or red -upregulation- circle at the top right hand corner of each icon).

This interaction network is a very powerful and useful tool, as it allows the identification of the most likely interacting partners of our genes of interest, in this case *S100A4.* Interestingly, Figure 5.1 shows that *S100A4* interacts with *HIF-1α*, with the direction of the interaction indicated by the arrow suggesting that *HIF-1α* could modulate *S100A4* expression.

5.2 HIF-1α as an interacting partner of S100A4

As detailed in chapter 1, section 1.6, pathological hypoxia can drive tissue dysfunction and disease development through immune cell dysregulation (Taylor *et al.* 2017). Hypoxia is a common issue found in obese patients, particularly in AT and pancreas, and is thought to be one of the mechanisms by which pro-inflammatory signalling is initiated and maintained (Gonzalez *et al.* 2018).

Because of the high demand for mitochondrial respiration under high glucose conditions, pancreatic β-cells consume large amounts of oxygen in a short time, inducing a hypoxic state (Sato *et al.* 2011). Accordingly, pancreatic islets of diabetic mice showed moderate levels of hypoxia through increased expression of HIF-1α and its target genes (Sato *et al.* 2014).

Adaptation to insufficient tissue oxygenation is regulated by HIFs, which are the key mediators of the cellular response to hypoxia. However, these factors are also associated with pathological conditions such as inflammation, bacteriological infection or cancer (Krzywinska *et al.* 2018). The HIF-1α transcription factor is activated in response to cellular hypoxia and induces transcription of various pro-inflammatory genes (Fakhruddin *et al.* 2017) and subsequent recruitment of inflammatory cells (Sun *et al.* 2012).

Interestingly, HIF-1α has been linked to regulation of *S100A4* expression in several different types of tumour cells after exposure to hypoxia. The mechanism by which this process takes place involves hypoxia-induced hypomethylation of the first intron of the *S100A4* gene, facilitating the binding of HIF-1α to HREs in the *S100A4* promoter region, thereby increasing *S100A4* transcription levels (Liu *et al.* 2010, Horiuchi *et al.* 2012, Fei *et al.* 2017).

Importantly, Figure 5.1 suggests that HIF-1α could also act as a transcription factor to modulate *S100A4* expression in the pancreatic β-cell in the glucolipotoxic environment characteristic of T2D.

5.3 Results

5.3.1 HIF-1α is upregulated in glucolipotoxic conditions

Firstly, in order to test the proposed hypothesis by which HIF-1α is involved in the upregulation of *S100A4* expression in INS-1 cells exposed to GLT, the RNAseq data was analysed to obtain readings for *HIF-1α* expression in glucolipotoxicity. A 1.25-fold increase in *HIF-1α* expression was observed, a moderate but significant increase (Figure 5.2).

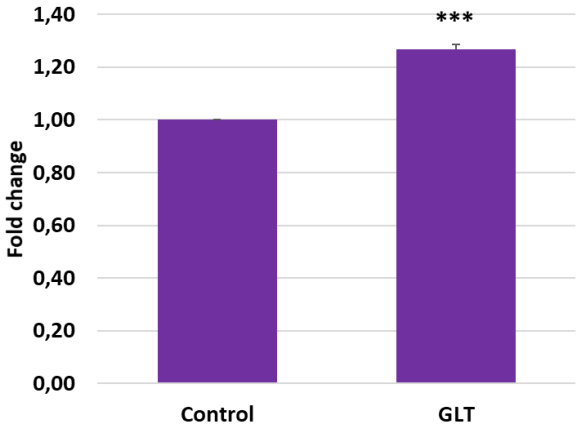


Figure 5.2: Differential expression of *HIF-1α* mRNA levels in GLT conditions compared to control obtained from the RNAseq data. Cells were incubated for 72 hours in control or GLT conditions. RNA was isolated and the sample converted to a library of cDNA fragments, which were then sequenced in a high-throughput manner to obtain short sequences. Resulting reads were aligned to a reference genome to produce a genome-scale transcription map including expression levels for each gene in the different conditions (Wang *et al.* 2009). Data is expressed as means + SEM of 3 independent experiments, ***p<0.0005.

In order to validate the RNAseq expression data for *HIF-1α*, INS-1 cells were incubated in control or GLT conditions for 72 hours. An extra control treatment consisting in adding deferoxamine (DFO) to RPMI media was used parallelly to the control RPMI and GLT treatments, in order to stabilise HIF-1α and examine whether the results obtained in GLT treated cells are comparable to the results obtained in the DFO control cells.

The HIF-1 transcription factor consists of 2 subunits: a constitutively active HIF-1β subunit and a highly regulated HIF-1α subunit. In normoxic conditions, the HIF-1α subunit is hydroxylated by prolyl hydroxylases (PHDs), which mark it for proteasomal degradation.

However, under hypoxic conditions, PHDs are inhibited, therefore HIF-1α is able to translocate to the nucleus, where it dimerises with HIF-1β and together they bind to HREs in the promoter region of target genes (Franke *et al.* 2013). As mentioned in chapter 1, section 1.6, HIF-1α is induced in diabetic pathological conditions (Sato *et al.* 2011). Therefore, the GLT treatment should increase HIF-1α expression and activity. In order to check whether GLT-induced changes are attributable to HIF signalling, DFO was used as a control treatment to stabilise HIF-1α.

In brief, INS-1 cells were treated with 100μM DFO, as described in chapter 2. This agent chelates the $Iron^{3+}$ ions necessary for the PHDs to hydroxylate the HIF-1α subunit (O'Rourke *et al.* 1996), thereby preventing HIF-1α degradation.

After both GLT and DFO treatments, cells were lysed, and total protein extracted and quantified as described in chapter 2. Protein extracts were electrophoresed on 4–20% Mini-PROTEAN® TGX™ precast polyacrylamide gradient gels (BioRad) to allow visualisation of both low and high molecular weight proteins (S100A4 is 14KDa, HIF-1α is 110KDa) (Figure 5.3).

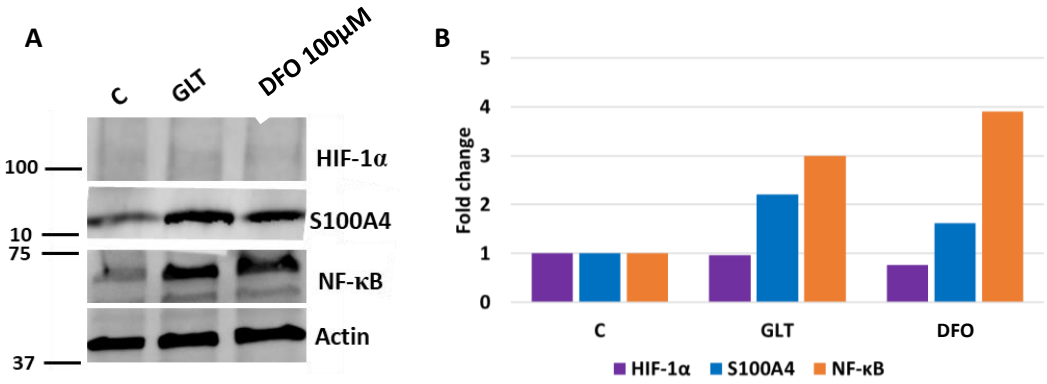


Figure 5.3: Effect of GLT and DFO on HIF-1α, S100A4 and NF-κB protein levels. INS-1 cells were incubated for 72 hours in control, GLT or DFO conditions, and subsequently lysed. Lysates were separated on 4–20% polyacrylamide gradient gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with HIF-1α, NF-κB and S100A4 specific antibodies. **A)** Western blot representative of several trials with different HIF-1α antibodies; **B)** Quantification of western blot bands, normalised to internal actin control.

As it can be observed from Figure 5.3, HIF-1α protein levels are undetectable through western blot, even after trying with different antibodies (only representative image shown). However, interestingly, results show that both S100A4 and NF-κB are upregulated in DFO conditions, as well as in GLT, as we saw earlier.

HIF-1α protein expression and subcellular localisation was then measured through indirect immunofluorescence (Figure 5.4). Interestingly, results show that HIF-1α expression and nuclear localisation increases both in GLT and DFO conditions.

All in all, these results show that the observable effect of the GLT treatment is similar to the effect produced by adding DFO to the cells, suggesting that GLT-induced changes could be driven by an increase in HIF-1α activity.

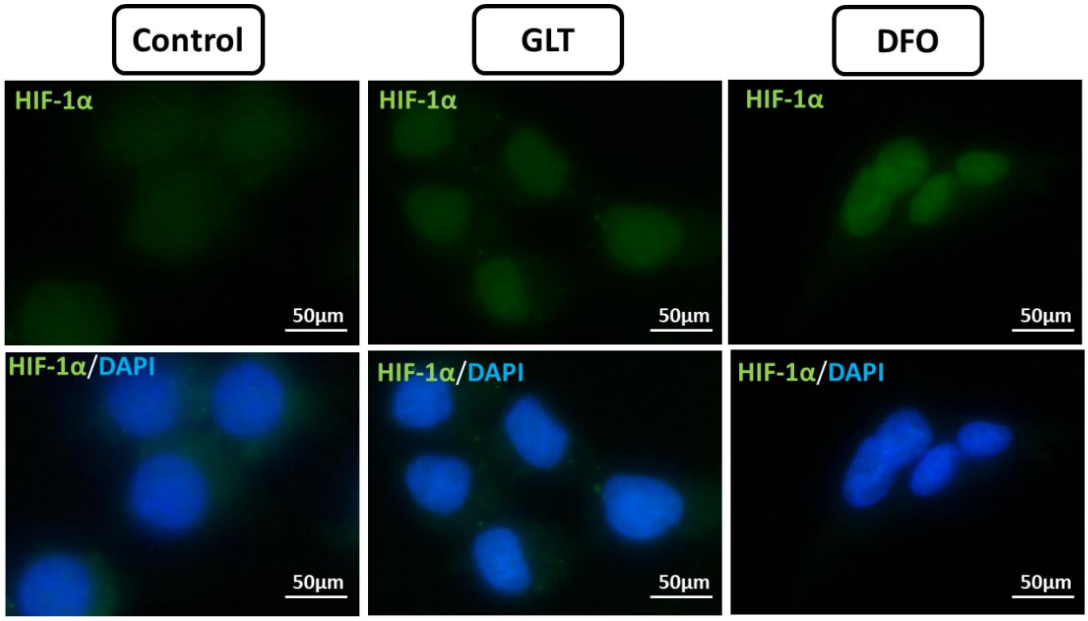


Figure 5.4: Effect of GLT and DFO on HIF-1α protein levels and subcellular localisation. INS-1 cells were cultured on coverslips. After 24 hours, media was removed and control, GLT or DFO conditions were applied for 72 hours. After fixing, cells were incubated with specific HIF-1α antibody, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. Figure shows representative images from 3 independent experiments. Scalebar 1cm:50µm.

5.3.2 HIF-1α is involved in the regulation of S100A4 expression

Our hypothesis states that HIF-1α acts as a transcription factor to activate *S100A4* expression in INS-1 β-cells. In order to test it, siRNA technology was used to transiently knockdown HIF-1α in GLT and DFO conditions, and subsequently measure S100A4 and NF-κB mRNA levels through qPCR, as well as their protein levels through immunofluorescence. mRNA levels were measured to verify the extent of HIF-1α knockdown as it could not be detected through western blot. Similarly, changes in protein levels were only assessed through immunofluorescence. Finally, the effect of *HIF-1α* knockdown on NF-κB activation in GLT compared to DFO and control conditions was also assessed by performing an NF-κB activity assay.

Firstly, the level of HIF-1α knockdown was validated through qPCR, given that its protein levels could not be detected though western blot. Figure 5.5 shows that mRNA levels of HIF-1α increase both in GLT and DFO treated cells in accordance with our immunofluorescence results (Figure 5.4), and more importantly that mRNA levels of HIF-1α decreased in siHIF-1α treated cells by 80%, 64% and 66% in control, GLT and DFO conditions respectively, proving that the knockdown was effective.

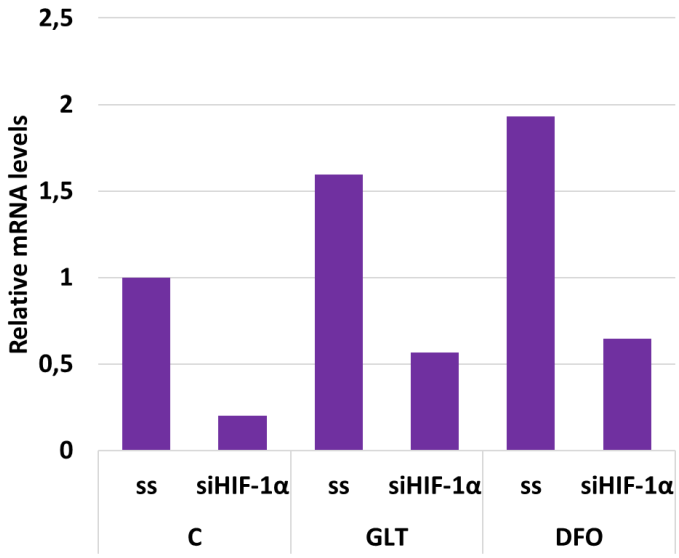


Figure 5.5: HIF-1α mRNA levels in ss/siHIF-1α treated cells in control, GLT and DFO conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control, GLT or DFO conditions were applied for 72 hours. RNA was then extracted, and qPCR conducted using specific primers for HIF-1α. Data represent ΔΔCt values expressed as a fold change compared to cells grown in control conditions and normalised to GAPDH internal control.

Given that mRNA levels do not always correlate with protein levels, and that it could not be detected through western blot, the level of HIF-1α knockdown was also validated through immunofluorescence. Figure 5.6 shows that HIF-1α protein expression and nuclear localisation increases in GLT and DFO, and decreases in siHIF-1α treated cells, in accordance with the qPCR results from the validation of HIF-1α knockdown shown in Figure 5.5.

It is worth mentioning that, despite of the background illumination being to some extent higher in the pictures corresponding to the GLT treatment, the increase in HIF-1α protein expression and nuclear localisation is still very significant compared to control conditions.

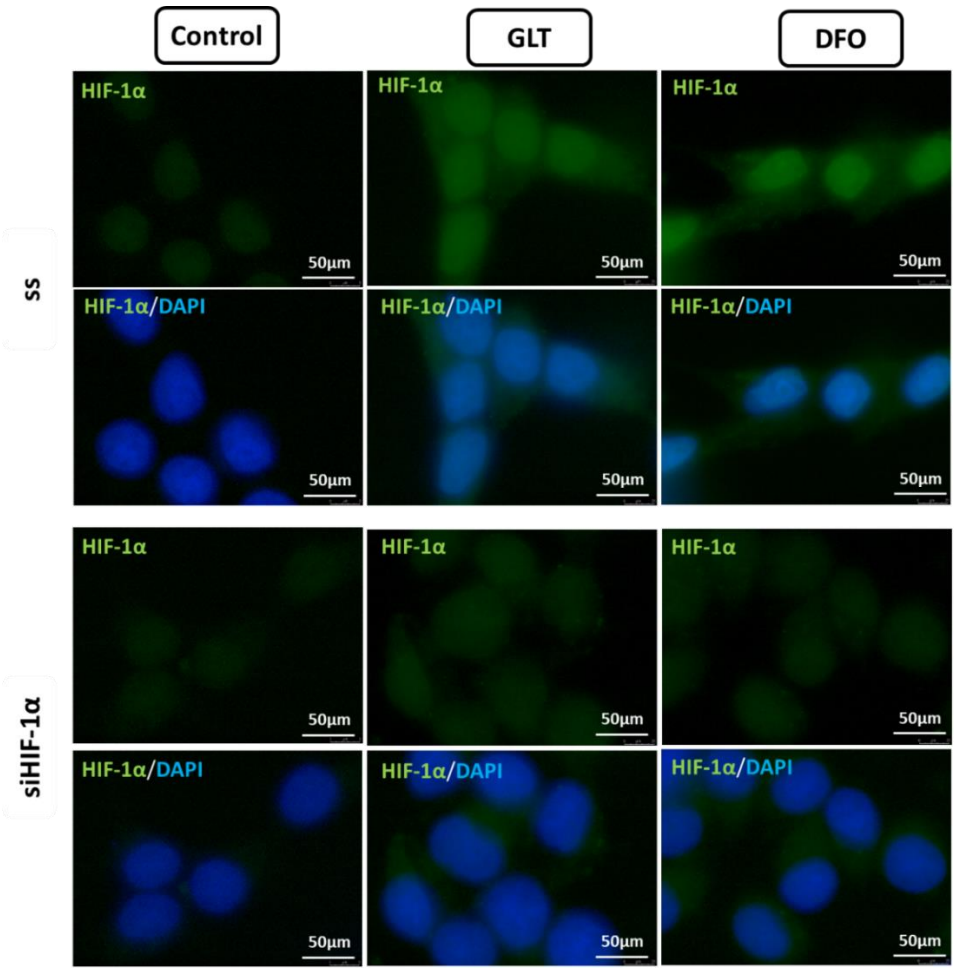


Figure 5.6: HIF-1α protein levels and subcellular localisation in HIF-1α-knocked down cells in control, GLT and DFO conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control, GLT or DFO conditions were applied for 72 hours. After fixing, cells were incubated with specific HIF-1α antibody, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. Figure shows representative images from 3 independent experiments. Scalebar 1cm:50µm.

Once HIF-1α knockdown had been validated, mRNA levels of S100A4 and NF-κB in HIF-1α-knocked down cells were measured (Figure 5.7).

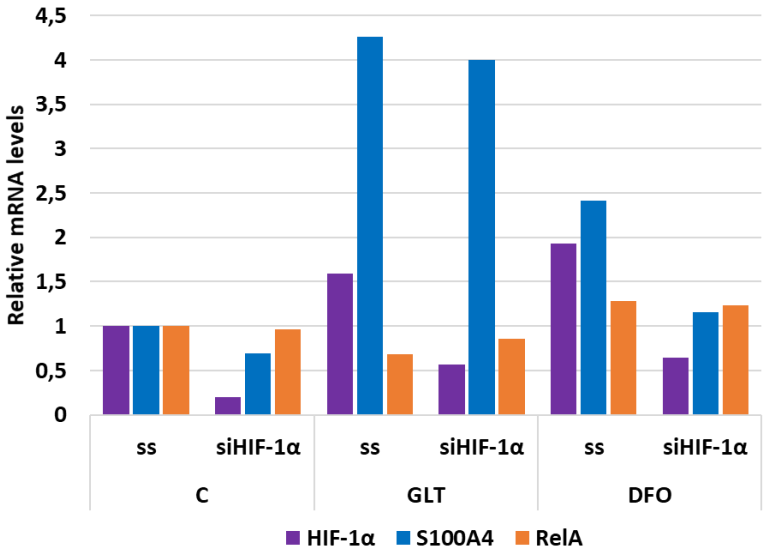
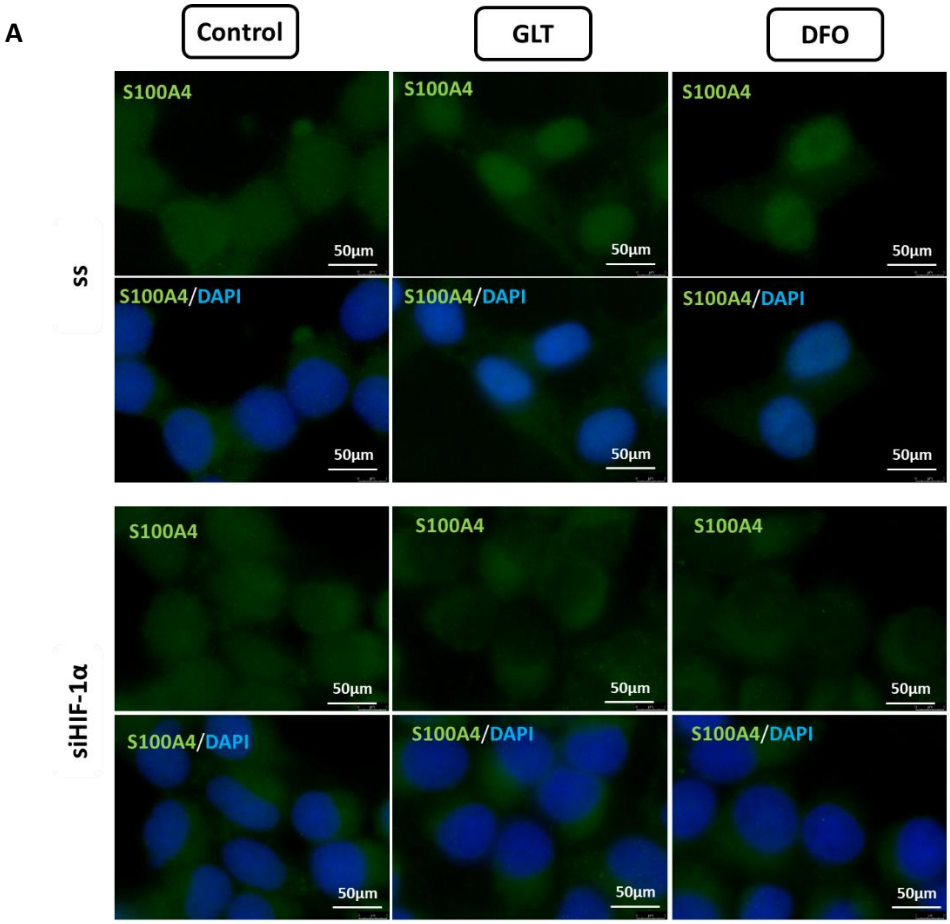


Figure 5.7: Effect of HIF-1α knockdown on S100A4 and NF-κB mRNA levels in control, GLT and DFO conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control, GLT or DFO conditions were applied for 72 hours. RNA was then extracted, and qPCR conducted using specific primers for HIF-1α, S100A4 or the NF-κB subunit RelA. Data represent ΔΔCt values expressed as a fold change compared to cells grown in control conditions and normalised to GAPDH internal control.

Figure 5.7 shows that HIF-1α knockdown decreases S100A4 mRNA levels in all conditions, which suggests that our hypothesis stating that HIF-1α acts as a transcription factor for *S100A4* expression is right. Interestingly, it can be observed that the increase in S100A4 expression in ssRNA-treated cells is much greater in GLT that in DFO, and that the decrease in S100A4 expression induced by HIF-1α knockdown is much greater in DFO than in GLT conditions. Both observations indicate that it may be driven by HIF-1α but that there must be some other component in GLT inducing its increase. Contrarily, the increase in NF-κB expression in ssRNA treated cells is greater in DFO than in GLT conditions, while its levels are unaffected by HIF-1α knockdown, which goes in line with our previous findings that NF-κB protein expression is not affected by S100A4 knockdown either (Figure 4.7). Anyhow, as mentioned earlier, NF-κB mRNA or protein levels do not correlate with its activity levels. These will be assessed further on.

In order to measure the effect of HIF-1α knockdown on S100A4 and NF-κB at the protein level, and given that I could not measure HIF-1α protein levels through western blot, I carried out indirect immunofluorescence on HIF-1α-knocked down cells and looked at the differential S100A4 (Figure 5.8A) and NF-κB (Figure 5.8B) protein expression and subcellular localisation.



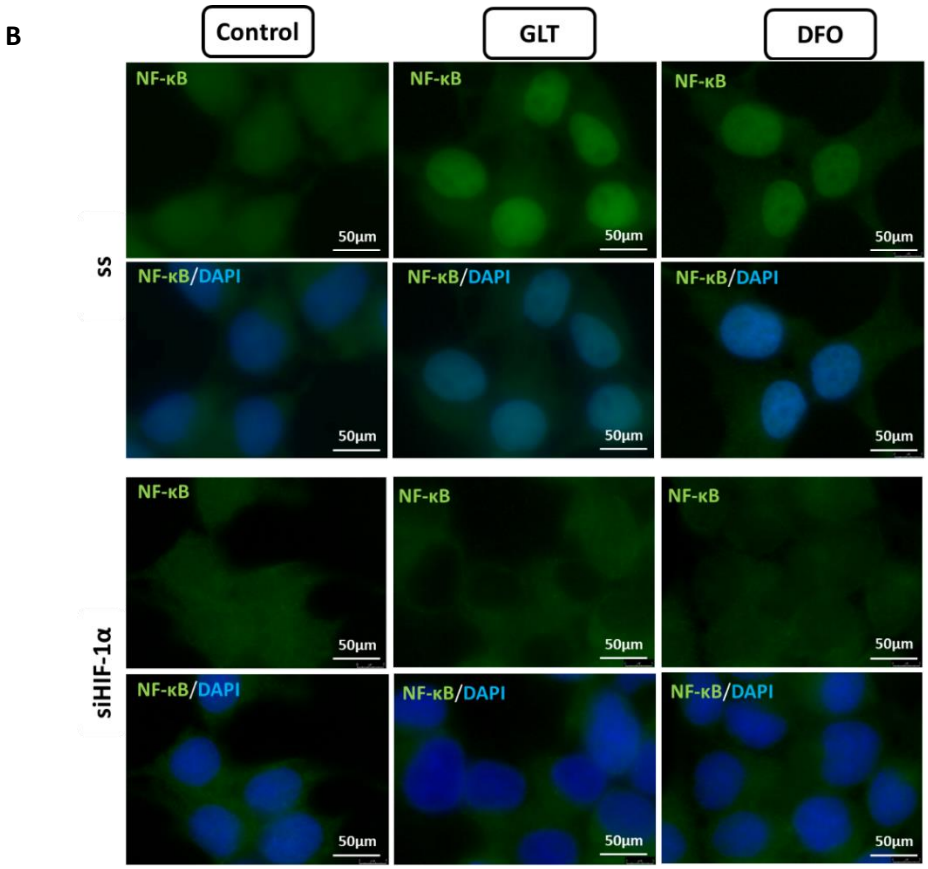


Figure 5.8: Effect of HIF-1α knockdown on S100A4 and NF-κB protein levels and subcellular localisation in control, GLT and DFO conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control, GLT or DFO conditions were applied for 72 hours. After fixing, cells were incubated with specific S100A4 **(A)** or NF-κB **(B)** antibody, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. Figure shows representative images from 3 independent experiments. Scalebar 1cm:50µm.

In accordance with our previous data, Figure 5.8A shows that S100A4 expression increases in GLT. Furthermore, it can be observed that its nuclear localisation also increases in GLT. And more importantly, it can also be observed that treatment with DFO induces the same changes, and that knockdown of HIF-1α reverses them, indicating that HIF-1α is involved in S100A4 expression. In the case of NF-κB, Figure 5.7 shows that mRNA levels were unaffected by HIF-1α knockdown. However, Figure 5.8B shows that its expression and nuclear localisation increases in GLT, as seen earlier (Figure 4.2), and that DFO produces the same effect. Importantly, this increase is reversed in HIF-1α-knocked down cells. This strengthens our theory of the proposed mechanism of action by which HIF-1α would act as a transcription factor for S100A4, which then activates NF-κB.

5.3.3 HIF-1α is involved in the induction of NF-κB activity through regulation of S100A4

Our results up until this point show that NF-κB mRNA or protein levels are not affected by S100A4 or HIF-1α knockdown. However, it is important to note that NF-κB is a transcription factor, therefore its ultimate regulating mechanism is its activity, not its mRNA or protein levels. Measurement of its nuclear localisation through immunofluorescence is however a good way of indirectly measuring its activity, and results indicate that NF-κB nuclear localisation increases both in GLT and DFO, and decreases when either S100A4 or HIF-1α are knocked down (Figures 4.2, 4.7 and 5.8).

A functional study measuring NF-κB activity is therefore needed in order to validate the aforementioned proposed mechanism of action. Accordingly, INS-1 cells were seeded, HIF-1α was knocked down, control, GLT or DFO conditions were applied as usual, and an NF-κB activity assay was conducted.

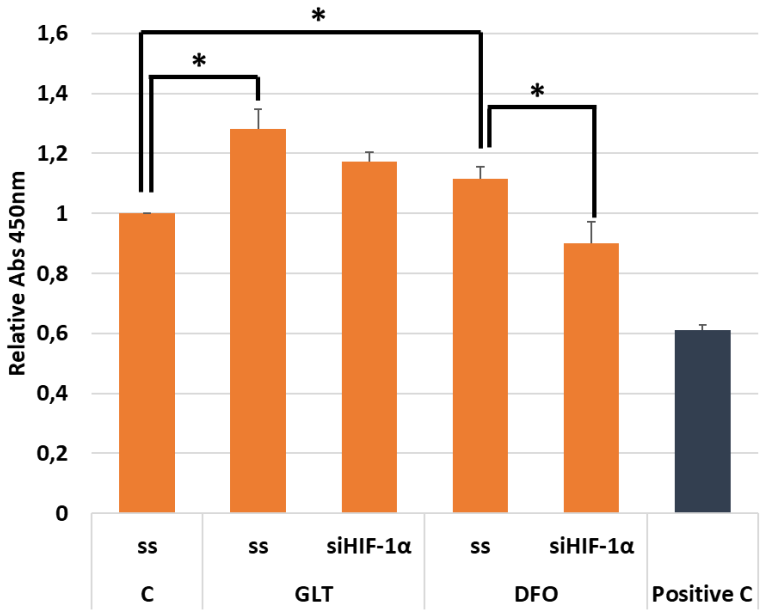


Figure 5.9: Effect of HIF-1α knockdown on NF-κB activity in control, GLT and DFO conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control, GLT or DFO conditions were applied for 72 hours, after which cells were lysed using a nuclear/cytosolic fractionation kit. The nuclear compartment was added to the oligonucleotide coated wells of the activity assay plate, and then incubated with specific NF-κB antibody and subsequent secondary antibody. Developing solution and subsequent stop solution was added to the wells, after which absorbance was read to measure NF-κB activity. Data is expressed as means + SEM of 3 independent experiments (*$p<0.05$).

Results in Figure 5.9 show that, as hypothesised, NF-κB activity increases in GLT and DFO treated cells (by 28% and 11% respectively), and this effect is reversed by HIF-1α knockdown (by 39% and 188% respectively), proving that HIF-1α induces expression of *S100A4*, which then activates NF-κB.

5.3.4 Identification of Karyopherin α2 (KPNA2) as an importin involved in HIF-1α nuclear translocation

Nuclear transport occurs through large nuclear pore complexes in the nuclear membrane. While small factors can diffuse passively through these pores, transport of large (~40 kDa) factors must be mediated by shuttle proteins, termed importins or karyopherins (Stewart 2007). The human genome encodes seven isoforms of importin α which are grouped into three subfamilies known as α1, α2 and α3 (Table 5.1) (Kelley *et al.* 2010). Karyopherin alpha 2 (KPNA2), also known as importin α1, is ~58 kDa and comprises 529 amino acids. It contains an N-terminal hydrophilic importin β-binding domain, a central hydrophobic region consisting of 10 armadillo repeats (which binds to the Nuclear Localisation Site - NLS- of the cargo protein), and a short acidic C-terminus with no reported function (Huang *et al.* 2013).

Table 5.1: The karyopherin α protein family.

Subfamily	Protein name	Alternative names	Gene name	Accession number
α1	**Importin α1**	**Karyopherin α2, hSRP1α, Rch1, Qip2, NPI-3**	***KPNA2***	**P52292**
	Importin α8	Karyopherin α7	*KPNA7*	A9QM74
α2	Importin α3	Karyopherin α4, Qip1	*KPNA4*	O00629
	Importin α4	Karyopherin α3, hSRP1γ, Qip2	*KPNA3*	O00505
α3	Importin α5	Karyopherin α1, NPI-1, SRP1, hSRP1	*KPNA1*	P52294
	Importin α6	Karyopherin α5	*KPNA5*	O15131
	Importin α7	Karyopherin α6, NPI-2	*KPNA6*	O60684

Despite their similarity in amino acid sequence and 3D structure, importin α isoforms display notable substrate specificity *in vivo* (Pumroy *et al.* 2015), conferring preferential nuclear entry of viral and cellular cargoes linked to human diseases. Furthermore, there are studies that link altered importin α to many forms of cancer (Kim *et al.* 2000). In most cases, the upregulation of the *KPNA2* gene indicates poor prognosis and poor survival rates, making it a useful biomarker (Zheng *et al.* 2010, Winnepenninckx *et al.* 2006). Accordingly, siRNA-mediated knockdown of importin α1 has proven effective in decreasing proliferation of cancerous cells in some cases (Wang *et al.* 2011).

Importantly, importin α1 (KPNA2) has also been linked to nuclear translocation of several members of the HIF factors family, including HIF-1α (Depping *et al.* 2008). If we go back to the interaction network in Figure 5.1, we can see that there is an importin α directly linked to HIF-1α, which is also directly linked to S100A4. Moreover, gene expression of this importin is upregulated as indicated by the red circle next to its symbol, suggesting an involvement of KPNA2 in HIF-1α nuclear transport in glucolipotoxic conditions.

In order to test this hypothesis, I first analysed the RNAseq data for *KPNA2* differential expression in glucolipotoxicity compared to control. As the interaction network does not specify which of all the importins α is involved in this link, I analysed the expression of all the importin α isoforms included in the RNAseq data (Figure 5.10).

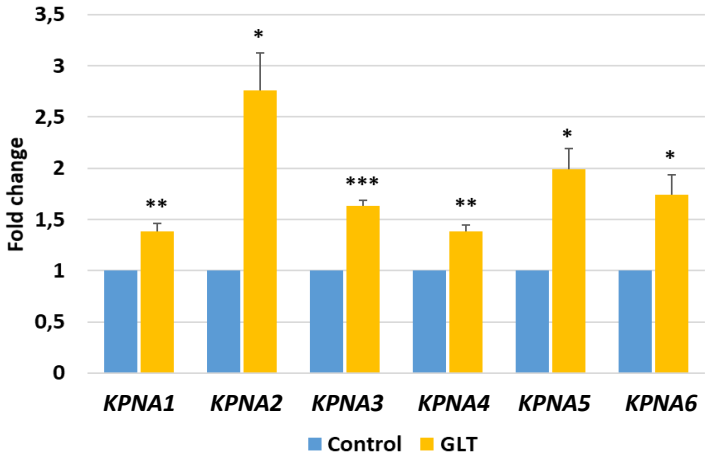


Figure 5.10: Differential expression of *KPNA* isoforms mRNA levels in GLT conditions compared to control obtained from the RNAseq data. Cells were incubated for 72 hours in control or GLT conditions, RNA was isolated, and the sample converted to a library of cDNA fragments, which were then sequenced in a high-throughput manner to obtain short sequences. Resulting reads were aligned to a reference genome to produce a genome-scale transcription map including expression levels for each gene in the different conditions (Wang *et al.* 2009). Data is expressed as means + SEM of 3 independent experiments, *p<0.05, **p<0.005, ***p<0.0005.

Interestingly, Figure 5.10 shows that *KPNA2* is the most highly upregulated isoform of the importin α family, with a 2.7-fold increase in glucolipotoxic conditions. It is therefore highly likely that this is the member that is included in the interaction network shown in Figure 5.1.

Subsequently, the increase in KPNA2 protein expression in INS-1 cells cultured in GLT, and DFO conditions was determined through western blot. Figure 5.11 shows that KPNA2 protein is slightly but significantly upregulated in both conditions, validating the RNAseq data regarding the GLT treatment, and suggesting that HIF-1α is also involved in the regulation of its expression.

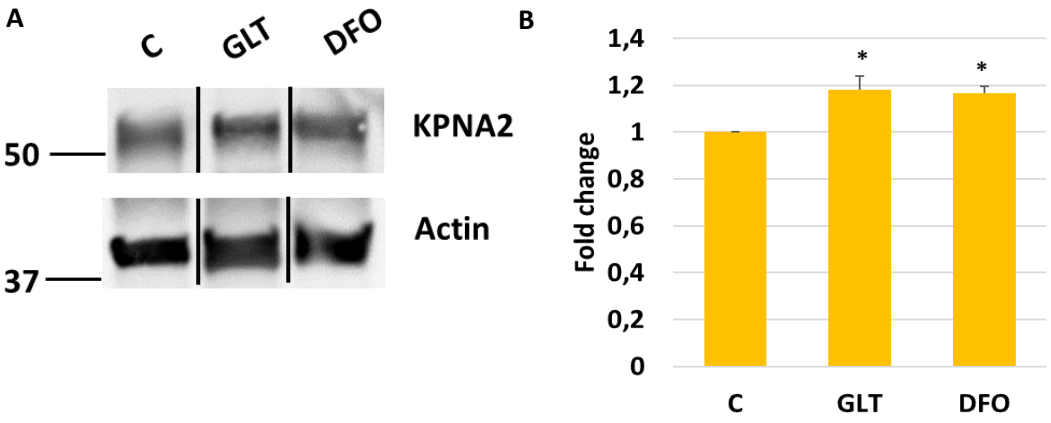


Figure 5.11: Effect of GLT and DFO on KPNA2 protein levels. INS-1 cells were incubated for 72 hours in control, GLT or DFO conditions, and subsequently lysed. Lysates were separated on 12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with KPNA2 specific antibody. **A)** Western blot, representative of 3 independent experiments; **B)** Quantification of anti-KPNA2 western blot bands, normalised to internal actin control. Data is expressed as means + SEM of 3 independent experiments (*p<0.05).

In order to determine whether KPNA2 is involved in HIF-1α translocation to the nucleus and subsequent activation of *S100A4* expression, siRNA technology was used to transiently knockdown KPNA2, and changes in HIF-1α, S100A4 and NF-κB protein expression and subcellular localisation were assessed through immunofluorescence. However, as we have seen, protein levels do not always correlate with transcription factor activity, therefore the effect of *KPNA2* knockdown on NF-κB activity was also measured with the use of an NF-κB activity assay.

Firstly, KPNA2 knockdown levels were validated at the protein level through western blot analusis. Figure 5.12 shows that a 28%, 24% and 23% knockdown was achieved in control, GLT and DFO treated cells respectively.

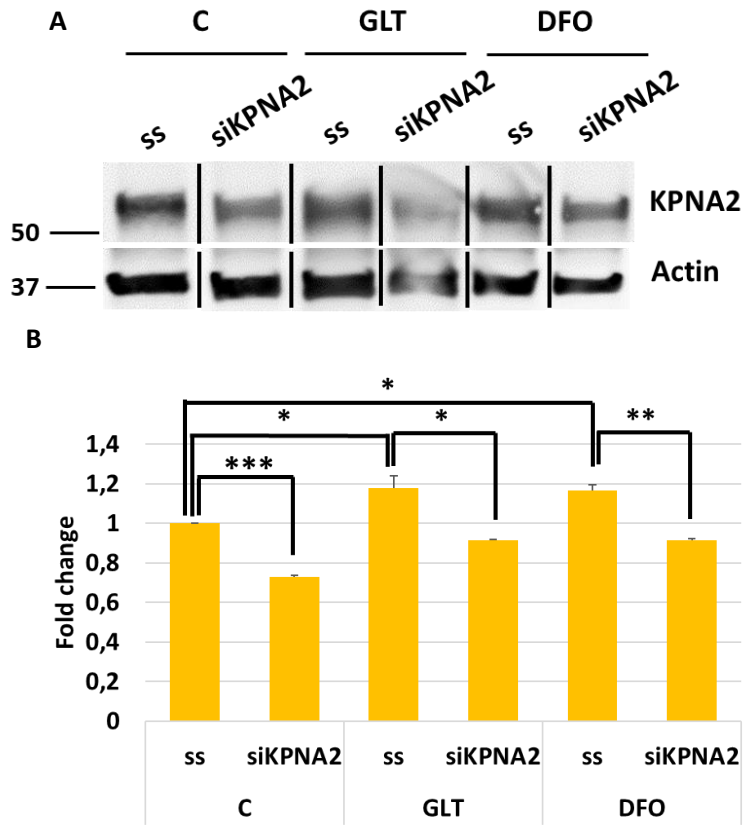


Figure 5.12: KPNA2 protein levels in ss/siKPNA2 treated cells in control, GLT and DFO conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control, GLT or DFO conditions were applied for 72 hours. Total protein was then extracted, and lysates were separated on 12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with KPNA2 specific antibody. **A)** Representative image of a western blot of KPNA2 knockdown; **B)** Quantification of anti-KPNA2 western blot bands, normalised to internal actin control. Data is expressed as means + SEM of 3 independent experiments (*p<0.05, **p<0.005, ***p<0.0005).

