The KINGS mouse as a model of beta cell endoplasmic reticulum (ER) stress and sex differences in diabetes.

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The KINGS mouse as a model of beta cell endoplasmic reticulum (ER) stress and sex differences in diabetes.

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ABBREVIATIONS:

17-beta-HSD1	17-beta-hydroxysteroid dehydrogenase 1
AAC	Area above the curve
ABCC8	ATP binding cassette subfamily C member 8
ADP	Adenosine monophosphate
ADT	Androgen deprivation therapy
ANOVA	Analysis of variance
AR	Androgen receptor
ASK-1	Apoptosis signal-regulating kinase 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
AU	Arbitrary unit
AUC	Area under the curve
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 Associated X-protein

BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BiP/GRP78	Binding immunoglobulin protein/ 78 kDa glucose-regulated protein
BMI	Body mass index
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
cAMP	Cyclic adenosine monophosphate
CD20	Cluster of differentiation 20
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cGMP	Cyclic guanosine monophosphate
CHO	Chinese hamster ovary
CHOP	C/EBP homologous protein
COPII	Cytosolic coat protein II
CPM	Counts per minute
CVB	Coxsackievirus B
DHT	Dihydrotestosterone
dNTP	Deoxynucleotide triphosphate
DPP-4	Dipeptidyl peptidase-4
DR4 and 5	Death receptor 4 and 5
DTT	Dithiothreitol
E1	Estrone
E2	Oestradiol
E3	Estriol
ETC	Electron transport chain
eIF2-alpha	Eukaryotic translation initiation factor 2 alpha
EIF2AK3	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3
ELISA	Enzyme-linked immunosorbent assay
ENU	N-ethyl-N-nitrosourea
ER	Endoplasmic reticulum

ER- alpha and beta	Oestrogen receptor alpha and beta
ERAD	ER associated degradation
ERE	Estrogen response elements
ERK	Extracellular signal-regulated kinases
ERO1-alpha	ER oxidoreductase 1 alpha
FBG	Fasted blood glucose
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FFA	Free fatty acid
FOXO1	Forkhead box protein O1
FOXP3	Forkhead box P3
FSH	Follicle stimulating hormone
g	Gram
GAD65	Glutamic acid decarboxylase 65
GADD34	Growth arrest and DNA damage-inducible protein 34
GC-A	Guanylyl cyclase A
GCK	Glucokinase
GDM	Gestational diabetes mellitus
GFP	Green fluorescent protein
GH	Growth hormone
GLP1	Glucagon-like peptide 1
GLS1 and GLS2	Golgi-localisation sequences 1 and 2
GLUT	Glucose transporter protein
GPER	G protein-coupled oestrogen receptor
GSIS	Glucose stimulated insulin secretion
GTT	Glucose tolerance test
GWAS	Genome wide associated studies
H&E	Haematoxylin and eosin
HFHS	High fat high sucrose
hIAPP	Human islet amyloid polypeptide
HLA	Human leukocyte antigens

HNF-1A	Hepatic nuclear factor 1 homeobox A
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
IA2	Insulinoma associated protein 2
IAPP	Islet amyloid polypeptide
ICR	Institute of Cancer Research
IF	Immunofluorescence
IFN-gamma	Interferon gamma
IL-1 alpha	Interleukin-1 alpha
INS1	INS1 Rat insulinoma cell line
IP_3R	Inositol-1, 4,5-trisphosphate receptor
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
iPSC	Induced pluripotent stem cells
IRE1	Inositol requiring enzyme 1
IRS2	Insulin receptor substrate 2
ITT	Insulin tolerance test
IU	International unit
JNK	c-Jun N-terminal kinase
KASP	Kompetitive Allele specific PCR
KATP	ATP sensitive potassium channel
KCNJ11	Potassium inwardly rectifying channel subfamily J member 11
Kg	Kilogram
KINGS	KCLIns2G32S
Kir6.2	Potassium subunit of the KATP channel
KO	Knockout
L-F1KO	Liver-specific Foxo1 knockout
LH	Luteinising hormone
MAFA	MAF BZIP Transcription Factor A
MAMs	Mitochondrial associated membranes
Mcl-1	Myeloid cell leukemia-1
MEM	Minimum essential media
MGI	Mouse genome informatics

MHC	Major histocompatibility complex
MIDY	Mutant INS-gene-induced Diabetes of Youth
MIN6	Mouse insulinoma 6 cell line
miR-17	MicroRNA-17
ml	Millilitre
mM	Millimolar
MODY	Monogenic diabetes of the young
mRNA	Messenger ribonucleic acid
mTORC1	Mammalian target of rapamycin complex 1
mV	Millivolts
NC	Normal chow
NCS	New-born calf serum
NDM	Neonatal diabetes mellitus
NeuroD1	Neuronal differentiation 1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFBG	Non-fasted blood glucose
Nkap	NF-kB activator protein
Nkx6.1	NK6 homeobox 1
NOD	Non-obese diabetic
Oma1	Overlapping proteolytic activity with m-AAA protease 1
OVX	Ovariectomy
PBA	Phenyl butyric acid
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
PDX1	Pancreatic and duodenal homeobox 1
PERK	Protein kinase R- like endoplasmic reticulum kinase
PKA	Protein kinase A
PKG	Protein kinase G
PP	Pancreatic polypeptide
PPIases	Prolyl peptidyl cis-trans isomerases
PVDF	Polyvinylidene fluoride