Validation of KPNA2 upregulation in GLT and DFO-treated cells, as well as the level of KPNA2 knockdown in siRNA treated cells was also assessed through immunofluorescence. Figure 5.13 shows that KPNA2 expression increases in GLT and DFO, and decreases in siKPNA2 treated cells, correlating with the western blot results.

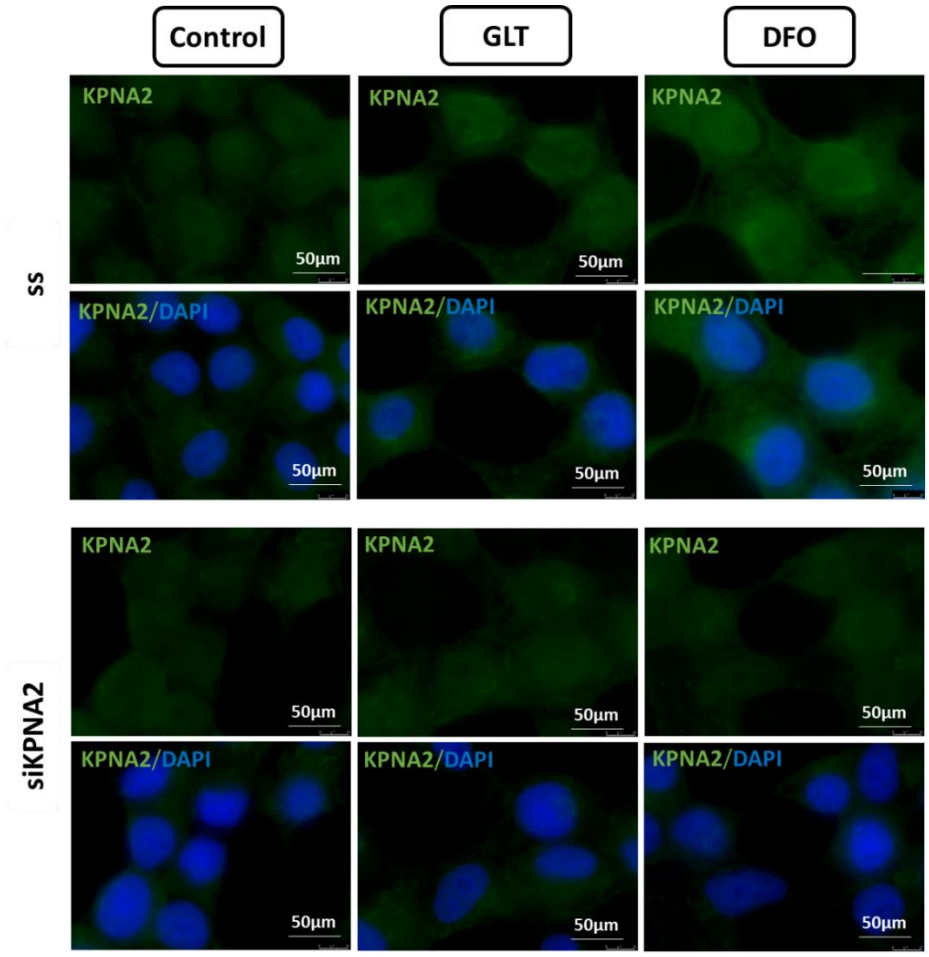
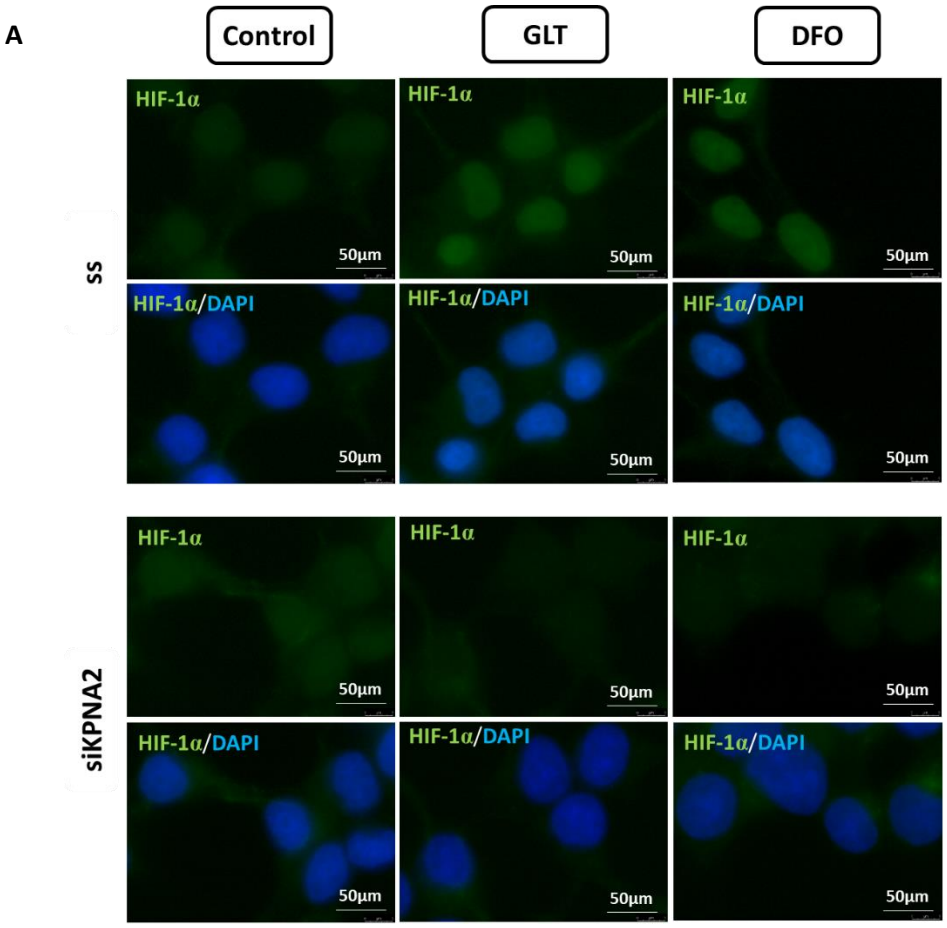
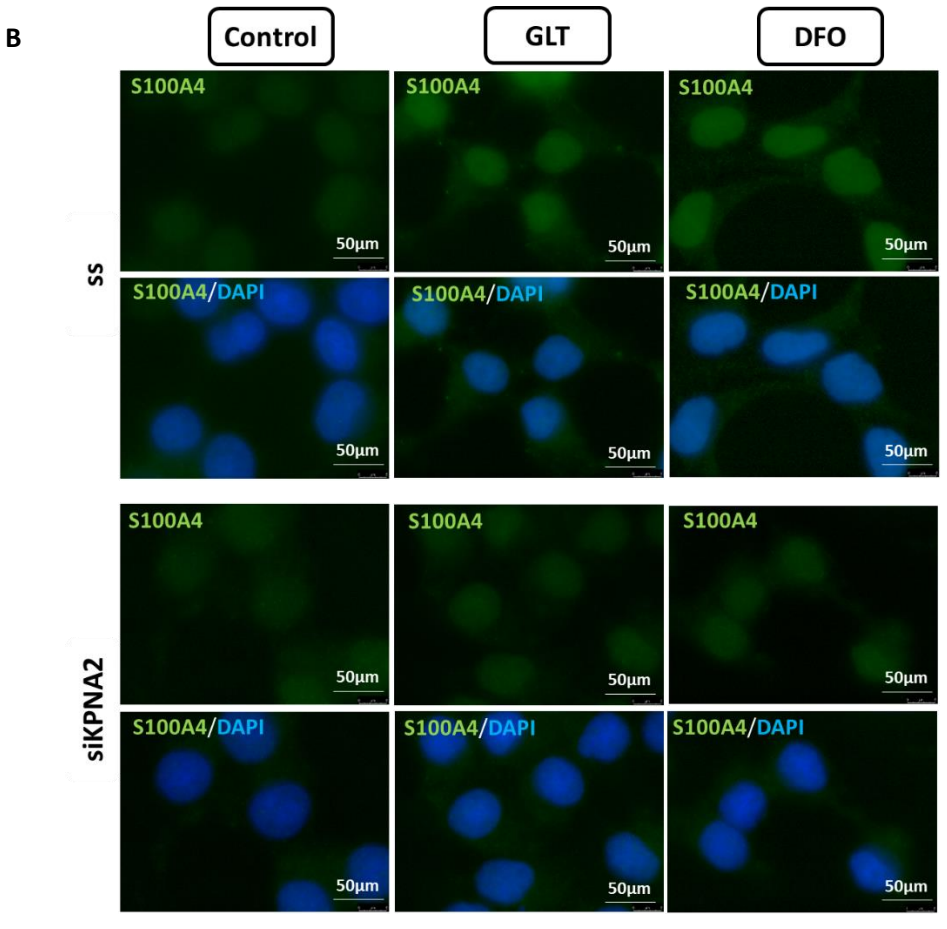


Figure 5.13: **KPNA2 protein levels and subcellular localisation in ss/siKPNA2 treated cells in control, GLT and DFO conditions**. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control, GLT or DFO conditions were applied for 72 hours. After fixing, cells were incubated with specific KPNA2 antibody, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. Figure shows representative images form 3 independent experiments. Scalebar 1cm:50µm.

Once established that I had achieved a significant level of knockdown of KPNA2 in all conditions, I next measured the changes that the decreased levels of KPNA2 triggered in HIF-1α (Figure 5.14A), S100A4 (Figure 5.14B) and NF-κB (Figure 5.14C) protein expression and subcellular localisation through immunofluorescence. Results show that KPNA2 knockdown reverses the GLT and DFO-driven increase in expression and nuclear localisation of HIF-1α (Figure 5.14A), which in turn causes a decrease in S100A4 protein expression (Figure 5.14B), which ultimately causes a decrease in NF-κB nuclear localisation (Figure 5.14C).


A
Control
GLT
DFO
ss
siKPNA2
HIF-1α
HIF-1α/DAPI
50μm


B
Control
GLT
DFO
ss
siKPNA2
S100A4
S100A4/DAPI
50μm

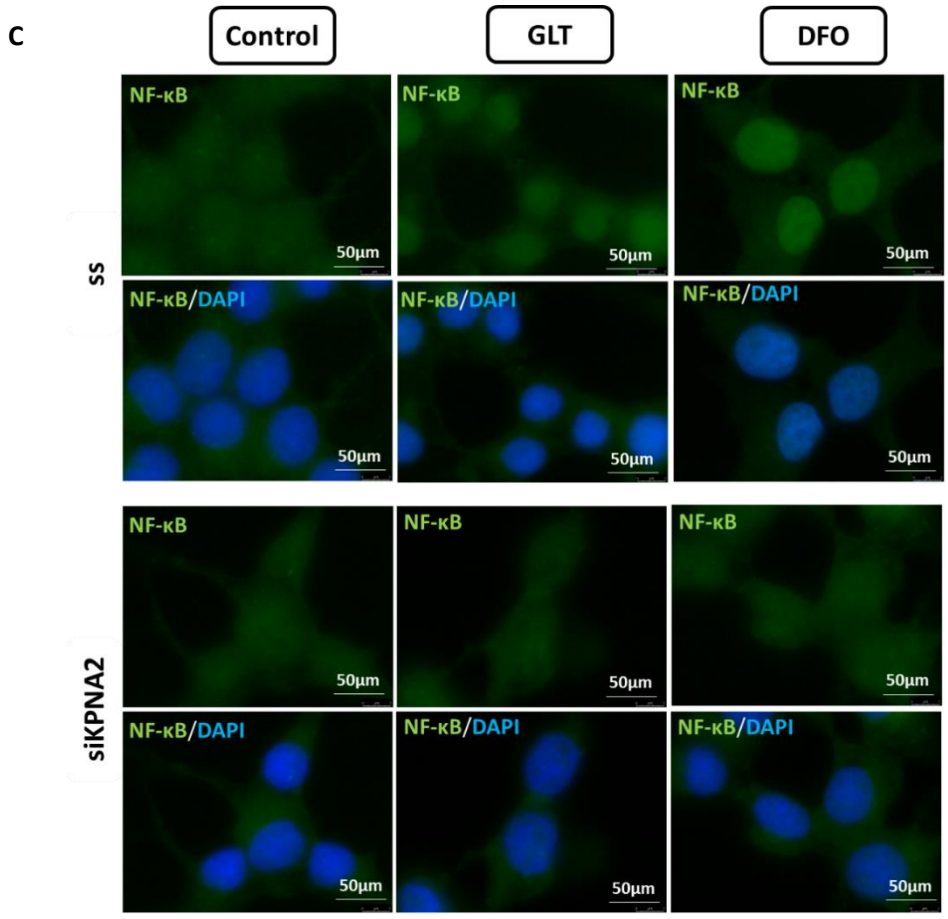


Figure 5.14: Effect of KPNA2 knockdown on HIF-1α, S100A4 and NF-κB protein expression and cellular localisation in control, GLT and DFO conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and replaced with control, GLT or DFO media for 72 hours. After fixing, cells were incubated with specific HIF-1α, S100A4 or NF-κB antibodies, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. Figure shows representative images of 3 independent experiments. Scalebar 1cm:50µm.

However, even though a decreased nuclear localisation of HIF-1α can be observed in KPNA2 knockdown cells (Figure 5.14A), if we are blocking HIF-1α translocation to the nucleus we should be seeing an increase in its cytoplasmic localisation. However, this is not particularly apparent from Figure 5.14A. It was therefore hypothesised that HIF-1α gets degraded as it accumulates in the cytoplasm. As mentioned in section 5.3.1, HIF-1α undergoes proteasomal degradation when marked by PHDs in normoxic conditions. I decided to use MG132, a proteasome inhibitor (Han *et al.* 2009, Goldberg 2012), to inhibit HIF-1α degradation, which would allow detection of HIF-1α in the cytoplasm.

In brief, a 10mM stock of MG132 was prepared in distilled water (Table 2.1). Cells were seeded, KPNA2 was knocked down and control, GLT or DFO conditions applied for 72 hours as usual. MG132 was then added at a final concentration of 10µM for the last six hours prior to cell fixation. Results in figure 5.15 show a modest increase in HIF-1α cytoplasmic localisation when cells were treated with MG132, compared to HIF-1α expression and localisation when MG132 is not added (Figure 5.14A).

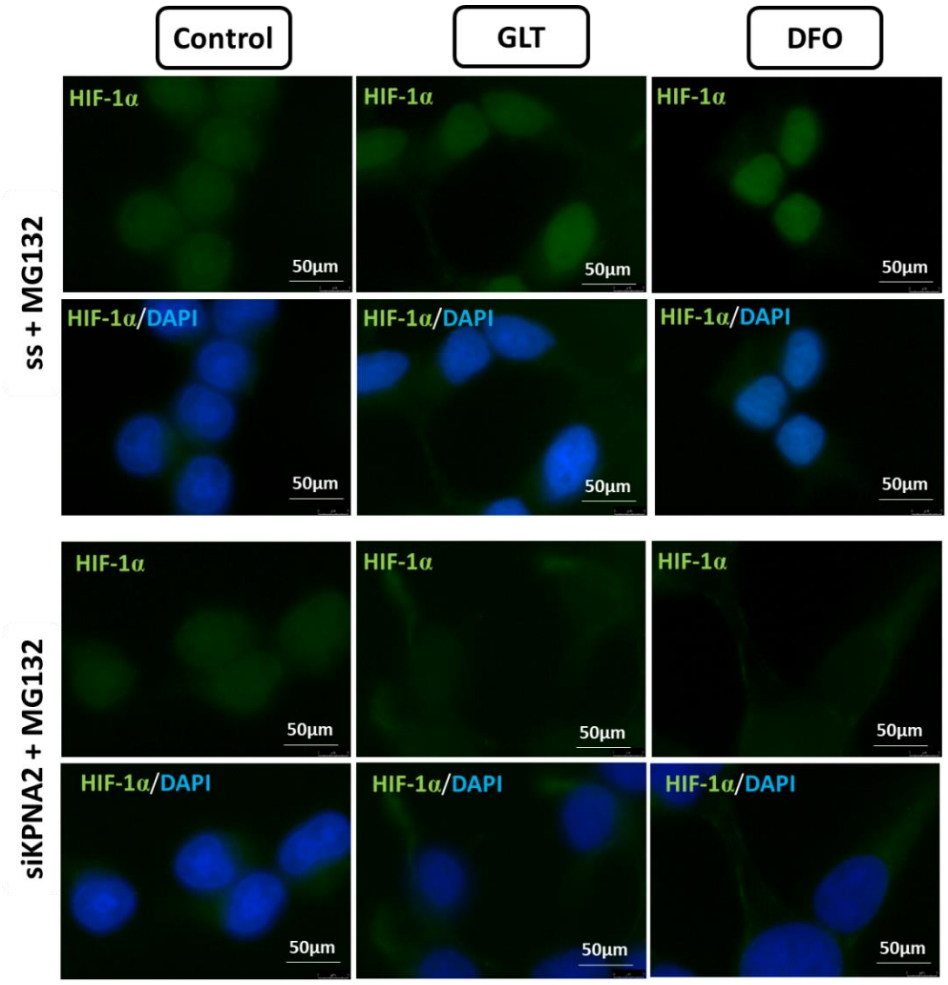


Figure 5.15: Effect of MG132 proteasome inhibitor on HIF-1α protein expression and subcellular localisation in KPNA2 knockdown cells in control, GLT and DFO conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control, GLT or DFO conditions were applied for 72 hours. For the last 6 hours of the incubation, MG132 was added to the cells at a final concentration of 10µM to inhibit HIF-1α proteasomal degradation. After fixing, cells were incubated with specific HIF-1α antibody, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. Figure shows representative images of 3 independent experiments. Scalebar 1cm:50µm.

Finally, a functional study is needed in order to confirm the effect of KPNA2 knockdown on NF-κB activity. INS-1 cells were seeded, KPNA2 was knocked down, control, GLT or DFO conditions applied as usual, and an NF-κB activity assay performed. Results in Figure 5.16 show that NF-κB activity increases in GLT and DFO treated cells (by 40% and 61% respectively) as seen before (Figure 4.4 and Figure 5.9), and that this effect is reversed by KPNA2 knockdown (by 80% and 72% respectively).

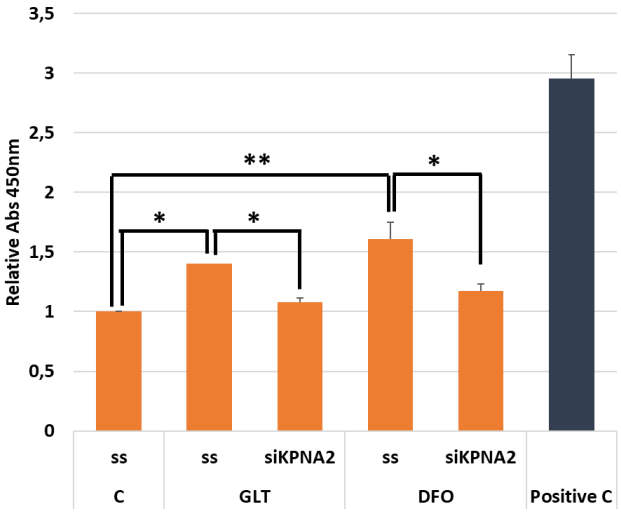


Figure 5.16: Effect of KPNA2 knockdown on NF-κB activity in control, GLT and DFO conditions. INS-1 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and control, GLT or DFO conditions were applied for 72 hours, after which cells were lysed using a nuclear/cytosolic fractionation kit. The nuclear compartment was added to the oligonucleotide coated wells of the activity assay plate, and then incubated with specific NF-κB antibody and subsequent secondary antibody. Developing solution and subsequent stop solution was added to the wells, after which absorbance was read to measure NF-κB activity. Data is expressed as means + SEM of 3 independent experiments (*$p<0.05$, **$p<0.005$).

5.4 Discussion and future directions

The study of the transcriptome has allowed a better understanding of the molecular changes involved in the onset of diabetic pathological conditions. Previously in our laboratory, RNAseq technology was used to characterise the molecular changes in the pancreatic β-cell after exposure to high concentrations of glucose and FAs, whilst in this thesis, the resulting RNAseq data was used to identify new molecules that could be involved in the impairment of β-cell function through pathway analysis.

Chapter 3 describes how S100A4 was identified as a protein involved in the GLT-induced activation of NF-κB and subsequent activation of inflammatory pathways. In order to avoid the onset of inflammation-driven T2D pathogenesis, S100A4-induced NF-κB activation could be targeted. One way to achieve this is through the downregulation or inhibition of S100A4 with the use of siRNA, microRNA or other inhibitors, as it has been done in several cancer and other studies involving different pathologies (Zhang *et al.* 2011, Liang *et al.* 2014, Zhang *et al.* 2016, Liu *et al.* 2012). As described earlier, the canonical pathway of NF-κB activation involves various steps, including the phosphorylation, ubiquitination, and degradation of IκBα. Thus, another way of regulating S100A4-induced NF-κB activation could be to use inhibitors or regulators of kinases as reported previously (Qu *et al.* 2015) to target IKK-mediated phosphorylation of IκBα. Finally, a third way of regulating S100A4-mediated activation of NF-κB activation could be to modulate *S100A4* at the transcriptional level, through the identification of molecules or transcription factors that directly interact with its regulatory regions.

In order to identify a wider network of interacting partners and pathways, an interaction network was constructed for *S100A4* together with our collaborators in Barts and The London School of Medicine and Dentistry based on our RNAseq data. This was done with use of Metacore™, which is able to identify genes directly linked to *S100A4* in our glucolipotoxic model of T2D. The resulting network was used to identify *HIF-1α* as a novel factor linked to *S100A4* in a diabetic scenario, and to infer the directionality of this link, by which *HIF-1α* acts on *S100A4*.

There is evidence that, in hypoxic conditions, HIF-1α binds to HREs in the promoter region of *S100A4* to activate its expression in some cancer cases (Horiuchi *et al.* 2012, Reimann *et al.* 2015) as well as in other types of cells when exposed to hypoxia (Liu et al. 2012). Herein, I wanted to determine whether HIF-1α could also regulate *S100A4* expression in hypoxic conditions characteristic of T2D, something that had not been proposed before. In order to do so, after validating HIF-1α increased expression and nuclear localisation in INS-1 cells exposed to GLT and DFO conditions, *HIF-1α* was knocked down, and S100A4 expression levels were measured. Results show that they decreased both at the mRNA and the protein level, indicating that HIF-1α could be acting as a transcription factor for S100A4 expression.

Given that HIF-1α knockdown decreased S100A4 expression levels, a decrease in NF-κB expression and activity would also be expected. Even though there was no change in NF-

κB mRNA levels after HIF-1α knockdown, mRNA levels do not necessarily equate protein levels or transcriptional activity. Therefore, indirect immunofluorescence was carried out, revealing that NF-κB nuclear localisation, which is indicative of transcriptional activity and which was seen to increase in GLT- and DFO-treated cells, decreased when HIF-1α was knocked down. Furthermore, the NF-κB activity assay reveals that its transcriptional activity also decreases when HIF-1α is diminished, strengthening our proposed mechanism of S100A4 induced-NF-κB activation via HIF-1α-mediated upregulation of *S100A4* expression in hypoxic conditions.

An alternative method of blocking HIF-1α-induced S100A4 expression other than knocking HIF-1α down was then explored. Interestingly, importin KPNA2 had previously been linked to nuclear translocation of several members of the HIF factors family, including HIF-1α (Depping *et al.* 2008). Moreover, KPNA2, which has been found to be uniformly upregulated in several cancer types (Rachidi *et al.* 2013), has recently been the subject in an oral squamous cell carcinoma (OSCC) study in which it has been knocked down, resulting in inhibition of autophagy and subsequent suppression of cell migration, by blocking p53 nuclear translocation (Lin *et al.* 2018).

Our interaction network for *S100A4* shows that there is an importin-α directly linked to *HIF-1α* and to *S100A4*. After analysing the RNAseq data, *KPNA2* was identified as the most highly upregulated importin α in the dataset, suggesting an involvement of KPNA2 in HIF-1α nuclear transport in glucolipotoxic conditions. I therefore decided to knockdown KPNA2 and determine the effects on HIF-1α subcellular localisation and consequently, on S100A4 protein expression and NF-κB activity.

After validating KPNA2 knockdown, a decrease in HIF-1α, S100A4 and NF-κB expression and nuclear localisation was observed, suggesting that KPNA2 is indeed involved in HIF-1α nuclear translocation.

However, as mentioned before, inhibition of HIF-1α nuclear translocation would imply its cytoplasmic accumulation, which is not obvious from our results in Figure 5.14A. It was hypothesised that, not being able to translocate to the nucleus, HIF-1α was subject to proteasomal degradation. Accordingly, it has been seen that during prolonged exposure to hypoxia, HIF-α increases mRNA levels of PHDs, constituting a direct, negative regulatory mechanism that leads to HIF-α degradation, which limits its cytoplasmic accumulation in chronic hypoxic conditions (Marxsen *et al.* 2004). In order to test this, MG132, a proteasome inhibitor (Han *et al.* 2009, Goldberg 2012), was used to block HIF-α

degradation. This would allow its detection the cytoplasm through immunofluorescence. Results in Figure 5.15 show a modest increase in HIF-1α cytoplasmic localisation after KPNA2 knockdown when cells were treated with MG132, suggesting that our hypothesis is correct.

Subsequently, in order to validate whether the decrease in NF-κB nuclear localisation after KPNA2 knockdown correlated with a decrease in its transcriptional activity, I carried out an NF-κB activity assay in KPNA2-knocked down cells in control, GLT and DFO conditions and observed an increase in its activity driven by both GLT and DFO treatment, which was reversed when KPNA2 was knocked down (by 80% and 72% respectively) (Figure 5.16).

However, it could be argued that the decrease in NF-κB nuclear localisation and transcriptional activity after KPNA2 knockdown could be due to the impairment of its own nuclear translocation through the KPNA2 importin, rather than due to the decreased HIF-1α induction of *S100A4* expression and subsequent NF-κB activation. However, nuclear translocation of the NF-κB (p50/p65) transcription factor has been seen to be mediated by importins α3, 4, 5 and 6 (Fagerlund *et al.* 2008, Fagerlund *et al.* 2005), but not α1, so by knocking down KPNA2 we are only blocking HIF-1α nuclear translocation without affecting that of NF-κB.

Although promising, these results are somewhat preliminary due to the inability to quantify changes in HIF-1α through western blot, as well as the unreliability posed by the eye-based quantification of all the immunofluorescence images. Therefore, further research is needed in order to validate the link between HIF-1α and *S100A4* other than blocking HIF-1α nuclear translocation through KPNA2 knockdown. Some experiments that could be done include inhibiting HIF-1α with the use of agents such as chetomin, a dithiodiketopiperazine metabolite that inhibits HIF binding to the transcriptional coactivator p300, thereby attenuating hypoxia-dependent transcription (Staab *et al.* 2007, Viziteu *et al.* 2016); or chrysin, an antioxidant flavonoid with anti-inflammatory and antitumor properties that inhibits HIF-1α protein synthesis through AKT signalling (Fu *et al.* 2007); or dimethyl-Bisphenol A, an endocrine-disrupting chemical that promotes degradation of HIF-1α protein by dissociating Hsp90 from HIF-1α (Kubo *et al.* 2004). Another possible experiment could be to induce methylation of the *S100A4* promoter region in order to block HIF-1α binding to its HREs (Horiuchi *et al.* 2012), thereby lowering its expression.

CHAPTER 6: Investigation of a potential link between T2D and cancer through HIF-1α and S100A4

6.1 Type 2 diabetes and cancer

6.1.1 Epidemiologic association between T2D and cancer

T2D and cancer are two heterogeneous, multifactorial, severe, and chronic diseases with alarming increasing incidence worldwide. Abundant epidemiologic data show that people with diabetes present a significantly higher risk for developing many different forms of cancer, although potential biologic links between the two diseases are only partly understood (Giovannucci *et al*. 2010).

The relative risks portrayed by T2D are greatest for pancreas, liver and endometrium cancers, and smaller but still significant for colon, rectum, breast and bladder cancers. On the other hand, it has been reported that presence of T2D correlates inversely with incidence of prostate cancer (Vigneri *et al*. 2009). Other cancers such as lung cancer do not appear to be influenced by the presence of diabetes, and evidence for other cancers such as kidney or lymphoma is inconclusive (Giovannucci *et al*. 2010).

As mentioned, several meta-analyses indicate that the greatest association is between T2D and pancreas and liver cancer. This is most likely due to the increased exposure of these organs to hyperinsulinemia, a characteristic trait of T2D, given the established role of insulin as a growth factor with well-known metabolic and mitogenic effects (Giovannucci *et al*. 2010). Accordingly, a recent study shows that, amongst T2D subjects, association with cancer incidence was more relevant for insulin-treated patients (Ballotari *et al*. 2017).

Some epidemiological studies suggest that on top of being a risk factor, presence of T2D may also significantly increase mortality in cancer patients (Barone *et al*. 2008, Lipscombe *et al*. 2008). Importantly, high pre-diagnosis CRP levels, an indirect marker of IR, have been associated with poor survival rates for prostate (Ma *et al*. 2008) and colorectal cancer (Wolpin *et al*. 2009).

Other factors associated with T2D such as obesity, hyperglycemia or increased oxidative stress have also been proposed to contribute to increased cancer risk (Giovannucci *et al*. 2010). However, it remains unclear whether the diabetes-cancer link is direct (for example

due to hyperglycemia), whether diabetes is a marker of underlying biological factors that influence cancer risk (for example IR and hyperinsulinemia), or whether this link is indirect and due to shared risk factors between both diseases, such as obesity (Giovannucci *et al*. 2010).

6.1.2 Common risk factors between T2D and cancer

Potential risk factors common to both cancer and T2D can be modifiable or non-modifiable. Non-modifiable factors include ageing (disease incidence increases with age) (de Magalhães 2013), sex (men have slightly higher risk of developing T2D and certain types of cancer) (Kim HI *et al*. 2018, Wu *et al*. 2018), and ethnicity (African Americans present the highest risk of T2D and some types of cancer due to socioeconomical and/or biological differences and genetic factors) (MD Anderson Cancer Centre). On the other hand, modifiable factors include obesity (it is considered the main cause for IR and T2D, and is associated with a much higher risk for many types of cancer) (Nguyen *et al*. 2011, Dobbins *et al*. 2013), diet (low red and processed meat and high vegetable, fruit and whole grain-based diets are associated with a protective role against T2D and a lower risk for many types of cancer, while energy-dense diets rich in sugary and highly-processed foods contribute to obesity and consequently T2D and cancer) (Barclay *et al*. 2008, Kastorini *et al*. 2009), physical activity (a protective role for increased physical activity in T2D and cancer has been established) (Hamasaki 2016, Clague *et al*. 2012), alcohol consumption (excess consumption has been linked to increased T2D risk, while a protective role has been described for moderate consumption; on the other hand, even moderate alcohol consumption has been seen to increase the risk of many types of cancer) (Howard *et al*. 2004, Baliunas *et al*. 2009, Connor 2017), and smoking (it has been associated with the development of T2D and its cardiovascular-related complications, while it accounts for 71% of all trachea, bronchus, and lung cancer deaths and is associated with many other types of cancer) (Chang 2012, Jacob *et al*. 2018).

6.1.3 Underlying mechanisms of the T2D-cancer link

As mentioned above, it remains unclear whether the link between T2D and cancer is due to their shared risk factors such as obesity, or whether diabetes itself, together with its characteristic metabolic imbalances (hyperinsulinemia, hyperglycemia and chronic

inflammation), and the changes induced by the use of anti-diabetic drugs, increase the risk for some types of cancer (Giovannucci *et al*. 2010).

a) Hyperinsulinemia

Insulin is a well-known growth factor with mitogenic effects (Giovannucci *et al*. 2010). Most cancer cells express insulin and IGF1 receptors, which, in addition to their metabolic functions, are also capable of stimulating cancer cell proliferation, protection from apoptotic stimuli, invasion and metastasis, upon insulin signalling (Denley *et al*. 2007). Furthermore, hyperinsulinemia could indirectly promote carcinogenesis through its effects on IGF1. Insulin decreases hepatic production of IGF binding proteins (Renehan *et al*. 2006), resulting in increased circulating levels of IGF1, a more potent mitogenic and anti-apoptotic factor than insulin (Weinstein *et al*. 2009). Accordingly, available epidemiological data indicates that insulin secretion rate and IGF1 levels influence cancer risk and/or progression (Pollak 2012).

b) Hyperglycemia

Glucose constitutes one of the most important sources of energy for tumour cells (Dang 2012). Cancer cells rewire their metabolism to promote growth, survival and proliferation through increased glucose uptake and its fermentation to lactate rather than the much more efficient oxidative phosphorylation pathway, even in the presence of functioning mitochondria and sufficient oxygen levels. This process is known as the Warburg effect, or aerobic glycolysis (Liberti *et al*. 2016). Interestingly, increased HIF-1 levels due to hypoxia or to increased growth factor stimulation have been seen to upregulate 9 of the 10 enzymes involved in the glycolytic pathway, favouring the conversion of pyruvate to lactate rather than its entry into the TCA cycle (Semenza 2003).

Hyperglycemia in T2D might be responsible for the excess glucose supply for tumour cells, also contributing to apoptosis resistance, oncogenesis, and resistance to chemotherapy (Duan *et al*. 2014). Additionally, the excess nutrient supply can lead to high production of ROS from increased mitochondrial respiration, which can lead to DNA damage and subsequent mutations (Zhang *et al*. 2007).

c) Chronic inflammation

Excess nutrient availability in the T2D milieu leads to chronic low-grade inflammation characterised by an increase in activated monocyte, macrophage and inflamed AT-secretion of cytokines such as TNFα and IL6 (Gonzalez *et al*. 2012, Roubicek *et al*. 2009),

both of which have been shown to promote tumour invasiveness and metastasis via secretion of MMPs (Kitamura *et al*. 2007). Moreover, IL6 is also able to enhance cancer cell proliferation, survival and invasion via activation of STAT signalling (Yu *et al*. 2009).

6.1.4 Influence of diabetes treatments on cancer risk

T2D is associated with overweight and obesity, and commonly progresses from a pre-diabetic state characterized by IR and hyperinsulinemia, to overt diabetes with chronic IR and subsequent β-cell failure and diminished insulin secretion. This gives rise to progressive hyperglycemia, making glucose control one of the central goals for effective diabetes management, resulting in increased use of pharmacologic agents over time and the eventual need for insulin therapy in approximately half of all T2D patients (Jabbour 2008).

There are several glucose-lowering therapeutic drugs currently available for treating T2D. The most widely used agent, metformin, lowers hepatic glucose production by acting on the liver via AMPK activation (Rena *et al*. 2017). Other agents increase endogenous insulin secretion by directly acting on pancreatic β-cells, as is the case of sulfonylureas (Stumvoll *et al*. 2005), or they increase the action of insulin secretion-inducing peptides, as is the case of incretin mimetics (Bloomgarden 2007). At late stage of T2D, insulin deficiency due to increased IR and progressive pancreatic β-cell failure makes the administration of exogenous insulin necessary (Mayfield *et al*. 2004).

a) Metformin and cancer risk

Besides its glucose-lowering and insulin-sensitising effects, metformin has been shown to inhibit cell proliferation, decrease colony formation, and cause partial cell cycle arrest in cancer cell lines *in vitro* (Liu *et al*. 2009, Dowling *et al*. 2007), and to decrease mammary tumour growth *in vivo* in rodent models (Anisimov *et al*. 2005). Interestingly, *in vivo* studies also show that the anti-neoplastic effect of metformin is not so obvious in mice on a control diet compared to its effect on mice on a high-energy diet associated with hyperinsulinemia (Algire *et al*. 2008), suggesting that the anti-neoplastic activity of metformin might be linked to its insulin-lowering action. More importantly, results from a growing number of observational human studies indicate that metformin treatment in T2D patients decreases incidence and mortality of several types of cancer compared to other anti-diabetes treatments (Currie *et al*. 2009, Decensi *et al*. 2010, Landman *et al*. 2010). Altough the mechanism underlying the anti-tumour effect of metformin has not

been well established, preclinical studies have determined that metformin impairs cellular metabolism and suppresses oncogenic signalling pathways such as the receptor tyrosine kinase, PI3K/Akt, and mTOR pathways (Yu *et al*. 2017). Collectively, these studies show that metformin constitutes a promising anti-cancer agent.

b) Thiazolidinediones and cancer risk

Thiazolidinediones (TZDs) are insulin-sensitizing peroxisome proliferator–activated receptor (PPAR)γ agonists that are usually used to treat T2D in combination with other treatments as they do not increase insulin secretion or lower glucose levels directly when used alone (Giovannucci *et al*. 2010).

In vitro studies indicate that PPARγ agonists have several anti-tumour activities, including inhibiting growth and inducing apoptosis and cell differentiation (Ohta *et al*. 2001). However, some *in vivo* studies with rodents indicate that other PPAR agonists can also potentiate tumorigenesis and metastasis (Rubenstrunk *et al*. 2007). Human data on cancer risk associated with TZDs treatment is inconclusive, as shown by several epidemiologic studies, which show that TZDs may increase, decrease, or have no effect on the risk of cancer incidence or progression (Koro *et al*. 2007, Colmers *et al*. 2012, Monami *et al*. 2014).

c) Secretagogues and cancer risk

Secretagogues such as sulfonylureas bind to specific cell receptors and directly stimulate insulin release from β-cells. While they constitute one of the most effective agents in lowering glycosylated haemoglobin levels, these drugs can cause hypoglycemia and weight gain (Bodmer *et al*. 2008).

A small number of observational studies revealed a higher incidence of cancer or cancer death among diabetic individuals treated with sulfonylureas compared with those treated with other anti-diabetes agents (Currie *et al*. 2009, Monami *et al*. 2009). Available data regarding the effect of secretagogues on cancer risk is however inconclusive.

d) Insulin and its analogues and cancer risk

Because of the progressive loss of β-cell function characteristic of T2D, between 40–80% of T2D patients will ultimately be considered for insulin therapy in order to treat hyperglycemia (Jabbour *et al*. 2008). Subcutaneous injection of insulin results in an effective and immediate attenuation of hyperglycemia, but also possibly amplifying the

link between hyperinsulinemia and cancer risk. As mentioned before, high levels of circulating insulin may enhance cancer initiation and propagation due to its mitogenic effects, and thus treatments with exogenous insulin and insulin secretagogues are likely to increase cancer risk.

6.1.5 Influence of cancer treatments on T2D risk

On the other hand, drugs used to treat cancer can also influence diabetes, either causing it or worsening it. Glucocorticoids are traditional anti-cancer treatments, and are well known to cause acute hyperglycemia (Hwangbo *et al*. 2017), resulting from either autoimmune destruction of β-cells or dysregulation of the insulin signalling pathway (Shariff *et al*. 2019). This will eventually lead to IR, worsening a condition of pre- or undiagnosed diabetes, or transforming mild diabetes into a clinically severe illness (Vigneri *et al*. 2009). Anti-androgens, another common anti-cancer drug, can also cause a variety of metabolic abnormalities including decreased insulin sensitivity and altered lipid profile, thereby increasing the risk of diabetes and cardiovascular disease (Saylor *et al*. 2009). An increasing number of targeted anti-cancer compounds are being tested for their ability to interfere with glucose metabolism, acting at different levels on the signalling substrates common to IGF1 and insulin receptors. However, since IGF1 signalling plays a key role in both tumour progression and glucose homeostasis, therapies targeting the IGF system for its pro-cancer effect may at the same time cause hyperglycemia (Vigneri *et al*. 2009).

6.2 Link between T2D and cancer through HIF-1α, S100A4 and inflammation

It has been widely recognised and studied that one of the most important common factors between T2D and cancer is obesity. Hyperinsulinemia and hyperglycemia are two predominant complications in both diseases. However, other factors such as abnormal circulating levels of adipokines (such as leptin and adiponectin) and cytokines (such as TNFα and interleukins), all of them important players in the regulation of inflammation, are also present in obesity and T2D, and their contribution to cancer risk and progression cannot be over-looked (Cohen *et al*. 2012).

For example, leptin is produced primarily by WAT and its levels correlate positively with WAT mass, which means that its levels are increased in obesity (Paz-Filho *et al*. 2011).

Leptin regulates energy homeostasis by mediating food intake and expenditure through its action on the hypothalamus (Morton 2007); however, it also stimulates cell growth, migration, and invasion (Garofalo *et al*. 2006) through several mechanisms, including angiogenesis (through the induction and activation of VEGF, FGF2, MMP2 and MMP9) (Park *et al*. 2001) and suppression of apoptosis (through a BCL2-dependent mechanism) (Artwohl *et al*. 2002). Additionally, leptin can increase macrophage production of cytokines, further stimulating cancer cells (Trayhurn *et al*. 2004).

Importantly, leptin expression is induced by hypoxia via HIF-1 signalling (Grosfeld *et al*. 2002), which, as we have seen, is increased both in tumour and diabetic environments. There is broad evidence linking hypoxia and HIF-1α to induction of inflammation, both in cancer (D'Ignazio *et al*. 2017) and diabetic environments (previously detailed in chapter 1, section 1.6), and this mechanism is very likely, at least in part, mediated by S100A4, as it has been seen that HIF-1α induces *S100A4* expression in cancer cells (Horiuchi *et al*. 2012), which in turn induces activation of NF-κB (Kim *et al*. 2017), which activates the broad inflammatory cascade characteristic of these pathologies.

Results so far indicate that HIF-1α-mediated induction of *S100A4* expression is responsible for the initiation of this inflammatory process through NF-κB activation in INS-1 β-pancreatic cells. In the following section, the PC3 prostate cancer cell line will be used to induce HIF-1α-mediated NF-κB activation via DFO-mediated stabilisation of HIF-1α. The aim is to explore how comparable the changes induced in both cell lines are, given that there is evidence that HIF-1α-induced expression of *S100A4* is able to activate NF-κB and the subsequent inflammatory cascade in several cancer cell lines.

6.3 Results

6.3.1 Cancer cell line screening for S100A4 expression

Different samples of various cancer cell lines, very kindly provided by several groups in the IBRC (Interdisciplinary Biomedical Research Centre, Nottingham Trent University), were collected to test for S100A4 expression levels. The obtained cell lines included the prostate cancer cell lines PC3, LNCaP and DU145 (provided by Dr. Elisabetta Verderio Edwards), the human osteosarcoma cell line U2OS (provided by Dr. Amanda Coutts) and the breast cancer cell line MCF-7 (provided by Dr. Selman Ali).

Cells were provided in the form of a pellet, which was lysed, and total protein extracted and quantified as described in chapter 2. Lysates were electrophoresed on 15% polyacrylamide gels and proteins were transferred to a nitrocellulose membrane. Specific antibody for S100A4 was used to incubate the membrane overnight. Figure 6.1 shows a significant S100A4 expression in the PC3 cell line and a moderate expression in the MCF-7 cell line, but no apparent expression in the rest of the cancer cell lines. I therefore decided to use the PC3 cancer cell line to carry out the experiments in this section.

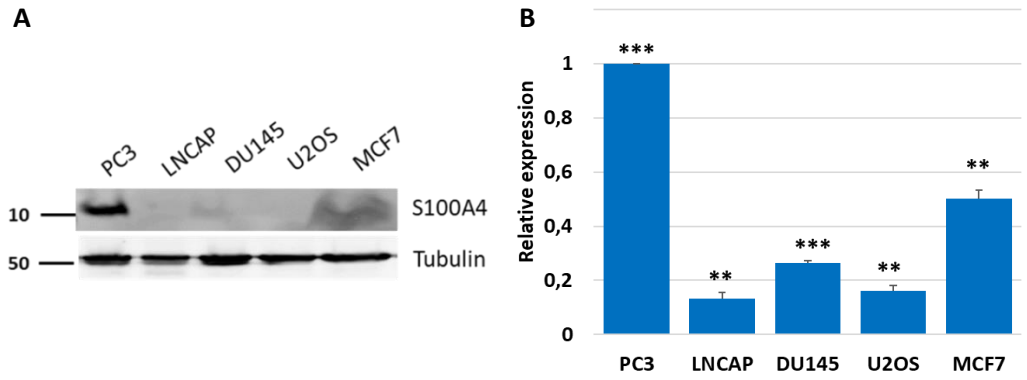


Figure 6.1: S100A4 relative protein expression in different cancer cell lines. Cell pellets were lysed, total protein was extracted, and lysates were separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with S100A4 specific antibody. **A)** Representative western blot of 3 independent experiments; **B)** Quantification of anti-S100A4 western blot bands, relative to tubulin expression, with PC3 expression normalised to 1. Data is expressed as means + SEM of 3 independent experiments (**p<0.005, ***p<0.0005).

6.3.2 Morphological effect of DFO treatment in PC3 cells

PC3 cells were treated with 100µM DFO for 72 hours as described in chapter 2 to stabilise HIF-1α, as in normal conditions HIF-1α would get degraded in contact with oxygen, in the same way as INS-1 cells were treated with GLT to induce inflammation and with DFO to stabilise HIF-1α and compare the effects of both treatments.

Cell viability was initially assessed though observation of morphological changes. Results in Figure 6.2 show that a 72 hours treatment with DFO induces morphological changes in PC3 cells (cells turn more rounded in shape and tend to aggregate and grow upwards rather than in the characteristic monolayer) without significantly decreasing cell viability (number of cells attached is comparable in both conditions), which is very similar to the effect that we observed in chapter 3 with the GLT treatment of INS-1 cells.

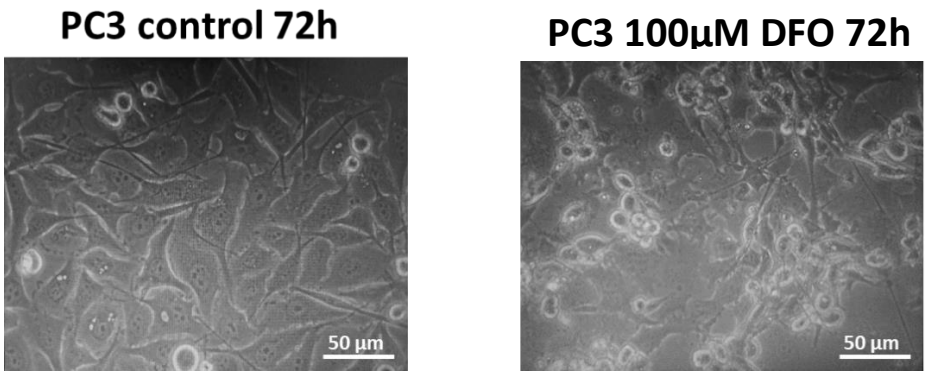


Figure 6.2: PC3 cells in control and DFO conditions. 100µM DFO is added 24 hours after passage and experimental procedures carried out 72 hours later. As the pictures indicate, cells are still viable after 72 hours both in control and DFO conditions, although they present phenotypic differences. Scalebar 1cm:50µm.

6.3.3 Effect of DFO on HIF-1α expression in PC3 cells

In order to determine whether HIF-1α is involved in the upregulation of S100A4 protein expression in PC3 cells as our results suggest is the case for INS-1 cells, the first step would be to look at HIF-1α expression. PC3 cells were incubated in DMEM or DMEM + DFO media (to stabilise HIF-1α) for 72 hours, after which cells were lysed, and total protein extracted and quantified as described in chapter 2. Protein extracts were electrophoresed on 4–20% Mini-PROTEAN® TGX™ precast polyacrylamide gradient gels (Bio-Rad) (Figure 6.3).

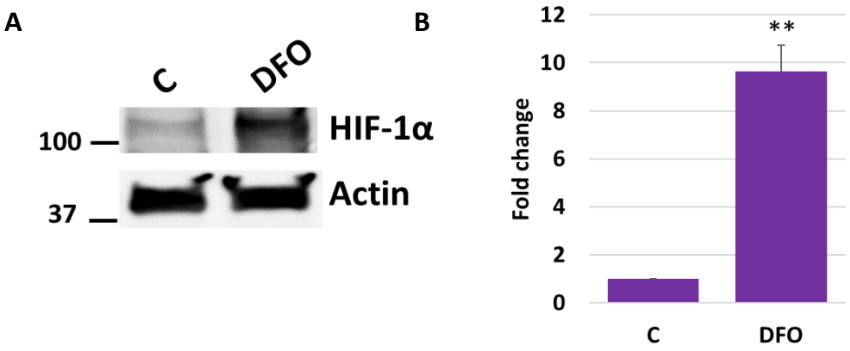


Figure 6.3: Effect of DFO treatment on HIF-1α protein levels. PC3 cells were incubated for 72 hours in control or 100µM DFO conditions and subsequently lysed. Lysates were separated on 4–20% polyacrylamide gradient gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with HIF-1α specific antibody. **A)** Western blot for HIF-1α, representative of 3 independent experiments; **B)** Quantification of western blot bands for HIF-1α, normalised to internal actin control. Data is expressed as means + SEM of 3 independent experiments (**p<0.005).

As we can observe in Figure 6.3, HIF-1α protein levels are practically undetectable in control conditions in PC3 cells, but when cells are treated with DFO, HIF-1α levels escalate dramatically (12-fold increase), showing that in absence of a HIF-1α stabiliser, HIF-1α gets degraded very rapidly in presence of oxygen.

Subsequently, PC3 cells were seeded on coverslips and treated in control or DFO conditions for 72 hours, after which they were subjected to an immunofluorescence assay in order to observe the previously mentioned changes in HIF-1α expression, as well as any changes in its subcellular localisation induced by the DFO treatment. Results in Figure 6.4 show that indeed, DFO induces an increase in HIF-1α expression and nuclear localisation, as predicted.

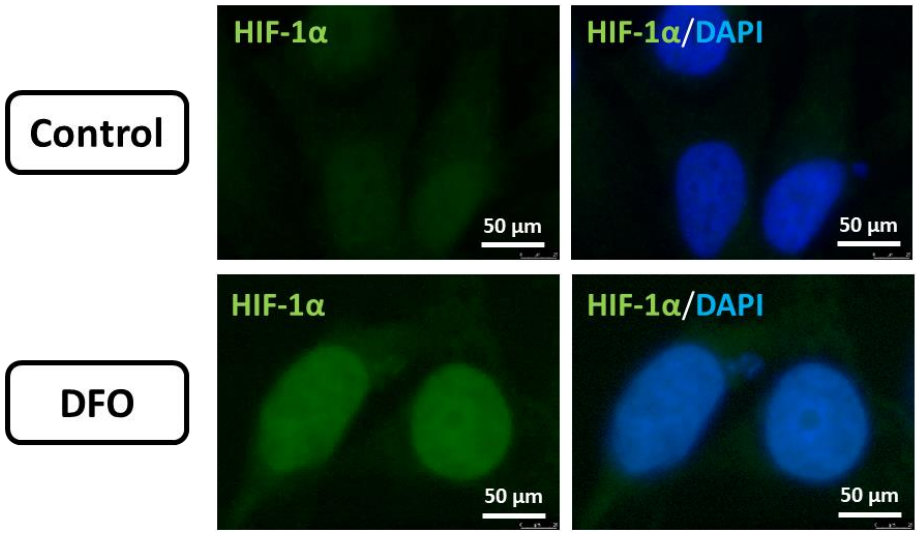


Figure 6.4: Effect of DFO treatment on HIF-1α protein levels and subcellular localisation. PC3 cells were cultured on coverslips and media replaced after 24 hours with DMEM or DMEM + 100µM DFO media for 72 hours. After fixing, cells were incubated with specific HIF-1α antibody, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. Figure shows representative images from 3 independent experiments. Scalebar 1cm:50µm.

6.3.4 Effect of DFO on S100A4 expression in PC3 cells

After validating the increased HIF-1α expression and nuclear localisation induced by DFO, S100A4 expression changes induced by this treatment were measured in PC3 cells. Results show that both mRNA (Figure 6.5A) and protein (Figures 6.5B and 6.5C) S100A4 levels increase in DFO-treated PC3 cells, correlating with the observed results in GLT- and DFO-

treated INS-1 cells (Figures 3.6, 3.7 in chapter 3, and Figure 5.3 in chapter 5 respectively), indicating that GLT-induced changes are comparable to HIF-1α activity-induced changes.

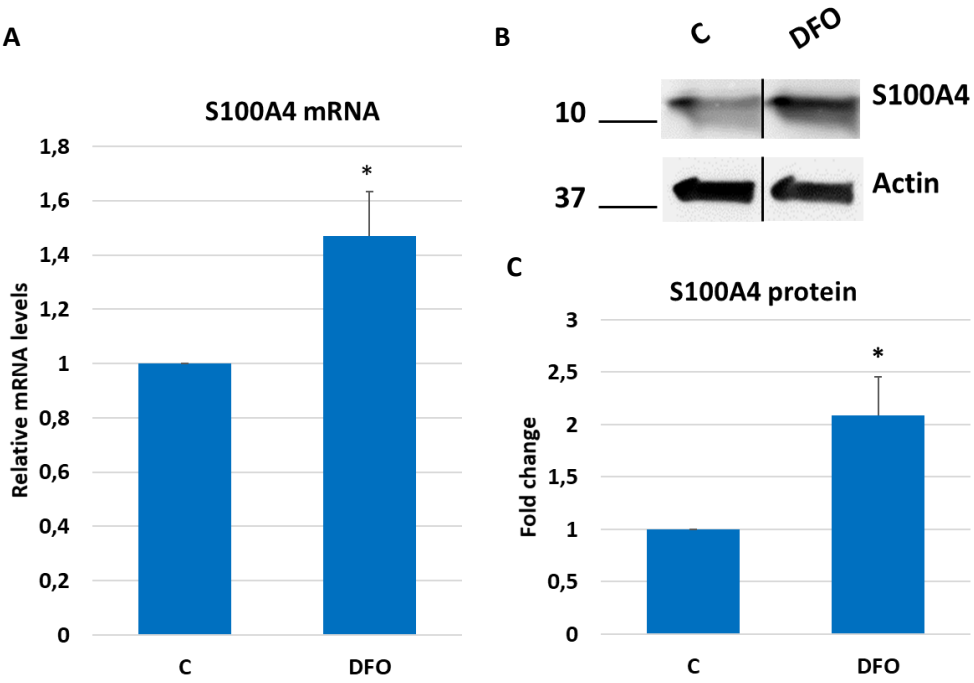


Figure 6.5: Effect of DFO treatment on S100A4 mRNA and protein levels. PC3 cells were incubated for 72 hours in DMEM or DMEM + 100µM DFO media. **A)** mRNA was extracted, and qPCR was performed using primers specific for S100A4. Data represent ΔΔCt values expressed as a fold change compared to cells grown in control conditions and normalised to GAPDH internal control. **B)** Cells were lysed, total protein extracted and separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with S100A4 specific antibody. Image shows a western blot representative of 3 independent experiments. **C)** Quantification of western blot bands normalised to internal actin control. Data is expressed as means + SEM of 3 independent experiments (*p<0.05).

6.3.5 NF-κB expression and activity in PC3 cells

After validating the increased expression of S100A4 induced by the DFO treatment, I wanted to determine whether this increase affected NF-κB protein expression, subcellular localisation, and transcriptional activity, as it did in INS-1 cells.

In order to do so, PC3 cells were cultured for 72 hours in control and DFO conditions, lysed and total protein extracted and quantified as described in chapter 2. Figure 6.6A show the increased NF-κB protein expression in DFO compared to control. When bands were

quantified and normalised to internal actin control, I observed a significant 2.4-fold increase ($p<0.05$) in NF-κB protein levels (Figure 6.6B). To observe any subcellular localisation changes induced by DFO, PC3 cells were also cultured on coverslips in control or DFO conditions for 72 hours and subsequently incubated with specific NF-κB antibody followed by incubation with fluorescent secondary antibody as described in chapter 2. Figure 6.6C shows increased expression and increased nuclear localisation of NF-κB in DFO-treated cells, which indicates that presence of HIF-1α induces nuclear translocation of NF-κB, suggesting an induction of its transcriptional activity, a mechanism probably mediated by the increase in S100A4 expression.

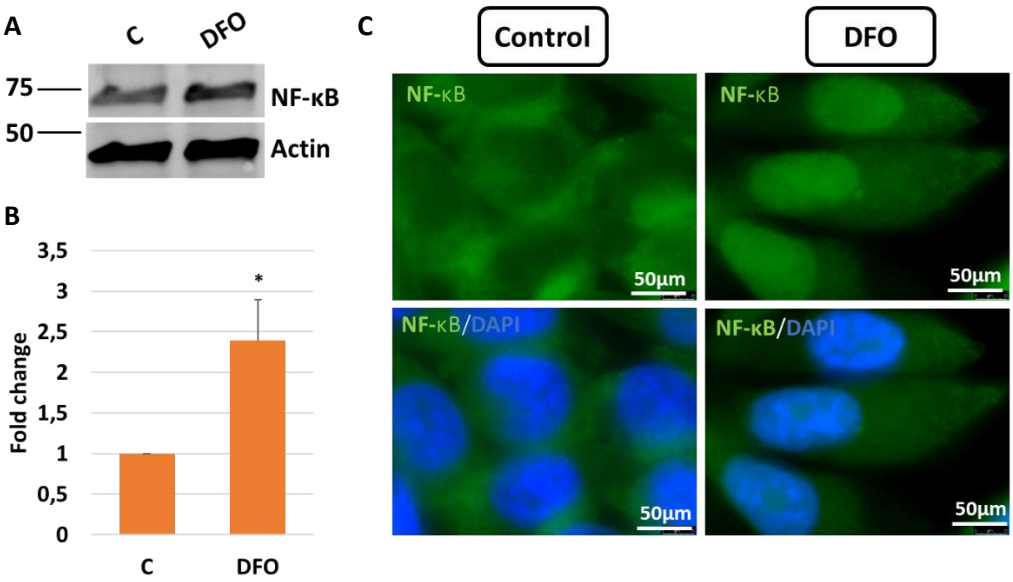


Figure 6.6: Effect of DFO treatment on NF-κB protein levels and nuclear localisation. PC3 cells were incubated for 72 hours in control or 100µM DFO conditions, subsequently lysed and total protein extracted. Lysates were electrophoresed on 12% polyacrylamide gels, and proteins were transferred to a nitrocellulose membrane. A specific antibody for NF-κB was used to incubate the membrane overnight. PC3 cells were also cultured on coverslips in control or DFO conditions for 72 hours, after which they were fixed and incubated with specific NF-κB antibody, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken **A)** Representative western blot of 3 independent experiments; **B)** Quantification of anti-NF-κB western blot bands, normalised to internal actin control. **C)** Representative images from 3 independent experiments. Scalebar 1cm:50µm. Data is expressed as means + SEM of 3 independent experiments (*$p<0.05$);

In order to determine whether the DFO-induced increase in NF-κB protein expression and nuclear localisation observed in Figure 6.6 correlates with an increase in NF-κB activity, PC3 cells were cultured in control and DFO conditions for 72 hours, after which their nuclear compartment was extracted using a nuclear/cytosol fractionation kit as described in chapter 2, and used for the assay. Data shows that there is a significant 82% increase in NF-κB activity resulting from exposure of PC3 cells to DFO, correlating with the observed increased nuclear expression and thereby confirming our hypothesis (Figure 6.7).

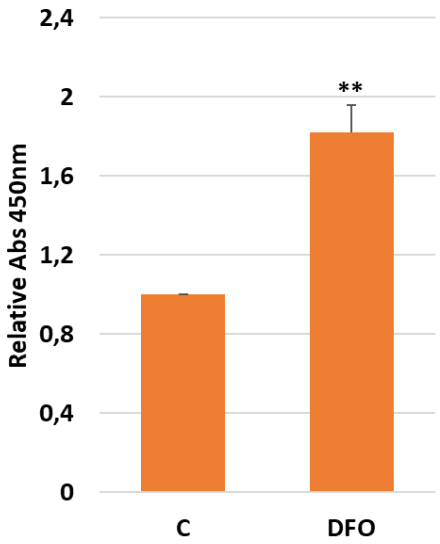


Figure 6.7: Effect of DFO treatment on NF-κB activity. PC3 cells were cultured in control or 100 μM DFO conditions for 72 hours, after which cells were lysed using a nuclear/cytosolic fractionation kit. The nuclear compartment was added to the oligonucleotide coated wells of the activity assay plate, and then incubated with specific NF-κB antibody and subsequent secondary antibody. Developing solution and subsequent stop solution was added to the wells, after which absorbance was read to measure NF-κB activity. Data is expressed as means + SEM of 3 independent experiments (**p<0.005).

6.3.6 S100A4 knockdown effect on NF-κB

Up until now, results show that HIF-1α stabilisation in PC3 cells results in an increase in S100A4 expression (Figure 6.5) and in increased NF-κB protein expression, nuclear localisation and transcriptional activity (Figures 6.6 and 6.7 respectively). In order to determine whether these changes are being triggered by increased HIF-1α-induced S100A4 expression as we observed in INS-1 cells, a transient knockdown of S100A4 was carried out using siRNA as described in chapter 2. Briefly, ssRNA/siRNA conjugated with

transfection reagent was added to PC3 cells 24 hours after seeding, for a period of 24 hours. Media was then replaced with DMEM or DMEM + DFO media for another 72 hours, after which either total protein or nuclear fraction was obtained for subsequent western blot analysis or NF-κB activity assay respectively. Cells were also cultured on coverslips to observe the effect of S100A4 knockdown on NF-κB subcellular localisation.

An optimisation process for S100A4 knockdown was initially carried out using the same three different transfection reagents that were used for the optimisation process of S100A4 knockdown in INS-1 cells (RNAiMAX, Transit-X2 and INTERFERin), and the same three different incubation periods (24, 48 and 72 hours), only in control conditions, in the same way as it was done for INS-1 cells (Figure 6.8).

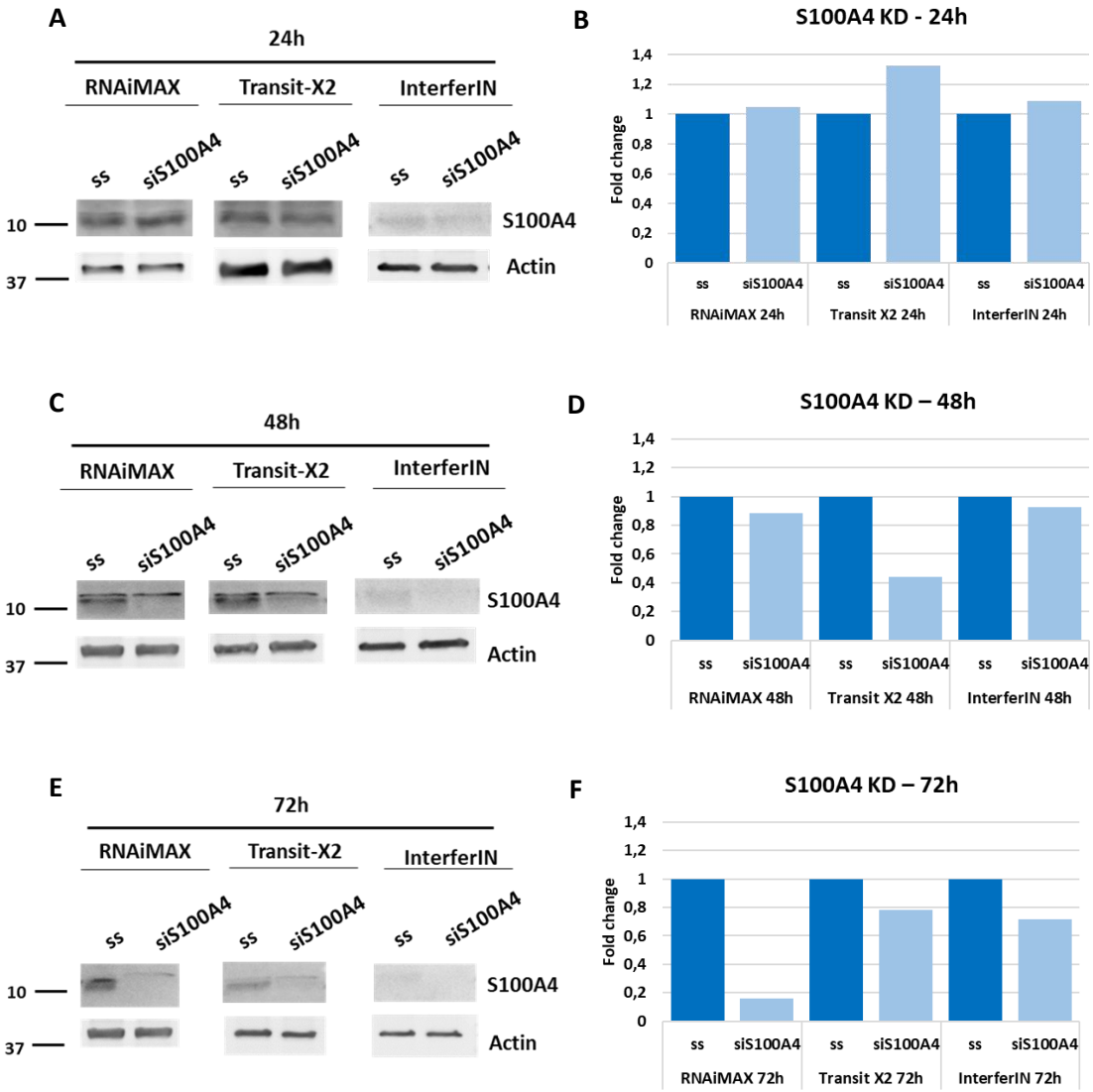


Figure 6.8: Optimisation process for S100A4 knockdown in PC3 cells. PC3 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and replaced with control DMEM media for 24, 48 or 72 hours. Total protein was then extracted, and lysates were separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with S100A4 specific antibody. **A, C, E)** Western blot of S100A4 after treatments for 24, 48 and 72 hours respectively, showing level of S100A4 knockdown with the 3 different transfection reagents; **B, D, F)** Quantification of anti-S100A4 western blot bands, normalised to internal actin control, for 24, 48 and 72 hours treatments respectively.

Preliminary results in Figure 6.8 show that a 24-hour incubation did not result in effective knockdown for any of the three transfection reagents (Figures 6.8A and B). A 48-hour incubation rendered no significant knockdown with RNAiMAX or INTERFERin, while it resulted in a 56% knockdown with Transit X2 (Figures 6.8C and D). Finally, a 72-hour incubation resulted in a 84% knockdown of S100A4 using RNAiMAX, while the other two transfection reagents were once more unsuccessful in knocking down S100A4 (Figures 6.8E and F). In conclusion, the most effective transfection reagent was RNAiMAX, and the optimal incubation period was 72 hours, which was convenient as these are the same conditions as the ones used with INS-1 cells, so experiments with both cell lines could be carried out simultaneously. All the experiments involving S100A4 knockdown will therefore be carried out using these conditions. These were therefore the conditions selected to carry out all S100A4 knockdown experiments in PC3 cells.

It is worth mentioning that some of the bar graph representations of the blot bands do not seem to relate to the corresponding bands. This is probably due to the low basal expression levels of S100A4, especially for those corresponding to the INTERFERin treatment. However, the most relevant results are those corresponding to the RNAiMAX treatment, as it resulted to be the most effective, and band quantification levels for this treatment seem much more accurate.

After the optimisation process, *S100A4* was knocked down in the optimum conditions as described above and subsequently, total protein was obtained to measure NF-κB protein expression through Western Blot, firstly only in control conditions (Figures 6.9A and 6.9B). PC3 cells were also cultured on coverslips in control conditions for 72 hours after S100A4 knockdown and subjected to incubation with fluorescent antibody for immunofluorescence imaging (Figure 6.9C).

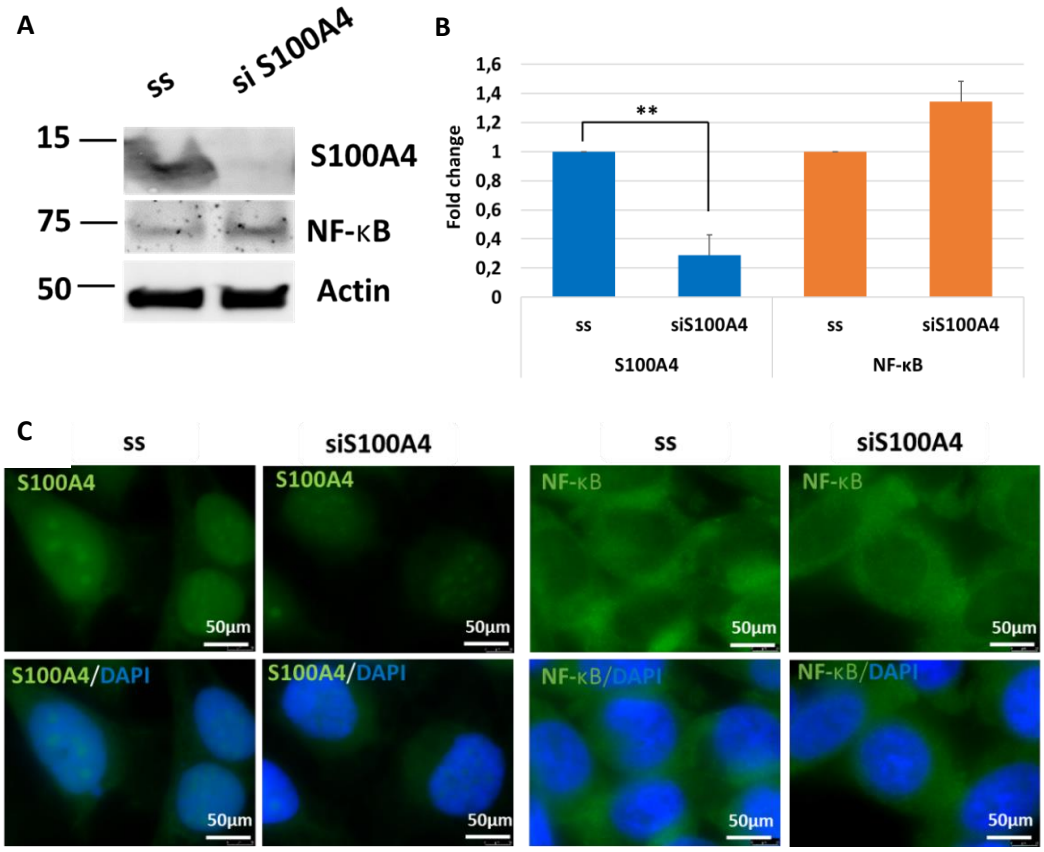


Figure 6.9: Effect of *S100A4* knockdown on NF-κB protein levels and subcellular localisation in control conditions. PC3 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and replaced with control media for 72 hours. Total protein was then extracted, and lysates were separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with S100A4 or NF-κB specific antibodies. PC3 cells were also cultured on coverslips, 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells, and media replaced after 24 hours with control media for 72 hours. After fixing, cells incubated with specific NF-κB antibody, followed by incubation with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken **A)** Representative western blot of 3 independent experiments; **B)** Quantification of anti-S100A4 and anti-NF-κB western blot bands, normalised to internal actin control. **C)** Representative images from 3 independent experiments. Scalebar 1cm:50µm. Data is expressed as means + SEM of 3 independent experiments (**p<0.005).

Results in Figures 6.9A and 6.9B show that following S100A4 knockdown (72%, *p<0.05) in control conditions there was no significant change in NF-κB protein expression. However, when looking at Figure 6.9C, we can observe that there is no apparent change in NF-κB protein expression or nuclear localisation, suggesting no induction of NF-κB activity.

DFO conditions compared to control were then used to try to increase NF-κB activity and in this way be able to perceive more clearly the changes induced by S100A4 knockdown. Again, S100A4 was knocked down as described above, this time adding DMEM or DMEM + DFO media for 72 hours after the 24 hours incubation with RNAiMAX-ss/siRNA complex, and subsequently, total protein was obtained to measure NF-κB protein expression through western blot (Figures 6.10A and 6.10B). PC3 cells were also cultured on coverslips in control and DFO conditions for 72 hours after S100A4 knockdown and subjected to incubation with S100A4 (Figure 6.10C) or NF-κB (Figure 6.10D) antibodies and subsequent secondary fluorescent antibody for immunofluorescence imaging (Figure 6.10C).

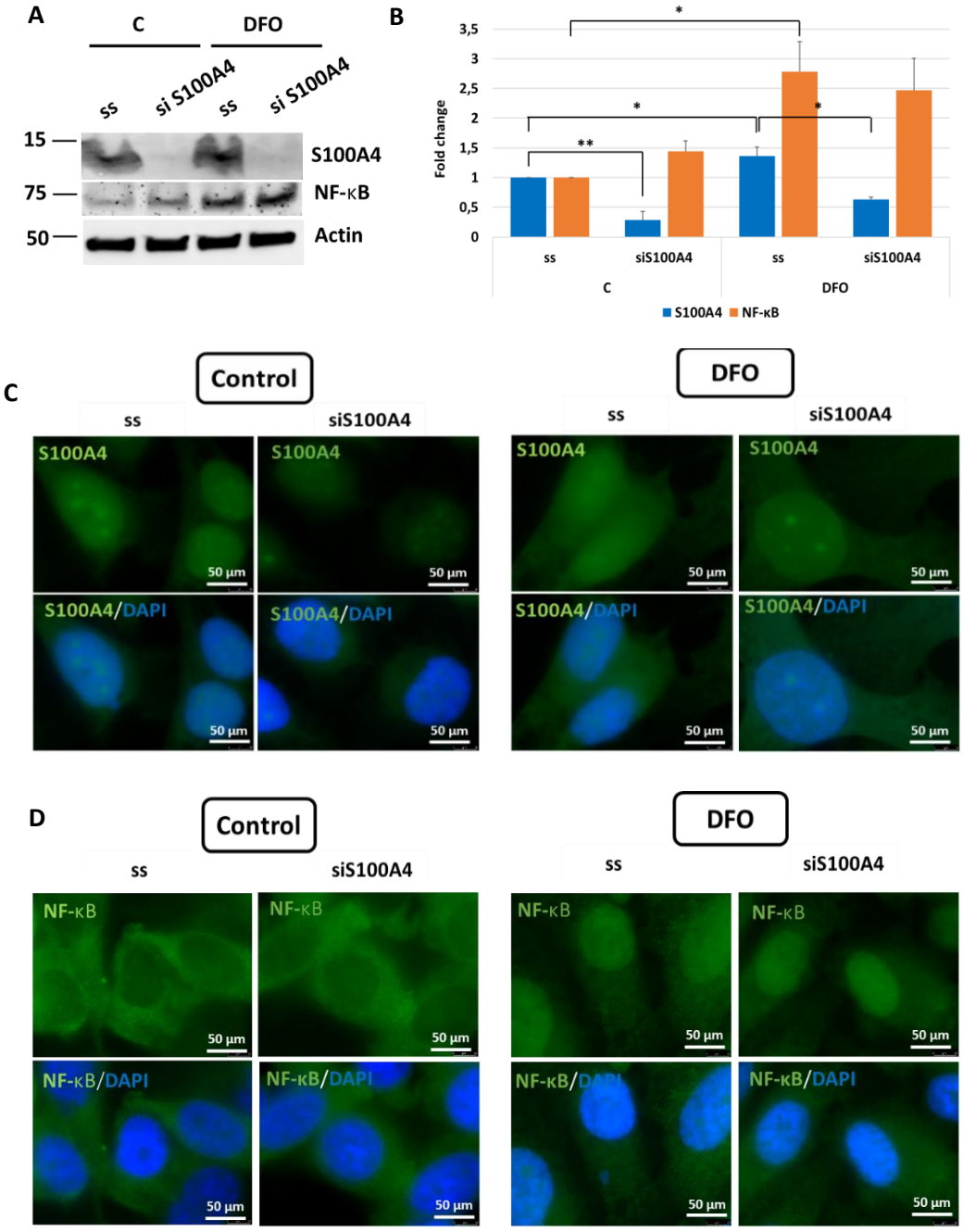


Figure 6.10: Effect of S100A4 knockdown on NF-κB protein levels and subcellular localisation in DFO conditions compared to control. PC3 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and replaced with DMEM or DMEM + 200µM DFO media for 72 hours. Total protein was then extracted, and lysates were separated on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with S100A4 or NF-κB specific antibodies. PC3 cells were also cultured on coverslips, 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells, and media replaced after 24 hours with DMEM or DMEM + 100µM DFO media for 72 hours. After fixing, cells were incubated with specific S100A4 or NF-κB antibody, followed by incubation

with fluorescent secondary antibody and nuclear DNA staining. Cells were visualised under a fluorescent microscope and pictures taken. **A)** Representative western blot of 3 independent experiments; **B)** Quantification of anti-S100A4 and anti-NF-κB western blot bands, normalised to internal actin control. **C, D)** Representative images for S100A4 and NF-κB from 3 independent experiments. Scalebar 1cm:50µm. Data is expressed as means + SD of 3 independent experiments (*p<0.05).

Results in Figure 6.10 show that NF-κB protein expression (A and B) and nuclear localisation (D) visibly increase in DFO-treated cells, as we saw earlier in Figure 6.6. However, S100A4 knockdown does not reverse this effect, neither at the protein level nor when looking at NF-κB nuclear localisation.

Together, these results do not offer a conclusive role for S100A4 on NF-κB activation in PC3 cells. However, as mentioned before, what is really important is the changes in NF-κB transcriptional activity induced by S100A4. This was therefore measured with the use of a NF-κB activity assay, for which nuclear extracts of S100A4-knocked down cells in control and DFO conditions were obtained. Results are shown in Figure 6.11.

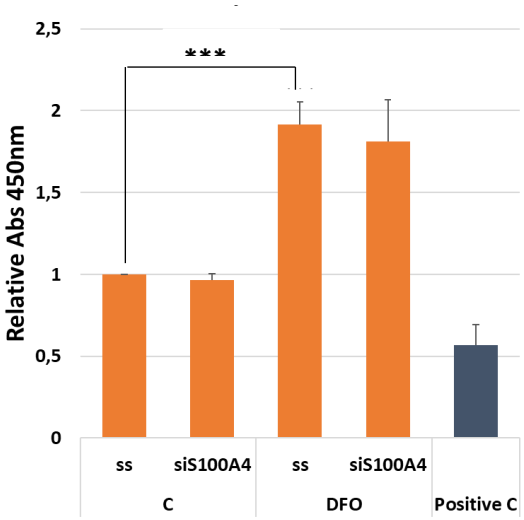


Figure 6.11: Effect of S100A4 knockdown on NF-κB activity in DFO conditions compared to control. PC3 cells were seeded, and 24 hours later the complex RNAiMAX-ssRNA/siRNA was added to the cells for 24 hours. Media was then removed and replaced with DMEM or DMEM + 200µM DFO media for 72 hours, after which cells were lysed using a nuclear/cytosolic fractionation kit. The nuclear compartment was added to the oligonucleotide coated wells of the activity assay plate, and then incubated with specific NF-κB antibody and subsequent secondary antibody. Developing solution and subsequent stop solution was added to the wells, after which absorbance was read to measure NF-κB activity. Data is expressed as means + SEM of 3 independent experiments (***p<0.0005).

Figure 6.11 shows that NF-κB activity increases in DFO conditions by over 90%, as shown earlier in Figure 6.7. However, as results in Figure 6.10 predicted, S100A4 knockdown does not reverse the increase in NF-κB activity, indicating that there must be some other mechanisms or molecules involved in NF-κB activation on PC3 cells other than S100A4.