RIA	Radioimmunoassay
RIDD	Regulated IRE1 dependent decay of mRNAs
RIP-LCMV-GP	RIP lymphocytic choriomeningitis virus glycoprotein
RIPA	Radioimmunoprecipitation assay
RNase	Ribonuclease
RNAseq	RNA sequencing
RNS	Reactive nitrogen species
ROI	Region of interest
ROS	Reactive oxygen species
S1P and S2P	Site 1 and 2 proteases
SDS	Laemmli sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERCA	Sarcoendoplasmic reticulum calcium ATPase
SERCA2b	Sarcoendoplasmic reticulum pump Ca2+ ATPase2b
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
SP	Signal peptide
SRP	Signal recognition particle
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline-tween
TCA	Tricarboxylic acid cycle
TNF-alpha	Tumour necrosis factor alpha
TRAF2	TBF receptor associated factor 2
TRB3	tribbles related 3
Tregs	T regulatory cells
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential cation channel subfamily V member 1
TUDCA	Tauroursodeoxycholic Acid

TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TXNIP	Thioredoxin-interacting protein
UPR	Unfolded protein response
uPRFs	Upstream open reading frames
WRS	Wolcott-Rallison syndrome
WT	Wildtype
XBP1	X-box binding protein 1
XBP1s	Spliced X-box binding protein 1
ZnT8	Zinc-transporter-8

LAY ABSTRACT:

Diabetes is a disease where the hormone responsible for storing glucose properly (insulin) is either not working or is absent, leading to high blood glucose levels. This can occur when the insulin-producing beta cells in the pancreas die or lose their function which may be in response to stress. At least 1 in 11 people have diabetes worldwide and it can lead to serious health complications, such as blindness and kidney damage.

Sex differences exist in diabetes, with pre-menopausal women less likely to develop diabetes compared to men. Some studies have suggested that this may be because female beta cells are better able to cope with stress. We have characterised a new mouse model (the KINGS mouse) that has a specific form of beta cell stress called 'endoplasmic reticulum stress' and that also shows a sex difference- males become diabetic but females do not. In this study, we used this model to test whether sex differences exist in response to beta cell stress and what factors can alleviate this stress and prevent diabetes in the males.

We found that females respond more favourably to beta cell stress and continue to be protected from diabetes even when this stress is worsened. We also found that sex hormones play some role in causing sex differences in beta cell stress and that certain drugs can prevent diabetes in male KINGS mice.

The results from this study are important because they provide more information about why sex differences exist in diabetes which may lead to more sex-tailored therapies.

ABSTRACT:

Background: The KINGS mouse is a novel model of beta cell endoplasmic reticulum (ER) stress which shows stark sex differences in diabetes, with males developing overt and progressive hyperglycaemia whilst females are protected. Beta cell ER stress has been implicated in many types of diabetes and underpins numerous factors known to drive beta cell failure. Sex differences also exist in diabetes in humans with premenopausal women having a lower diabetes incidence compared to men. Further characterisation of the KINGS mice may provide valuable insight into these phenomena.

Aims: The objectives of this thesis were to 1) further characterise beta cell ER stress and associated cellular response in the KINGS mice, 2) investigate the influence of sex hormones and beta cell ER stress manipulation on glycaemic control in the KINGS mice and 3) investigate whether diabetes development can be prevented in the male KINGS mice.

Methods: Western blotting and immunofluorescent staining were used to investigate the expression of ER stress and unfolded protein response (UPR) markers in KINGS islets, as well as beta cell turnover and mass. To determine the influence of oestradiol on the KINGS phenotype, endogenous oestradiol was removed from female mice via ovariectomy, and exogenous oestradiol was delivered to male KINGS mice through implantation of oestradiol-containing capsules. A western diet was used to exacerbate beta cell ER stress in female KINGS mice, whilst liraglutide administration, TUDCA administration and removal of endogenous testosterone (via orchidectomy) was used in an attempt to reduce ER stress and prevent diabetes in the male KINGS mice. For all *in vivo* studies, glycaemic control was assessed through blood glucose concentration monitoring, glucose tolerance testing and insulin tolerance testing.

Results: Male KINGS mice developed diabetes by 5-6 weeks of age whereas female KINGS mice were protected, in line with previous studies. Protein

markers of ER stress and the UPR were observed in KINGS islets from 4 weeks of age and a sex difference was observed in expression profiles with males largely showing an increased expression of markers. Despite this, we did not observe a loss of beta cell mass in either male or female KINGS mice. However, subtle changes in beta cell proliferation and apoptosis in the male KINGS mice are suggestive of mild changes to beta cell turnover which may contribute to diabetes development.

A western diet exacerbated beta cell ER stress in female KINGS mice, however this only led to a mild impairment in glycaemic control which was not as severe as that seen in male KINGS mice. This may suggest that even under conditions of further ER stress, female mice are still able to respond adaptively. Removal of endogenous oestradiol also exacerbated beta cell ER stress, however again this was only associated with a subtle impairment in glycaemic control. On the contrary, exogenous oestradiol delivery in the male KINGS mice prevented the development of overt diabetes.

Treatment with liraglutide was used in an attempt to alleviate ER stress in the male KINGS mice. Although liraglutide prevented the development of diabetes and reduced blood glucose concentrations once diabetes was established, this protection only lasted during the treatment window and cessation of treatment was associated with increases in blood glucose concentrations. In addition, liraglutide had no effect on beta cell ER stress levels. Treatment with TUDCA, a chemical chaperone previously found to reduce beta cell ER stress, had no impact on blood glucose concentrations in the KINGS mice. However, removal of endogenous testosterone through orchidectomy prevented the development of overt diabetes.

Conclusion: In this study we have confirmed that the KINGS mutation drives beta cell ER stress and that sex differences exist in beta cell response to this. Interestingly, an adaptive response to beta cell ER stress was still maintained in female KINGS mice when ER stress was exacerbated through a western diet. We also found that whilst oestradiol likely contributes in-part to sex differences in diabetes, it cannot be the sole mediator and other factors must

be involved. Indeed, we found that endogenous testosterone removal prevented the development of diabetes in male mice. Liraglutide treatment also prevented diabetes development in male mice, however this was likely to be mediated through mechanisms unrelated to beta cell ER stress. Further study is required to investigate how testosterone removal and liraglutide protect male mice.

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I dedicate this thesis to my dad who I wish could have been there to share this journey with me.

CHAPTER 1: GENERAL INTRODUCTION:

1.1 BLOOD GLUCOSE HOMEOSTASIS:

1.1.1 MECHANISMS REGULATING BLOOD GLUCOSE CONCENTRATIONS:

Glucose represents the primary substrate for energy production in most cells, however, maintenance of its concentration in the blood within a narrow range (4-6mM) is paramount since hypoglycaemia and hyperglycaemia are associated with a variety of health complications and can have lethal consequences (Giri et al., 2018; Kalra et al., 2013; Mathew et al., 2023). The regulation of blood glucose homeostasis is complex and is orchestrated by the neurohormonal system which involves glucoregulatory hormones and neuropeptides predominantly derived from the pancreas, brain, adipose, muscle and intestine (Röder et al., 2016). The pancreas is a significant component of this network, responsible for the synthesis of insulin and glucagon from the islets of Langerhans which have opposing and balanced actions to maintain normoglycemia (Göke, 2008).

The islets of Langerhans are comprised of clusters of endocrine cells and are dispersed throughout the exocrine tissue of the pancreas, contributing to 1-2% of the total organ mass (Röder et al., 2016). Among the five types of endocrine cell present in the islets, insulin secreting beta cells are the most abundant, making up ~60% of the islet in humans and ~80% in mice (Brissova et al., 2005). Glucagon-producing alpha cells contribute 15-20%, somatostatin-producing delta cells contribute 3-10% and epsilon cells and PP cells, which produce ghrelin and pancreatic polypeptide (PP) respectively, make up 3-6% (Brissova et al., 2005). Organisation of these cell types within islets is species specific (Brissova et al., 2005; Steiner et al., 2010). In mice, islet cytoarchitecture is highly structured with beta cells located at the core whilst alpha/delta cells reside at the periphery (Figure 1). This organisation favours beta-beta cell interactions which is thought to be important in optimal insulin secretion and synthesis. Islet architecture is markedly different in humans with cell organisation seemingly random, however it has been

suggested that human islets have an organisation that resembles a collection of murine islets (Bonner-Weir et al., 2015; Cabrera et al., 2006).

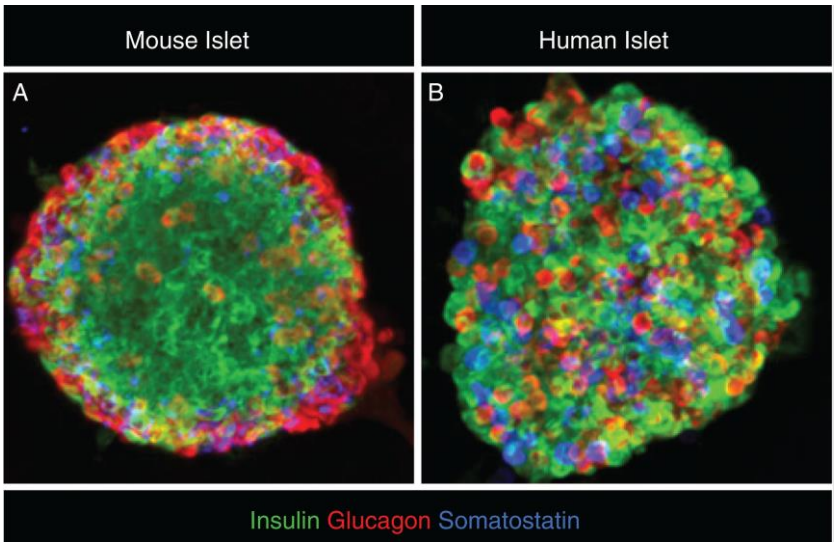


Figure 1: *Mouse and human islet structure.*
Immunofluorescent images of individual mouse (A) and human (B) islets where insulin (green), glucagon (red) and somatostatin (blue) indicate beta, alpha and delta cells, respectively. Image adapted from (Aamodt & Powers, 2017).

The endocrine hormones produced from pancreatic islets have distinct functions in glucose homeostasis (Göke, 2008). Insulin is responsible for lowering blood glucose concentration when this rises post-prandially. It does this through inducing glucose uptake at peripheral sites via glucose transporters (GLUT), but also through promoting the storage of glucose as glycogen (glycogenesis) and stimulating lipogenesis and protein synthesis (Krycer et al., 2020; Wilcox, 2005). In contrast, glucagon's function is catabolic, acting to increase blood glucose concentrations in the fasting state through promoting hepatic glycogenolysis and gluconeogenesis (Zeigerer et al., 2021). Somatostatin acts to finely tune glucose homeostasis, inhibiting both glucagon and insulin secretion (Hauge-Evans et al., 2009). PP has also been implicated in inhibiting glucagon secretion as well as somatostatin secretion and has roles in reducing appetite, whereas ghrelin stimulates glucagon secretion and increases appetite (Mani et al., 2019; Zhu et al., 2023). Dense vascularisation of islets (up to ten times that of the surrounding exocrine

tissue) permits rapid transport of these hormones to the blood, allowing for a prompt response to blood glucose fluctuations (Muratore et al., 2021).