6.4 Discussion and future directions

The main purpose of this section was to compare the effects of S100A4 on NF-κB activation in a cancer cell line, in this case using the prostate cancer cell line PC3, to its effects on the pancreatic β-cell line INS-1, given that evidence for a link between cancer and T2D is rapidly gaining strength (Giovannucci *et al*. 2010), with S100A4 being a very strong candidate as one of the main factors driving this cancer-T2D link.

There is broad evidence linking hypoxia and HIF-1α to induction of inflammation, both in cancer (D'Ignazio *et al*. 2017) and diabetic environments (detailed in chapter 1, section 1.6), and this mechanism is very likely mediated by S100A4, as it has been seen that HIF-1α induces *S100A4* expression in various cancer, including ovarian cancer (Horiuchi *et al*. 2012), which in turn induces activation of NF-κB in several cancer cell lines, including osteoblasts (Kim *et al*. 2017), which activates the broad inflammatory cascade characteristic of both pathologies.

It is known that binding of S100A4 to RAGE induces activation of NF-κB in human cancer cell lines (Boye *et al*. 2008, Kim *et al*. 2017). However, very little is known about the role of S100A4 in the activation of the inflammatory processes mediated by NF-κB in other disorders, including T2D. In this chapter, the effects of S100A4 on NF-κB activation were therefore compared between both cell lines, to try to point at S100A4 as the main inducer of the inflammatory process characteristic of both pathologies.

After screening a number of cancer cell lines for S100A4 protein expression, I decided to use the PC3 prostate cancer cell line as it was the one which showed the highest expression levels of S100A4. DFO was used as a HIF-1α stabiliser for its ability to chelate the $Iron^{3+}$ ions necessary for hydroxylation by PHDs (allowing subsequent ubiquitination and proteasomal degradation of HIF-1α) (O'Rourke *et al.* 1996), in order to induce an inflammatory state, comparable to the effect of the GLT treatment on INS-1 cells.

Results indicate that DFO is able to stabilise and increase expression and nuclear localisation of HIF-1α in PC3 cells, as demonstrated for other cancer cell lines (Zhang *et al.* 2014) as well as in *in vivo* studies of other pathologies (Li *et al.* 2014).

The results obtained in this section also reveal that S100A4 mRNA and protein expression increase in DFO conditions (by 1.5 and 2.1 times respectively), indicating that HIF-1α might be involved in the upregulation of *S100A4* by acting as a transcription factor that binds to HREs in its promoter region. Subsequently, I show that DFO is also able to induce an increase in NF-κB protein expression (2.4-fold increase) and nuclear localisation, as well as an increase in its activity (80% activity increase), as shown by the NF-κB activity assay results.

In order to determine whether S100A4 plays a role in this DFO-induced expression and activation of NF-κB, siRNA was used to transiently knockdown S100A4, to observe the effects this has on NF-κB protein expression, subcellular localisation and activation. After a knockdown optimisation process in which the best conditions to knockdown S100A4 were identified, results showed that a 72% knockdown in control conditions did not cause any significant change in NF-κB protein expression or nuclear localisation. Furthermore, when cells were treated with DFO, S100A4 knockdown was still not able to reverse the DFO-induced increase in NF-κB protein expression or nuclear localisation.

A possible explanation for these results might be that, being a cancer cell line, NF-κB will be active even in control conditions. Given that HIF-1α is barely detectable without the use of its stabiliser DFO, and that NF-κB expression and activity is still considerable in these conditions, there must be some other mechanisms involved in the activation of NF-κB other than HIF-1α-induced S100A4. Consequently, the knockdown of *S100A4* will not produce a significant decrease in NF-κB expression or activity.

Furthermore, as mentioned before, NF-κB has only been seen to be activated by S100A4 in several cancer cell lines, while no S100A4-mediated activation of NF-κB has been detected in other cancer cell lines (Boye *et al.* 2008). Therefore, it is possible that S100A4 is not involved in NF-κB activation in PC3 cells either.

Overall, our results do not offer a conclusive role for HIF-1α-induced S100A4 on NF-κB activation in PC3 cells, and further research is necessary in order to establish a firmer relationship.

Other experiments that could be carried out to test this hypothesis include measuring IKK protein levels and IκBα phosphorylation levels in control and DFO conditions +/- si*S100A4*, given that there is evidence that S100A4 mediates NF-κB activation through induction of IKK-mediated IκBα phosphorylation and subsequent degradation in many cancer cell lines (Boye *et al*. 2008), and that we have seen that this is how S100A4 induces NF-κB activation in INS-1 cells (Figure 4.8).

As mentioned in the INS-1 section, another way to measure the effect of S100A4 on NF-κB activity could be to carry out a reporter assay based on NF-κB responsive promoter elements driving expression of a secreted luciferase (Badr *et al.* 2009). A fluorescent reader would then be used to measure expression levels of the luciferase following *S100A4* knockdown, with fluorescence signal positively correlating with NF-κB activity. Also, measurement of RAGE mRNA and protein expression levels in PC3 cells in control and DFO conditions, followed by knockdown or inhibition of RAGE signalling with the use of anti-RAGE antibodies could also be carried out in order to block S100A4-mediated activation of NF-κB. However, as mentioned before, S100A4 is also able to signal through other receptors such as EGFR, TLR4 and IL10R to activate NF-κB (Grotterød *et al*. 2010), so this approach would most likely offer no conclusive results for the role of S100A4 on NF-κB activation.

Finally, experiments involving the knockdown of HIF-1α and KPNA2 and subsequent measurement of S100A4 expression and NF-κB expression, localisation and activity, as it was done with the INS-1 cells, would reveal whether KPNA2 is responsible for the nuclear translocation of HIF-1α, and whether HIF-1α acts as a transcription factor to induce *S100A4* expression in PC3 cells.

CHAPTER 7: Summary of findings and implications of research

Diabetes is one of the main causes of death worldwide, contributing to almost 10% of global mortality (Cho *et al.* 2018). Furthermore, the rate at which diabetes incidence and prevalence is increasing is alarming. Over the last decades, our laboratory and many others around the globe have heavily contributed to our understanding of the mechanisms involved in the onset and development of this disease; however, an even greater global effort is needed in order to fully understand the pathogenesis of diabetes in order to develop efficient new treatments for its eradication, or at least, to slow down its progress. The main goal of this PhD project was to help discover new factors involved in the pathogenesis of T2D, and more precisely in the inflammatory process that is characteristic of the disease. The findings and results presented here expand our knowledge on this topic.

In the first part of the thesis, S100A4 was identified as an upregulated factor in pancreatic INS-1 cells cultured in conditions of glucolipotoxicity, which was used in this project to mimic the diabetic environment. S100A4 had previously been extensively studied in a cancer environment, in which it functions to increase tumour progression and metastasis through enhancement of chemotactic behaviour, stimulation of angiogenesis, attraction of immune cells and promotion of secretion of cytokines and growth factors (Fei *et al.* 2017). However, knowledge on its role on the pathogenesis of T2D is limited. Interestingly, evidence indicated that S100A4 could play an important role in diabetes, as it had been associated with some comorbidities of T2D such as diabetic retinopathy (Abu El-Asrar *et al.* 2014), as well as with other inflammatory diseases including rheumatoid arthritis, systemic sclerosis, allergy, psoriasis and cancer (Ambartsumian *et al.* 2019).

Initial data presented in this thesis confirms that S100A4 is upregulated in glucolipotoxic conditions. Subsequent knockdown experiments reveal that S100A4 is able to induce inflammation through activation of the main proinflammatory transcription factor, NF-κB, whose activity was increased in glucolipotoxic conditions and decreased after S100A4 knockdown. These results indicate that S100A4 is potentially an important player in the development of diabetes as a proinflammatory disease. These findings are also consistent with *in vivo* studies which have demonstrated that S100A4 mediates macrophage recruitment to sites of inflammation and chemotaxis (Li *et al.* 2010).

Through bioinformatic analysis and interaction pathway construction based on the RNAseq data obtained previously in our laboratory, a possible mechanism by which S100A4 upregulation might be mediated in glucolipotoxic conditions has been identified. The interaction maps generated indicate a link between S100A4 and HIF-1α, a transcription factor which functions as the main hypoxia effector, and whose role in T2D is incompletely defined. Our findings show that HIF-1α is also upregulated in glucolipotoxic conditions, in all probability as a consequence of increased excess nutrient-induced metabolic stress and subsequent ROS production (Cerychova *et al.* 2018). Importantly, HIF-1α knockdown experiments show that S100A4 decreases, suggesting that its upregulation could be mediated through HIF-1α-induced transcriptional activity. This had been seen before in some cancer cell lines (Horiuchi *et al.* 2012), and implies that S100A4 expression could be modulated with the use of HIF-1α inhibitors, an approach that is currently under investigation as anti-cancer therapy (Yu *et al.* 2017, Ban *et al.* 2017).

My research sought to uncover the mechanism by which HIF-1α translocates to the nucleus to activate S100A4 transcription. There is evidence pointing at KPNA2 as the nuclear transporter responsible for HIF-1α translocation in other cell types (Depping *et al.* 2008). The KPNA2 knockdown experiments presented here indicate that it might also be involved in HIF-1α transport in INS-1 pancreatic cells, however further experiments are needed in order to confirm this. Future lines of research should focus on validating these preliminary results in primary islets, which, if proven true, could open a range of possibilities for therapy mechanisms focused on blocking HIF-1α transport and consequently its transcriptional activity.

The latter part of this thesis focused on finding a link between T2D and cancer through HIF-1α and S100A4, in order to compare the effects of S100A4 in both diseases, and with the hope of being able to extrapolate what has already been done in cancer therapy to T2D treatment such as the use of HIF-1α inhibitors (Yu *et al.* 2017). Firstly, a cancer cell-line was identified in which S100A4 basal expression was high, namely the prostate cancer cell line PC3. Treatment with DFO was added in order to induce a hypoxic state through stabilisation of HIF-1α, comparable to the glucolipotoxic treatment that was used with the INS-1 cells. Results revealed that HIF-1α stabilisation (evidenced by increased protein expression and nuclear localisation) results in S100A4 upregulation, suggesting the involvement of HIF-1α in the induction of S100A4 expression. S100A4 was then knocked down and NF-κB activity measured. Results revealed that, even though NF-κB activity

increased after DFO treatment, the knockdown of S100A4 was not able to reverse its activation, most probably due to the fact that NF-κB was already constitutively active in the cells' basal state, or that it was being overactivated by some other stimuli in the proinflammatory milieu characteristic of cancer cells. Therefore, further research is necessary to confirm the link between S100A4 and NF-κB in the PC3 cancer cell-line.

In conclusion, the findings presented in this thesis have contributed to our understanding of the causes of T2D, and provide further insight into the molecular mechanisms responsible for the pathogenesis of the worldwide pandemic that T2D represents. By discovering novel factors and mechanisms implicated in these processes, this work opens the door to a range of new possibilities for future research in molecular therapy.

References

Abdellatif AM, Ogata K, Kudo T, Xiafukaiti G, Chang YH, Katoh MC, El-Morsy SE, Oishi H, Takahashi S. 2015. "Role of Large MAF Transcription Factors in the Mouse Endocrine Pancreas." *Experimental animals / Japanese Association for Laboratory Animal Science* 64(3): 305–12.

Abedini A, Schmidt AM. 2013. "Mechanisms of Islet Amyloidosis Toxicity in Type 2 Diabetes." *FEBS Letters* 587(8): 1119–27.

Abu El-Asrar AM, Nawaz MI, De Hertogh G, Alam K, Siddiquei MM, Van den Eynde K, Mousa A, Mohammad G, Geboes K, Opdenakker G. 2014. "S100A4 Is Upregulated in Proliferative Diabetic Retinopathy and Correlates with Markers of Angiogenesis and Fibrogenesis." *Molecular vision* (20): 1209–24.

Acosta-Montaño P, García-González V. 2018. "Effects of Dietary Fatty Acids in Pancreatic Beta Cell Metabolism, Implications in Homeostasis." *Nutrients* 10(4): E393.

Aguilar-Morante D, Morales-Garcia JA, Santos A, Perez-Castillo A. 2015. "CCAAT/Enhancer Binding Protein Beta Induces Motility and Invasion of Glioblastoma Cells through Transcriptional Regulation of the Calcium Binding Protein S100A4." *Oncotarget* 6(6): 4369–84.

Aldukhayel A. 2017. "Prevalence of Diabetic Nephropathy among Type 2 Diabetic Patients in Some of the Arab Countries." *International Journal of Health Science* 11(1): 1–4.

Alexandraki K, Piperi C, Kalofoutis C, Singh J, Alaveras A, Kalofoutis A. 2006. "Inflammatory Process in Type 2 Diabetes: The Role of Cytokines." *Annals of the New York Academy of Sciences* 1084(1): 89–117.

Alfadhli EM. 2015. "Gestational Diabetes Mellitus." *Saudi Med J.* 36(4): 399–406.

Algire C, Zakikhani M, Blouin MJ, Shuai JH, Pollak M. 2008. "Metformin Attenuates the Stimulatory Effect of a High-Energy Diet on in Vivo LLC1 Carcinoma Growth." *Endocrine-Related Cancer* 15(3): 833–39.

Allagnat F, Cunha D, Moore F, Vanderwinden JM, Eizirik DL, Cardozo AK. 2011. "Mcl-1 Downregulation by pro-Inflammatory Cytokines and Palmitate Is an Early Event Contributing to B-Cell Apoptosis." *Cell Death and Differentiation* 18(2): 328–37.

Alonso LC, Yokoe T, Zhang P, Scott DK, Kim SK, O'Donnell CP, Garcia-Ocaña A. 2007. "Glucose Infusion in Mice: A New Model to Induce β-Cell Replication." *Diabetes* 56(7): 1792–1801.

Ambartsumian N, Klingelhöfer J, Grigorian M, Christensen C, Kriajevska M, Tulchinsky E, Georgiev G, Berezin V, Bock E, Rygaard J, Cao R, Cao Y, Lukanidin E. 2001. "The Metastasis-Associated Mts1 (S100A4) Protein Could Act as an Angiogenic Factor." *Oncogene* 20(34): 4685–95.

Ambartsumian N, Klingelhöfer J, Grigorian M. 2019. "The Multifaceted S100A4 Protein in Cancer and Inflammation." *Methods in Molecular Biology* 1929: 339–65.

Ambartsumian N, Grigorian M. 2016. "S100A4, a Link between Metastasis and Inflammation." *Молекулярная биология* 50(4): 577–88.

Ambartsumian NS, Grigorian MS, Larsen IF, Karlstrøm O, Sidenius N, Rygaard J, Georgiev G, Lukanidin E. 1996. “Metastasis of Mammary Carcinomas in GRS/A Hybrid Mice Transgenic for the Mts1 Gene.” *Oncogene* 13(8): 1621–30.

Amrani A, Jafarian-Tehrani M, Mormède P, Durant S, Pleau JM, Haour F, Dardenne M, Homo-Delarche F. 1996. “Interleukin-1 Effect on Glycemia in the Non-Obese Diabetic Mouse at the Pre-Diabetic Stage.” *Journal of Endocrinology* 148(1): 139–48.

Andersen K, Smith-Sørensen B, Pedersen KB, Hovig E, Myklebost O, Fodstad Ø, Maelandsmo GM. 2003. “Interferon-γ Suppresses S100A4 Transcription Independently of Apoptosis or Cell Cycle Arrest.” *British Journal of Cancer* 88(12): 1995–2001.

Anderson BM, Ma DW. 2009. “Are All N-3 Polyunsaturated Fatty Acids Created Equal?” *Lipids in Health and Disease* 8(33).

Anisimov VN, Berstein LM, Egormin PA, Piskunova TS, Popovich IG, Zabezhinski MA, Kovalenko IG, Poroshina TE, Semenchenko AV, Provinciali M, Re F, Franceschi C. 2005. “Effect of Metformin on Life Span and on the Development of Spontaneous Mammary Tumors in HER-2/Neu Transgenic Mice.” *Experimental Gerontology* 40(8–9): 685–93.

Arcuri C, Giambanco I, Bianchi R, Donato R. 2002. “Annexin V, Annexin VI, S100A1 and S100B in Developing and Adult Avian Skeletal Muscles.” *Neuroscience* 109(2): 371–88.

Arner E, Westermark PO, Spalding KL, Britton T, Rydén M, Frisén J, Bernard S, Arner P. 2010. “Adipocyte Turnover: Relevance to Human Adipose Tissue Morphology.” *Diabetes* 59(1): 105–9.

Arner P, Petrus P, Esteve D, Boulomié A, Näslund E, Thorell A, Gao H, Dahlman I, Rydén M. 2018. “Screening of Potential Adipokines Identifies S100A4 as a Marker of Pernicious Adipose Tissue and Insulin Resistance.” *International Journal of Obesity* 42(12): 2047–56.

Artwohl M, Roden M, Hölzenbein T, Freudenthaler A, Waldhäusl W, Baumgartner-Parzer SM. 2002. “Modulation by Leptin of Proliferation and Apoptosis in Vascular Endothelial Cells.” *International Journal of Obesity* 26(4): 577–80.

Arumugam T, Logsdon CD. 2011. “S100P: A Novel Therapeutic Target for Cancer.” *Amino Acids* 41(4): 893–839.

Ashcroft FM, Rorsman P. 2012. “Diabetes Mellitus and the β Cell: The Last Ten Years.” *Cell* 148(6): 1160–71.

Assefa Z, Lavens A, Steyaert C, Stangé G, Martens GA, Ling Z, Hellemans K, Pipeleers D. 2014. “Glucose Regulates Rat Beta Cell Number through Age-Dependent Effects on Beta Cell Survival and Proliferation.” *PLoS ONE* 9(1): e85174.

Association, American Diabetes. 2017. “Classification and Diagnosis of Diabetes.” *Diabetes Care* 40(Suppl 1): S11–24.

Austermann J, Nazmi AR, Müller-Tidow C, Gerke V. 2008. “Characterization of the Ca2+-Regulated Ezrin-S100P Interaction and Its Role in Tumor Cell Migration.” *Journal of Biological Chemistry* 283(43): 29331–40.

Austermann J, Spiekermann C, Roth J. 2018. “S100 Proteins in Rheumatic Diseases.” *Nature Reviews Rheumatology* 14(9): 528–241.

Averill MM, Barnhart S, Becker L, Li X, Heinecke JW, Leboeuf RC, Hamerman JA, Sorg C, Kerkhoff C, Bornfeldt KE. 2011. “S100A9 Differentially Modifies Phenotypic States of Neutrophils, Macrophages, and Dendritic Cells: Implications for Atherosclerosis and Adipose Tissue Inflammation.” *Circulation* 123(11): 1216–26.

Averill MM, Kerkhoff C, Bornfeldt KE. 2012. “S100A8 and S100A9 in Cardiovascular Biology and Disease.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 32(2): 223–29.

Babu A, Fogelfeld L. 2006. “Metabolic Syndrome and Prediabetes.” *Disease-a-Month* 52(2–3): 55–144.

Badr CE, Niers JM, Tjon-Kon-Fat LA, Noske DP, Wurdinger T, Tannous BA. 2009. “Real-Time Monitoring of Nuclear Factor KB Activity in Cultured Cells and in Animal Models.” *Molecular Imaging* 8(5): 278–90.

Baena-Díez JM, Peñafiel J, Subirana I, Ramos R, Elosua R, Marín-Ibañez A, Guembe MJ, Rigo F, Tormo-Díaz MJ, Moreno-Iribas C, Cabré JJ, Segura A, García-Lareo M, Gómez de la Cámara A, Lapetra J, Quesada M, Marrugat J, Medrano MJ, Berjón J, Frontera G, Gavr, Grau M. 2016. “Risk of Cause-Specific Death in Individuals with Diabetes: A Competing Risks Analysis.” *Diabetes Care* 39(11): 1987–95.

Bagnati M, Ogunkolade BW, Marshall C, Tucci C, Hanna K, Jones TA, Bugliani M, Nedjai B, Caton PW, Kieswich J, Yaqoob MM, Ball GR, Marchetti P, Hitman GA, Turner MD. 2016. “Glucolipotoxicity Initiates Pancreatic β-Cell Death through TNFR5/CD40-Mediated STAT1 and NF-KB Activation.” *Cell Death and Disease* 7(8): e2329.

Baillet A, Trocmé C, Berthier S, Arlotto M, Grange L, Chenau J, Quétant S, Sève M, Berger F, Juvin R, Morel F, Gaudin P. 2010. “Synovial Fluid Proteomic Fingerprint: S100A8, S100A9 and S100A12 Proteins Discriminate Rheumatoid Arthritis from Other Inflammatory Joint Diseases.” *Rheumatology (Oxford, England)* 49(4): 671–82.

Bajor M, Zaręba-Kozioł M, Zhukova L, Goryca K, Poznański J, Wysłouch-Cieszyńska A. 2016. “An Interplay of S-Nitrosylation and Metal Ion Binding for Astrocytic S100B Protein.” *PLoS ONE* 11(5): e0154822.

Baldi P, Long AD. 2001. “A Bayesian Framework for the Analysis of Microarray Expression Data: Regularized t-Test and Statistical Inferences of Gene Changes.” *Bioinformatics* 17(6): 509–19.

Baliunas DO, Taylor BJ, Irving H, Roerecke M, Patra J, Mohapatra S, Rehm J. 2009. “Alcohol as a Risk Factor for Type 2 Diabetes: A Systematic Review and Meta-Analysis.” *Diabetes Care* 32(11): 2123–32.

Ballotari P, Vicentini M, Manicardi V, Gallo M, Chiatamone Ranieri S, Greci M, Giorgi Rossi P. 2017. “Diabetes and Risk of Cancer Incidence: Results from a Population-Based Cohort Study in Northern Italy.” *BMC Cancer* 17(1): 703.

Ban HS, Kim BK, Lee H, Kim HM, Harmalkar D, Nam M, Park SK, Lee K, Park JT, Kim I, Lee K, Hwang GS, Won M. 2017. “The Novel Hypoxia-Inducible Factor-1α Inhibitor IDF-11774 Regulates Cancer Metabolism, Thereby Suppressing Tumor Growth.” *Cell death & disease* 8(6): e2843.

Barbé-Tuana FM, Klein D, Ichii H, Berman DM, Coffey L, Kenyon NS, Ricordi C, Pastori RL.

2006. “CD40-CD40 Ligand Interaction Activates Proinflammatory Pathways in Pancreatic Islets.” *Diabetes* 55(9): 2437–45.

Barclay AW, Petocz P, McMillan-Price J, Flood VM, Prvan T, Mitchell P, Brand-Miller JC. 2008. “Glycemic Index, Glycemic Load, and Chronic Disease Risk - A Metaanalysis of Observational Studies.” *American Journal of Clinical Nutrition* 87(3): 627–37.

Barone BB, Yeh HC, Snyder CF, Peairs KS, Stein KB, Derr RL, Wolff AC, Brancati FL. 2008. “Long-Term All-Cause Mortality in Cancer Patients with Preexisting Diabetes Mellitus: A Systematic Review and Meta-Analysis.” *JAMA - Journal of the American Medical Association* 300(23): 2754–64.

Bartolome A, Guillen C, Benito M. 2012. “Autophagy Plays a Protective Role in Endoplasmic Reticulum Stress-Mediated Pancreatic β Cell Death.” *Autophagy* 8(12): 1757–68.

Bensellam M, Maxwell EL, Chan JY, Luzuriaga J, West PK, Jonas JC, Gunton JE, Laybutt DR. 2016. “Hypoxia Reduces ER-to-Golgi Protein Trafficking and Increases Cell Death by Inhibiting the Adaptive Unfolded Protein Response in Mouse Beta Cells.” *Diabetologia* 59(7): 1492–1502.

Bensellam M, Van Lommel L, Overbergh L, Schuit FC, Jonas JC. 2009. “Cluster Analysis of Rat Pancreatic Islet Gene MRNA Levels after Culture in Low-, Intermediate- and High-Glucose Concentrations.” *Diabetologia* 52(3): 463–76.

Bernard C, Berthault MF, Saulnier C, Ktorza A. 1999. “Neogenesis vs. Apoptosis As Main Components of Pancreatic Beta Cell Ass Changes in Glucose-Infused Normal and Mildly Diabetic Adult Rats.” *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 13(10): 1195–1205.

Bhattacharya S, Large E, Heizmann CW, Hemmings B, Chazin WJ. 2003. “Structure of the Ca2+/S100B/NDR Kinase Peptide Complex: Insights into S100 Target Specificity and Activation of the Kinase.” *Biochemistry* 42(49): 14416–26.

Bian Y, Guo J, Qiao L, Sun X. 2018. “MiR-3189-3p Mimics Enhance the Effects of S100A4 SiRNA on the Inhibition of Proliferation and Migration of Gastric Cancer Cells by Targeting CFL2.” *International Journal of Molecular Sciences* 19(1): pii: E236.

Bianchi C, Miccoli R, Trombetta M, Giorgino F, Frontoni S, Faloia E, Marchesini G, Dolci MA, Cavalot F, Cavallo G, Leonetti F, Bonadonna RC, Del Prato S. 2013. “Elevated 1-Hour Postload Plasma Glucose Levels Identify Subjects with Normal Glucose Tolerance but Impaired β-Cell Function, Insulin Resistance, and Worse Cardiovascular Risk Profile: The GENFIEV Study.” *Journal of Clinical Endocrinology and Metabolism* 98(5): 2100–2105.

Biddlestone J, Bandarra D, Rocha S. 2015. “The Role of Hypoxia in Inflammatory Disease (Review).” *International Journal of Molecular Medicine* 35(4): 859–69.

Biden TJ, Boslem E, Chu KY, Sue N. 2014. “Lipotoxic Endoplasmic Reticulum Stress, β Cell Failure, and Type 2 Diabetes Mellitus.” *Trends in Endocrinology and Metabolism* 25(8): 389–98.

Björk P, Björk A, Vogl T, Stenström M, Liberg D, Olsson A, Roth J, Ivars F, Leanderson T. 2009. “Identification of Human S100A9 as a Novel Target for Treatment of Autoimmune Disease via Binding to Quinoline-3-Carboxamides.” *PLoS Biology* 7(4): e97.

Bjørnland K, Winberg JO, Odegaard OT, Hovig E, Loennechen T, Aasen AO, Fodstad O, Maelandsmo GM. 1999. “S100A4 Involvement in Metastasis: Deregulation of Matrix Metalloproteinases and Tissue Inhibitors of Matrix Metalloproteinases in Osteosarcoma Cells Transfected with an Anti-S100A4 Ribozyme.” *Cancer Research* 59(18): 4702–8.

Blas E, Kurup AS. 2010. “Synergy for Equity.” *Equity Social Determinants and Public Health Programmes*: 77–94.

Bloomgarden ZT. 2007. “Exploring Treatment Strategies for Type 2 Diabetes.” *Diabetes Care* 30(10): 2737–45.

Bodmer M, Meier C, Krähenbühl S, Jick SS, Meier CR. 2008. “Metformin, Sulfonylureas, or Other Antidiabetes Drugs and the Risk of Lactic Acidosis or Hypoglycemia a Nested Case-Control Analysis.” *Diabetes Care* 31(11): 2086–91.

Bond M, Chase AJ, Baker AH, Newby AC. 2001. “Inhibition of Transcription Factor NF-KB Reduces Matrix Metalloproteinase-1, -3 and -9 Production by Vascular Smooth Muscle Cells.” *Cardiovascular Research* 50(3): 556–65.

Böni-Schnetzler M, Meier DT. 2019. “Islet Inflammation in Type 2 Diabetes.” *Seminars in Immunopathology* 41(4): 501–13.

Bonner-Weir, S. 2000. “Islet Growth and Development in the Adult.” In *Journal of Molecular Endocrinology*,.

Borghaei RC, Gorski G, Javadi M; Mariah Chambers. 2009. “NF-KB and ZBP-89 Regulate MMP-3 Expression via a Polymorphic Site in the Promoter.” *Biochemical and Biophysical Research Communications* 382(2): 269–73.

Boslem E, Meikle PJ, Biden TJ. 2012. “Roles of Ceramide and Sphingolipids in Pancreatic β-Cell Function and Dysfunction.” *Islets* 4(3): 177–187.

Boucher J, Kleinridders A, Kahn CR. 2014. “Insulin Receptor Signaling in Normal and Insulin-Resistant States.” *Cold Spring Harbor Perspectives in Biology* 6(1): pii: a009191.

Boyd PJ, Doyle J, Gee E, Pallan S, Haas TL. 2005. “MAPK Signaling Regulates Endothelial Cell Assembly into Networks and Expression of MT1-MMP and MMP-2.” *American Journal of Physiology - Cell Physiology* 288(3): C659-668.

Boye K, Grotterød I, Aasheim HC, Hovig E, Maelandsmo GM. 2008. “Activation of NF-KB by Extracellular S100A4: Analysis of Signal Transduction Mechanisms and Identification of Target Genes.” *International Journal of Cancer* 123(6): 1301–10.

Boye K, Maelandsmo GM. 2010. “S100A4 and Metastasis: A Small Actor Playing Many Roles.” *American Journal of Pathology* 176(2): 528–35.

Bresnick AR, Weber DJ, Zimmer DB. 2015. “S100 Proteins in Cancer.” *Nature Reviews Cancer* 15(2): 96–109.

Brett J, Schmidt AM, Yan SD, Zou YS, Weidman E, Pinsky D, Nowygrod R, Neeper M, Przysiecki C, Shaw A. 1993. “Survey of the Distribution of a Newly Characterized Receptor for Advanced Glycation End Products in Tissues.” *The American journal of pathology* 143(6): 1699–1712.

Briaud I, Harmon JS, Kelpe CL, Segu VB, Poitout V. 2001. “Lipotoxicity of the Pancreatic β-Cell Is Associated with Glucose-Dependent Esterification of Fatty Acids into

Neutral Lipids." *Diabetes* 50(2): 315–21.

Briaud I, Kelpe CL, Johnson LM, Tran PO, Poitout V. 2002. "Differential Effects of Hyperlipidemia on Insulin Secretion in Islets of Langerhans from Hyperglycemic versus Normoglycemic Rats." *Diabetes* 51(3): 662–68.

Brini M, Ottolini D, Calì T, Carafoli E. 2013. "Calcium in Health and Disease." *Metal Ions in Life Sciences* (13): 81–137.

Brink C, Chowdhury K, Gruss P. 2001. "Pax4 Regulatory Elements Mediate Beta Cell Specific Expression in the Pancreas." *Mechanisms of Development* 100(1): 37–43.

Brissova M, Shiota M, Nicholson WE, Gannon M, Knobel SM, Piston DW, Wright CV, Powers AC. 2002. "Reduction in Pancreatic Transcription Factor PDX-1 Impairs Glucose-Stimulated Insulin Secretion." *Journal of Biological Chemistry* 277(13): 11225–32.

Brufsky A. 2010. "Trastuzumab-Based Therapy for Patients with HER2-Positive Breast Cancer: From Early Scientific Development to Foundation of Care." *American Journal of Clinical Oncology: Cancer Clinical Trials* 33(2): 186–95.

Burke AP, Kolodgie FD, Zieske A, Fowler DR, Weber DK, Varghese PJ, Farb A, Virmani R. 2004. "Morphologic Findings of Coronary Atherosclerotic Plaques in Diabetics: A Postmortem Study." *Arteriosclerosis, Thrombosis, and Vascular Biology* 24(7): 1266–71.

Burkhardt K, Schwarz S, Pan C, Stelter F, Kotliar K, Von Eynatten M, Sollinger D, Lanzl I, Heemann U, Baumann M. 2009. "Myeloid-Related Protein 8/14 Complex Describes Microcirculatory Alterations in Patients with Type 2 Diabetes and Nephropathy." *Cardiovascular Diabetology* 8(10).

Busch AK, Gurisik E, Cordery DV, Sudlow M, Denyer GS, Laybutt DR, Hughes WE, Biden TJ. 2005. "Increased Fatty Acid Desaturation and Enhanced Expression of Stearoyl Coenzyme A Desaturase Protects Pancreatic β-Cells from Lipoapoptosis." *Diabetes* 54(10): 2917–24.

Buteau J, Foisy S, Joly E, Prentki M. 2003. "Glucagon-like Peptide 1 Induces Pancreatic β-Cell Proliferation via Transactivation of the Epidermal Growth Factor Receptor." *Diabetes* 52(1): 124–32.

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. 2003. "β-Cell Deficit and Increased β-Cell Apoptosis in Humans with Type 2 Diabetes." *Diabetes* 52(1): 102–10.

Butler PC, Meier JJ, Butler AE, Bhushan A. 2007. "The Replication of β Cells in Normal Physiology, in Disease and for Therapy." *Nature Clinical Practice Endocrinology and Metabolism* 3(11): 758–68.

Byrne BG, Dubuisson JF, Joshi AD, Persson JJ, Swanson MS. 2013. "Inflammasome Components Coordinate Autophagy and Pyroptosis as Macrophage Responses to Infection." *mBio* 4(1): e00620-12.

Cafaro RP. 1960. "Hypoxia: Its Causes and Symptoms." *J Am Dent Soc Anesthesiol.* 7(4): 4–8.

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D,

Keshert E. 1998. “Role of HIF-1α in Hypoxiamediated Apoptosis, Cell Proliferation and Tumour Angiogenesis.” *Nature* 394(6692): 485–90.

Carta G, Murru E, Banni S, Manca C. 2017. “Palmitic Acid: Physiological Role, Metabolism and Nutritional Implications.” *Frontiers in Physiology* 8(902).

Carta G, Murru E, Lisai S, Sirigu A, Piras A, Collu M, Batetta B, Gambelli L, Banni S. 2015. “Dietary Triacylglycerols with Palmitic Acid in the Sn-2 Position Modulate Levels of N-Acylethanolamides in Rat Tissues.” *PLoS ONE* 10(3): e0120424.

Castro AM, Macedo-de la Concha LE, and Pantoja-Meléndez CA. 2017. “Low-Grade Inflammation and Its Relation to Obesity and Chronic Degenerative Diseases.” *Revista Médica del Hospital General de México*.

Cecil DL, Terkeltaub R. 2008. “Transamidation by Transglutaminase 2 Transforms S100A11 Calgranulin into a Procatabolic Cytokine for Chondrocytes.” *The Journal of Immunology* 180(12): 8378–85.

Cerezo L, Šumová B, Prajzlerová K, Veigl D, Damgaard D, Nielsen CH, Pavelka K, Vencovský J, Šenolt L. 2017. “Calgizzarin (S100A11): A Novel Inflammatory Mediator Associated with Disease Activity of Rheumatoid Arthritis.” *Arthritis Research and Therapy* 19(1): 79.

Cerezo LA, Kuncová K, Mann H, Tomcík M, Zámecník J, Lukanidin E, Neidhart M, Gay S, Grigorian M, Vencovsky J, Senolt L. 2011. “The Metastasis Promoting Protein S100A4 Is Increased in Idiopathic Inflammatory Myopathies.” *Rheumatology* 50(10): 1766–72.

Cerezo LA, Remáková M, Tomčik M, Gay S, Neidhart M, Lukanidin E, Pavelka K, Grigorian M, Vencovský J, Šenolt L. 2014. “The Metastasis-Associated Protein S100A4 Promotes the Inflammatory Response of Mononuclear Cells via the TLR4 Signalling Pathway in Rheumatoid Arthritis.” *Rheumatology (United Kingdom)* 53(8): 1520–26.

Cerychova R, Pavlinkova G. 2018. “HIF-1, Metabolism, and Diabetes in the Embryonic and Adult Heart.” *Frontiers in Endocrinology* 9: 460.

Chaabane C, Heizmann CW, Bochaton-Piallat ML. 2015. “Extracellular S100A4 Induces Smooth Muscle Cell Phenotypic Transition Mediated by RAGE.” *Biochimica et Biophysica Acta - Molecular Cell Research* 1853(9): 2144–57.

Chang-Chen KJ, Mullur R, Bernal-Mizrachi E. 2008. “β-Cell Failure as a Complication of Diabetes.” *Reviews in Endocrine and Metabolic Disorders* 9(4): 329–43.

Chang SA. 2012. “Smoking and Type 2 Diabetes Mellitus.” *Diabetes and Metabolism Journal* 36(6): 399–403.

Chang YC, Wu WC. 2013. “Dyslipidemia and Diabetic Retinopathy.” *Review of Diabetic Studies* 10(2–3): 121–32.

Chaves ML, Camozzato AL, Ferreira ED, Piazenski I, Kochhann R, Dall’Igna O, Mazzini GS, Souza DO, Portela LV. 2010. “Serum Levels of S100B and NSE Proteins in Alzheimer’s Disease Patients.” *Journal of Neuroinflammation* 7(6).

Chazin WJ. 2011. “Relating Form and Function of EF-Hand Calcium Binding Proteins.” *Accounts of Chemical Research* 44(3): 171–79.

Chen C, Cohrs CM, Stertmann J, Bozsak R, Speier S. 2017. “Human Beta Cell Mass and

Function in Diabetes: Recent Advances in Knowledge and Technologies to Understand Disease Pathogenesis." *Molecular Metabolism* 6(9): 943–57.

Chen C, Ma X, Yang C, Nie W, Zhang J, Li H, Rong P, Yi S, Wang W. 2018. "Hypoxia Potentiates LPS-Induced Inflammatory Response and Increases Cell Death by Promoting NLRP3 Inflammasome Activation in Pancreatic β Cells." *Biochemical and Biophysical Research Communications* 495(4): 2512–18.

Chen H, Xu C, Jin Q, Liu Z. 2014. "S100 Protein Family in Human Cancer." *American journal of cancer research* 4(2): 89–115.

Chen H, Yu D, Luo A, Tan W, Zhang C, Zhao D, Yang M, Liu J, Lin D, Liu Z. 2009. "Functional Role of S100A14 Genetic Variants and Their Association with Esophageal Squamous Cell Carcinoma." *Cancer Research* 69(8): 3451–57.

Chen J, Saxena G, Mungrue IN, Lusis AJ, Shalev A. 2008. "Thioredoxin-Interacting Protein A Critical Link between Glucose Toxicity and β-Cell Apoptosis." *Diabetes* 57(4): 938–44.

Chen L, Magliano DJ, Zimmet PZ. 2012. "The Worldwide Epidemiology of Type 2 Diabetes Mellitus - Present and Future Perspectives." *Nature Reviews Endocrinology* 8(4): 228–36.

Cheng P, Corzo CA, Luetteke N, Yu B, Nagaraj S, Bui MM, Ortiz M, Nacken W, Sorg C, Vogl T, Roth J, Gabrilovich DI. 2008. "Inhibition of Dendritic Cell Differentiation and Accumulation of Myeloid-Derived Suppressor Cells in Cancer Is Regulated by S100A9 Protein." *The Journal of Experimental Medicine* 205(10): 2235–49.

Cho NH, Shaw JE, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge AW, Malanda B. 2018. "IDF Diabetes Atlas: Global Estimates of Diabetes Prevalence for 2017 and Projections for 2045." *Diabetes Research and Clinical Practice* 138: 271–82.

Chow KH, Park HJ, George J, Yamamoto K, Gallup AD, Graber JH, Chen Y, Jiang W, Steindler DA, Neilson EG, Kim BYS, Yun K. 2017. "S100A4 Is a Biomarker and Regulator of Glioma Stem Cells That Is Critical for Mesenchymal Transition in Glioblastoma." *Cancer Research* 77(19): 5360–73.

Christian F, Smith E, Carmody R. 2016. "The Regulation of NF-KB Subunits by Phosphorylation." *Cells* 5(1): 12.

Clague J, Bernstein L. 2012. "Physical Activity and Cancer." *Curr Oncol Rep.* 14(6): 550–58.

Cnop M, Abdulkarim B, Bottu G, Cunha DA, Igoillo-Esteve M, Masini M, Turatsinze JV, Griebel T, Villate O, Santin I, Bugliani M, Ladriere L, Marselli L, McCarthy MI, Marchetti P, Sammeth M, Eizirik DL. 2014. "RNA Sequencing Identifies Dysregulation of the Human Pancreatic Islet Transcriptome by the Saturated Fatty Acid Palmitate." *Diabetes* 63(6): 1978–93.