1.1.2 Insulin biosynthesis, storage, and release from beta cells:

Regulation of insulin biosynthesis, storage and release is crucial for maintaining blood glucose concentrations within a healthy range. The insulin molecule is comprised of an alpha and a beta chain (21 and 30 amino acids long, respectively) which are linked by disulphide bonds; one between A7 and B7, and the other between A20 and B19 (Figure 2) (De Meyts, 2004). A further disulphide bond is intrachain, between A6 to A11. These disulphide bonds are critical for the tertiary structure of insulin. Although insulin itself is 51 amino acids long, the insulin gene encodes a 110-amino acid long protein called preproinsulin, the precursor of insulin. Preproinsulin must be processed to form insulin in a series of stages which occur within the endoplasmic reticulum (ER, preproinsulin to proinsulin) as well as in immature secretory vesicles (proinsulin to insulin) (Figure 3) (Liu et al., 2018).

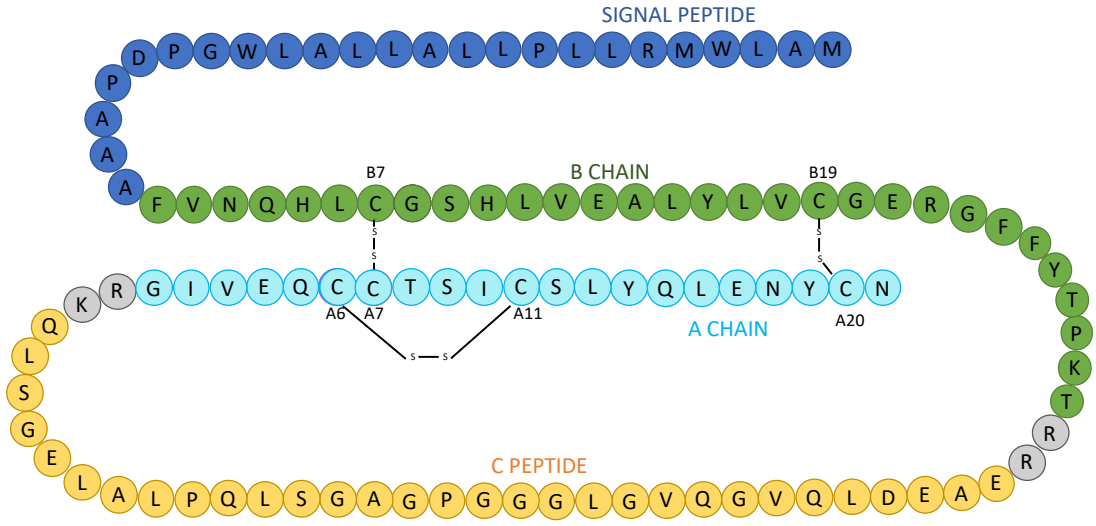


FIGURE 2: *Amino acid sequence of preproinsulin:*

Amino acid sequence of preproinsulin indicating the three disulphide bridges (B7-A7, B19-A20 and A6-A11) critical for the three-dimensional folding of the protein. The signal peptide (dark blue) and the C-chain (yellow) are cleaved

from the B chain (green) and A chain (teal) to form mature insulin. Image taken and adapted from (Austin et al., 2020).

Preproinsulin is directed into the ER through co-translational translocation; where translocation is concomitant with protein synthesis (Guo et al., 2018; Liu et al., 2018). This is achieved through interaction of preproinsulin's N-terminal signal peptide (SP) with the ER signal recognition particle (SRP) as it emerges from the ribosome, allowing the elongating peptide to enter the ER lumen through the Sec61 translocon. However, there is evidence to suggest that translocation of preproinsulin via this route is highly inefficient, resulting in newly translated and unstable preproinsulin being left in the cytosol (Guo et al., 2018). Therefore, post-translational translocation is likely also used to rescue the un-translocated nascent protein.

Once in the ER lumen, signal peptidase cleaves the SP to form proinsulin which is subsequently folded (Liu et al., 2018). Formation of the disulphide bridges discussed above are crucial in determining proinsulin folding, and these occur sequentially with A20-B19 forming first, which kinetically enables A7-B7 disulphide bridge formation followed by A6-A11 (Sun et al., 2015). Interestingly, the latter seem to have differential importance in determining the folding of proinsulin. *In vitro* studies where these disulphide bonds were selectively deleted showed that A20-B19 ablation severely disrupted protein tertiary structure *in vitro*, whilst A7-B7 ablation led to a more moderate disruption and A6-A11 ablation led to a mild disruption (Chang et al., 2003). Moreover, the investigation of proinsulin misfolded intermediates *in vitro* found that loss of either A20-B19 or A7-B7 results in impaired export from the ER whilst A6-A11 disruption impairs receptor binding but does not impact insulin secretion efficiency (Liu et al., 2005; Sun et al., 2015).

Although the precise mechanisms underlying appropriate formation of proinsulin's disulphide bonds and the disruption of inappropriate ones are unclear, this is critically dependent upon the environment within the ER and this includes an abundance of protein disulphide isomerases (PDIs) which act to both oxidise disulphide links and reduce inappropriate disulphide links (Sun

et al., 2015), (Liu et al., 2018). Accordingly, mRNA of members of the PDI family are expressed at high levels in pancreatic islets (Lan et al., 2004). When the tertiary structure of proinsulin is achieved, proinsulin is transported to the Golgi apparatus to be packaged into immature secretory vesicles (Figure 3). Here, the C chain is cleaved to form C-peptide and insulin which forms zinc-bound hexamers. Insulin hexamers, C-peptide and other secretory proteins (such as amyloid polypeptide) are stored together in secretory granules (Fu et al., 2012).