Cohen DH, LeRoith D. 2012. "Obesity, Type 2 Diabetes, and Cancer: The Insulin and IGF Connection." *Endocrine-Related Cancer* 19(5): F27-45.

Collier JJ, Sparer TE, Karlstad MD, Burke SJ. 2017. "Pancreatic Islet Inflammation: An Emerging Role for Chemokines." *Journal of Molecular Endocrinology* 59(1): R33-46.

Colmers IN, Bowker SL, Majumdar SR, Johnson JA. 2012. "Use of Thiazolidinediones and the Risk of Bladder Cancer among People with Type 2 Diabetes: A Meta-Analysis."

CMAJ 184(12): E675-683.

Connor J. 2017. “Alcohol Consumption as a Cause of Cancer.” *Addiction* 112(2): 222–28.

Costa V, Angelini C, De Feis I, Ciccodicola A. 2010. “Uncovering the Complexity of Transcriptomes with RNA-SEQ.” *The Role of Bioinformatics in Agriculture* 2010: 853–916.

Cremers NAJ, van den Bosch MHJ, van Dalen S, Di Ceglie I, Ascone G, van de Loo F, Koenders M, van der Kraan P, Sloetjes A, Vogl T, Roth J, Geven EJW, Blom AB, van Lent PLEM. 2017. “S100A8/A9 Increases the Mobilization of pro-Inflammatory Ly6Chigh Monocytes to the Synovium during Experimental Osteoarthritis.” *Arthritis Research and Therapy* 19(1): 217.

Currie CJ, Poole CD, Gale EA. 2009. “The Influence of Glucose-Lowering Therapies on Cancer Risk in Type 2 Diabetes.” *Diabetologia* 52(9): 1766–77.

D’Ignazio L, Batie M, Rocha S. 2017. “Hypoxia and Inflammation in Cancer, Focus on HIF and NF-KB.” *Biomedicines* 9(2): E21.

Dahlmann M, Kobelt D, Walther W, Mudduluru G, Stein U. 2016. “S100A4 in Cancer Metastasis: Wnt Signaling-Driven Interventions for Metastasis Restriction.” *Cancers* 8(6): pii: E59.

Dahlmann M, Okhrimenko A, Marcinkowski P, Osterland M, Herrmann P, Smith J, Heizmann CW, Schlag PM, Stein U. 2014. “RAGE Mediates S100A4-Induced Cell Motility via MAPK/ERK and Hypoxia Signaling and Is a Prognostic Biomarker for Human Colorectal Cancer Metastasis.” *Oncotarget* 5(10): 3220–33.

Dahlmann M, Sack U, Herrmann P, Lemm M, Fichtner I, Schlag PM, Stein U. 2012. “Systemic ShRNA Mediated Knock-down of S100A4 in Colorectal Cancer Xenografted Mice Reduces Metastasis Formation.” *Oncotarget* 3(8): 783–97.

Dakhel S, Padilla L, Adan J, Masa M, Martinez JM, Roque L, Coll T, Hervas R, Calvis C, Messeguer R, Mitjans F, Hernández JL. 2014. “S100P Antibody-Mediated Therapy as a New Promising Strategy for the Treatment of Pancreatic Cancer.” *Oncogenesis* 3(3): e92.

Dandekar RC, Kingaonkar AV, Dhabekar GS. 2011. “Role of Macrophages in Malignancy.” *Annals of maxillofacial surgery* 1(2): 150–54.

Dandona P, Aljada A, Bandyopadhyay A. 2004. “Inflammation: The Link between Insulin Resistance, Obesity and Diabetes.” *Trends in Immunology* 25(1): 4–7.

Dang CV. 2012. “Links between Metabolism and Cancer.” *Genes and Development* 26(9): 877–90.

Dardano A, Penno G, Del Prato S, Miccoli R. 2014. “Optimal Therapy of Type 2 Diabetes: A Controversial Challenge.” *Aging* 6(3): 187–206.

Davies BR, Barraclough R, Rudland PS. 1994. “Induction of Metastatic Ability in a Stably Diploid Benign Rat Mammary Epithelial Cell Line by Transfection with DNA from Human Malignant Breast Carcinoma Cell Lines.” *Cancer Research* (54): 2785–93.

Davies MP, Rudland PS, Robertson L, Parry EW, Jolicoeur P, Barraclough R. 1996. “Expression of the Calcium-Binding Protein S100A4 (P9Ka) in MMTV-Neu Transgenic Mice Induces Metastasis of Mammary Tumours.” *Oncogene* 13(8): 1631–37.

Decensi A, Puntoni M, Goodwin P, Cazzaniga M, Gennari A, Bonanni B, Gandini S. 2010. "Metformin and Cancer Risk in Diabetic Patients: A Systematic Review and Meta-Analysis." *Cancer Prevention Research* 3(11): 1451–61.

Dejardin E. 2006. "The Alternative NF-KB Pathway from Biochemistry to Biology: Pitfalls and Promises for Future Drug Development." *Biochemical Pharmacology* 72(9): 1161–79.

Demirtas L, Guclu A, Erdur FM, Akbas EM, Ozcicek A, Onk D, Turkmen K. 2016. "Apoptosis, Autophagy & Endoplasmic Reticulum Stress in Diabetes Mellitus." *Indian Journal of Medical Research* 144(4): 515–24.

Demozay D, Tsunekawa S, Briaud I, Shah R, Rhodes CJ. 2011. "Specific Glucose-Induced Control of Insulin Receptor Substrate-2 Expression Is Mediated via Ca 2+-Dependent Calcineurin/NFAT Signaling in Primary Pancreatic Islet β-Cells." *Diabetes* 60(11): 2892–2902.

Denley A, Carroll JM, Brierley GV, Cosgrove L, Wallace J, Forbes B, Roberts CT Jr. 2007. "Differential Activation of Insulin Receptor Substrates 1 and 2 by Insulin-Like Growth Factor-Activated Insulin Receptors." *Molecular and Cellular Biology* 27(10): 3569–77.

Depping R, Steinhoff A, Schindler SG, Friedrich B, Fagerlund R, Metzen E, Hartmann E, Köhler M. 2008. "Nuclear Translocation of Hypoxia-Inducible Factors (HIFs): Involvement of the Classical Importin α/β Pathway." *Biochimica et Biophysica Acta - Molecular Cell Research* 1783(3): 394–404.

van Dieck J, Teufel DP, Jaulent AM, Fernandez-Fernandez MR, Rutherford TJ, Wyslouch-Cieszynska A, Fersht AR. 2009. "Posttranslational Modifications Affect the Interaction of S100 Proteins with Tumor Suppressor P53." *Journal of Molecular Biology* 394(5): 922–30.

Diedisheim M, Oshima M, Albagli O, Wennberg-Huldt C, Ahlstedt I, Clausen M, Menon S, Aivazidis A, Andreasson AC, Haynes WG, Marchetti P, Marselli L, Armanet M, Chimienti F, Scharfmann R. 2018. "Modeling Human Pancreatic Beta Cell Dedifferentiation." *Molecular Metabolism* 10: 74–86.

Dobbins M, Decorby K, Choi BC. 2013. "The Association between Obesity and Cancer Risk: A Meta-Analysis of Observational Studies from 1985 to 2011." *ISRN Preventive Medicine* (2013): 680536.

Donath MY, Böni-Schnetzler M, Ellingsgaard H, Ehses JA. 2009. "Islet Inflammation Impairs the Pancreatic β-Cell in Type 2 Diabetes." *Physiology* (24): 325–31.

Donath MY, Dalmas É, Sauter NS, Böni-Schnetzler M. 2013. "Inflammation in Obesity and Diabetes: Islet Dysfunction and Therapeutic Opportunity." *Cell Metabolism* 17(6): 860–72.

Donath MY. 2014. "Targeting Inflammation in the Treatment of Type 2 Diabetes: Time to Start." *Nature Reviews Drug Discovery* 15(Suppl. 3): 193–96.

Donato R, Cannon BR, Sorci G, Riuzzi F, Hsu K, Weber DJ, Geczy CL. 2013. "Functions of S100 Proteins." *Current Molecular Medicine* 13(1): 24–57.

Donato R, Sorci G, Riuzzi F, Arcuri C, Bianchi R, Brozzi F, Tubaro C, Giambanco I. 2009. "S100B's Double Life: Intracellular Regulator and Extracellular Signal." *Biochimica et Biophysica Acta - Molecular Cell Research* 1793(6): 1008–22.

Donato R. 2003. “Intracellular and Extracellular Roles of S100 Proteins.” *Microscopy Research and Technique* 60(6): 540–41.

Donato R. 2001. “S100: A Multigenic Family of Calcium-Modulated Proteins of the EF-Hand Type with Intracellular and Extracellular Functional Roles.” *International Journal of Biochemistry and Cell Biology* 33(7): 637–68.

Dong N, Shi H, Xu B, Cai Y. 2015. “Increased Plasma S100A12 Levels Are Associated with Diabetic Retinopathy and Prognostic Biomarkers of Macrovascular Events in Type 2 Diabetic Patients.” *Investigative Ophthalmology and Visual Science* 56(8): 4177–85.

Dor Y, Glaser B. 2013. “Beta-Cell Dedifferentiation and Type 2 Diabetes.” *New England Journal of Medicine* 368(6): 572–73.

Dou C, Liu Z, Xu M, Jia Y, Wang Y, Li Q, Yang W, Zheng X, Tu K, Liu Q. 2016. “MiR-187-3p Inhibits the Metastasis and Epithelial–Mesenchymal Transition of Hepatocellular Carcinoma by Targeting S100A4.” *Cancer Letters* 381(2): 380–90.

Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. 2007. “Metformin Inhibits Mammalian Target of Rapamycin-Dependent Translation Initiation in Breast Cancer Cells.” *Cancer Research* 67(22): 10804–12.

Duan W, Shen X, Lei J, Xu Q, Yu Y, Li R, Wu E, Ma Q. 2014. “Hyperglycemia, a Neglected Factor during Cancer Progression.” *BioMed Research International* (2014): 461917.

Dulyaninova NG, Malashkevich VN, Almo SC, Bresnick AR. 2005. “Regulation of Myosin-IIA Assembly and Mts1 Binding by Heavy Chain Phosphorylation.” *Biochemistry* 44(18): 6867–76.

Ebralidze A, Tulchinsky E, Grigorian M, Afanasyeva A, Senin V, Revazova E, Lukanidin E. 1989. “Isolation and Characterization of a Gene Specifically Expressed in Different Metastatic Cells and Whose Deduced Gene Product Has a High Degree of Homology to a Ca2+-Binding Protein Family.” *Genes & development* 3(7): 1086–93.

Eddy D, Schlessinger L, Kahn R, Peskin B, Schiebinger R. 2009. “Relationship of Insulin Resistance and Related Metabolic Variables to Coronary Artery Disease: A Mathematical Analysis.” *Diabetes Care* 32(2): 361–66.

Eeg-Olofsson K, Cederholm J, Nilsson PM, Zethelius B, Svensson AM, Gudbjörnsdóttir S, Eliasson B. 2010. “New Aspects of HbA1c as a Risk Factor for Cardiovascular Diseases in Type 2 Diabetes: An Observational Study from the Swedish National Diabetes Register (NDR).” *Journal of Internal Medicine* 268(5): 471–82.

Eguchi K, Nagai R. 2017. “Islet Inflammation in Type 2 Diabetes and Physiology.” *Journal of Clinical Investigation* 127(1): 14–23.

Ehses JA, Lacraz G, Giroix MH, Schmidlin F, Coulaud J, Kassis N, Irminger JC, Kergoat M, Portha B, Homo-Delarche F, Donath MY. 2009. “IL-1 Antagonism Reduces Hyperglycemia and Tissue Inflammation in the Type 2 Diabetic GK Rat.” *Proceedings of the National Academy of Sciences* 106(33): 13998–3.

Eichler EE, Flint J, Gibson G, Kong A, Leal SM, Moore JH, Nadeau JH. 2010. “Missing Heritability and Strategies for Finding the Underlying Causes of Complex Disease.” *Nature Reviews Genetics* 11(6): 446–50.

El-Assaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L, Prentki M. 2003. “Saturated Fatty Acids Synergize with Elevated

Glucose to Cause Pancreatic β-Cell Death." *Endocrinology* 144(9): 4154–63.

Elley CR, Kenealy T, Robinson E, Drury PL. 2008. "Glycated Haemoglobin and Cardiovascular Outcomes in People with Type 2 Diabetes: A Large Prospective Cohort Study." *Diabetic Medicine* 25(11): 1295–1301.

Elsner M, Gehrmann W, Lenzen S. 2011. "Peroxisome-Generated Hydrogen Peroxide as Important Mediator of Lipotoxicity in Insulin-Producing Cells." *Diabetes* 60(1): 200–208.

Emberley ED, Niu Y, Curtis L, Troup S, Mandal SK, Myers JN, Gibson SB, Murphy LC, Watson PH. 2005. "The S100A7-c-Jun Activation Domain Binding Protein 1 Pathway Enhances Prosurvival Pathways in Breast Cancer." *Cancer Research* 65(13): 5696–5702.

Eue I, Pietz B, Storck J, Klempt M, Sorg C. 2000. "Transendothelial Migration of 27E10+ Human Monocytes." *International Immunology* 12(11): 1593–1604.

Fagerlund R, Kinnunen L, Köhler M, Julkunen I, Melén K. 2005. "NF-KB Is Transported into the Nucleus by Importin A3 and Importin A4." *Journal of Biological Chemistry* 280(16): 15942–51.

Fagerlund R, Melén K, Cao X, Julkunen I. 2008. "NF-KB P52, RelB and c-Rel Are Transported into the Nucleus via a Subset of Importin α Molecules." *Cellular Signalling* 20(8): 1442–51.

Fakhruddin S, Alanazi W, Jackson KE. 2017. "Diabetes-Induced Reactive Oxygen Species: Mechanism of Their Generation and Role in Renal Injury." *Journal of Diabetes Research*: 8379327.

Fei F, Qu J, Li C, Wang X, Li Y, Zhang S. 2017. "Role of Metastasis-Induced Protein S100A4 in Human Non-Tumor Pathophysiologies." *Cell and Bioscience* 7(64).

Fei F, Qu J, Zhang M, Li Y, Zhang S. 2017. "S100A4 in Cancer Progression and Metastasis: A Systematic Review." *Oncotarget* 8(42): 73219–39.

Fernandez-Fernandez MR, Veprintsev DB, Fersht AR. 2005. "Proteins of the S100 Family Regulate the Oligomerization of P53 Tumor Suppressor." *Proceedings of the National Academy of Sciences* 102(13): 4735–40.

Ferrannini E, Balkau B, Coppack SW, Dekker JM, Mari A, Nolan J, Walker M, Natali A, Beck-Nielsen H. 2007. "Insulin Resistance, Insulin Response, and Obesity as Indicators of Metabolic Risk." *Journal of Clinical Endocrinology and Metabolism* 92(8): 2885–92.

Flannick J, Florez JC. 2016. "Type 2 Diabetes: Genetic Data Sharing to Advance Complex Disease Research." *Nature Reviews Genetics* 17(9): 535–49.

Flier SN, Kulkarni RN, Kahn CR. 2001. "Evidence for a Circulating Islet Cell Growth Factor in Insulin-Resistant States." *Proceedings of the National Academy of Sciences* 98(13): 7475–80.

Foell D, Seeliger S, Vogl T, Koch HG, Maschek H, Harms E, Sorg C, Roth J. 2003. "Expression of S100A12 (EN-RAGE) in Cystic Fibrosis." *Thorax* 58(7): 613–17.

Foell D, Wittkowski H, Kessel C, Lüken A, Weinhage T, Varga G, Vogl T, Wirth T, Viemann D, Björk P, van Zoelen MA, Gohar F, Srikrishna G, Kraft M, Roth J. 2013. "Proinflammatory S100A12 Can Activate Human Monocytes via Toll-like Receptor

4." *American Journal of Respiratory and Critical Care Medicine* 187(12): 1324–34.

Foell D, Wittkowski H, Vogl T, Roth J. 2007. "S100 Proteins Expressed in Phagocytes: A Novel Group of Damage-Associated Molecular Pattern Molecules." *Journal of Leukocyte Biology* 81(1): 28–37.

Fonseca SG, Gromada J, Urano F. 2011. "Endoplasmic Reticulum Stress and Pancreatic β-Cell Death." *Trends in Endocrinology and Metabolism* 22(7): 266–74.

Fonseca VA. 2009. "Defining and Characterizing the Progression of Type 2 Diabetes." *Diabetes care* 32(Suppl 2): S151-156.

Ford HL, Silver DL, Kachar B, Sellers JR, Zain SB. 1997. "Effect of Mts1 on the Structure and Activity of Nonmuscle Myosin II." *Biochemistry* 36(51): 16321–27.

Franke K, Gassmann M, Wielockx B. 2013. "Erythrocytosis: The HIF Pathway in Control." *Blood* 122(7): 1122–28.

Franz MJ, MacLeod J, Evert A, Brown C, Gradwell E, Handu D, Reppert A, Robinson M. 2017. "Academy of Nutrition and Dietetics Nutrition Practice Guideline for Type 1 and Type 2 Diabetes in Adults: Nutrition Intervention Evidence Reviews and Recommendations." *Journal of the Academy of Nutrition and Dietetics* 117(10): 1659–79.

Fratantonio D, Cimino F, Speciale A, Virgili F. 2018. "Need (More than) Two to Tango: Multiple Tools to Adapt to Changes in Oxygen Availability." *BioFactors* 44(3): 207–18.

Frayn KN, Karpe F, Fielding BA, Macdonald IA, Coppack SW. 2003. "Integrative Physiology of Human Adipose Tissue." *International Journal of Obesity* 27(8): 875–88.

Friedrichsen BN, Richter HE, Hansen JA, Rhodes CJ, Nielsen JH, Billestrup B, Møldrup A. 2003. "Signal Transducer and Activator of Transcription 5 Activation Is Sufficient to Drive Transcriptional Induction of Cyclin D2 Gene and Proliferation of Rat Pancreatic β-Cells." *Molecular Endocrinology* 17(5): 945–58.

Frykberg RG, Zgonis T, Armstrong DG, Driver VR, Giurini JM, Kravitz SR, Landsman AS, Lavery LA, Moore JC, Schuberth JM, Wukich DK, Andersen C, Vanore JV. 2006. "Diabetic Foot Disorders. A Clinical Practice Guideline (2006 Revision)." *Journal of Foot and Ankle Surgery* 45(5 suppl): S1-66.

Fu B, Xue J, Li Z, Shi X, Jiang BH, Fang J. 2007. "Chrysin Inhibits Expression of Hypoxia-Inducible Factor-1 through Reducing Hypoxia-Inducible Factor-1 Stability and Inhibiting Its Protein Synthesis." *Molecular Cancer Therapeutics* 6(1): 220–26.

Fujimoto K, Polonsky KS. 2009. "Pdx1 and Other Factors That Regulate Pancreatic β-Cell Survival." *Diabetes, Obesity and Metabolism* 11(Suppl4): 30–37.

Fujiwara K, Maekawa F, Yada T. 2005. "Oleic Acid Interacts with GPR40 to Induce Ca 2+ Signaling in Rat Islet β-Cells: Mediation by PLC and L-Type Ca 2+ Channel and Link to Insulin Release." *American Journal of Physiology-Endocrinology and Metabolism* 289(4): E670-677.

Fujiya A, Nagasaki H, Seino Y, Okawa T, Kato J, Fukami A, Himeno T, Uenishi E, Tsunekawa S, Kamiya H, Nakamura J, Oiso Y, Hamada Y. 2014. "The Role of S100B in the Interaction between Adipocytes and Macrophages." *Obesity* 22(2): 371–79.

Gardam S, Sierro F, Basten A, Mackay F, Brink R. 2008. “TRAF2 and TRAF3 Signal Adapters Act Cooperatively to Control the Maturation and Survival Signals Delivered to B Cells by the BAFF Receptor.” *Immunity* 28(3): 391–401.

Garofalo C, Surmacz E. 2006. “Leptin and Cancer.” *Journal of Cellular Physiology* 207(1): 12–22.

Garrett SC, Varney KM, Weber DJ, Bresnick AR. 2006. “S100A4, a Mediator of Metastasis.” *Journal of Biological Chemistry* 281(2): 677–80.

Gheith O, Farouk N, Nampoory N, Halim MA, Al-Otaibi T. 2016. “Diabetic Kidney Disease: World Wide Difference of Prevalence and Risk Factors.” *J Nephropharmacol.* 5(1): 49–56.

Giacca A, Xiao C, Oprescu AI, Carpentier AC, Lewis GF. 2011. “Lipid-Induced Pancreatic -Cell Dysfunction: Focus on in Vivo Studies.” *AJP: Endocrinology and Metabolism* 300(2): E255-262.

Giacco F, Brownlee M. 2010. “Oxidative Stress and Diabetic Complications.” *Circulation Research* 107(9): 1058–70.

Gianni M, Terao M, Kurosaki M, Paroni G, Brunelli L, Pastorelli R, Zanetti A, Lupi M, Acquavita A, Bolis M, Fratelli M, Rochette-Egly C, Garattini E. 2018. “S100A3 a Partner Protein Regulating the Stability/Activity of RARα and PML-RARα in Cellular Models of Breast/Lung Cancer and Acute Myeloid Leukemia.” *Oncogene*.

Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, Pollak M, Regensteiner JG, Yee D. 2010. “Diabetes and Cancer: A Consensus Report.” *Diabetes Care* 33(7): 1674–85.

Gläser R, Meyer-Hoffert U, Harder J, Cordes J, Wittersheim M, Kobliakova J, Fölster-Holst R, Proksch E, Schröder JM, Schwarz T. 2009. “The Antimicrobial Protein Psoriasin (S100a7) Is Upregulated in Atopic Dermatitis and after Experimental Skin Barrier Disruption.” *Journal of Investigative Dermatology* 129(3): 641–49.

Glick D, Barth S, Macleod KF. 2010. “Autophagy: Cellular and Molecular Mechanisms.” *Journal of Pathology* 221(1): 3–12.

Gnacińska M, Małgorzewicz S, Stojek M, Łysiak-Szydłowska W, Sworczak K. 2009. “Role of Adipokines in Complications Related to Obesity. A Review.” *Advances in Medical Sciences* 54(2): 150–57.

Goldberg AL. 2012. “Development of Proteasome Inhibitors as Research Tools and Cancer Drugs.” *Journal of Cell Biology* 199(4): 583–88.

Golson ML, Misfeldt AA, Kopsombut UG, Petersen CP, Gannon M. 2010. “High Fat Diet Regulation of β-Cell Proliferation and β-Cell Mass.” *The Open Endocrinology Journal* 4.

Gongoll S, Peters G, Mengel M, Piso P, Klempnauer J, Kreipe H, von Wasielewski R. 2002. “Prognostic Significance of Calcium-Binding Protein S100A4 in Colorectal Cancer.” *Gastroenterology* 123(5): 1478–84.

Gonzalez LL, Garrie K, Turner MD. 2018. “Type 2 Diabetes – An Autoinflammatory Disease Driven by Metabolic Stress.” *Biochimica et Biophysica Acta - Molecular Basis of Disease* 1864(11): 3805–23.

Gonzalez Y, Herrera MT, Soldevila G, Garcia-Garcia L, Fabián G, Pérez-Armendariz EM,

Bobadilla K, Guzmán-Beltrán S, Sada E, Torres M. 2012. “High Glucose Concentrations Induce TNF-α Production through the down-Regulation of CD33 in Primary Human Monocytes.” *BMC Immunology* 13(19).

Greenbaum D, Colangelo C, Williams K, Gerstein M. 2003. “Comparing Protein Abundance and MRNA Expression Levels on a Genomic Scale.” *Genome Biology* 4(9): 117.

Gribenko AV, Hopper JE, Makhatadze GI. 2001. “Molecular Characterization and Tissue Distribution of a Novel Member of the S100 Family of EF-Hand Proteins.” *Biochemistry* 40(51): 15538–48.

Grigorian M, Andresen S, Tulchinsky E, Kriajevska M, Carlberg C, Kruse C, Cohn M, Ambartsumian N, Christensen A, Selivanova G, Lukanidin E. 2001. “Tumor Suppressor P53 Protein Is a New Target for the Metastasis-Associated Mts1/S100A4 Protein. Functional Consequences of Their Interaction.” *Journal of Biological Chemistry* 276(25): 22699–708.

Grimbaldeston MA, Geczy CL, Tedla N, Finlay-Jones JJ, Hart PH. 2003. “S100A8 Induction in Keratinocytes by Ultraviolet A Irradiation Is Dependent on Reactive Oxygen Intermediates.” *Journal of Investigative Dermatology* 121(5): 1168–74.

Gromada J, Franklin I, Wollheim CB. 2007. “α-Cells of the Endocrine Pancreas: 35 Years of Research but the Enigma Remains.” *Endocrine Reviews* 28(1): 84–116.

Grosfeld A, Andre J, Hauguel-De Mouzon S, Berra E, Pouyssegur J, Guerre-Millo M. 2002. “Hypoxia-Inducible Factor 1 Transactivates the Human Leptin Gene Promoter.” *Journal of Biological Chemistry* 277(45): 42953–57.

Gross SR, Sin CG, Barraclough R, Rudland PS. 2014. “Joining S100 Proteins and Migration: For Better or for Worse, in Sickness and in Health.” *Cellular and Molecular Life Sciences* 71(9): 1551–79.

Grotterød I, Maelandsmo GM, Boye K. 2010. “Signal Transduction Mechanisms Involved in S100A4-Induced Activation of the Transcription Factor NF-KB.” *BMC Cancer* 10(241).

Grum-Schwensen B, Klingelhöfer J, Beck M, Bonefeld CM, Hamerlik P, Guldberg P, Grigorian M, Lukanidin E, Ambartsumian N. 2015. “S100A4-Neutralizing Antibody Suppresses Spontaneous Tumor Progression, Pre-Metastatic Niche Formation and Alters T-Cell Polarization Balance.” *BMC Cancer* 15(44).

Gu G, Dubauskaite J, Melton DA. 2002. “Direct Evidence for the Pancreatic Lineage : NGN3 + Cells Are Islet Progenitors and Are Distinct from Duct Progenitors.” *Development* 129(10): 2447–57.

Guarner V, Rubio-Ruiz ME. 2014. “Low-Grade Systemic Inflammation Connects Aging, Metabolic Syndrome and Cardiovascular Disease.” *Aging and Health - A Systems Biology Perspective* 40: 99–106.

Del Guerra S, Lupi R, Marselli L, Masini M, Bugliani M, Sbrana S, Torri S, Pollera M, Boggi U, Mosca F, Del Prato S, Marchetti P. 2005. “Functional and Molecular Defects of Pancreatic Islets in Human Type 2 Diabetes.” *Diabetes* 54(3): 727–35.

Guo Y, Xiao P, Lei S, Deng F, Xiao GG, Liu Y, Chen X, Li L, Wu S, Chen Y, Jiang H, Tan L, Xie J, Zhu X, Liang S, Deng H. 2008. “How Is MRNA Expression Predictive for Protein Expression? A Correlation Study on Human Circulating Monocytes.” *Acta*

Biochimica et Biophysica Sinica 40(5): 426–36.

Haase VH. 2012. “Hypoxia-Inducible Factor Signaling in the Development of Kidney Fibrosis.” *Fibrogenesis Tissue Repair* 5(Suppl 1)(S16).

Hagman DK, Hays LB, Parazzoli SD, Poitout V. 2005. “Palmitate Inhibits Insulin Gene Expression by Altering PDX-1 Nuclear Localization and Reducing MafA Expression in Isolated Rat Islets of Langerhans.” *Journal of Biological Chemistry* 280(37): 32413–18.

Halban PA, Polonsky KS, Bowden DW, Hawkins MA, Ling C, Mather KJ, Powers AC, Rhodes CJ, Sussel L, Weir GC. 2014. “β-Cell Failure in Type 2 Diabetes: Postulated Mechanisms and Prospects for Prevention and Treatment.” *Journal of Clinical Endocrinology and Metabolism* 37(6): 1751–58.

Halban PA, Praz GA, Wollheim CB. 1983. “Abnormal Glucose Metabolism Accompanies Failure of Glucose to Stimulate Insulin Release from a Rat Pancreatic Cell Line (RINm5F).” *The Biochemical journal* 212(2): 439–43.

Hamasaki H. 2016. “Daily Physical Activity and Type 2 Diabetes: A Review.” *World Journal of Diabetes* 7(12): 243–51.

Han JC, Lawlor DA, Kimm SY. 2010. “Childhood Obesity - 2010: Progress and Challenges.” *Lancet* 375(9727): 1737–48.

Han YH, Moon HJ, You BR, Park WH. 2009. “The Effect of MG132, a Proteasome Inhibitor on HeLa Cells in Relation to Cell Growth, Reactive Oxygen Species and GSH.” *Oncology Reports* 22(1): 215–21.

Hanley SC, Austin E, Assouline-Thomas B, Kapeluto J, Blaichman J, Moosavi M. 2010. “β-Cell Mass Dynamics and Islet Cell Plasticity in Human Type 2 Diabetes.” *Endocrinology* 151(4): 1462–72.

Hara T, Mahadevan J, Kanekura K, Hara M, Lu S, Urano F. 2014. “Calcium Efflux from the Endoplasmic Reticulum Leads to β-Cell Death.” *Endocrinology* 155(3): 758–68.

Harja E, Bu DX, Hudson BI, Chang JS, Shen X, Hallam K, Kalea AZ, Lu Y, Rosario RH, Oruganti S, Nikolla Z, Belov D, Lalla E, Ramasamy R, Yan SF, Schmidt AM. 2008. “Vascular and Inflammatory Stresses Mediate Atherosclerosis via RAGE and Its Ligands in ApoE-/- Mice.” *Journal of Clinical Investigation* 118(1): 183–94.

Heil A, Nazmi AR, Koltzscher M, Poeter M, Austermann J, Assard N, Baudier J, Kaibuchi K, Gerke V. 2011. “S100P Is a Novel Interaction Partner and Regulator of IQGAP1.” *Journal of Biological Chemistry* 286(9): 7227–38.

Heit JJ, Apelqvist AA, Gu X, Winslow MM, Neilson JR, Crabtree GR, Kim SK. 2006. “Calcineurin/NFAT Signalling Regulates Pancreatic β-Cell Growth and Function.” *Nature* 443(7109): 345–49.

Heizmann CW, Fritz G, Schäfer BW. 2002. “S100 Proteins: Structure, Functions and Pathology.” *Frontiers in Bioscience* (7): d1356-1368.

Helfman DM, Kim EJ, Lukanidin E, Grigorian M. 2005. “The Metastasis Associated Protein S100A4: Role in Tumour Progression and Metastasis.” *British Journal of Cancer* 92(11): 1955–58.

Hennige AM, Burks DJ, Ozcan U, Kulkarni RN, Ye J, Park S, Schubert M, Fisher TL, Dow MA, Leshan R, Zakaria M, Mossa-Basha M, White MF. 2003. “Upregulation of

Insulin Receptor Substrate-2 in Pancreatic β Cells Prevents Diabetes." *Journal of Clinical Investigation* 112(10): 1521–32.

Herath H, Herath R, Rajitha Wickremasinghe R. 2017. "Gestational Diabetes Mellitus and Risk of Type 2 Diabetes 10 Years after the Index Pregnancy in Sri Lankan Women - A Community Based Retrospective Cohort Study." *PLoS ONE* 12(6): e0179647.

Hernández-Ochoa EO, Prosser BL, Wright NT, Contreras M, Weber DJ, Schneider MF. 2009. "Augmentation of Ca v 1 Channel Current and Action Potential Duration after Uptake of S100A1 in Sympathetic Ganglion Neurons." *American Journal of Physiology-Cell Physiology* 297(4): C955-970.

Hernández JL, Padilla L, Dakhel S, Coll T, Hervas R, Adan J, Masa M, Mitjans F, Martinez JM, Coma S, Rodríguez L, Noé V, Ciudad CJ, Blasco F, Messeguer R. 2013. "Therapeutic Targeting of Tumor Growth and Angiogenesis with a Novel Anti-S100A4 Monoclonal Antibody." *PLoS ONE* 8(9): e72480.

Hiratsuka S, Watanabe A, Aburatani H, Maru Y. 2006. "Tumour-Mediated Upregulation of Chemoattractants and Recruitment of Myeloid Cells Predetermines Lung Metastasis." *Nature Cell Biology* 8(12): 1369–75.

Hiratsuka S, Watanabe A, Sakurai Y, Akashi-Takamura S, Ishibashi S, Miyake K, Shibuya M, Akira S, Aburatani H, Maru Y. 2008. "The S100A8-Serum Amyloid A3-TLR4 Paracrine Cascade Establishes a Pre-Metastatic Phase." *Nature Cell Biology* 10(11): 1349–55.

Hodson L, Humphreys SM, Karpe F, Frayn KN. 2013. "Metabolic Signatures of Human Adipose Tissue Hypoxia in Obesity." *Diabetes* 62(5): 1417–25.

Hoffmann A, Natoli G, Ghosh G. 2006. "Transcriptional Regulation via the NF-KB Signaling Module." *Oncogene* 25(51): 6706–16.

Hofmann Bowman M, Wilk J, Heydemann A, Kim G, Rehman J, Lodato JA, Raman J, McNally EM. 2010. "S100A12 Mediates Aortic Wall Remodeling and Aortic Aneurysm." *Circulation Research* 106(1): 145–54.

Hofmann K, Clauder AK, Manz RA. 2018. "Targeting B Cells and Plasma Cells in Autoimmune Diseases." *Frontiers in Immunology* 9(835).

Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, Avila C, Kambham N, Bierhaus A, Nawroth P, Neurath MF, Slattery T, Beach D, McClary J, Nagashima M, Morser J, Stern D, Schmidt AM. 1999. "RAGE Mediates a Novel Proinflammatory Axis: A Central Cell Surface Receptor for S100/Calgranulin Polypeptides." *Cell* 97(7).

Horiuchi A, Hayashi T, Kikuchi N, Hayashi A, Fuseya C, Shiozawa T, Konishi I. 2012. "Hypoxia Upregulates Ovarian Cancer Invasiveness via the Binding of HIF-1α to a Hypoxia-Induced, Methylation-Free Hypoxia Response Element of S100A4 Gene." *International Journal of Cancer* 131(8): 1755–67.

Hou S, Tian T, Qi D, Sun K, Yuan Q, Wang Z, Qin Z, Wu Z, Chen Z, Zhang J. 2018. "S100A4 Promotes Lung Tumor Development through β-Catenin Pathway-Mediated Autophagy Inhibition Article." *Cell Death and Disease* 9(3): 277.

Howard AA, Arnsten JH, Gourevitch MN. 2004. "Effect of Alcohol Consumption on Diabetes Mellitus: A Systematic Review." *Annals of Internal Medicine* 140(3): 211–19.

Hsieh HL, Schäfer BW, Weigle B, Heizmann CW. 2004. “S100 Protein Translocation in Response to Extracellular S100 Is Mediated by Receptor for Advanced Glycation Endproducts in Human Endothelial Cells.” *Biochemical and Biophysical Research Communications* 316(3): 949–59.

Hsu K, Champaiboon C, Guenther BD, Sorenson BS, Khammanivong A, Ross KF, Geczy CL, Herzberg MC. 2012. “Anti-Infective Protective Properties of S100 Calgranulins.” *Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry* 8(4): 290–305.

Huang CJ, Lin CY, Haataja L, Gurlo T, Butler AE, Rizza RA, Butler PC. 2007. “High Expression Rates of Human Islet Amyloid Polypeptide Induce Endoplasmic Reticulum Stress-Mediated β-Cell Apoptosis, a Characteristic of Humans with Type 2 but Not Type 1 Diabetes.” *Diabetes* 56(8): 2016–27.

Huang JS, Gottschalk M, Norman GJ, Calfas KJ, Sallis JF, Patrick K. 2011. “Compliance with Behavioral Guidelines for Diet, Physical Activity and Sedentary Behaviors Is Related to Insulin Resistance among Overweight and Obese Youth.” *BMC Research Notes* 4(29).

Huang L, Wang HY, Li JD, Wang JH, Zhou Y, Luo RZ, Yun JP, Zhang Y, Jia WH, Zheng M. 2013. “KPNA2 Promotes Cell Proliferation and Tumorigenicity in Epithelial Ovarian Carcinoma through Upregulation of C-Myc and Downregulation of FOXO3a.” *Cell Death and Disease* 4(e745).

Huang Y, Cai X, Mai W, Li M, Hu Y. 2016. “Association between Prediabetes and Risk of Cardiovascular Disease and All Cause Mortality: Systematic Review and Meta-Analysis.” *BMJ (Online)* 355: i5953.

Huang Y, Cai X, Qiu M, Chen P, Tang H, Hu Y, Huang Y. 2014. “Prediabetes and the Risk of Cancer: A Meta-Analysis.” *Diabetologia* 57(11): 2261–69.

Huang Y, Chang Y. 2014. “Regulation of Pancreatic Islet Beta-Cell Mass by Growth Factor and Hormone Signaling.” *Progress in Molecular Biology and Translational Science* 121: 321–49.

Hudish LI, Reusch JE, Sussel L. 2019. “β Cell Dysfunction during Progression of Metabolic Syndrome to Type 2 Diabetes.” *Journal of Clinical Investigation* 129(10): 4001–8.

Hügl SR, White MF, Rhodes CJ. 1998. “Insulin-like Growth Factor I (IGF-I)-Stimulated Pancreatic Beta-Cell Growth Is Glucose-Dependent. Synergistic Activation of Insulin Receptor Substrate-Mediated Signal Transduction Pathways by Glucose and IGF-I in INS-1 Cells.” *Journal of Biological Chemistry* 273(28): 17771–79.

Hur KY, Jung HS, Lee MS. 2010. “Role of Autophagy in β-Cell Function and Mass.” *Diabetes, Obesity and Metabolism* Suppl 2: 20–26.

Huttunen HJ, Kuja-Panula J, Sorci G, Agneletti AL, Donato R, Rauvala H. 2000. “Coregulation of Neurite Outgrowth and Cell Survival by Amphoterin and S100 Proteins through Receptor for Advanced Glycation End Products (RAGE) Activation.” *Journal of Biological Chemistry* 275(51): 40096–105.

Huxford T, Ghosh G. 2009. “A Structural Guide to Proteins of the NF-KappaB Signaling Module.” *Cold Spring Harbor perspectives in biology* 1(3): a000075.

Hwangbo Y, Lee EK. 2017. “Acute Hyperglycemia Associated with Anti-Cancer Medication.” *Endocrinology and Metabolism* 32(1): 23–29.

Ichikawa M, Williams R, Wang L, Vogl T, Srikrishna G. 2011. “S100A8/A9 Activate Key Genes and Pathways in Colon Tumor Progression.” *Molecular Cancer Research* 9(2): 133–48.

Igoillo-Esteve M, Marselli L, Cunha DA, Ladrière L, Ortis F, Grieco FA, Dotta F, Weir GC, Marchetti P, Eizirik DL, Cnop M. 2010. “Palmitate Induces a Pro-Inflammatory Response in Human Pancreatic Islets That Mimics CCL2 Expression by Beta Cells in Type 2 Diabetes.” *Diabetologia* 53(7): 1395–1405.

Illán-Gómez F, Gonzálvez-Ortega M, Orea-Soler I, Alcaraz-Tafalla MS, Aragón-Alonso A, Pascual-Díaz M, Pérez-Paredes M, Lozano-Almela ML. 2012. “Obesity and Inflammation: Change in Adiponectin, C-Reactive Protein, Tumour Necrosis Factor-Alpha and Interleukin-6 after Bariatric Surgery.” *Obesity Surgery* 22(6): 950–55.

Imamura M, Maeda S. 2011. “Genetics of Type 2 Diabetes: The GWAS Era and Future Perspectives [Review].” *Endocrine Journal* 58(9): 723–39.

Inaishi J, Saisho Y, Sato S, Kou K, Murakami R, Watanabe Y. 2016. “Effects of Obesity and Diabetes on α-and β-Cell Mass in Surgically Resected Human Pancreas.” *Journal of Clinical Endocrinology and Metabolism* 101(7): 2874–82.

Ingersoll MA, Spanbroek R, Lottaz C, Gautier EL, Frankenberger M, Hoffmann R, Lang R, Haniffa M, Collin M, Tacke F, Habenicht AJ, Ziegler-Heitbrock L, Randolph GJ. 2009. “Comparison of Gene Expression Profiles between Human and Mouse Monocyte Subsets.” *Blood* 115(3): e10-19.

Ionescu-Tirgoviste C, Gagniuc PA, Gubceac E, Mardare L, Popescu I, Dima S, Militaru M. 2015. “A 3D Map of the Islet Routes throughout the Healthy Human Pancreas.” *Scientific Reports* 5(14634).

Ionita MG, Vink A, Dijke IE, Laman JD, Peeters W, van der Kraak PH, Moll FL, de Vries JP, Pasterkamp G, de Kleijn DP. 2009. “High Levels of Myeloid-Related Protein 14 in Human Atherosclerotic Plaques Correlate with the Characteristics of Rupture-Prone Lesions.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 29(8): 1220–27.

Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H, Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M. 2003. “Free Fatty Acids Regulate Insulin Secretion from Pancreatic β Cells through GPR40.” *Nature* 422(6928): 173–76.

Jabbour S. 2008. “Primary Care Physicians and Insulin Initiation: Multiple Barriers, Lack of Knowledge or Both?” *International Journal of Clinical Practice* 62(6): 845–47.

Jacob L, Freyn M, Kalder M, Dinas K, Kostev K. 2018. “Impact of Tobacco Smoking on the Risk of Developing 25 Different Cancers in the UK: A Retrospective Study of 422,010 Patients Followed for up to 30 Years.” *Oncotarget* 9(25): 17420–29.

Jacqueminet S, Briaud I, Rouault C, Reach G, Poitout V. 2000. “Inhibition of Insulin Gene Expression by Long-Term Exposure of Pancreatic β Cells to Palmitate Is Dependent on the Presence of a Stimulatory Glucose Concentration.” *Metabolism: Clinical and Experimental* 49(9): 532–36.

Jang MK, Son Y, Jung MH. 2013. “ATF3 Plays a Role in Adipocyte Hypoxia-Mediated Mitochondria Dysfunction in Obesity.” *Biochemical and Biophysical Research Communications* 431(3): 421–27.

Jarad G, Miner JH. 2009. “Update on the Glomerular Filtration Barrier.” *Current Opinion in Nephrology and Hypertension* 18(3): 226–32.

Jelinek HF, Osman WM, Khandoker AH, Khalaf K, Lee S, Almahmeed W, Alsafar HS. 2017. “Clinical Profiles, Comorbidities and Complications of Type 2 Diabetes Mellitus in Patients from United Arab Emirates.” *BMJ Open Diabetes Research and Care* 5(1): e000427.

Jenny M, Uhl C, Roche C, Duluc I, Guillermin V, Guillemot F, Jensen J, Kedinger M, Gradwohl G. 2002. “Neurogenin3 Is Differentially Required for Endocrine Cell Fate Specification in the Intestinal and Gastric Epithelium.” *EMBO Journal* 21(23): 6338–47.

Ježek P, Jabůrek M, Holendová B, Plecitá-Hlavatá L. 2018. “Fatty Acid-Stimulated Insulin Secretion vs. Lipotoxicity.” *Molecules* 23(6): 1483.

Jiang C, Qu A, Matsubara T, Chanturiya T, Jou W, Gavrilova O, Shah YM, Gonzalez FJ. 2011. “Disruption of Hypoxia-Inducible Factor 1 in Adipocytes Improves Insulin Sensitivity and Decreases Adiposity in High-Fat Diet-Fed Mice.” *Diabetes* 60(10): 2484–95.

Jin K, Li T, van Dam H, Zhou F, Zhang L. 2017. “Molecular Insights into Tumour Metastasis: Tracing the Dominant Events.” *Journal of Pathology* 241(5): 567–77.

Jin Q, Chen H, Luo A, Ding F, Liu Z. 2011. “S100A14 Stimulates Cell Proliferation and Induces Cell Apoptosis at Different Concentrations via Receptor for Advanced Glycation End Products (RAGE).” *PLoS ONE* 6(4): e19375.

Jo Y, Okazaki H, Moon YA, Zhao T. 2016. “Regulation of Lipid Metabolism and Beyond.” *International Journal of Endocrinology* 5415767.

Johnson AR, Milner JJ, Makowski L. 2012. “The Inflammation Highway: Metabolism Accelerates Inflammatory Traffic in Obesity.” *Immunological Reviews* 249(1): 218–38.

Jonas JC, Bensellam M, Duprez J, Elouil H, Guiot Y, Pascal SM. 2009. “Glucose Regulation of Islet Stress Responses and β-Cell Failure in Type 2 Diabetes.” *Diabetes, Obesity and Metabolism* 11(Suppl 4): 65–81.

Jung UJ, Choi MS. 2014. “Obesity and Its Metabolic Complications: The Role of Adipokines and the Relationship between Obesity, Inflammation, Insulin Resistance, Dyslipidemia and Nonalcoholic Fatty Liver Disease.” *International Journal of Molecular Sciences* 15(4): 6184–6223.

Jurgens CA, Toukatly MN, Fligner CL, Udayasankar J, Subramanian SL, Zraika S, Aston-Mourney K, Carr DB, Westermark P, Westermark GT, Kahn SE, Hull RL. 2011. “β-Cell Loss and β-Cell Apoptosis in Human Type 2 Diabetes Are Related to Islet Amyloid Deposition.” *American Journal of Pathology* 178(6): 2632–40.

Kahan S, Manson JE. 2017. “Nutrition Counseling in Clinical Practice: How Clinicians Can Do Better.” *JAMA - Journal of the American Medical Association* 318(12): 1101–2.

Kahanovitz L, Sluss PM, Russell SJ. 2017. “Type 1 Diabetes-a Clinical Perspective.” *Point of Care* 16(1): 37–40.

Kahn SE, D’Alessio DA, Schwartz MW, Fujimoto WY, Ensinck JW, Taborsky GJ Jr, Porte D Jr. 1990. “Evidence of Cosecretion of Islet Amyloid Polypeptide and Insulin by β-

Cells." *Diabetes* 39(5): 634–38.

Kahn SE, Hull RL, Utzschneider KM. 2006. "Mechanisms Linking Obesity to Insulin Resistance and Type 2 Diabetes." *Nature* 444(7121): 840–46.

Kahn SE, Prigeon RL, McCulloch DK, Boyko EJ, Bergman RN, Schwartz MW. 1993. "Quantification of the Relationship between Insulin Sensitivity and β- Cell Function in Human Subjects: Evidence for a Hyperbolic Function." *Diabetes* 42(11): 1663–72.

Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. 1979. "Establishment and Characterization of a Human Prostatic Carcinoma Cell Line (PC-3)." *Investigative Urology* 17(1): 16–23.

Kalluri R, Weinberg RA. 2009. "The Basics of Epithelial-Mesenchymal Transition." *Journal of Clinical Investigation* 119(6): 1420–28.

Karunakaran U, Park KG. 2013. "A Systematic Review of Oxidative Stress and Safety of Antioxidants in Diabetes: Focus on Islets and Their Defense." *Diabetes and Metabolism Journal* 37(2): 106–12.

Kastorini CM, Panagiotakos DB. 2009. "Dietary Patterns and Prevention of Type 2 Diabetes: From Research to Clinical Practice; A Systematic Review." *Current Diabetes Reviews* 5(4): 221–27.

Keirsebilck A, Bonné S, Bruyneel E, Vermassen P, Lukanidin E, Mareel M, van Roy F. 1998. "E-Cadherin and Metastasin (Mts-1/S100A4) Expression Levels Are Inversely Regulated in Two Tumor Cell Families." *Cancer Research* 58(20): 4587–91.

Kelley JB, Talley AM, Spencer A, Gioeli D, Paschal BM. 2010. "Karyopherin A7 (KPNA7), a Divergent Member of the Importin α Family of Nuclear Import Receptors." *BMC Cell Biology* 11(63).

Kessel C, Holzinger D, Foell D. 2013. "Phagocyte-Derived S100 Proteins in Autoinflammation: Putative Role in Pathogenesis and Usefulness as Biomarkers." *Clinical Immunology* 147(3): 229–41.

Kharroubi I, Ladrière L, Cardozo AK, Dogusan Z, Cnop M, Eizirik DL. 2004. "Free Fatty Acids and Cytokines Induce Pancreatic β-Cell Apoptosis by Different Mechanisms: Role of Nuclear Factor-KB and Endoplasmic Reticulum Stress." *Endocrinology* 145(11): 5087–5096.

Kim H, Lee YD, Kim MK, Kwon JO, Song MK, Lee ZH, Kim HH. 2017. "Extracellular S100A4 Negatively Regulates Osteoblast Function by Activating the NF-KB Pathway." *BMB Reports* 50(2): 97–102.

Kim HI, Lim H, Moon A. 2018. "Sex Differences in Cancer: Epidemiology, Genetics and Therapy." *Biomolecules and Therapeutics* 26(4): 335–42.

Kim IS, Kim DH, Han SM, Chin MU, Nam HJ, Cho HP, Choi SY, Song BJ, Kim ER, Bae YS, Moon YH. 2000. "Truncated Form of Importin α Identified in Breast Cancer Cell Inhibits Nuclear Import of P53." *Journal of Biological Chemistry* 275(30): 23139–45.

Kim SH, Reaven GM. 2008. "Insulin Resistance and Hyperinsulinemia: You Can't Have One without the Other." *Diabetes Care* 31(7): 1433–38.

Kim WH, Lee JW, Suh YH, Hong SH, Choi JS, Lim JH, Song JH, Gao B, Jung MH. 2005. "Exposure to Chronic High Glucose Induces β-Cell Apoptosis through Decreased Interaction of Glucokinase with Mitochondria: Downregulation of Glucokinase in

Pancreatic β-Cells." *Diabetes* 54(9): 2602–11.

Kiryushko D, Novitskaya V, Soroka V, Klingelhofer J, Lukanidin E, Berezin V, Bock E. 2006. "Molecular Mechanisms of Ca2+ Signaling in Neurons Induced by the S100A4 Protein." *Molecular and Cellular Biology* 26(9): 3625–38.

Kiss B, Duelli A, Radnai L, Kékesi KA, Katona G, Nyitray L. 2012. "Crystal Structure of the S100A4-Nonmuscle Myosin IIA Tail Fragment Complex Reveals an Asymmetric Target Binding Mechanism." *Proceedings of the National Academy of Sciences* 109(16): 6048–53.

Kitamura T, Kometani K, Hashida H, Matsunaga A, Miyoshi H, Hosogi H, Aoki M, Oshima M, Hattori M, Takabayashi A, Minato N, Taketo MM. 2007. "SMAD4-Deficient Intestinal Tumors Recruit CCR1+ Myeloid Cells That Promote Invasion." *Nature Genetics* 39(4): 467–75.

Kizawa K, Takahara H, Troxler H, Kleinert P, Mochida U, Heizmann CW. 2008. "Specific Citrullination Causes Assembly of a Globular S100A3 Homotetramer: A Putative Ca2+ Modulator Matures Human Hair Cuticle." *Journal of Biological Chemistry* 283(3): 5004–13.

Klingelhöfer J, Grum-Schwensen B, Beck MK, Knudsen RS, Grigorian M, Lukanidin E, Ambartsumian N. 2012. "Anti-S100A4 Antibody Suppresses Metastasis Formation by Blocking Stroma Cell Invasion." *Neoplasia (United States)* 14(12): 1260–68.

Klingelhöfer J, Senolt L, Baslund B, Nielsen GH, Skibshøj I, Pavelka K, Neidhart M, Gay S, Ambartsumian N, Hansen BS, Petersen J, Lukanidin E, Grigorian M. 2007. "Up-Regulation of Metastasis-Promoting S100A4 (Mts-1) in Rheumatoid Arthritis: Putative Involvement in the Pathogenesis of Rheumatoid Arthritis." *Arthritis and Rheumatism* 56(3): 779–89.

Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM. 2002. "Reduction in the Incidence of Type 2 Diabetes with Lifestyle Intervention or Metformin." *The New England Journal of Medicine* 346(6): 393–403.

Kohya N, Kitajima Y, Jiao W, Miyazaki K. 2003. "Effects of E-Cadherin Transfection on Gene Expression of a Gallbladder Carcinoma Cell Line: Repression of MTS1/S100A4 Gene Expression." *International Journal of Cancer* 104(1): 44–53.

Komada T, Araki R, Nakatani K, Yada I, Naka M, Tanaka T. 1996. "Novel Specific Chemotactic Receptor for S100L Protein on Guinea Pig Eosinophils." *Biochemical and Biophysical Research Communications* 220(3): 871–74.

König M, Rharbaoui F, Aigner S, Dälken B, Schüttrumpf J. 2016. "Tregalizumab – A Monoclonal Antibody to Target Regulatory T Cells." *Frontiers in Immunology* 7(11).

Konikoff MR, Denson LA. 2006. "Role of Fecal Calprotectin as a Biomarker of Intestinal Inflammation in Inflammatory Bowel Disease." *Inflammatory Bowel Diseases* 12(6): 524–34.

Koro C, Barrett S, Qizilbash N. 2007. "Cancer Risks in Thiazolidinedione Users Compared to Other Anti-Diabetic Agents." *Pharmacoepidemiology and Drug Safety* 16(5): 485–92.

Krzywinska E, Stockmann C. 2018. "Hypoxia, Metabolism and Immune Cell Function." *Biomedicines* 6(2): pii: E56.

Ku GM, Kim H, Vaughn IW, Hangauer MJ, Myung Oh C, German MS, McManus MT. 2012. "Research Resource: RNA-Seq Reveals Unique Features of the Pancreatic β-Cell Transcriptome." *Molecular Endocrinology* 26(10): 1783–92.

Kubo T, Maezawa N, Osada M, Katsumura S, Funae Y, Imaoka S. 2004. "Bisphenol A, an Environmental Endocrine-Disrupting Chemical, Inhibits Hypoxic Response via Degradation of Hypoxia-Inducible Factor 1α (HIF-1α): Structural Requirement of Bisphenol A for Degradation of HIF-1α." *Biochemical and Biophysical Research Communications* 318(4): 1006–11.

Kubota N, Tobe K, Terauchi Y, Eto K, Yamauchi T, Suzuki R, Tsubamoto Y, Komeda K, Nakano R, Miki H, Satoh S, Sekihara H, Sciacchitano S, Lesniak M, Aizawa S, Nagai R, Kimura S, Akanuma Y, Taylor SI, Kadowaki T. 2000. "Disruption of Insulin Receptor Substrate 2 Causes Type 2 Diabetes Because of Liver Insulin Resistance and Lack of Compensatory β-Cell Hyperplasia." *Diabetes* 49(11): 1880–89.

Kushner JA1, Ye J, Schubert M, Burks DJ, Dow MA, Flint CL, Dutta S, Wright CV, Montminy MR, White MF. 2002. "Pdx1 Restores β Cell Function in Irs2 Knockout Mice." *Journal of Clinical Investigation* 109(9): 1193–1201.

Lackey DE, Olefsky JM. 2016. "Regulation of Metabolism by the Innate Immune System." *Nature Reviews Endocrinology* 12(1): 15–28.

Laforest S, Labrecque J, Michaud A, Cianflone K, Tchernof A. 2015. "Adipocyte Size as a Determinant of Metabolic Disease and Adipose Tissue Dysfunction." *Critical Reviews in Clinical Laboratory Sciences* 52(6): 301–13.

Lagasse E, Weissman IL. 1992. "Mouse MRP8 and MRP14, Two Intracellular Calcium-Binding Proteins Associated with the Development of the Myeloid Lineage." *Blood* 79(8): 1907–15.

Lall, Kristi et al. 2016. "Personalized Risk Prediction for Type 2 Diabetes: The Potential of Genetic Risk Scores." *Personalized risk prediction for type 2 diabetes: The potential of genetic risk scores* 19(3): 322–29.

Landman GW, Kleefstra N, van Hateren KJ, Groenier KH, Gans RO, Bilo HJ. 2010. "Metformin Associated with Lower Cancer Mortality in Type 2 Diabetes: Zodiac-16." *Diabetes Care* 33(2): 322–26.

Landriscina M, Soldi R, Bagalá C, Micucci I, Bellum S, Tarantini F, Prudovsky I, Maciag T. 2001. "S100A13 Participates in the Release of Fibroblast Growth Factor 1 in Response to Heat Shock in Vitro." *Journal of Biological Chemistry* 276(25): 22544–52.

Laplante M, Sabatini DM. 2012. "MTOR Signaling in Growth Control and Disease." *Cell* 149(2): 274–93.

Leclerc E, Fritz G, Vetter SW, Heizmann CW. 2009. "Binding of S100 Proteins to RAGE: An Update." *Biochimica et Biophysica Acta - Molecular Cell Research* 1793(6): 993–1007.

Lee AH, Iwakoshi NN, Glimcher LH. 2003. "XBP-1 Regulates a Subset of Endoplasmic Reticulum Resident Chaperone Genes in the Unfolded Protein Response." *Molecular and cellular biology* 23(21): 7448–59.

Lee BC, Lee J. 2014. "Cellular and Molecular Players in Adipose Tissue Inflammation in the Development of Obesity-Induced Insulin Resistance." *Biochimica et Biophysica*

Acta - Molecular Basis of Disease 1842(3): 446–62.

Lee MS. 2014. "Role of Islet β Cell Autophagy in the Pathogenesis of Diabetes." *Trends in Endocrinology and Metabolism* 25(12): 620–27.

Lee R, Wong TY, Sabanayagam C. 2015. "Epidemiology of Diabetic Retinopathy, Diabetic Macular Edema and Related Vision Loss." *Eye and Vision* 2(17): 1–25.

Lee YT, Dimitrova YN, Schneider G, Ridenour WB, Bhattacharya S, Soss SE, Caprioli RM, Filipek A, Chazin WJ. 2008. "Structure of the S100A6 Complex with a Fragment from the C-Terminal Domain of Siah-1 Interacting Protein: A Novel Mode for S100 Protein Target Recognition." *Biochemistry* 47(41): 10921–32.

van Leiden HA, Dekker JM, Moll AC, Nijpels G, Heine RJ, Bouter LM, Stehouwer CD, Polak BC. 2002. "Blood Pressure, Lipids, and Obesity Are Associated with Retinopathy: The Hoorn Study." *Diabetes Care* 25(8): 1320–25.

Lenarčič Živković M, Zaręba-Kozioł M, Zhukova L, Poznański J, Zhukov I, Wysłouch-Cieszyńska A. 2012. "Post-Translational S-Nitrosylation Is an Endogenous Factor Fine Tuning the Properties of Human S100A1 Protein." *Journal of Biological Chemistry* 287(48): 40457–70.

LeRoith D. 2002. "β-Cell Dysfunction and Insulin Resistance in Type 2 Diabetes: Role of Metabolic and Genetic Abnormalities." *The American Journal of Medicine* 113(Suppl 6A): S3-11.

Leśniak W, Słomnicki ŁP, Filipek A. 2009. "S100A6 - New Facts and Features." *Biochemical and Biophysical Research Communications* 390(4): 1087–92.

Levine B, Kroemer G. 2008. "Autophagy in the Pathogenesis of Disease." *Cell* 132(1): 27–42.

Li L, Yin X, Ma N, Lin F, Kong X, Chi J, Feng Z. 2014. "Desferrioxamine Regulates HIF-1 Alpha Expression in Neonatal Rat Brain after Hypoxia-Ischemia." *American Journal of Translational Research* 6(4): 377–83.

Li Y, Cao X, Li LX, Brubaker PL, Edlund H, Drucker DJ. 2005. "β-Cell Pdx1 Expression Is Essential for the Glucoregulatory, Proliferative, and Cytoprotective Actions of Glucagon-like Peptide-1." *Diabetes* 54(2): 482–91.

Li ZH, Dulyaninova NG, House RP, Almo SC, Bresnick AR. 2010. "S100A4 Regulates Macrophage Chemotaxis." *Molecular Biology of the Cell* 21(5): 2598–2610.

Liang DP, Huang TQ, Li SJ, Chen ZJ. 2014. "Knockdown of S100A4 Chemosensitizes Human Laryngeal Carcinoma Cells in Vitro through Inhibition of Slug." *European Review for Medical and Pharmacological Sciences* 18(22): 3484–90.

Liberti MV, Locasale JW. 2016. "The Warburg Effect: How Does It Benefit Cancer Cells?" *Trends in Biochemical Sciences* 41(3): 211–18.

Licatalosi DD, Darnell RB. 2010. "RNA Processing and Its Regulation: Global Insights into Biological Networks." *Nature Reviews Genetics* 11(1): 75–87.

Liemburg-Apers DC, Willems PH, Koopman WJ, Grefte S. 2015. "Interactions between Mitochondrial Reactive Oxygen Species and Cellular Glucose Metabolism." *Archives of Toxicology* 89(8): 1209–26.

Lim SY, Raftery M, Cai H, Hsu K, Yan WX, Hseih HL, Watts RN, Richardson D, Thomas S,

Perry M, Geczy CL. 2014. “S-Nitrosylated S100A8: Novel Anti-Inflammatory Properties.” *The Journal of Immunology* 181(8): 5627–36.

Lim SY, Raftery MJ, Geczy CL. 2010. “Oxidative Modifications of DAMPs Suppress Inflammation: The Case for S100A8 and S100A9.” *Antioxidants & Redox Signaling* 15(8): 2235–48.

Lima VV, Spitler K, Choi H, Webb RC, Tostes RC. 2012. “O-GlcNAcylation and Oxidation of Proteins: Is Signalling in the Cardiovascular System Becoming Sweeter?” *Clinical Science* 123(8): 473–86.

Lin F, Gao L, Su Z, Cao X, Zhan Y, Li Y, Zhang B. 2018. “Knockdown of KPNA2 Inhibits Autophagy in Oral Squamous Cell Carcinoma Cell Lines by Blocking P53 Nuclear Translocation.” *Oncology Reports* 40(1): 179–94.

Lindberg J, Lundeberg J. 2010. “The Plasticity of the Mammalian Transcriptome.” *Genomics* 95(1): 1–6.

Lindsey JC, Lusher ME, Anderton JA, Gilbertson RJ, Ellison DW, Clifford SC. 2007. “Epigenetic Deregulation of Multiple S100 Gene Family Members by Differential Hypomethylation and Hypermethylation Events in Medulloblastoma.” *British Journal of Cancer* 97(2): 267–74.

Lingohr MK, Briaud I, Dickson LM, McCuaig JF, Alárcon C, Wicksteed BL, Rhodes CJ. 2006. “Specific Regulation of IRS-2 Expression by Glucose in Rat Primary Pancreatic Islet β-Cells.” *Journal of Biological Chemistry* 281(23): 15884–14892.

Lipscombe LL, Goodwin PJ, Zinman B, McLaughlin JR, Hux JE. 2008. “The Impact of Diabetes on Survival Following Breast Cancer.” *Breast Cancer Research and Treatment* 109(2): 389–95.

Lipson KL, Fonseca SG, Ishigaki S, Nguyen LX, Foss E, Bortell R, Rossini AA, Urano F. 2006. “Regulation of Insulin Biosynthesis in Pancreatic Beta Cells by an Endoplasmic Reticulum-Resident Protein Kinase IRE1.” *Cell Metabolism* 4(3): 245–54.

Liu AY, Silvestre MP, Poppitt SD. 2015. “Prevention of Type 2 Diabetes through Lifestyle Modification: Is There a Role for Higher-Protein Diets?” *Advances in Nutrition: An International Review Journal* 6(6): 665–73.

Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE, Thor AD. 2009. “Metformin Induces Unique Biological and Molecular Responses in Triple Negative Breast Cancer Cells.” *Cell Cycle* 8(13): 2031–40.

Liu B, Sun WY, Zhi CY, Lu TC, Gao HM, Zhou JH, Yan WQ, Gao HC. 2013. “Role of S100A3 in Human Colorectal Cancer and the Anticancer Effect of Cantharidinate.” *Experimental and Therapeutic Medicine* 6(6): 1499–1503.

Liu J, Guo Y, Fu S, Yang M, Sun KL, Fu WN. 2010. “Hypomethylation-Induced Expression of S100A4 Increases the Invasiveness of Laryngeal Squamous Cell Carcinoma.” *Oncology Reports* 23(4): 1101–7.

Liu J, Wang H, Zhang L, Xu Y, Deng W, Zhu H, Qin C. 2011. “S100B Transgenic Mice Develop Features of Parkinson’s Disease.” *Archives of Medical Research* 42(1): 1–7.

Liu J, Xu ZM, Qiu GB, Zheng ZH, Sun KL, Fu WN. 2014. “S100A4 Is Upregulated via the Binding of C-Myb in Methylation-Free Laryngeal Cancer Cells.” *Oncology Reports* 31(1): 442–49.

Liu T, Li Y, Lin K, Yin H, He B, Zheng M, Wang G. 2012. “Regulation of S100A4 Expression via the JAK2-STAT3 Pathway in Rhomboid-Phenotype Pulmonary Arterial Smooth Muscle Cells Exposure to Hypoxia.” *International Journal of Biochemistry and Cell Biology* 44(8): 1337–45.

Liu W, Shen SM, Zhao XY, Chen GQ. 2012. “Targeted Genes and Interacting Proteins of Hypoxia Inducible Factor-1.” *International Journal of Biochemistry and Molecular Biology* 3(2): 165–78.

Liu Y, Zhang R, Xin J, Sun Y, Li J, Wei D, Zhao AZ. 2011. “Identification of S100A16 as a Novel Adipogenesis Promoting Factor in 3T3-L1 Cells.” *Endocrinology* 152(3): 903–11.

Lorenzo A, Razzaboni B, Weir GC, Yankner BA. 1994. “Pancreatic Islet Cell Toxicity of Amylin Associated with Type-2 Diabetes Mellitus.” *Nature* 368(6473): 756–60.

Lorenzo O, Picatoste B, Ares-Carrasco S, Ramírez E, Egido J, Tuñón J. 2011. “Potential Role of Nuclear Factor Kb in Diabetic Cardiomyopathy.” *Mediators of Inflammation* (2011): 652097.

Low Wang CC, Hess CN, Hiatt WR, Goldfine AB. 2016. “Clinical Update: Cardiovascular Disease in Diabetes Mellitus: Atherosclerotic Cardiovascular Disease and Heart Failure in Type 2 Diabetes Mellitus – Mechanisms, Management, and Clinical Considerations.” *Circulation* 133(24): 2459–2502.

Low Wang, Cecilia C, Connie N Hess, William R Hiatt, and Allison B Goldfine. 2016. “Clinical Update: Cardiovascular Disease in Diabetes Mellitus: Atherosclerotic Cardiovascular Disease and Heart Failure in Type 2 Diabetes Mellitus - Mechanisms, Management, and Clinical Considerations.” *Circulation* 133(24): 2459–2502.

Lu J, Esposito G, Scuderi C, Steardo L, Delli-Bovi LC, Hecht JL, Dickinson BC, Chang CJ, Mori T, Sheen V. 2011. “S100B and APP Promote a Gliocentric Shift and Impaired Neurogenesis in down Syndrome Neural Progenitors.” *PLoS ONE* 6(7): e22126.

Luu KC, Nie GY, Salamonsen LA. 2004. “Endometrial Calbindins Are Critical for Embryo Implantation: Evidence from in Vivo Use of Morpholino Antisense Oligonucleotides.” *Proceedings of the National Academy of Sciences* 101(21): 8028–33.

Ma J, Li H, Giovannucci E, Mucci L, Qiu W, Nguyen PL, Gaziano JM, Pollak M, Stampfer MJ. 2008. “Prediagnostic Body-Mass Index, Plasma C-Peptide Concentration, and Prostate Cancer-Specific Mortality in Men with Prostate Cancer: A Long-Term Survival Analysis.” *The Lancet Oncology* 9(11): 1039–47.

Ma ZA, Zhao Z, Turk J. 2012. “Mitochondrial Dysfunction and β-Cell Failure in Type 2 Diabetes Mellitus.” *Experimental Diabetes Research* 2012: 703538.

Mackay F, Tangye SG. 2004. “The Role of the BAFF/APRIL System in B Cell Homeostasis and Lymphoid Cancers.” *Current Opinion in Pharmacology* 4(4): 347–54.

Madureira PA, O’Connell PA, Surette AP, Miller VA, Waisman DM. 2012. “The Biochemistry and Regulation of S100A10: A Multifunctional Plasminogen Receptor Involved in Oncogenesis.” *Journal of Biomedicine and Biotechnology* 2012(353687).

Maedler K, Schumann DM, Schulthess F, Oberholzer J, Bosco D, Berney T, Donath MY. 2006. “Aging Correlates with Decreased β-Cell Proliferative Capacity and Enhanced

Sensitivity to Apoptosis: A Potential Role for Fas and Pancreatic Duodenal Homeobox-1." *Diabetes* 55(9): 2455–62.

Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY. 2002. "Glucose-Induced β Cell Production of IL-1β Contributes to Glucotoxicity in Human Pancreatic Islets." *Journal of Clinical Investigation* 110(6): 851–60.

Maedler K, Spinas GA, Lehmann R, Sergeev P, Weber M, Fontana A, Kaiser N, Donath MY. 2001. "Glucose Induces β-Cell Apoptosis Via Upregulation of the Fas Receptor in Human Islets." *Diabetes* 50(8): 1683–90.

Maelandsmo GM, Hovig E, Skrede M, Engebraaten O, Flørenes VA, Myklebost O, Grigorian M, Lukanidin E, Scanlon KJ, Fodstad O. 1996. "Reversal of the in Vivo Metastatic Phenotype of Human Tumor Cells by an Anti-CAPL (Mts1) Ribozyme." *Cancer Research* 56(23): 5490–98.

de Magalhães JP. 2013. "How Ageing Processes Influence Cancer." *Nature Reviews Cancer* 13(5): 357–65.

Maijer KI, Noort AR, de Hair MJ, van der Leij C, van Zoest KP, Choi IY, Gerlag DM, Maas M, Tak PP, Tas SW. 2015. "Nuclear Factor-KB-Inducing Kinase Is Expressed in Synovial Endothelial Cells in Patients with Early Arthritis and Correlates with Markers of Inflammation: A Prospective Cohort Study." *Journal of Rheumatology* 42(9): 1573–81.

Malashkevich VN, Varney KM, Garrett SC, Wilder PT, Knight D, Charpentier TH, Ramagopal UA, Almo SC, Weber DJ, Bresnick AR. 2008. "Structure of Ca2+-Bound S100A4 and Its Interaction with Peptides Derived from Nonmuscle Myosin-IIA." *Biochemistry* 47(18): 5111–26.

Malone JH, Oliver B. 2011. "Microarrays, Deep Sequencing and the True Measure of the Transcriptome." *BMC Biology* 9(34).

Marchetti P, Bugliani M, Lupi R, Marselli L, Masini M, Boggi U, Filipponi F, Weir GC, Eizirik DL, Cnop M. 2007. "The Endoplasmic Reticulum in Pancreatic Beta Cells of Type 2 Diabetes Patients." *Diabetologia* 50(12): 2486–94.

Marenholz I, Heizmann CW, Fritz G. 2004. "S100 Proteins in Mouse and Man: From Evolution to Function and Pathology (Including an Update of the Nomenclature)." *Biochemical and Biophysical Research Communications* 322(4): 1111–22.

Marmugi A, Parnis J, Chen X, Carmichael L, Hardy J, Mannan N, Marchetti P, Piemonti L, Bosco D, Johnson P, Shapiro JA, Cruciani-Guglielmacci C, Magnan C, Ibberson M, Thorens B, Valdivia HH, Rutter GA, Leclerc I. 2016. "Sorcin Links Pancreatic β-Cell Lipotoxicity to ER Ca2+ Stores." *Diabetes* 65(4): 1009–21.

Martel A, Antony L, Gerelli Y, Porcar L, Fluitt A, Hoffmann K, Kiesel I, Vivaudou M, Fragneto G, de Pablo JJ. 2017. "Membrane Permeation versus Amyloidogenicity: A Multitechnique Study of Islet Amyloid Polypeptide Interaction with Model Membranes." *Journal of the American Chemical Society* 139(1): 137–48.

Martino L, Masini M, Novelli M, Beffy P, Bugliani M, Marselli L, Masiello P, Marchetti P, De Tata V. 2012. "Palmitate Activates Autophagy in INS-1E β-Cells and in Isolated Rat and Human Pancreatic Islets." *PLoS ONE* 7(5): e36188.

Marxsen JH, Stengel P, Doege K, Heikkinen P, Jokilehto T, Wagner T, Jelkmann W,

Jaakkola P, Metzen E. 2004. “Hypoxia-Inducible Factor-1 (HIF-1) Promotes Its Degradation by Induction of HIF-α-Prolyl-4-Hydroxylases.” *Biochemical Journal* 381(Pt.3): 761–67.

Mason TM, Goh T, Tchipashvili V, Sandhu H, Gupta N, Lewis GF, Giacca A. 1999. “Prolonged Elevation of Plasma Free Fatty Acids Desensitizes the Insulin Secretory Response to Glucose in Vivo in Rats.” *Diabetes* 48(3): 524–30.

Matsuura I, Lai CY, Chiang KN. 2010. “Functional Interaction between Smad3 and S100A4 (Metastatin-1) for TGF-β-Mediated Cancer Cell Invasiveness.” *Biochemical Journal* 426(3): 327–35.

Mayfield JA, White RD. 2004. “Insulin Therapy for Type 2 Diabetes: Rescue, Augmentation, and Replacement of Beta-Cell Function.” *American Family Physician* 70(3): 489–500.

Miao EA, Rajan JV, Aderem A. 2011. “Caspase-1-Induced Pyroptotic Cell Death.” *Immunological Reviews* 243(1): 206–14.

Miao S, Qiu T, Zhao Y, Wang H, Sun X, Wang Y, Xuan Y, Qin Y, Jiao W. 2018. “Overexpression of S100A13 Protein Is Associated with Tumor Angiogenesis and Poor Survival in Patients with Early-Stage Non-Small Cell Lung Cancer.” *Thoracic Cancer* 9(9): 1136–44.

Miller P, Kidwell KM, Thomas D, Sabel M, Rae JM, Hayes DF, Hudson BI, El-Ashry D, Lippman ME. 2017. “Elevated S100A8 Protein Expression in Breast Cancer Cells and Breast Tumor Stroma Is Prognostic of Poor Disease Outcome.” *Breast Cancer Research and Treatment* 166(1): 85–94.

Miranda KJ, Loeser RF, Yammani RR. 2010. “Sumoylation and Nuclear Translocation of S100A4 Regulate IL-1β-Mediated Production of Matrix Metalloproteinase-13.” *Journal of Biological Chemistry* 285(41): 31517–24.

Monami M, Dicembrini I, Mannucci E. 2014. “Thiazolidinediones and Cancer: Results of a Meta-Analysis of Randomized Clinical Trials.” *Acta Diabetologica* 51(1): 91–101.

Monami M, Lamanna C, Balzi D, Marchionni N, Mannucci E. 2009. “Sulphonylureas and Cancer: A Case-Control Study.” *Acta Diabetologica* 46(4): 279–84.

Morán I, Akerman I, van de Bunt M, Xie R, Benazra M, Nammo T, Arnes L, Nakić N, García-Hurtado J, Rodríguez-Seguí S, Pasquali L, Sauty-Colace C, Beucher A, Scharfmann R, van Arensbergen J, Johnson PR, Berry A, Lee C, Harkins T, Gmyr V, Pattou F, Kerr-Cont, Ferrer J. 2012. “Human β Cell Transcriptome Analysis Uncovers LncRNAs That Are Tissue-Specific, Dynamically Regulated, and Abnormally Expressed in Type 2 Diabetes.” *Cell Metabolism* 16(4): 435–48.

Mori T, Koyama N, Arendash GW, Horikoshi-Sakuraba Y, Tan J, Town T. 2010. “Overexpression of Human S100B Exacerbates Cerebral Amyloidosis and Gliosis in the Tg2576 Mouse Model of Alzheimer’s Disease.” *GLIA* 58(3): 300–314.

Morris DH, Khunti K, Achana F, Srinivasan B, Gray LJ, Davies MJ, Webb D. 2013. “Progression Rates from HbA1c6.0-6.4% and Other Prediabetes Definitions to Type 2 Diabetes: A Meta-Analysis.” *Diabetologia* 56(7): 1489–93.

Mortensen OH, Nielsen AR, Erikstrup C, Plomgaard P, Fischer CP, Krogh-Madsen R, Lindegaard B, Petersen AM, Taudorf S, Pedersen BK. 2009. “Calprotectin - A Novel Marker of Obesity.” *PLoS ONE* 4(10): e7419.

Morton GJ. 2007. “Hypothalamic Leptin Regulation of Energy Homeostasis and Glucose Metabolism.” *Journal of Physiology* 583(Pt.2): 437–43.

Movassat J, Saulnier C, Portha B. 1997. “Insulin Administration Enhances Growth of the β-Cell Mass in Streptozotocin-Treated Newborn Rats.” *Diabetes* 46(9): 1445–52.

Mueller A, Bächi T, Höchli M, Schäfer BW, Heizmann CW. 1999. “Subcellular Distribution of S100 Proteins in Tumor Cells and Their Relocation in Response to Calcium Activation.” *Histochemistry and Cell Biology* 111(6): 453–59.

Mueller A, Schäfer BW, Ferrari S, Weibel M, Makek M, Höchli M, Heizmann CW. 2005. “The Calcium-Binding Protein S100A2 Interacts with P53 and Modulates Its Transcriptional Activity.” *Journal of Biological Chemistry* 280(32): 29186–93.

Murea M, Ma L, Freedman BI. 2012. “Genetic and Environmental Factors Associated with Type 2 Diabetes and Diabetic Vascular Complications.” *Review of Diabetic Studies* 9(1): 6–22.

Murzik U, Hemmerich P, Weidtkamp-Peters S, Ulbricht T, Bussen W, Hentschel J, von Eggeling F, Melle C. 2008. “Rad54B Targeting to DNA Double-Strand Break Repair Sites Requires Complex Formation with S100A11.” *Molecular Biology of the Cell* 19(7): 2926–35.

Narres M, Kvitkina T, Claessen H, Droste S, Morbach S, Kuss O, Icks A. 2015. “Incidence of Lower Extremity Amputation in the Diabetic Compared to the Non-Diabetic Population: A Systematic Review Protocol.” *Systematic Reviews* 12(8): e0182081.

Nather A, Bee CS, Huak CY, Chew JL, Lin CB, Neo S, Sim EY. 2008. “Epidemiology of Diabetic Foot Problems and Predictive Factors for Limb Loss.” *Journal of Diabetes and its Complications* 22(2): 77–82.

Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ. 1997. “Diabetes, Defective Pancreatic Morphogenesis, and Abnormal Enteroendocrine Differentiation in BETA2/NeuroD-Deficient Mice.” *Genes and Development* 11(18): 2323–34.

Neerman-Arbez M, Halban PA. 1993. “Novel, Non-Crinophagic, Degradation of Connecting Peptide in Transformed Pancreatic Beta Cells.” *Journal of Biological Chemistry* 268(22): 16248–52.

Németh J, Stein I, Haag D, Riehl A, Longerich T, Horwitz E, Breuhahn K, Gebhardt C, Schirmacher P, Hahn M, Ben-Neriah Y, Pikarsky E, Angel P, Hess J. 2009. “S100A8 and S100A9 Are Novel Nuclear Factor Kappa b Target Genes during Malignant Progression of Murine and Human Liver Carcinogenesis.” *Hepatology* 50(4): 1251–62.