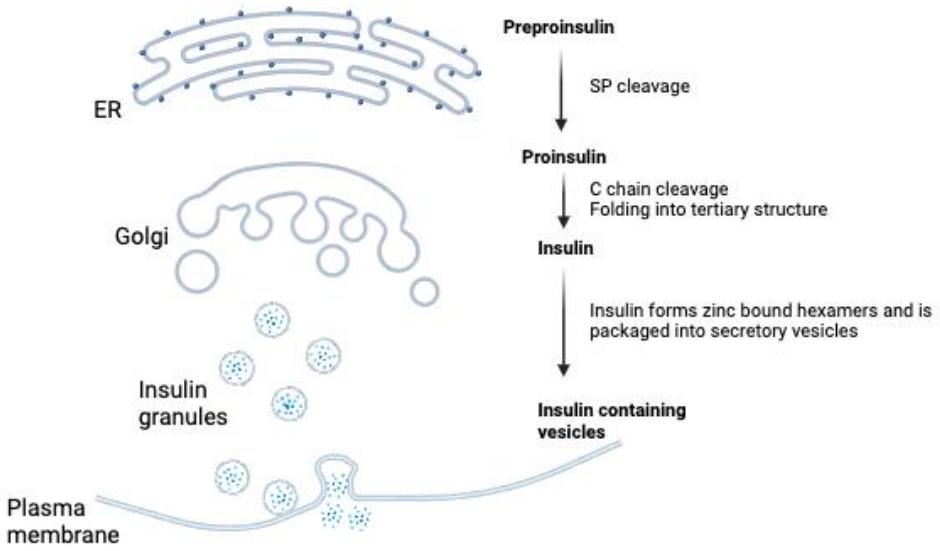


FIGURE 3: *Schematic of insulin biosynthesis:*
Schematic illustrating the different stages involved in insulin biosynthesis. Preproinsulin is translated and directed into the ER through co-translational translocation and once in the ER lumen its N-terminal signal peptide (SP) is cleaved to form proinsulin. Proinsulin is folded into its tertiary structure through the formation of three disulphide bridges within the ER. Once properly folded, proinsulin is transported to the Golgi apparatus where it is packaged into immature secretory vesicles and cleaved to form insulin. Zinc bound hexamers of insulin and C-peptide are stored in secretory vesicles. Image created with BioRender.com

Insulin secretion from beta cells is stimulated by many factors which include hormones (such as gastrointestinal-derived incretins), nutrients (such as fatty

acids) and neural input (Preitner et al., 2004; Röder et al., 2016; Thorens, 2011). However, elevated blood glucose concentrations (>5mM) represent the primary stimulant (Figure 4). Glucose is taken up by beta cells through low-affinity binding glucose transporters (mainly GLUT2 in rodents and GLUT1 in humans) which mediates its facilitated diffusion into the cytoplasm (De Vos et al., 1995; Navale & Paranjape, 2016). Cytoplasmic glucose is phosphorylated by glucokinase into glucose-6-phophate which is converted into pyruvate in the glycolytic pathway. Pyruvate then enters the tricarboxylic acid cycle (TCA) within the mitochondria resulting in its conversion to ATP (Komatsu et al., 2013). This influences the ATP:ADP ratio within the beta cell. Increased ATP is associated with enhanced ATP binding to the ATP-regulated potassium channels (K_{ATP} channels), thus causing their closure. Since potassium efflux maintains a resting membrane potential (-70mV), potassium channel closure results in membrane depolarisation. Membrane depolarisation leads to an opening of voltage-gated L-type calcium channels which causes a subsequent influx of calcium ions into the beta cell (Komatsu et al., 2013). The latter drives the exocytosis of insulin granules through SNAP and SNARE protein coupled fusion of granules with the plasma membrane.

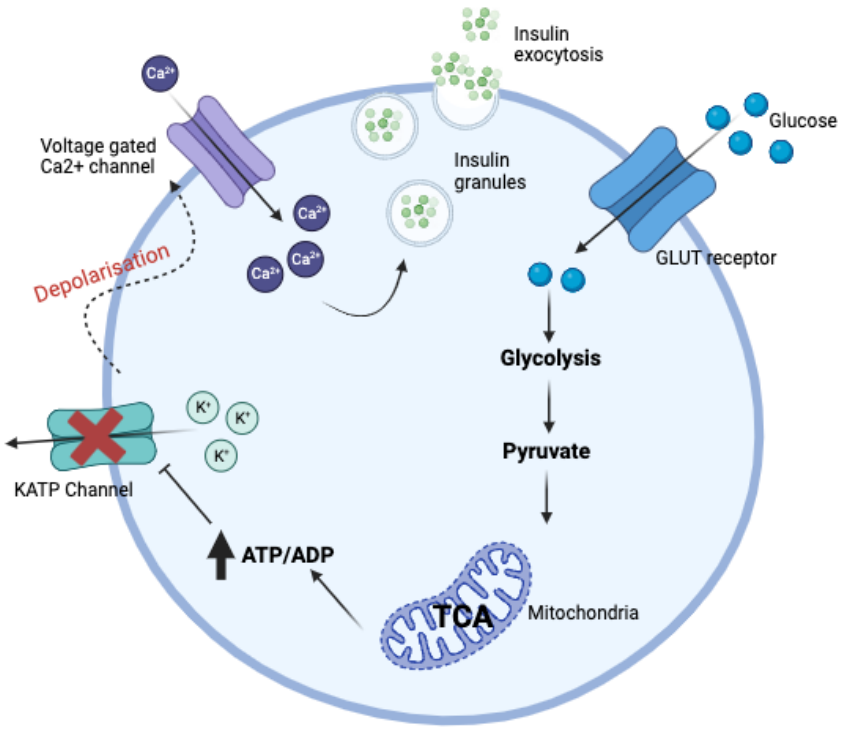


FIGURE 4: *Schematic of beta cell insulin secretion:*

Schematic illustrating the different stages involved in beta cell insulin secretion in response to glucose. Glucose is taken into the beta cell via glucose transporters. Through glycolysis it is converted into pyruvate which enters the tricarboxylic acid cycle (TCA) in the mitochondria to form ATP. Increases in the ATP:ADP ratio drives the closure of ATP potassium channels (K_{ATP} channels) *preventing the efflux of positively charged potassium ions out of the cell and therefore depolarising the membrane. Voltage gated calcium channels open when the membrane is depolarised, and this results in the influx of calcium ions into the cell which drives the exocytosis of insulin granules. Image created with BioRender.com*