Nguyen NT, Nguyen XM, Lane J, Wang P. 2011. “Relationship between Obesity and Diabetes in a US Adult Population: Findings from the National Health and Nutrition Examination Survey, 1999-2006.” *Obesity Surgery* 21(3): 351–55.

Nicholls SJ, Tuzcu EM, Kalidindi S, Wolski K, Moon KW, Sipahi I, Schoenhagen P, Nissen SE. 2008. “Effect of Diabetes on Progression of Coronary Atherosclerosis and Arterial Remodeling. A Pooled Analysis of 5 Intravascular Ultrasound Trials.” *Journal of the American College of Cardiology* 52(4): 255–62.

Nieto-Vazquez I, Fernández-Veledo S, de Alvaro C, Lorenzo M. 2008. “Dual Role of Interleukin-6 in Regulating Insulin Sensitivity in Murine Skeletal Muscle.” *Diabetes*

57(12): 3211–21.

Ning Q, Li F, Wang L, Li H, Yao Y, Hu T, Sun Z. 2018. “S100A4 Amplifies TGF-β-Induced Epithelial–Mesenchymal Transition in a Pleural Mesothelial Cell Line.” *Journal of Investigative Medicine* 66(2): 334–39.

Nishimura W, Bonner-Weir S, Sharma A. 2009. “Expression of MafA in Pancreatic Progenitors Is Detrimental for Pancreatic Development.” *Developmental Biology* 333(1): 108–20.

Nordmann TM, Dror E, Schulze F, Traub S, Berishvili E, Barbieux C, Böni-Schnetzler M, Donath MY. 2017. “The Role of Inflammation in β-Cell Dedifferentiation.” *Scientific Reports* 7(1): 68–85.

Novitskaya V, Grigorian M, Kriajevska M, Tarabykina S, Bronstein I, Berezin V, Bock E, Lukanidin E. 2000. “Oligomeric Forms of the Metastasis-Related Mts1 (S100A4) Protein Stimulate Neuronal Differentiation in Cultures of Rat Hippocampal Neurons.” *Journal of Biological Chemistry* 275(52): 41278–86.

Novotny GW, Lundh M, Backe MB, Christensen DP, Hansen JB, Dahllöf MS, Pallesen EM, Mandrup-Poulsen T. 2012. “Transcriptional and Translational Regulation of Cytokine Signaling in Inflammatory β-Cell Dysfunction and Apoptosis.” *Archives of Biochemistry and Biophysics* 528(2): 171–84.

O’Connell JT, Sugimoto H, Cooke VG, MacDonald BA, Mehta AI, LeBleu VS, Dewar R, Rocha RM, Brentani RR, Resnick MB, Neilson EG, Zeisberg M, Kalluri R. 2011. “VEGF-A and Tenascin-C Produced by S100A4+ Stromal Cells Are Important for Metastatic Colonization.” *Proceedings of the National Academy of Sciences* 108(38): 16002–7.

O’Connell PA, Surette AP, Liwski RS, Svenningsson P, Waisman DM. 2010. “S100A10 Regulates Plasminogen-Dependent Macrophage Invasion.” *Blood* 116(7): 1136–46.

O’Rourke JF, Pugh CW, Bartlett SM, Ratcliffe PJ. 1996. “Identification of Hypoxically Inducible MRNAs in HeLa Cells Using Differential-Display PCR - Role of Hypoxia-Inducible Factor-1.” *European Journal of Biochemistry* 241(2): 403–10.

Oeckinghaus A, Ghosh S. 2009. “The NF-KappaB Family of Transcription Factors and Its Regulation.” *Cold Spring Harbor perspectives in biology* 1(4): a000034.

Oesterle A, Bowman MA. 2015. “S100A12 and the S100/Calgranulins: Emerging Biomarkers for Atherosclerosis and Possibly Therapeutic Targets.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 35(12): 2496–2507.

Oh YS, Bae GD, Baek DJ, Park EY, Jun HS. 2018. “Fatty Acid-Induced Lipotoxicity in Pancreatic Beta-Cells during Development of Type 2 Diabetes.” *Frontiers in Endocrinology* 9(384).

Oh YS, Lee YJ, Kang Y, Han J, Lim OK, Jun HS. 2013. “Exendin-4 Inhibits Glucolipotoxic ER Stress in Pancreatic β Cells via Regulation of SREBP1c and C/EBPβ Transcription Factors.” *Journal of Endocrinology* 216(3): 343–52.

Oh YS. 2015. “Mechanistic Insights into Pancreatic Beta-Cell Mass Regulation by Glucose and Free Fatty Acids.” *Anatomy & Cell Biology* 48(1): 16–24.

Ohsugi M, Cras-Méneur C, Zhou Y, Bernal-Mizrachi E, Johnson JD, Luciani DS, Polonsky KS, Permutt MA. 2005. “Reduced Expression of the Insulin Receptor in Mouse

Insulinoma (MIN6) Cells Reveals Multiple Roles of Insulin Signaling in Gene Expression, Proliferation, Insulin Content, and Secretion." *Journal of Biological Chemistry* 280(6): 4992–5003.

Ohta K, Endo T, Haraguchi K, Hershman JM, Onaya T. 2001. "Ligands for Peroxisome Proliferator-Activated Receptor γ Inhibit Growth and Induce Apoptosis of Human Papillary Thyroid Carcinoma Cells." *Journal of Clinical Endocrinology and Metabolism* 86(5): 2170–77.

Okada H, Danoff TM, Kalluri R, Neilson EG. 2017. "Early Role of Fsp1 in Epithelial-Mesenchymal Transformation." *American Journal of Physiology-Renal Physiology* 273(4Pt2): F563-574.

Olokoba AB, Obateru OA, Olokoba LB. 2012. "Type 2 Diabetes Mellitus: A Review of Current Trends." *Oman Medical Journal* 27(4): 269–273.

Oprescu AI, Bikopoulos G, Naassan A, Allister EM, Tang C, Park E, Uchino H, Lewis GF, Fantus IG, Rozakis-Adcock M, Wheeler MB, Giacca A. 2007. "Free Fatty Acid-Induced Reduction in Glucose-Stimulated Insulin Secretion: Evidence for a Role of Oxidative Stress in Vitro and in Vivo." *Diabetes* 56(12): 2927–37.

Ortega A, Berná G, Rojas A, Martín F, Soria B. 2017. "Gene-Diet Interactions in Type 2 Diabetes: The Chicken and Egg Debate." *International Journal of Molecular Sciences* 18(6): 1188.

Otani K, Kulkarni RN, Baldwin AC, Krutzfeldt J, Ueki K, Stoffel M, Kahn CR, Polonsky KS. 2003. "Reduced Beta-Cell Mass and Altered Glucose Sensing Impair Insulin-Secretory Function in IRKO Mice." *AJP: Endocrinology and Metabolism* 286(1): E41-49.

Ott C, Jacobs K, Haucke E, Navarrete Santos A, Grune T, Simm A. 2014. "Role of Advanced Glycation End Products in Cellular Signaling." *Redox Biology* 9(2): 411–29.

Otterbein LR, Kordowska J, Witte-Hoffmann C, Wang CL, Dominguez R. 2002. "Crystal Structures of S100A6 in the Ca2+-Free and Ca2+-Bound States: The Calcium Sensor Mechanism of S100 Proteins Revealed at Atomic Resolution." *Structure* 10(4): 557–67.

Ozorowski G, Milton S, Luecke H. 2013. "Structure of a C-Terminal AHNAK Peptide in a 1:2:2 Complex with S100A10 and an Acetylated N-Terminal Peptide of Annexin A2." *Acta Crystallographica Section D: Biological Crystallography* 69(Pt1): 92–104.

Padilla L, Dakhel S, Adan J, Masa M, Martinez JM, Roque L, Coll T, Hervas R, Calvis C, Llinas L, Buenestado S, Castellsague J, Messeguer R, Mitjans F, Hernandez JL. 2017. "S100A7: From Mechanism to Cancer Therapy." *Oncogene* 36(49): 6749–61.

Park HY, Kwon HM, Lim HJ, Hong BK, Lee JY, Park BE, Jang Y, Cho SY, Kim HS. 2001. "Potential Role of Leptin in Angiogenesis: Leptin Induces Endothelial Cell Proliferation and Expression of Matrix Metalloproteinases in Vivo and in Vitro." *Experimental & Molecular Medicine* 33(2): 95–102.

Parzych KR, Klionsky DJ. 2013. "An Overview of Autophagy: Morphology, Mechanism, and Regulation." *Antioxidants & Redox Signaling* 20(3): 460–73.

Pascoe J, Hollern D, Stamateris R, Abbasi M, Romano LC, Zou B, O'Donnell CP, Garcia-Ocana A, Alonso LC. 2012. "Free Fatty Acids Block Glucose-Induced β-Cell

Proliferation in Mice by Inducing Cell Cycle Inhibitors P16 and P18." *Diabetes* 61(3): 632–41.

Paz-Filho G, Lim EL, Wong ML, Licinio J. 2011. "Associations between Adipokines and Obesity-Related Cancer." *Frontiers in bioscience (Landmark edition)* (16): 1634–50.

Pedruzzi LM, Stockler-Pinto MB, Leite M Jr, Mafra D. 2012. "Nrf2-Keap1 System versus NF-KB: The Good and the Evil in Chronic Kidney Disease?" *Biochimie* 94(12): 2461–66.

Peraldi P, Hotamisligil GS, Buurman WA, White MF, Spiegelman BM. 1996. "Tumor Necrosis Factor (TNF)-β Inhibits Insulin Signaling through Stimulation of the P55 TNF Receptor and Activation of Sphingomyelinase." *Journal of Biological Chemistry* 271(22): 13018–22.

Pereira FO, Frode TS, Medeiros YS. 2006. "Evaluation of Tumour Necrosis Factor Alpha, Interleukin-2 Soluble Receptor, Nitric Oxide Metabolites, and Lipids as Inflammatory Markers in Type 2 Diabetes Mellitus." *Mediators of Inflammation* 2006(1): 39062.

Perfetti R, Zhou JIE, Doyle ME, Egan JM. 2000. "Glucagon-like Peptide-1 Induces Cell Proliferation and Pancreatic-Duodenum Homeobox-1 Expression and Increases Endocrine Cell Mass in the Pancreas of Old, Glucose-Intolerant Rats." *Endocrinology* 141(12): 4600–4605.

Perkins JM, Joy NG, Tate DB, Davis SN. 2015. "Acute Effects of Hyperinsulinemia and Hyperglycemia on Vascular Inflammatory Biomarkers and Endothelial Function in Overweight and Obese Humans." *American Journal of Physiology-Endocrinology and Metabolism* 309(2): 168–76.

Peters AL, Stunz LL, Bishop GA. 2009. "CD40 and Autoimmunity: The Dark Side of a Great Activator." *Seminars in Immunology* 21(5): 293–300.

Di Pino A, Urbano F, Piro S, Purrello F, Rabuazzo AM. 2016. "Update on Pre-Diabetes: Focus on Diagnostic Criteria and Cardiovascular Risk." *World Journal of Diabetes* 7(18): 423–32.

Poitout V, Amyot J, Semache M, Zarrouki B, Hagman D, Fontés G. 2010. "Glucolipotoxicity of the Pancreatic Beta Cell." *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1801(3): 289–98.

Poitout V, Robertson RP. 2008. "Glucolipotoxicity: Fuel Excess and β-Cell Dysfunction." *Endocrine Reviews* 29(3): 351–66.

Pollak M. 2012. "The Insulin and Insulin-like Growth Factor Receptor Family in Neoplasia: An Update." *Nature Reviews Cancer* 18(1): 40–50.

Prasad RB, Groop L. 2015. "Genetics of Type 2 Diabetes—Pitfalls and Possibilities." *Genes* 6(1): 87–123.

Prasadan K, Shiota C, Xiangwei X, Ricks D, Fusco J, Gittes G. 2016. "A Synopsis of Factors Regulating Beta Cell Development and Beta Cell Mass." *Cellular and Molecular Life Sciences* 73(19): 3623–37.

Preston AM, Gurisik E, Bartley C, Laybutt DR, Biden TJ. 2009. "Reduced Endoplasmic Reticulum (ER)-to-Golgi Protein Trafficking Contributes to ER Stress in Lipotoxic Mouse Beta Cells by Promoting Protein Overload." *Diabetologia* 52(11): 2369–73.

Pugazhenthi, S. 2014. “Pancreatic Beta Cell Autophagy and Islet Transplantation.” In *Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging*, , 238–47.

Pulgaron ER, Delamater AM. 2014. “Obesity and Type 2 Diabetes in Children: Epidemiology and Treatment.” *Current Diabetes Reports* 14(8): 508.

Pumroy RA, Cingolani G. 2015. “Diversification of Importin-α Isoforms in Cellular Trafficking and Disease States.” *Biochemical Journal* 466(1): 13–28.

Qin H, Lerman B, Sakamaki I, Wei G, Cha SC, Rao SS, Qian J, Hailemichael Y, Nurieva R, Dwyer KC, Roth J, Yi Q, Overwijk WW, Kwak LW. 2014. “Generation of a New Therapeutic Peptide That Depletes Myeloid-Derived Suppressor Cells in Tumor-Bearing Mice.” *Nature Medicine* 20(6): 676–81.

Qin W, Ho L, Wang J, Peskind E, Pasinetti GM. 2009. “S100A7, a Novel Alzheimer’s Disease Biomarker with Non-Amyloidogenic α-Secretase Activity Acts via Selective Promotion of ADAM-10.” *PLoS ONE* 4(1): e4181.

Qu L, Ji Y, Zhu X, Zheng X. 2015. “HCINAP Negatively Regulates NF-KB Signaling by Recruiting the Phosphatase PP1 to Deactivate IKK Complex.” *Journal of Molecular Cell Biology* 7(6): 529–42.

Rachidi SM, Qin T, Sun S, Zheng WJ, Li Z. 2013. “Molecular Profiling of Multiple Human Cancers Defines an Inflammatory Cancer-Associated Molecular Pattern and Uncovers KPNA2 as a Uniform Poor Prognostic Cancer Marker.” *PLoS ONE* 8(3): e57911.

Rahimi F, Hsu K, Endoh Y, Geczy CL. 2005. “FGF-2, IL-1β and TGF-β Regulate Fibroblast Expression of S100A8.” *FEBS Journal* 272(11): 2811–27.

Ravasi T, Hsu K, Goyette J, Schroder K, Yang Z, Rahimi F, Miranda LP, Alewood PF, Hume DA, Geczy C. 2004. “Probing the S100 Protein Family through Genomic and Functional Analysis.” *Genomics* 84(1): 10–22.

Reiber GE, Vileikyte L, Boyko EJ, del Aguila M, Smith DG, Lavery LA, Boulton AJ. 1999. “Causal Pathways for Incident Lower-Extremity Ulcers in Patients with Diabetes from Two Settings.” *Diabetes care* 22(1): 157–62.

Reimann S, Fink L, Wilhelm J, Hoffmann J, Bednorz M, Seimetz M, Dessureault I, Troesser R, Ghanim B, Klepetko W, Seeger W, Weissmann N, Kwapiszewska G. 2015. “Increased S100A4 Expression in the Vasculature of Human COPD Lungs and Murine Model of Smoke-Induced Emphysema.” *Respiratory Research* 16(127).

Rena G, Hardie DG, Pearson ER. 2017. “The Mechanisms of Action of Metformin.” *Diabetologia* 60(9): 1577–85.

Renehan AG, Frystyk J, Flyvbjerg A. 2006. “Obesity and Cancer Risk: The Role of the Insulin-IGF Axis.” *Trends in Endocrinology and Metabolism* 17(8): 328–36.

Reppel M, Sasse P, Piekorz R, Tang M, Roell W, Duan Y, Kletke A, Hescheler J, Nürnberg B, Fleischmann BK. 2005. “S100A1 Enhances the L-Type Ca2+ Current in Embryonic Mouse and Neonatal Rat Ventricular Cardiomyocytes.” *Journal of Biological Chemistry* 280(43): 36019–28.

Rescher U, Gerke V. 2008. “S100A10/P11: Family, Friends and Functions.” *Pflugers Archiv European Journal of Physiology* 455(4): 575–82.

Réty S, Sopkova J, Renouard M, Osterloh D, Gerke V, Tabaries S, Russo-Marie F, Lewit-Bentley A. 1999. “The Crystal Structure of a Complex of P11 with the Annexin II N-Terminal Peptide.” *Nature Structural Biology* 6(1): 89–95.

Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG. 2009. “Islet-Associated Macrophages in Type 2 Diabetes.” *Diabetologia* 52(8): 1686–88.

Ritz E, Orth SR. 1999. “Nephropathy in Patients with Type 2 Diabetes Mellitus.” *New England Journal of Medicine* 245(2): 111–26.

Robertson RP, Zhang HJ, Pyzdrowski KL, Walseth TF. 1992. “Preservation of Insulin MRNA Levels and Insulin Secretion in HIT Cells by Avoidance of Chronic Exposure to High Glucose Concentrations.” *Journal of Clinical Investigation* 90(2): 320–25.

Robertson RP. 2004. “Chronic Oxidative Stress as a Central Mechanism for Glucose Toxicity in Pancreatic Islet Beta Cells in Diabetes.” *Journal of Biological Chemistry* 279(41): 42351–54.

Robertson RP, Harmon JS. 2007. “Pancreatic Islet β-Cell and Oxidative Stress: The Importance of Glutathione Peroxidase.” *FEBS Letters* 581(19): 3743–48.

Röder PV, Wu B, Liu Y, Han W. 2016. “Pancreatic Regulation of Glucose Homeostasis.” *Experimental & molecular medicine* 48(3): e209.

Roh J, Knight S, Chung JY, Eo SH, Goggins M, Kim J, Cho H, Yu E, Hong SM. 2014. “S100A4 Expression Is a Prognostic Indicator in Small Intestine Adenocarcinoma.” *Journal of Clinical Pathology* 67(3): 216–21.

Rohde D, Ritterhoff J, Voelkers M, Katus HA, Parker TG, Most P. 2010. “S100A1: A Multifaceted Therapeutic Target in Cardiovascular Disease.” *Journal of Cardiovascular Translational Research* 3(5): 525–37.

Rojas J, Bermudez V, Palmar J, Martínez MS, Olivar LC, Nava M, Tomey D, Rojas M, Salazar J, Garicano C, Velasco M. 2018. “Pancreatic Beta Cell Death: Novel Potential Mechanisms in Diabetes Therapy.” *Journal of Diabetes Research* 2018: 9601801.

Rojas LB, Gomes MB. 2013. “Metformin: An Old but Still the Best Treatment for Type 2 Diabetes.” *Diabetology and Metabolic Syndrome* 5(6).

Roubicek T, Bartlova M, Krajickova J, Haluzikova D, Mraz M, Lacinova Z, Kudla M, Teplan V, Haluzik M. 2009. “Increased Production of Proinflammatory Cytokines in Adipose Tissue of Patients with End-Stage Renal Disease.” *Nutrition* 25(7–8): 762–68.

Rubenstrunk A, Hanf R, Hum DW, Fruchart JC, Staels B. 2007. “Safety Issues and Prospects for Future Generations of PPAR Modulators.” *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1771(8): 1065–81.

Rudland PS, Platt-Higgins A, Renshaw C, West CR, Winstanley JH, Robertson L, Barraclough R. 2000. “Prognostic Significance of the Metastasis-Inducing Protein S100A4 (P9Ka) in Human Breast Cancer.” *Cancer Research* 60(6): 1595–1603.

Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. 2003. “Proinflammatory Activities of S100: Proteins S100A8, S100A9, and S100A8/A9 Induce Neutrophil Chemotaxis and Adhesion.” *The Journal of Immunology* 170(6): 3233–42.

Sabatini DM. 2006. “MTOR and Cancer: Insights into a Complex Relationship.” *Nature Reviews Cancer* 6(9): 729–34.

Sack U, Walther W, Scudiero D, Selby M, Aumann J, Lemos C, Fichtner I, Schlag PM, Shoemaker RH, Stein U. 2011. “S100A4-Induced Cell Motility and Metastasis Is Restricted by the Wnt/β-Catenin Pathway Inhibitor Calcimycin in Colon Cancer Cells.” *Molecular Biology of the Cell* 22(18): 3344–54.

Sack U, Walther W, Scudiero D, Selby M, Kobelt D, Lemm M, Fichtner I, Schlag PM, Shoemaker RH, Stein U. 2011. “Novel Effect of Antihelminthic Niclosamide on S100a4-Mediated Metastatic Progression in Colon Cancer.” *Journal of the National Cancer Institute* 103(13): 1018–36.

Sade-Feldman M, Kanterman J, Ish-Shalom E, Elnekave M, Horwitz E, Baniyash M. 2013. “Tumor Necrosis Factor-α Blocks Differentiation and Enhances Suppressive Activity of Immature Myeloid Cells during Chronic Inflammation.” *Immunity* 38(3): 541–54.

Saïd-Sadier N, Ojcius DM. 2012. “Alarmins, Inflammasomes and Immunity.” *Biomedical journal* 35(6): 437–49.

Saif MW. 2013. “Anti-VEGF Agents in Metastatic Colorectal Cancer (MCRC): Are They All Alike?” *Cancer Management and Research* (5): 103–15.

Saleem M, Kweon MH, Johnson JJ, Adhami VM, Elcheva I, Khan N, Bin Hafeez B, Bhat KM, Sarfaraz S, Reagan-Shaw S, Spiegelman VS, Setaluri V, Mukhtar H. 2006. “S100A4 Accelerates Tumorigenesis and Invasion of Human Prostate Cancer through the Transcriptional Regulation of Matrix Metalloproteinase 9.” *Proceedings of the National Academy of Sciences* 103(40): 14825–30.

Sander M, Sussel L, Conners J, Scheel D, Kalamaras J, Dela Cruz F, Schwitzgebel V, Hayes-Jordan A, German M. 2000. “Homeobox Gene Nkx6.1 Lies Downstream of Nkx2.2 in the Major Pathway of Beta-Cell Formation in the Pancreas.” *Development (Cambridge, England)* 127(24): 5533–40.

Santamaria-Kisiel L, Rintala-Dempsey AC, Shaw GS. 2006. “Calcium-Dependent and -Independent Interactions of the S100 Protein Family.” *Biochemical Journal* 396(2): 201–14.

Sanz AB, Sanchez-Niño MD, Ramos AM, Moreno JA, Santamaria B, Ruiz-Ortega M, Egido J, Ortiz A. 2010. “NF-KB in Renal Inflammation.” *J Am Soc Nephrol.* 21(8): 1254–62.

Sapkota D, Bruland O, Costea DE, Haugen H, Vasstrand EN, Ibrahim SO. 2011. “S100A14 Regulates the Invasive Potential of Oral Squamous Cell Carcinoma Derived Cell-Lines in Vitro by Modulating Expression of Matrix Metalloproteinases, MMP1 and MMP9.” *European Journal of Cancer* 47(4): 600–610.

Sato Y, Endo H, Okuyama H, Takeda T, Iwahashi H, Imagawa A, Yamagata K, Shimomura I, Inoue M. 2011. “Cellular Hypoxia of Pancreatic β-Cells Due to High Levels of Oxygen Consumption for Insulin Secretion in Vitro.” *Journal of Biological Chemistry* 286(14): 12524–32.

Sato Y, Inoue M, Yoshizawa T, Yamagata K. 2014. “Moderate Hypoxia Induces β-Cell Dysfunction with HIF-1-Independent Gene Expression Changes.” *PLoS ONE* 9(12): e114868.

Saylor PJ, Smith MR. 2009. “Metabolic Complications of Androgen Deprivation Therapy for Prostate Cancer.” *Journal of Urology* 181(5): 1998–2006.

Scarlatti F, Granata R, Meijer AJ, Codogno P. 2009. “Does Autophagy Have a License to Kill Mammalian Cells?” *Cell Death and Differentiation* 16(1): 12–20.

Schaf, Débora V. et al. 2005. “S100B and NSE Serum Levels in Patients with Parkinson’s Disease.” *Parkinsonism and Related Disorders* 11(1): 39–43.

Schäfer BW, Heizmann CW. 1996. “The S100 Family of EF-Hand Calcium-Binding Proteins: Functions and Pathology.” *Trends in Biochemical Sciences* 21(4): 134–40.

Schenten V, Plançon S, Jung N, Hann J, Bueb JL, Bréchard S, Tschirhart EJ, Tolle F. 2018. “Secretion of the Phosphorylated Form of S100A9 from Neutrophils Is Essential for the Proinflammatory Functions of Extracellular S100A8/A9.” *Frontiers in Immunology* 9(447).

Scheuner D, Kaufman RJ. 2008. “The Unfolded Protein Response: A Pathway That Links Insulin Demand with β-Cell Failure and Diabetes.” *Endocrine Reviews* 29(3): 317–33.

Schlueter C, Hauke S, Flohr AM, Rogalla P, Bullerdiek J. 2003. “Tissue-Specific Expression Patterns of the RAGE Receptor and Its Soluble Forms - A Result of Regulated Alternative Splicing?” *Biochimica et Biophysica Acta - Gene Structure and Expression* 1630(1): 1–6.

Schmidt-Hansen B, Ornås D, Grigorian M, Klingelhöfer J, Tulchinsky E, Lukanidin E, Ambartsumian N. 2004. “Extracellular S100A4(Mts1) Stimulates Invasive Growth of Mouse Endothelial Cells and Modulates MMP-13 Matrix Metalloproteinase Activity.” *Oncogene* 23(32): 5487–95.

Schober E, Rami B, Grabert M, Thon A, Kapellen T, Reinehr T, Holl RW. 2009. “Phenotypical Aspects of Maturity-Onset Diabetes of the Young (MODY Diabetes) in Comparison with Type 2 Diabetes Mellitus (T2DM) in Children and Adolescents: Experience from a Large Multicentre Database.” *Diabetic Medicine* 26(5): 446–73.

Schroeter ML, Sacher J, Steiner J, Schoenknecht P, Mueller K. 2013. “Serum S100B Represents a New Biomarker for Mood Disorders.” *Current Drug Targets* 14(11): 1237–48.

Segerstolpe Å, Palasantza A, Eliasson P, Andersson EM, Andréasson AC, Sun X, Picelli S, Sabirsh A, Clausen M, Bjursell MK, Smith DM, Kasper M, Ämmälä C, Sandberg R. 2016. “Single-Cell Transcriptome Profiling of Human Pancreatic Islets in Health and Type 2 Diabetes.” *Cell Metabolism* 24(4): 593–607.

Seijkens T, Kusters P, Engel D, Lutgens E. 2013. “CD40-CD40L: Linking Pancreatic, Adipose Tissue and Vascular Inflammation in Type 2 Diabetes and Its Complications.” *Diabetes and Vascular Disease Research* 10(2): 115–22.

Seino Y, Nanjo K, Tajima N, Kadowaki T, Kashiwagi A, Araki E, Ito C, Inagaki N, Iwamoto Y, Kasuga M, Hanafusa T, Haneda M. 2010. “Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus.” *Journal of Diabetes Investigation* 1(5): 212–28.

Semenza GL. 2003. “Targeting HIF-1 for Cancer Therapy.” *Nature Reviews Cancer* 3(10): 721–32.

Semov A, Moreno MJ, Onichtchenko A, Abulrob A, Ball M, Ekiel I, Pietrzynski G, Stanimirovic D, Alakhov V. 2005. “Metastasis-Associated Protein S100A4 Induces Angiogenesis through Interaction with Annexin II and Accelerated Plasmin Formation.” *Journal of Biological Chemistry* 280(21): 20833–41.

Serrano A, Apolloni S, Rossi S, Lattante S, Sabatelli M, Peric M, Andjus P, Michetti F, Carrì

MT, Cozzolino M, D'Ambrosi N. 2019. "The S100A4 Transcriptional Inhibitor Niclosamide Reduces Pro-Inflammatory and Migratory Phenotypes of Microglia: Implications for Amyotrophic Lateral Sclerosis." *Cells* 8(10): pii: E1261.

Shahbazian H, Rezaii I. 2013. "Diabetic Kidney Disease; Review of the Current Knowledge." *Journal of renal injury prevention* 2(2): 73–80.

Shariff AI, Syed S, Shelby RA, Force J, Clarke JM, D'Alessio D, Corsino L. 2019. "Novel Cancer Therapies and Their Association with Diabetes." *Journal of molecular endocrinology* 62(2): R187–99.

Sharma RB, Alonso LC. 2014. "Lipotoxicity in the Pancreatic Beta Cell: Not Just Survival and Function, but Proliferation as Well?" *Current Diabetes Reports* 14(6): 492.

Sheng L, Zhou Y, Chen Z, Ren D, Cho KW, Jiang L, Shen H, Sasaki Y, Rui L. 2012. "NF-KB-Inducing Kinase (NIK) Promotes Hyperglycemia and Glucose Intolerance in Obesity by Augmenting Glucagon Action." *Nature Medicine* 18(6): 943–49.

Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. 2006. "TLR4 Links Innate Immunity and Fatty Acid-Induced Insulin Resistance." *Journal of Clinical Investigation* 116(11): 3015–25.

Shih VF, Tsui R, Caldwell A, Hoffmann A. 2011. "A Single NFκB System for Both Canonical and Non-Canonical Signaling." *Cell Research* 21(1): 86–102.

Shimabukuro M, Higa M, Zhou YT, Wang MY, Newgard CB, Unger RH. 1998. "Lipoapoptosis in Beta-Cells of Obese Prediabetic Fa/Fa Rats. Role of Serine Palmitoyltransferase Overexpression." *Journal of Biological Chemistry* 273(49): 32487–90.

Shishibori T, Oyama Y, Matsushita O, Yamashita K, Furuichi H, Okabe A, Maeta H, Hata Y, Kobayashi R. 1999. "Three Distinct Anti-Allergic Drugs, Amlexanox, Cromolyn and Tranilast, Bind to S100A12 and S100A13 of the S100 Protein Family." *Biochemical Journal* 338(Pt 3): 583–89.

Short, Kevin R. 2013. "Regulation of Glycemic Control by Physical Activity: A Role for Mitochondria?" *Diabetes* 62(1): 34–35.

Sica A, Bronte V. 2007. "Altered Macrophage Differentiation and Immune Dysfunction in Tumor Development." *Journal of Clinical Investigation* 117(5): 1155–66.

Skelin M, Rupnik M, Cencic A. 2010. "Pancreatic Beta Cell Lines and Their Applications in Diabetes Mellitus Research." *ALTEX* 27(2): 105–13.

Das SL, Kennedy JI, Murphy R, Phillips AR, Windsor JA, Petrov MS. 2014. "Relationship between the Exocrine and Endocrine Pancreas after Acute Pancreatitis." *World Journal of Gastroenterology* 20(45): 17196–205.

Smith BN, Bhowmick NA. 2016. "Role of EMT in Metastasis and Therapy Resistance." *Journal of Clinical Medicine* 5(2): pii: E17.

Snodgrass RG, Huang s, Namgaladze D, Jandali O, Shao T, Sama S, Brüne B, Hwang DH. 2016. "Docosahexaenoic Acid and Palmitic Acid Reciprocally Modulate Monocyte Activation in Part through Endoplasmic Reticulum Stress." *Journal of Nutritional Biochemistry* 32: 39–45.

Sokolowska E, Blachnio-Zabielska A. 2019. "The Role of Ceramides in Insulin Resistance." *Frontiers in Endocrinology* 10(577): eCollection 2019.

Somesh BP, Verma MK, Sadasivuni MK, Mammen-Oommen A, Biswas S, Shilpa PC, Reddy AK, Yateesh AN, Pallavi PM, Nethra S, Smitha R, Neelima K, Narayanan U, Jagannath MR. 2013. “Chronic Glucolipotoxic Conditions in Pancreatic Islets Impair Insulin Secretion Due to Dysregulated Calcium Dynamics, Glucose Responsiveness and Mitochondrial Activity.” *BMC Cell Biology* 14(31).

Sone H, Kagawa Y. 2005. “Pancreatic Beta Cell Senescence Contributes to the Pathogenesis of Type 2 Diabetes in High-Fat Diet-Induced Diabetic Mice.” *Diabetologia* 48(1): 58–67.

Sorci G, Giovannini G, Riuzzi F, Bonifazi P, Zelante T, Zagarella S, Bistoni F, Donato R, Romani L. 2011. “The Danger Signal S100B Integrates Pathogen- and Danger-Sensing Pathways to Restrain Inflammation.” *PLoS Pathogens* 7(3): e1001315.

Sorci G, Riuzzi F, Giambanco I, Donato R. 2013. “RAGE in Tissue Homeostasis, Repair and Regeneration.” *Biochimica et Biophysica Acta - Molecular Cell Research* 1833(1): 101–9.

Spijker HS, Ravelli RBG, Mommaas-Kienhuis AM, Van Apeldoorn AA, Engelse MA, Zaldumbide A, Bonner-Weir S, Rabelink TJ, Hoeben RC, Clevers H, Mummery CL, Carlotti F, De Koning EJP. 2013. “Conversion of Mature Human β-Cells into Glucagon- Producing α-Cells.” *Diabetes* 62(7): 2471–80.

Srikrishna G, Freeze HH. 2011. “S100 Protein Family and Tumorigenesis.” *Atlas of Genetics and Cytogenetics in Oncology and Haematology*.

Srinivasan S, Bernal-Mizrachi E, Ohsugi M, Permutt MA. 2002. “Glucose Promotes Pancreatic Islet β-Cell Survival through a PI 3-Kinase/Akt-Signaling Pathway.” *American Journal of Physiology - Endocrinology And Metabolism* 283(4): E784-793.

Staab A, Loeffler J, Said HM, Diehlmann D, Katzer A, Beyer M, Fleischer M, Schwab F, Baier K, Einsele H, Flentje M, Vordermark D. 2007. “Effects of HIF-1 Inhibition by Chetomin on Hypoxia-Related Transcription and Radiosensitivity in HT 1080 Human Fibrosarcoma Cells.” *BMC Cancer* 13(7): 213.

Stein U, Arlt F, Smith J, Sack U, Herrmann P, Walther W, Lemm M, Fichtner I, Shoemaker RH, Schlag PM. 2011. “Intervening in Beta-Catenin Signaling by Sulindac Inhibits S100A4-Dependent Colon Cancer Metastasis.” *Neoplasia* 13(2): 131–44.

Stein U, Arlt F, Walther W, Smith J, Waldman T, Harris ED, Mertins SD, Heizmann CW, Allard D, Birchmeier W, Schlag PM, Shoemaker RH. 2006a. “The Metastasis-Associated Gene S100A4 Is a Novel Target of β-Catenin/T-Cell Factor Signaling in Colon Cancer.” *Gastroenterology* 131(5): 1486–1500.

———. 2006b. “The Metastasis-Associated Gene S100A4 Is a Novel Target of β-Catenin/T-Cell Factor Signaling in Colon Cancer.” *Gastroenterology* 131(5): 1486–1500.

Steinckwich N, Schenten V, Melchior C, Bréchard S, Tschirhart EJ. 2011. “An Essential Role of STIM1, Orai1, and S100A8-A9 Proteins for Ca2+ Signaling and Fc R-Mediated Phagosomal Oxidative Activity.” *The Journal of Immunology* 186(4): 2182–91.

Stewart M. 2007. “Molecular Mechanism of the Nuclear Protein Import Cycle.” *Nature Reviews Molecular Cell Biology* 8(3): 195–208.

Striegel DA, Hara M, Periwal V. 2015. “The Beta Cell in Its Cluster: Stochastic Graphs of

Beta Cell Connectivity in the Islets of Langerhans." *PLoS Computational Biology* 11(8): e1004423.

Stumvoll M, Goldstein BJ, van Haeften TW. 2005. "Type 2 Diabetes: Principles of Pathogenesis and Therapy." *Lancet* 365(9467): 1333–46.

Sturchler E, Cox JA, Durussel I, Weibel M, Heizmann CW. 2006. "S100A16, a Novel Calcium-Binding Protein of the EF-Hand Superfamily." *Journal of Biological Chemistry* 281(50): 38905–17.

Sun K, Halberg N, Khan M, Magalang UJ, Scherer PE. 2012. "Selective Inhibition of Hypoxia-Inducible Factor 1 Ameliorates Adipose Tissue Dysfunction." *Molecular and Cellular Biology* 33(5): 904–17.

Sun SC. 2011. "Non-Canonical NF-KB Signaling Pathway." *Cell Research* 21(1): 71–85.

———. 2017. "The Non-Canonical NF-KB Pathway in Immunity and Inflammation." *Nature reviews. Immunology* 17(9): 545–58.

Sunahori K, Yamamura M, Yamana J, Takasugi K, Kawashima M, Yamamoto H, Chazin WJ, Nakatani Y, Yui S, Makino H. 2006. "The S100A8/A9 Heterodimer Amplifies Proinflammatory Cytokine Production by Macrophages via Activation of Nuclear Factor Kappa B and P38 Mitogen-Activated Protein Kinase in Rheumatoid Arthritis." *Arthritis Research and Therapy* 8(3): R69.

Surette AP, Madureira PA, Phipps KD, Miller VA, Svenningsson P, Waisman DM. 2011. "Regulation of Fibrinolysis by S100A10 in Vivo." *Blood* 118(11): 3172–81.

Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL, German MS. 1998. "Mice Lacking the Homeodomain Transcription Factor Nkx2.2 Have Diabetes Due to Arrested Differentiation of Pancreatic Beta Cells." *Development (Cambridge, England)* 125(12): 2213–21.

Suzuki D, Toyoda M, Yamamoto N, Miyauchi M, Katoh M, Kimura M, Maruyama M, Honma M, Umezono T, Yagame M. 2006. "Relationship between the Expression of Advanced Glycation End-Products (AGE) and the Receptor for AGE (RAGE) MRNA in Diabetic Nephropathy." *Internal Medicine* 45(7): 435–41.

Svenningsson P, Chergui K, Rachleff I, Flajolet M, Zhang X, El Yacoubi M, Vaugeois JM, Nomikos GG, Greengard P. 2006. "Alterations in 5-HT1B Receptor Function by P11 in Depression-like States." *Science* 311(5757): 77–80.

Taft RJ, Pheasant M, Mattick JS. 2007. "The Relationship between Non-Protein-Coding DNA and Eukaryotic Complexity." *BioEssays* 29(3): 288–99.

Tajeddine N, Louis M, Vermylen C, Gala JL, Tombal B, Gailly P. 2008. "Tumor Associated Antigen PRAME Is a Marker of Favorable Prognosis in Childhood Acute Myeloid Leukemia Patients and Modifies the Expression of S100A4, Hsp 27, P21, IL-8 and IGFBP-2 in Vitro and in Vivo." *Leukemia and Lymphoma* 49(6): 1123–31.

Takenaga K, Kozlova EN. 2006. "Role of Intracellular S100A4 for Migration of Rat Astrocytes." *GLIA* 53(3): 313–21.

Talchai C, Xuan S, Lin HV, Sussel L, Accili D. 2012. "Pancreatic β Cell Dedifferentiation as a Mechanism of Diabetic β Cell Failure." *Cell* 150(6): 1223–34.

Tan PK, Downey TJ, Spitznagel EL Jr, Xu P, Fu D, Dimitrov DS, Lempicki RA, Raaka BM, Cam MC. 2003. "Evaluation of Gene Expression Measurements from Commercial

Microarray Platforms." *Nucleic Acids Research* 31(19): 5676–84.

Tanaka Y, Gleason CE, Tran PO, Harmon JS, Robertson RP. 1999. "Prevention of Glucose Toxicity in HIT-T15 Cells and Zucker Diabetic Fatty Rats by Antioxidants." *Proceedings of the National Academy of Sciences* 96(19): 10857–62.

Tanaka Y, Tran PO, Harmon J, Robertson RP. 2002. "A Role for Glutathione Peroxidase in Protecting Pancreatic Cells against Oxidative Stress in a Model of Glucose Toxicity." *Proceedings of the National Academy of Sciences* 99(19): 12363–68.

Tang WH, Martin KA, Hwa J. 2012. "Aldose Reductase, Oxidative Stress, and Diabetic Mellitus." *Frontiers in Pharmacology* 3(87).

Tao R, Wang ZF, Qiu W, He YF, Yan WQ, Sun WY, Li HJ. 2017. "Role of S100A3 in Human Hepatocellular Carcinoma and the Anticancer Effect of Sodium Cantharidinate." *Experimental and Therapeutic Medicine* 13(6): 2812–18.

Tarabykina S, Griffiths TR, Tulchinsky E, Mellon JK, Bronstein IB, Kriajevska M. 2007. "Metastasis-Associated Protein S100A4: Spotlight on Its Role in Cell Migration." *Current Cancer Drug Targets* 7(3): 217–28.

Taylor CT, Colgan SP. 2017. "Regulation of Immunity and Inflammation by Hypoxia in Immunological Niches." *Nature Reviews Immunology* 17(12): 774–85.

Taylor S, Herrington S, Prime W, Rudland PS, Barraclough R. 2002. "S100A4 (P9Ka) Protein in Colon Carcinoma and Liver Metastases: Association with Carcinoma Cells and T-Lymphocytes." *British Journal of Cancer* 86(3): 409–16.

Teshima Y, Takahashi N, Nishio S, Saito S, Kondo H, Fukui A, Aoki K, Yufu K, Nakagawa M, Saikawa T. 2014. "Production of Reactive Oxygen Species in the Diabetic Heart." *Circulation Journal* 78(2): 300–306.

Thelin EP, Jeppsson E, Frostell A, Svensson M, Mondello S, Bellander BM, Nelson DW. 2016. "Utility of Neuron-Specific Enolase in Traumatic Brain Injury; Relations to S100B Levels, Outcome, and Extracranial Injury Severity." *Critical Care* 20(285).

Tidehag V, Hammarsten P, Egevad L, Granfors T, Stattin P, Leanderson T, Wikström P, Josefsson A, Hägglöf C, Bergh A. 2014. "High Density of S100A9 Positive Inflammatory Cells in Prostate Cancer Stroma Is Associated with Poor Outcome." *European Journal of Cancer* 50(10): 1829–35.

Tiedge M, Lortz S, Drinkgern J, Lenzen S. 1997. "Relation between Antioxidant Enzyme Gene Expression and Antioxidative Defense Status of Insulin-Producing Cells." *Diabetes* 46(11): 1733–42.

Tiwari S, Pratyush DD, Dwivedi A, Gupta SK, Rai M, Singh SK. 2012. "Microbiological and Clinical Characteristics of Diabetic Foot Infections in Northern India." *Journal of Infection in Developing Countries* 6(4): 329–32.

Tkacova R, Ukropec J, Skyba P, Ukropcova B, Pobeha P, Kurdiova T, Joppa P, Klimes I, Tkac I, Gasperikova D. 2013. "Effects of Hypoxia on Adipose Tissue Expression of NFκB, IκBα, IKKγ and IKAP in Patients with Chronic Obstructive Pulmonary Disease." *Cell Biochemistry and Biophysics* 66(1): 7–12.

Tomcik M, Palumbo-Zerr K, Zerr P, Avouac J, Dees C, Sumova B, Distler A, Beyer C, Cerezo LA, Becvar R, Distler O, Grigorian M, Schett G, Senolt L, Distler JH. 2015. "S100A4 Amplifies TGF-β-Induced Fibroblast Activation in Systemic Sclerosis."

Annals of the Rheumatic Diseases 74(9): 1748–55.

Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. 2015. "Globan Cancer Statistics, 2012." *CA: A Cancer Journal for Clinicians* 65(2): 87–108.

Trayhurn P, Wood IS. 2004. "Adipokines: Inflammation and the Pleiotropic Role of White Adipose Tissue." *The British journal of nutrition* 92(3): 347–55.

Tsonkova VG, Sand FW, Wolf XA, Grunnet LG, Kirstine Ringgaard A, Ingvorsen C, Winkel L, Kalisz M, Dalgaard K, Bruun C, Fels JJ, Helgstrand C, Hastrup S, Öberg FK, Vernet E, Sandrini MPB, Shaw AC, Jessen C, Grønborg M, Hald J, Willenbrock H, Madsen D, Wern, Frogne T. 2018. "The EndoC-BH1 Cell Line Is a Valid Model of Human Beta Cells and Applicable for Screenings to Identify Novel Drug Target Candidates." *Molecular Metabolism* (8): 144–57.

Tsukamoto N, Egawa S, Akada M, Abe K, Saiki Y, Kaneko N, Yokoyama S, Shima K, Yamamura A, Motoi F, Abe H, Hayashi H, Ishida K, Moriya T, Tabata T, Kondo E, Kanai N, Gu Z, Sunamura M, Unno M, Horii A. 2013. "The Expression of S100A4 in Human Pancreatic Cancer Is Associated with Invasion." *Pancreas* 42(6): 1027–33.

Tumova J, Andel M, Trnka J. 2016. "Excess of Free Fatty Acids as a Cause of Metabolic Dysfunction in Skeletal Muscle." *Physiological Research* 65(2): 193–207.

Tuomilehto J, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Laakso M, Louheranta A, Rastas M, Salminen V, Uusitupa M. 2001. "Prevention of Type 2 Diabetes Mellitus by Changes in Lifestyle among Subjects with Impaired Glucose Tolerance." *New England Journal of Medicine* 344(18): 1343–50.

Turner MD, Nedjai B, Hurst T, Pennington DJ. 2014. "Cytokines and Chemokines: At the Crossroads of Cell Signalling and Inflammatory Disease." *Biochimica et Biophysica Acta - Molecular Cell Research* 1843(11): 2563–82.

Udayasankar J, Kodama K, Hull RL, Zraika S, Aston-Mourney K, Subramanian SL, Tong J, Faulenbach MV, Vidal J, Kahn SE. 2009. "Amyloid Formation Results in Recurrence of Hyperglycaemia Following Transplantation of Human IAPP Transgenic Mouse Islets." *Diabetologia* 52(1): 145–53.

Unwin N, Shaw J, Zimmet P, Alberti KG. 2002. "Impaired Glucose Tolerance and Impaired Fasting Glycaemia: The Current Status on Definition and Intervention." *Diabetic Medicine* 19(9): 708–23.

Vachharajani V, Granger DN. 2009. "Adipose Tissue: A Motor for the Inflammation Associated with Obesity." *IUBMB Life* 61(4): 424–30.

Vasavada RC, Gonzalez-Pertusa JA, Fujinaka Y, Fiaschi-Taesch N, Cozar-Castellano I, Garcia-Ocaña A. 2006. "Growth Factors and Beta Cell Replication." *International Journal of Biochemistry and Cell Biology* 38(5–6): 931–50.

Véret J, Bellini L, Giussani P, Ng C, Magnan C, Le Stunff H. 2014. "Roles of Sphingolipid Metabolism in Pancreatic β Cell Dysfunction Induced by Lipotoxicity." *Journal of Clinical Medicine* 3(2): 646–62.

Véret J, Coant N, Berdyshev EV, Skobeleva A, Therville N, Bailbé D, Gorshkova I, Natarajan V, Portha B, Le Stunff H. 2011. "Ceramide Synthase 4 and de Novo Production of Ceramides with Specific N-Acyl Chain Lengths Are Involved in Glucolipotoxicity-Induced Apoptosis of INS-1 β-Cells." *Biochemical Journal* 438(1):

177–89.

Vigneri P, Frasca F, Sciacca L, Pandini G, Vigneri R. 2009. “Diabetes and Cancer.” *Endocr Relat Cancer.* 16(4): 1103–23.

Villarroya F, Cereijo R, Gavaldà-Navarro A, Villarroya J, Giralt M. 2018. “Inflammation of Brown/Beige Adipose Tissues in Obesity and Metabolic Disease.” *Journal of Internal Medicine* 248(5): 492–504.

Viziteu E, Grandmougin C, Goldschmidt H, Seckinger A, Hose D, Klein B, Moreaux J. 2016. “Chetomin, Targeting HIF-1α/P300 Complex, Exhibits Antitumour Activity in Multiple Myeloma.” *British Journal of Cancer* 114(5): 519–23.

Vogl T, Ludwig S, Goebeler M, Strey A, Thorey IS, Reichelt R, Foell D, Gerke V, Manitz MP, Nacken W, Werner S, Sorg C, Roth J. 2004. “MRP8 and MRP14 Control Microtubule Reorganization during Transendothelial Migration of Phagocytes.” *Blood* 104(13): 4260–68.

Vogl T, Pröpper C, Hartmann M, Strey A, Strupat K, van den Bos C, Sorg C, Roth J. 1999. “S100A12 Is Expressed Exclusively by Granulocytes and Acts Independently from MRP8 and MRP14.” *Journal of Biological Chemistry* 274(36): 25291–96.

Vogl T, Stratis A, Wixler V, Völler T, Thurainayagam S, Jorch SK, Zenker S, Dreiling A, Chakraborty D, Fröhling M, Paruzel P, Wehmeyer C, Hermann S, Papantonopoulou O, Geyer C, Loser K, Schäfers M, Ludwig S, Stoll M, Leanderson T, Schultze JL, König S, Pa, Roth J. 2018. “Autoinhibitory Regulation of S100A8/S100A9 Alarmin Activity Locally Restricts Sterile Inflammation.” *Journal of Clinical Investigation* 128(5): 1852–66.

Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, Nacken W, Foell D, van der Poll T, Sorg C, Roth J. 2007. “Mrp8 and Mrp14 Are Endogenous Activators of Toll-like Receptor 4, Promoting Lethal, Endotoxin-Induced Shock.” *Nature Medicine* 13(9): 1042–49.

Volmer-Thole M, Lobmann R. 2016. “Neuropathy and Diabetic Foot Syndrome.” *International Journal of Molecular Sciences* 17(6): 917.

Volmer R, van der Ploeg K, Ron D. 2013. “Membrane Lipid Saturation Activates Endoplasmic Reticulum Unfolded Protein Response Transducers through Their Transmembrane Domains.” *Proceedings of the National Academy of Sciences* 110(12): 4628–33.

Wafer LN, Tzul FO, Pandharipande PP, Makhatadze GI. 2013. “Novel Interactions of the TRTK12 Peptide with S100 Protein Family Members: Specificity and Thermodynamic Characterization.” *Biochemistry* 52(34): 5844–56.

Walter I, Wolfesberger B, Miller I, Mair G, Burger S, Gallè B, Steinborn R. 2014. “Human Osteosarcoma Cells Respond to Sorafenib Chemotherapy by Downregulation of the Tumor Progression Factors S100A4, CXCR4 and the Oncogene FOS.” *Oncology Reports* 31(3): 1147–56.

Wang CI, Wang CL, Wang CW, Chen CD, Wu CC, Liang Y, Tsai YH, Chang YS, Yu JS, Yu CJ. 2011. “Importin Subunit Alpha-2 Is Identified as a Potential Biomarker for Non-Small Cell Lung Cancer by Integration of the Cancer Cell Secretome and Tissue Transcriptome.” *International Journal of Cancer* 128(10): 2364–72.

Wang H, Duan L, Zou Z, Li H, Yuan S, Chen X, Zhang Y, Li X, Sun H, Zha H, Zhang Y, Zhou L.

2014. “Activation of the PI3K/Akt/MTOR/P70S6K Pathway Is Involved in S100A4-Induced Viability and Migration in Colorectal Cancer Cells.” *International Journal of Medical Sciences* 11(8): 841–49.

Wang LC, Zhang HY, Shao L, Chen L, Liu ZH, He X, Gong WX. 2013. “S100A12 Levels in Synovial Fluid May Reflect Clinical Severity in Patients with Primary Knee Osteoarthritis.” *Biomarkers* 18(3): 216–20.

Wang Q, Williamson M, Bott S, Brookman-Amissah N, Freeman A, Nariculam J, Hubank MJ, Ahmed A, Masters JR. 2007. “Hypomethylation of WNT5A, CRIP1 and S100P in Prostate Cancer.” *Oncogene* 26(45): 6560–65.

Wang XH, Zhang LH, Zhong XY, Xing XF, Liu YQ, Niu ZJ, Peng Y, Du H, Zhang GG, Hu Y, Liu N, Zhu YB, Ge SH, Zhao W, Lu AP, Li JY, Ji JF. 2010. “S100A6 Overexpression Is Associated with Poor Prognosis and Is Epigenetically Up-Regulated in Gastric Cancer.” *American Journal of Pathology* 177(2): 586–97.

Wang Z, Gerstein M, Snyder M. 2009. “RNA-Seq: A Revolutionary Tool for Transcriptomics.” *Nature Reviews Genetics* 10(1): 57–63.

Warner-Schmidt JL, Chen EY, Zhang X, Marshall JJ, Morozov A, Svenningsson P, Greengard P. 2010. “A Role for P11 in the Antidepressant Action of Brain-Derived Neurotrophic Factor.” *Biological Psychiatry* 68(6): 528–35.

Watanabe Y, Usuda N, Tsugane S, Kobayashi R, Hidaka H. 1992. “Calvasculin, an Encoded Protein from MRNA Termed PEL-98, 18A2, 42A, or P9Ka, Is Secreted by Smooth Muscle Cells in Culture and Exhibits Ca2+- Dependent Binding to 36-KDa Microfibril-Associated Glycoprotein.” *Journal of Biological Chemistry* 267(24): 17136–40.

Waters MJ, Brooks AJ. 2015. “JAK2 Activation by Growth Hormone and Other Cytokines.” *Biochemical Journal* 466(Pt1): 1–11.

Weinstein D, Simon M, Yehezkel E, Laron Z, Werner H. 2009. “Insulin Analogues Display IGF-I-like Mitogenic and Anti-Apoptotic Activities in Cultured Cancer Cells.” *Diabetes/Metabolism Research and Reviews* 25(1): 41–49.

Weir GC, Bonner-Weir S. 2007. “A Dominant Role for Glucose in β Cell Compensation of Insulin Resistance.” *Journal of Clinical Investigation* 117(1): 81–83.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. 2003. “Obesity Is Associated with Macrophage Accumulation in Adipose Tissue.” *Journal of Clinical Investigation* 112(12): 1796–1808.

Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW. 1995. “Mechanism of Autoxidative Glycosylation: Identification of Glyoxal and Arabinose as Intermediates in the Autoxidative Modification of Proteins by Glucose.” *Biochemistry* 34(11): 3702–9.

West NR, Watson PH. 2010. “S100A7 (Psoriasin) Is Induced by the Proinflammatory Cytokines Oncostatin-M and Interleukin-6 in Human Breast Cancer.” *Oncogene* 29(14): 2083–92.

Westermark P, Andersson A, Westermark GT. 2011a. “Islet Amyloid Polypeptide, Islet Amyloid, and Diabetes Mellitus.” *Physiological reviews* 91(3): 795–826.

———. 2011b. “Islet Amyloid Polypeptide, Islet Amyloid, and Diabetes Mellitus.”

Physiological Reviews 91(3): 795–826.

Westwell-Roper CY, Chehroudi CA, Denroche HC, Courtade JA, Ehses JA, Verchere CB. 2014. “IL-1 Mediates Amyloid-Associated Islet Dysfunction and Inflammation in Human Islet Amyloid Polypeptide Transgenic Mice.” *Diabetologia* 58(3): 575–85.

Wheeler E, Barroso I. 2011. “Genome-Wide Association Studies and Type 2 Diabetes.” *Briefings in Functional Genomics* 10(2): 52–60.

Wheeler LC, Harms MJ. 2017. “Human S100A5 Binds Ca2+ and Cu2+ Independently.” *BMC Biophysics* 10(8).

Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy MA, Simonson DC, Creager MA. 1998. “Acute Hyperglycemia Attenuates Endothelium-Dependent Vasodilation in Humans in Vivo.” *Circulation* 97(17): 1695–1701.

Winnepenninckx V, Lazar V, Michiels S, Dessen P, Stas M, Alonso SR, Avril MF, Ortiz Romero PL, Robert T, Balacescu O, Eggermont AM, Lenoir G, Sarasin A, Tursz T, van den Oord JJ, Spatz A. 2006. “Gene Expression Profiling of Primary Cutaneous Melanoma and Clinical Outcome.” *Journal of the National Cancer Institute* 98(7): 472–82.

Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF. 1998. “Disruption of IRS-2 Causes Type 2 Diabetes in Mice.” *Nature* 391(6670): 900–904.

Wojtczak L, Schönfeld P. 1993. “Effect of Fatty Acids on Energy Coupling Processes in Mitochondria.” *BBA - Bioenergetics* 1183(1): 41–57.

Wolf R, Lewerenz V, Büchau AS, Walz M, Ruzicka T. 2007. “Human S100A15 Splice Variants Are Differentially Expressed in Inflammatory Skin Diseases and Regulated through Th1 Cytokines and Calcium.” *Experimental Dermatology* 16(8): 685–91.

Wolf R, Ruzicka T, Yuspa SH. 2011. “Novel S100A7 (Psoriasin)/S100A15 (Koebnerisin) Subfamily: Highly Homologous but Distinct in Regulation and Function.” *Amino Acids* 41(4): 789–96.

Wolff SP, Dean RT. 1987. “Glucose Autoxidation and Protein Modification. The Potential Role of ‘Autoxidative Glycosylation’ in Diabetes.” *Biochemical Journal* 245(1): 243–50.

Wolpin BM, Meyerhardt JA, Chan AT, Ng K, Chan JA, Wu K, Pollak MN, Giovannucci EL, Fuchs CS. 2009. “Insulin, the Insulin-like Growth Factor Axis, and Mortality in Patients with Nonmetastatic Colorectal Cancer.” *Journal of Clinical Oncology* 27(2): 176–85.

Wu S, McCormick JB, Curran JE, Fisher-Hoch SP. 2017. “Transition from Pre-Diabetes to Diabetes and Predictors of Risk in Mexican-Americans.” *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 10: 491–503.

Wu S, Zhu W, Thompson P, Hannun YA. 2018. “Evaluating Intrinsic and Non-Intrinsic Cancer Risk Factors.” *Nature Communications* 9(1): 3490.

Du X, Matsumura T, Edelstein D, Rossetti L, Zsengellér Z, Szabó C, Brownlee M. 2003. “Inhibition of GAPDH Activity by Poly (ADP-Ribose) Polymerase Activates Three Major Pathways of Hyperglycemic Damage in Endothelial Cells.” *Journal of Clinical Investigation* 112(7): 1049–57.

Xia C, Braunstein Z, Toomey AC, Zhong J, Rao X. 2018. “S100 Proteins as an Important Regulator of Macrophage Inflammation.” *Frontiers in Immunology* 8: 1908.

Xia Y, Pauza ME, Feng L, Lo D. 1997. “RelB Regulation of Chemokine Expression Modulates Local Inflammation.” *The American journal of pathology* 151(2): 375–87.

Xin Y, Kim J, Okamoto H, Ni M, Wei Y, Adler C, Murphy AJ, Yancopoulos GD, Lin C, Gromada J. 2016. “RNA Sequencing of Single Human Islet Cells Reveals Type 2 Diabetes Genes.” *Cell Metabolism* 24(4): 608–15.

Xu X, Su B, Xie C, Wei S, Zhou Y, Liu H, Dai W, Cheng P, Wang F, Xu X, Guo C. 2014. “Sonic Hedgehog-Gli1 Signaling Pathway Regulates the Epithelial Mesenchymal Transition (EMT) by Mediating a New Target Gene, S100A4, in Pancreatic Cancer Cells.” *PLoS ONE* 9(7): e96441.

Yagihashi S. 2012. “Clinical Staging of Type 2 Diabetes: The Time Has Come.” *Journal of Diabetes Investigation* 3(1): 1–2.

Yammani RR, Carlson CS, Bresnick AR, Loeser RF. 2006. “Increase in Production of Matrix Metalloproteinase 13 by Human Articular Chondrocytes Due to Stimulation with S100A4: Role of the Receptor for Advanced Glycation End Products.” *Arthritis and Rheumatism* 54(9): 2901–11.

Yan SF, Ramasamy R, Schmidt AM. 2009. “Receptor for AGE (RAGE) and Its Ligands-Cast into Leading Roles in Diabetes and the Inflammatory Response.” *Journal of Molecular Medicine* 87(3): 235–47.

Yang JS, Lu CC, Kuo SC, Hsu YM, Tsai SC, Chen SY, Chen YT, Lin YJ, Huang YC, Chen CJ, Lin WD, Liao WL, Lin WY, Liu YH, Sheu JC, Tsai FJ. 2017. “Autophagy and Its Link to Type II Diabetes Mellitus.” *BioMedicine* 2(8).

Yang X, Feng L, Li C, Li Y. 2014. “Tranilast Alleviates Endothelial Dysfunctions and Insulin Resistance via Preserving Glutathione Peroxidase 1 in Rats Fed a High-Fat Emulsion.” *Journal of Pharmacological Sciences* 124(1): 18–30.

Yang Z, Tao T, Raftery MJ, Youssef P, Di Girolamo N, Geczy CL. 2001. “Proinflammatory Properties of the Human S100 Protein S100A12.” *Journal of leukocyte biology* 69(6): 986–94.

Yang Z, Yan WX, Cai H, Tedla N, Armishaw C, Di Girolamo N, Wang HW, Hampartzoumian T, Simpson JL, Gibson PG, Hunt J, Hart P, Hughes JM, Perry MA, Alewood PF, Geczy CL. 2007a. “S100A12 Provokes Mast Cell Activation: A Potential Amplification Pathway in Asthma and Innate Immunity.” *Journal of Allergy and Clinical Immunology* 119(1): 106–14.

———. 2007b. “S100A12 Provokes Mast Cell Activation: A Potential Amplification Pathway in Asthma and Innate Immunity.” *Journal of Allergy and Clinical Immunology* 119(1): 106–14.

Yao D, Brownlee M. 2010. “Hyperglycemia-Induced Reactive Oxygen Species Increase Expression of the Receptor for Advanced Glycation End Products (RAGE) and RAGE Ligands.” *Diabetes* 59(1): 249–55.

Yao R, Lopez-Beltran A, Maclennan GT, Montironi R, Eble JN, Cheng L. 2007. “Expression of S100 Protein Family Members in the Pathogenesis of Bladder Tumors.” *Anticancer Research* 27(5A): 3051–58.

Yoshida H, Matsui T, Hosokawa N, Kaufman RJ, Nagata K, Mori K. 2003. “A Time-Dependent Phase Shift in the Mammalian Unfolded Protein Response.” *Developmental cell* 4(2): 265–71.

Yu H, Pardoll D, Jove R. 2009. “STATs in Cancer Inflammation and Immunity: A Leading Role for STAT3.” *Nature Reviews Cancer* 9(11): 798–809.

Yu J, Zhou X, Nakaya M, Jin W, Cheng X, Sun SC. 2014. “T Cell-Intrinsic Function of the Noncanonical NF- B Pathway in the Regulation of GM-CSF Expression and Experimental Autoimmune Encephalomyelitis Pathogenesis.” *The Journal of Immunology* 193(1): 422–30.

Yu T, Tang B, Sun X. 2017. “Development of Inhibitors Targeting Hypoxia-Inducible Factor 1 and 2 for Cancer Therapy.” *Yonsei Medical Journal* 58(3): 489–96.

Yu X, Mao W, Zhai Y, Tong C, Liu M, Ma L, Yu X, Li S. 2017. “Anti-Tumor Activity of Metformin: From Metabolic and Epigenetic Perspectives.” *Oncotarget* 8(3): 5619–28.

Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE. 2001. “Reversal of Obesity- and Diet-Induced Insulin Resistance with Salicylates or Targeted Disruption of Ikkβ.” *Science* 293(5535): 1673–77.

Zakaria R, Platt-Higgins A, Rathi N, Crooks D, Brodbelt A, Chavredakis E, Lawson D, Jenkinson MD, Rudland PS. 2016. “Metastasis-Inducing Proteins Are Widely Expressed in Human Brain Metastases and Associated with Intracranial Progression and Radiation Response.” *British Journal of Cancer* 114(10): 1101–8.

Zeisberg M, Neilson EG. 2009. “Biomarkers for Epithelial-Mesenchymal Transitions.” *Journal of Clinical Investigation* 119(61429–1437).

Zhang G, Li M, Jin J, Bai Y, Yang C. 2011. “Knockdown of S100A4 Decreases Tumorigenesis and Metastasis in Osteosarcoma Cells by Repression of Matrix Metalloproteinase-9.” *Asian Pac J Cancer Prev* 12(8): 2075–80.

Zhang J, Zhang K, Jiang X, Zhang J. 2014. “S100A6 as a Potential Serum Prognostic Biomarker and Therapeutic Target in Gastric Cancer.” *Digestive Diseases and Sciences* 59(9): 2136–44.

Zhang K, Yu M, Hao F, Dong A, Chen D. 2016. “Knockdown of S100A4 Blocks Growth and Metastasis of Anaplastic Thyroid Cancer Cells in Vitro and in Vivo.” *Cancer Biomarkers* 17(3): 281–91.

Zhang R, Fu H, Chen D, Hua J, Hu Y, Sun K, Sun X. 2010. “Subcellular Distribution of S100A4 and Its Transcriptional Regulation under Hypoxic Conditions in Gastric Cancer Cell Line BGC823.” *Cancer Science* 101(5): 1141–46.

Zhang W, Wu Y, Yan Q, Ma F, Shi X, Zhao Y, Peng Y, Wang J, Jiang B. 2014. “Deferoxamine Enhances Cell Migration and Invasion through Promotion of HIF-1α Expression and Epithelial-Mesenchymal Transition in Colorectal Cancer.” *Oncology Reports* 31(1): 111–16.

Zhang Y, Zhou J, Wang T, Cai L. 2007. “High Level Glucose Increases Mutagenesis in Human Lymphoblastoid Cells.” *International Journal of Biological Sciences* 3(6): 375–79.

Zhao S, Fung-Leung WP, Bittner A, Ngo K, Liu X. 2014. “Comparison of RNA-Seq and

Microarray in Transcriptome Profiling of Activated T Cells." *PLoS ONE* 9(1): e78644.

Zheng M, Tang L, Huang L, Ding H, Liao WT, Zeng MS, Wang HY. 2010. "Overexpression of Karyopherin-2 in Epithelial Ovarian Cancer and Correlation with Poor Prognosis." *Obstetrics and Gynecology* 116(4): 884–91.

Zhou L, Cai X, Han X, Ji L. 2014. "P38 Plays an Important Role in Glucolipotoxicity-Induced Apoptosis in INS-1 Cells." *Journal of Diabetes Research* 2014: 834528.

Zhou M, Li ZQ, Wang ZL. 2015. "S100A4 Upregulation Suppresses Tissue Ossification and Enhances Matrix Degradation in Experimental Periodontitis Models." *Acta Pharmacologica Sinica* 36(11): 1388–94.

Zhou YP, Grill VE. 1994. "Long-Term Exposure of Rat Pancreatic Islets to Fatty Acids Inhibits Glucose-Induced Insulin Secretion and Biosynthesis through a Glucose Fatty Acid Cycle." *Journal of Clinical Investigation* 93(2): 870–76.

Zimmer DB, Eubanks JO, Ramakrishnan D, Criscitiello MF. 2013. "Evolution of the S100 Family of Calcium Sensor Proteins." *Cell Calcium* 53(3): 170–79.

Appendix 1

S100 protein	Other names	Expression	Function	Interactions	Associated pathologies	Regulation	References
S100A1	S100 alpha	Skeletal muscle fibres, cardiomyocytes and certain neuronal populations.	Extracell: internalized into neurons and delivered to endosomes, Golgi and lysosomes. Enhances Ca^{2+} influx in cardiomyocytes. Intracell: associates with cytoskeletal components, interacts with SR Ca^{2+}-ATPase and RyR2 in the heart, improving contractile performance.	Extracell: RAGE. Intracell: SERCA, RyR1 & 2, Fructose-bisphosphate aldolase	S100A1 deficiency results in abnormal SR Ca^{2+} content and fluxes, deterioration of cardiac performance and heart failure	Inhibitory transcription factors downstream of GPCRs and PKC	Rohde *et al.* 2010, Hernández-Ochoa *et al.* 2009, Reppel *et al.* 2005
S100A2	S100L, CAN19	Urothelium, respiratory, gastrointestinal and squamous epithelium.	Extracell: chemotactic factor for eosinophils. Intracell: binds to p53 and potentiates tumour-suppressing activity.	Extracell: RAGE.	Downregulated in many cancers, but upregulated in others	-	van Dieck *et al.* 2009, Komada *et al.* 1996
S100A3	S100E	Hair root cells and some astrocytomas	Epithelial cell differentiation and Ca^{2+}-dependent hair cuticular barrier formation.	RARα	Involved in HCC tumorigenesis and tumour aggressiveness	-	Kizawa *et al.* 2008, Tao *et al.* 2017, Gianni *et al.* 2018
S100A4	Metastasin1 (Mts1), Calvasculin	Tumour and stromal cells, myeloid cells, adipocytes, fibroblasts, immunocytes, vascular cells.	Extracell: key role in tumour cell survival and metastasis. Activates NF-κB, inducing production of pro-inflammatory cytokines and migration of neutrophils, monocytes, and macrophages. Activates ERK1/2, modulating growth and survival. Intracell: induces MMP expression and interacts with cytoskeletal proteins NMIIA, tropomyosin and actin to promote cell migration.	Extracell: RAGE, EGFR, Gαq-coupled receptor. Intracell: NMIIA, tropomyosin, actin, p53, S100A1, annexin2	Upregulated in many cancers	Upregulated by β-catenin/T-cell factor complex	Boye *et al.* 2010, Stein *et al.* 2006a, Kiryushko et al 2006
S100A5	S100D	-	-	RAGE	Upregulated in bladder cancer and recurrent grade I meningiomas	-	Yao *et al.* 2007
S100A6	Calcyclin (CACY)	Epithelial cells, fibroblasts and different kinds of cancer cells	Extracell: activates RAGE and promotes apoptosis and generation of ROS. Stimulates insulin release from pancreatic islet cells. Intracell: interacts with caldesmon, calponin, tropomyosin and kinesin to modulate cell proliferation, cytoskeletal dynamics and tumorigenesis.	Extracell: RAGE	Overexpressed in AT.	-	Leśniak *et al.* 2009
S100A7	Psoriasin1 (PSOR1)	Keratinocytes	Extracell: signals through RAGE to activate NF-κB, inducing production of pro-inflammatory cytokines and migration of neutrophils, monocytes, and macrophages. Intracell: promotes aggressive features in breast cancer by stimulating Akt and NF-κB.	Extracell: RAGE	Overexpression induces leukocyte infiltration linked to inflammatory skin diseases such as psoriasis.	Upregulated in breast cancer by proinflammatory cytokines and in keratinocytes by IL-17, IL-22 and flagellin.	Emberley *et al.* 2005, West *et al.* 2010

S100A8	Calgranulin-A (CAGA), Calprotectin 1L1	Macrophages, dendritic cells, microvascular endothelial cells, epithelial cells and fibroblasts upon activation	Extracell: regulates inflammation. Chemotactic factor for neutrophils. Induces cell differentiation and TNF-α and IL-1β production in myeloid cells. Scavenges intracellular ROS and stabilizes NO in neutrophils, protecting from oxidative damage in inflammatory lesions. Intracell: stimulates keratinocyte differentiation and exerts anti-inflammatory effects.	Extracell: GPCR, TLR4, Scavenger receptor CD36. Intracell: telomerase	Overexpressed in inflammatory and autoimmune conditions.	Induced by pro-inflammatory stimuli, TLR agonists and oxidative stress in an IL-10-dependent manner.	Hsu *et al.* 2012, Lagasse *et al.* 1992, Lim *et al.* 2014, Averill *et al.* 2012
S100A9	Calgranulin-B (CAGB), Calprotectin L1H	Monocytes, neutrophils and dendritic cells; Fibroblasts, mature macrophages, vascular endothelial cells and keratinocytes upon activation	Extracell: involved in leukocyte migration, chemotactic for neutrophils. Induces TNF-α, IL-1β, IL-6 and IL-8 in macrophages via NF-κB activation. Intracell: inhibits myeloid differentiation and accumulation of myeloid-derived suppressor cells via ROS generation, contributing to tumour growth. Regulates S100A8/S100A9 activities.	Extracell: RAGE, TLR4, Scavenger receptor CD36	Anti-inflammatory in healthy state, while oxidative stress activates its pro-inflammatory functions. Contributes to the pathogenesis of autoimmune diseases.	Upregulated by oxidative stress, corticosteroids, cytokines and growth factors.	Hsu *et al.* 2012, Vogl *et al.* 2004, Ryckman *et al.* 2003, Sunahori *et al.* 2006
S100A8/ S100A9	Calprotectin	Monocytes, neutrophils and dendritic cells; Fibroblasts, mature macrophages, vascular endothelial cells and keratinocytes upon activation	Extracell: anti-microbial properties. Chemotactic for neutrophils. Regulates inflammation, cell proliferation, differentiation and tumour development via NF-κB-mediated pro-inflammatory cytokine production in monocytes and macrophages. Intracell: inhibits myeloid cell differentiation. Facilitates FA transport. Cytoplasmic Ca^{2+}sensor linking Ca^{2+}influx to phagosomal ROS production. Induces microtubule polymerization and F-actin cross-linking.	Extracell: RAGE, Scavenger receptor	Overexpression promotes resistance to TNF-α-induced apoptosis and induces malignant progression through ROS production. Mediates differentiation of psoriatic keratinocytes. Overexpressed in atherosclerotic lesions and cardiovascular events.	Regulated through an autoinhibitory process resulting in restriction of inflammation.	Steinckwich *et al.* 2011, Németh *et al*. 2009, *Averill et al.* 2012, Vogl *et al.* 2018
S100A10	Calpactin-1 (CAL-1L)	Macrophages	Regulator of cellular plasmin production: plasminogen receptor, mediates macrophage recruitment into tumour sites in response to inflammatory stimuli. Bound to annexin 2, serves as binding scaffold for pathogens and host proteins, assisting their trafficking and anchorage to the plasma membrane. Plays important roles in angiogenesis and endothelial cell function.	Annexin2, serotonin 1B receptor	Downregulated in depressive-like states. Implicated in the action of antidepressant drugs and electroconvulsive seizures due to its interaction with serotonin receptors.	Induced by EGF, TGF-α, IFN-γ, NGF, KGF, RA and thrombin, and by the oncogenes PML-RARα and Kras.	Rescher *et al.* 2008, Warner-Schmidt *et al.* 2010, Surette *et al.* 2011, Madureira *et al.* 2012
S100A11	S100C, Calgizzarin	Chondrocytes, luteal cells, oviductal epithelial cells	Extracell: promotes chondrocyte hypertrophic differentiation and stimulates RAGE-dependent type X collagen and IL-8 production. Intracell: When phosphorylated by PKC-α, Ca^{2+}-bound S100A11 inhibits cell growth through activation of the cell cycle modulator p21WAF1/CIP1.	Extracell: RAGE. Intracell: Nucleolin, Rad54B	Signal through RAGE to activate p38 MAPK, accelerating chondrocyte hypertrophy and matrix catabolism to promote osteoarthritis progression.	Induced/released by chondrocytes exposed to IL-1β, TNF-α, and CXCL8	Murzik *et al*. 2008, Cecil *et al.* 2008.

S100A12	Calgranulin-C (CAGC)	Constitutively expressed in neutrophils, monocytes and early macrophages. Induced in endothelial and epithelial cells and proinflammatory macrophages under inflammatory condition.	Extracell: Activates NF-κB, inducing production of pro-inflammatory cytokines, TNF-α, and chemokines for neutrophil, monocyte and lymphocyte recruitment. Intracell: modulates interactions between cytoskeletal elements and membranes. Inhibits aggregation of aldolase and GAPDH.	Extracell: RAGE, GPCR, Scavenger receptor. Intracell: Aldolase, Nox-1.	Expression in epithelial cells is associated with growth arrest. Overexpression causes VSMC dysfunction and aortic aneurysms, linked to leukocyte influx.	TNF-α, IL-6 and endotoxin induce its expression in monocytes/macrophages; LPS in smooth muscle cells.	Vogl *et al.* 1999, Yang *et al.* 2001, Yang *et al.* 2007, Hofmann *et al.* 2010
S100A13	-	Fibroblasts, osteoblasts and melanoma cells	Involved in stress-induced release of FGF-1 and IL-1α from several cell types. Promotes its own intracellular translocation, possibly via RAGE signalling. Plays a key role in tumour growth, angiogenesis and metastasis.	Extracell: RAGE. Intracell: FGF-1, p40 Syt1	Overexpression associated with high intratumoral angiogenesis and poor prognosis in patients with stage I NSCLC.	Induced by FGF1 and IL-1α upon intracellular stress conditions.	Landriscina *et al.* 2001, Hsieh *et al.* 2004, Miao *et al.* 2018
S100A14	-	Lymphocytes, epithelial cells	Extra: at low doses stimulates proliferation, at high doses stimulates apoptosis in ESCC cells via RAGE signalling. Intracell: may function as a cancer suppressor affecting the p53 pathway and modulating expression of MMP1, MMP2 and MMP9.	Extracell: RAGE. Intracell: p53	Ectopic overexpression promotes motility and invasiveness of ESCC cells.	Induced by EGF through p-ERK signalling pathway in breast cancer cells	Chen *et al.* 2009, Sapkota *et al.* 2011, Jin *et al*. 2011
S100A15	S100A7A	Keratinocytes in inflamed skin	Putative functional role in innate immunity, epidermal cell maturation, and epithelial tumorigenesis. Acts as chemotactic factor for monocytes and granulocytes. Acts synergistically with S100A7 in leukocyte recruitment *in vitro* and *in vivo.*	GPCR	Potential therapeutic target for various human disorders including arthritis, cancer, and AD.	Induced by LPS, IL-1β and Th-1 cytokines	Wolf *et al.* 2011, Wolf *et al.* 2007, Gläser *et al.* 2009
S100A16	S100F	Astrocytes, preadipocytes	Acts as a novel adipogenesis-promoting factor, affecting negatively insulin sensitivity.	p53	Upregulated in several tumours	Increased expression in AT of diet-induced obese mice	Sturchler *et al.* 2006, Liu *et al*. 2011
S100B	-	Astrocytes, Schwann cells, melanocytes, chondrocytes, adipocytes, skeletal myofibers, certain dendritic cell and lymphocyte populations	Extracell: Released from astrocytes, signals through RAGE. At low concentrations stimulates proliferation through ERK1/2 and NF-κB-mediated upregulation of Bcl-2. At high concentrations promotes inflammatory activities and kills neurons through ROS production. Intracell: interacts with nuclear NDR kinase and blocks recruitment of its substrates. May maintain cell proliferation with beneficial effects during development and tissue regeneration, and detrimental effects during tumorigenesis.	Extracell: RAGE, FGFR. Intracell: Tubulin, actin-binding proteins, annexin 6, Rac1, SRC kinase, NDR kinase, p53, intermediates upstream of IKKβ/NF-κB.	Involved in brain, cartilage and muscle repair, activation of astrocytes in brain damage and neurodegenerative processes, cardiomyocyte remodelling after infarction and in melanomagenesis and gliomagenesis.	NF-κB, EGF and IFN-γ regulate S100B expression in several cell types.	Donato *et al.* 2009, Sorci *et al.* 2011, Liu *et al.* 2011, Mori *et al.* 2010
S100G	Calbindin-D9K (CABP9K)	Epithelial cells	Acts as cytosolic Ca^{2+} buffer in many tissues, resulting in modulation of Ca^{2+} adsorption.	-	-	-	Luu *et al.* 2004

S100P	S100E	Lymphocytes, epithelial cells	Extracell: mediates tumour growth and cancer cell drug resistance through RAGE signalling. Intracell: promotes transendothelial migration of tumour cells through a decrease in focal adhesion sites.	Extracell: RAGE. Intracell: ezrin/radixin/moesin, IQGAP1	Overexpressed in clinically isolated tumours, associated with metastasis, drug resistance, and poor clinical outcome.	-	Austermann *et al.* 2008, Heil *et al.* 2011, Arumugam *et al.* 2011
S100Z	-	Lymphocytes	-	-	Downregulated in several tumours	-	Gribenko *et al.* 2001