Insulin secretion in response to glucose is biphasic, with a rapid initial transient spike in insulin named the 'first phase response' lasting ~10 minutes, followed by a slower but more sustained secretion of insulin (lasting 60 minutes) termed the second-phase insulin response (Rorsman et al., 2000). The latter facilitates a response to increased blood glucose levels that is simultaneously rapid but that can also be sustained. It is thought that the biphasic response is orchestrated by two distinct intracellular populations of insulin granules (Hao et al., 2005). The first phase response is associated with the release of insulin from readily releasable reserves (contributing to 1-5% of all insulin granules), which can be rapidly released as they are pre-docked to the beta cell plasma membrane (Olofsson et al., 2002). However, the second phase response is associated with mobilisation of reserve pools (which are non-releasable) to refill the sites of the rapid release pools (Hou et al., 2009). The mechanisms by which reserve pools are mobilised requires further study, however this process requires ATP and calcium with the latter possibly being derived mainly from extracellular stores (Hao et al., 2005).

1.2 Diabetes mellitus:

Maintenance of blood glucose concentrations within a normoglycemic range is critically dependent upon the action of insulin. Inadequate insulin secretion or tissue response to insulin causes a group of metabolic disorders collectively referred to as diabetes mellitus, which are characterised by hyperglycaemia. As such, the criteria for the diagnosis of diabetes includes a fasted blood

glucose concentration exceeding 7mM and a non-fasted blood glucose concentration exceeding 11.1mM (Schleicher et al., 2022).

Although hyperglycaemia is the hallmark characteristic of all types of diabetes, the underlying pathology can differ, and therefore diabetes can be subdivided into distinct types. This includes the most prevalent type 2 diabetes mellitus (T2DM), which impacts over 6% of the global population and is caused by a relative deficit in insulin (a result of peripheral insulin resistance and beta cell dysfunction) (Khan et al., 2019). Type 1 diabetes mellitus (T1DM) on the other hand affects 0.1% of the global population (contributing to 5-10% of all diabetes cases) and is characterised by a loss of beta cells resulting from autoimmune destruction (Mobasseri et al., 2020). Less common types of diabetes also include gestational diabetes mellitus (GDM) which is caused by the development of relative insulin deficiency only during pregnancy, and monogenic forms of diabetes such as neonatal diabetes mellitus (NDM) and maturity onset diabetes of the young (MODY) (Modzelewski et al., 2022). NDM is caused by a severe or total lack of insulin usually before 6 months of age due to single gene mutations that drive malformation of the pancreas or abnormalities in beta cell function/survival (Beltrand et al., 2020). MODY is observed in children and young adults and is caused by single gene mutations which drive an impairment in beta cell insulin secretion.

Although a wide range of treatment options are available which attempt to maintain blood glucose concentrations within a normal physiological range in diabetic patients, constant maintenance of this is difficult. Since acute and chronic hyperglycaemia are associated with a range of health complications, including microvascular complications (such as retinopathies, neuropathies and nephropathies), macrovascular complications (such as cardiovascular disease), reduced immune system function and increased risk of cancer, diabetes is linked to an overall increased risk of mortality (Calcutt et al., 2009; Marcovecchio et al., 2011).

1.2.1 Aetiology of type 2 diabetes:

Type 2 diabetes (T2DM) is by far the most prevalent form of diabetes mellitus, estimated to contribute up to 90% of cases (Wu et al., 2014). Although numerous genetic and environmental factors have been associated with T2DM development, it is fundamentally caused by a failure of the beta cells to adapt to an increased insulin demand driven by insulin resistance and can thus be considered a disease of beta cell dysfunction and insulin resistance (Kahn et al., 2006).

Obesity represents an important driver of insulin resistance and as such is the most significant risk factor associated with T2DM. Indeed, close to 80-90% of individuals with T2DM are either overweight or obese (Nianogo & Arah, 2022). Obesity has also been associated with directly mediating beta cell dysfunction. These effects of obesity have been at least in-part attributed to factors released through the expansion of adipose depots, including pro-inflammatory cytokines, glycerol, free fatty acids, and hormones (Nianogo & Arah, 2022). Healthy obese individuals compensate for periphery insulin insensitivity through increasing beta cell insulin secretion, hypertrophy, and proliferation. Indeed, obesity has been associated with ~50% increase in beta cell mass (Saisho et al., 2013). However, in individuals where underlying beta cell dysfunction impairs these compensatory responses to insulin resistance, glucose homeostasis becomes dysregulated (Prentki, 2006). Beta cell dysfunction is further exacerbated by glucotoxicity and beta cell exhaustion. Longitudinal studies have supported the notion that beta cell dysfunction predates diabetes, finding that defects in beta cell insulin secretion in a population of Pima Indians were apparent prior to diabetes development (Weyer et al., 1999).

T2DM is caused by a complex interaction of environmental and genetic factors and numerous gene mutations have been implicated in pre-existing beta cell dysfunction which may contribute to T2DM development. For example, a missense polymorphism in the gene encoding the Kir6.2 subunit of the ATP-sensitive potassium channel has been found to reduce insulin secretion and is associated with increased risk of T2DM development (Gloyn et al., 2003). Environmental factors also contribute to beta cell dysfunction, and this

includes high plasma levels of free fatty acids (FFAs) which is associated with obesity (Kahn et al., 2006). Beta cells exposed chronically to FFAs both *in vivo* and *in vitro* show reduced insulin biosynthesis and secretion (Sako & Grill, 1990; Y. P. Zhou & Grill, 1994). This is despite FFAs being necessary in a physiologically healthy state for optimal insulin secretion. Interestingly, whilst acute lipid infusion in humans is associated with a beneficial increase in beta cell insulin secretion, this effect is lost upon prolonged infusion (Carpentier et al., 1999). It is important to note however that the direct causality between FFAs and beta cell dysfunction has been questioned. This is because it is unknown whether concentrations of FFAs used in *in vitro* studies mimic the concentrations experienced by beta cells *in vivo*, and FFA concentrations used for *in vivo* lipid infusion studies have typically been higher than what is observed in obese individuals (Weir, 2020).

1.2.2 Aetiology of type 1 diabetes:

Type 1 diabetes contributes to 5-10% of diabetes cases and its prevalence is increasing worldwide (You & Henneberg, 2016). In the vast majority of cases (70-90%), T1DM is caused by the autoimmune destruction of beta cells. However, T1DM can also be idiopathic with no clear cause driving beta cell destruction (Hameed et al., 2011). In both cases, a progressive decline in beta cell mass results in an inadequate supply of insulin to meet demand and chronic hyperglycaemia develops usually in childhood or early adulthood. Clinical symptoms do not manifest until beta cell mass is reduced by 70-80% and at this point exogenous insulin is required to maintain glucose homeostasis (Paschou et al., 2018). Here we will focus on the more common autoimmune-mediated form.

The pathological mechanisms driving autoimmune diabetes are unclear, however beta cell destruction is thought to be primarily driven by T lymphocytes. CD4+ T cells harbour autoreactivity towards beta cell antigens as a result of autoantigen presentation by antigen presenting cells (macrophages and dendritic cells) through the major histocompatibility complex (MHC). Common autoantigens include glutamic acid decarboxylase 65 (GAD65), insulinoma-associated protein 2 (IA-2) and zinc-transporter-8

(ZnT8) among others (Purcell et al., 2019). Autoreactive CD4+ T cells then go on to activate CD8+ cytotoxic T cells. Cytotoxic T cells can mediate beta cell death through various mechanisms, including the release of perforin and granzyme, inflammatory cytokines (IFN-gamma, TNF-alpha) and via Fas-FasLigand interactions (Atkinson et al., 2015). A destructive positive feedback loop is subsequently set up whereby destruction of beta cells results in release of further beta cell antigens, causing further production of autoreactive T cells.

Interestingly, insulitis profiles of individuals with T1DM are heterogeneous and as such it has been proposed that autoimmune T1DM does not represent a single disease but rather can be divided up into distinct endotypes. This distinction has been made based on the level of islet CD20 B cell infiltration with Arif *et al.* finding that pancreas samples from individuals with T1DM could be grouped based on their insulitis profile into CD20-high and CD20-low (Arif et al., 2014). Intriguingly, these endotypes were found to correlate with disease aggressiveness (Leete et al., 2016). The CD20-high endotype is associated with increased CD8 cell islet infiltration, increased islet autoantibodies as well as an earlier age of onset (<7-years) and a reduced residual beta cell mass. In contrast, the CD20-low endotype is associated with a superior residual beta cell mass, older age of diabetes onset (>13-years) and reduced islet infiltration of other immune cells.

How autoimmune destruction of beta cells is initiated in T1DM remains elusive and a topic of debate. However, it is widely believed that this event is triggered through an interaction of genetic and environmental factors. Indeed, studies have indicated that T1DM concordance is ~50% in monozygotic twins, suggesting an interplay between these factors (Redondo et al., 1999). Variants in the HLA (human leukocyte antigen, human MHC) class II region are among the most commonly associated with T1DM, with 90% of patients displaying variants of the HLA-DR and HLA-DQ genes (Pociot & Lernmark, 2016; Roark et al., 2014). Products of the HLA class II region are responsible for presenting exogenous antigens to T cells, and these variants have been associated with altering the nature of antigen peptides that can bind and be presented, although it is not understood how this contributes to beta cell autoreactivity.

Numerous environmental factors associated with T1DM have also been proposed, including viral infection, diet, childhood obesity, sanitation, and intestinal microbiota (Xia et al., 2019). Coxsackie virus B (CVB) infection, for example, is thought to represent a considerable environmental risk factor. It can be found in pancreatic tissue of T1DM patients and CVB RNA can be detected in blood during T1DM development (Dotta et al., 2007; Andréoletti et al., 1997). Although evidence does not confirm a causal link between CVB and T1DM, various models have been proposed to explain how infection may contribute to beta cell autoreactivity including molecular mimicry and viral-induced beta cell death initiating autoantigen presentation (Xia et al., 2019). More recently, it has been suggested that the novel virus COVID-19 may initiate the development of T1DM (Wang et al., 2023). Indeed, COVID-19 has been found to infect human beta cells via the neuropilin-1 receptor and induce death (Wu et al., 2021). In accordance with this, T1DM cases increased during the COVID-19 pandemic (Wang et al., 2023).

1.2.3 Aetiology of monogenic diabetes:

The categories of diabetes listed above are the result of a complex interaction between environmental and genetic factors, however, a subset of diabetes cases are caused by single gene mutations. This includes neonatal diabetes (NDM), which occurs before 6 months of age, and maturity onset diabetes of the young (MODY) which typically develops during adolescence or in young adulthood. Although NDM is rare, predicted to occur in 1 in 10,000 births, MODY is thought to contribute to 1-4% of diabetes cases under the age of 30 (Misra & Owen, 2018). Both MODY and NDM are associated with single gene mutations that impact insulin processing/biosynthesis and secretion, but NDM is also associated with mutations impacting beta cell death and pancreatic development. As a result, both can give crucial insight into factors important in maintaining a functional beta cell mass.

MODY is typically diagnosed by the presentation of hyperglycaemia prior to 25 years of age, in the absence of islet autoantibodies and with a sustained secretion of endogenous insulin (Urakami, 2019). MODY has been associated

with mutations in at least 14 different genes and based on these, MODY can be sub-categorised. Since the mutated gene and mutation type differs between MODY patients, it represents a very heterogeneous disease. MODY2 is one of the most common subtypes (contributing to 20-50% of all cases) and is caused by mutations in glucokinase (GCK) whose action converts glucose into glucose-6-phosphate early on in glucose metabolism which ultimately drives insulin secretion (Urakami, 2019). Heterozygous loss of function mutations in GCK are associated with mild sub-clinical diabetes where the threshold for insulin secretion is slightly increased. However, MODY3 which also contributes to 20-50% of MODY cases, causes progressive hyperglycaemia with a disease course similar to that of T2DM (Peng et al., 2023; Urakami, 2019). This is because it is caused by heterozygous mutations in hepatic nuclear factor 1 homeobox A (HNF-1A), a transcription factor responsible for the regulation of pancreatic and duodenal homeobox 1 (PDX1) expression which is critical for beta cell function (Miyachi et al., 2022). Specific HNF-1A gene variants have also been associated with the development of T2DM.

NDM is characterised by hyperglycaemia occurring before 6 months of age, is associated with a lack or relative lack of insulin and can be categorised based on whether the disease is permanent or transient (Beltrand et al., 2020). NDM occurs due to single gene mutations that either lead to the malformation of the pancreas or impairments in beta cell function. Many genes implicated in MODY have also been associated with NDM, for example homozygous mutations in GCK have been associated with permanent NDM (Osbak et al., 2009). Activating mutations in genes encoding components of the ATP-sensitive potassium channel (*ABCC8* which encodes the outer subunit, SUR1, and *KCNJ11* which encodes the inner subunit, Kir6.2) represent the most common cause of NDM (Babenko et al., 2006; Gloyn et al., 2004). These mutations prevent potassium channel closure, precluding control of beta cell membrane depolarisation and thus impairing beta cell insulin secretion. Other known mutations implicated in NDM include those of the insulin gene itself (Edghill et al., 2008; Støy et al., 2007). These mutations impact insulin folding and cause beta cell dysfunction and death as a result of misfolded protein

accumulation within the endoplasmic reticulum (Meur et al., 2010; Støy et al., 2010). The existence of the latter emphasises the importance of optimal protein homeostasis in beta cell function and the control of blood glucose concentrations.

1.3 Beta cell protein homeostasis and ER stress:

As discussed previously, proinsulin is folded and processed within the endoplasmic reticulum (ER) of the beta cell. This places an enormous burden upon this organelle because insulin demand constantly fluctuates as blood glucose levels change, and so the folding capacity of the ER is continually altered to match these demands (Eizirik & Cnop, 2010). On top of this, proinsulin synthesis can take up more than half of all protein synthesis in the beta cell at any given time and proinsulin has a high propensity to mis-fold (Alam et al., 2021; Arunagiri et al., 2019; Dhayalan et al., 2021; Scheuner & Kaufman, 2008; Schuit et al., 1988). Proinsulin biosynthesis is therefore thought to lead to the accumulation of misfolded and unfolded proteins within the ER as it struggles to match folding capacity with demand, and this leads to a phenomenon called ER stress. Indeed, beta cells likely experience low levels of ER stress continuously (Chen et al., 2022; Eizirik & Cnop, 2010). This stress is likely further exacerbated by the long-lived and low proliferative nature of beta cell, which means that individual beta cells must adapt to changes in insulin demand which occur throughout the organism's life as a result of age or obesity, for example.

Under physiologically healthy circumstances, beta cells are able to meet these high demands and maintain protein homeostasis through activation of various signalling pathways collectively known as the unfolded protein response (UPR) which is activated upon detection of ER stress. However, in pathological states, where ER stress in unable to be resolved through adaptive mechanisms, the same UPR pathways can be co-opted to promote beta cell dysfunction and death. Here we shall briefly discuss the function of the ER in more detail before exploring adaptive and pathological activation of ER stress response pathways in beta cells.

1.3.1 The function of the endoplasmic reticulum (ER):

The ER is a specialist organelle with various functions. One function is its role in the processing and folding of proteins. It also has a central role in lipid biosynthesis and represents a major storage site for calcium, with calcium concentrations 4-5 fold that of the cytosol, a gradient which is sustained via sarcoendoplasmic reticulum calcium ATPase (SERCA) proteins (Eizirik et al., 2008).

Within the ER lumen exist a plethora of protein chaperones and foldase enzymes which interact with nascent polypeptides to facilitate protein folding and prevent protein aggregation. Broadly speaking, chaperones prevent inappropriate intra and inter polypeptide interaction and provide an appropriate environment whereby foldases can interact with polypeptide chains. These can be grouped into classical chaperones (heat shock protein chaperones), or lectin chaperones (Braakman & Hebert, 2013). Classical chaperones bind directly to polypeptide chains at exposed hydrophobic segments and prevent inappropriate polypeptide aggregation. Of the classical chaperones, immunoglobulin heavy chain binding protein (BiP or GRP78) is the most important and is regarded as a master regulator of the ER. It not only has a chaperone role, but it also plays an important role in targeting mis/un-folded proteins for degradation and calcium store maintenance (Pobre et al., 2019). Lectin chaperones (which includes calnexin and calreticulin) recognise and bind glycans on nascent polypeptide chains which are added early on in the polypeptide folding process (Braakman & Hebert, 2013). Protein chaperones not only have function in folding nascent polypeptides, but they also have a role in quality control by retaining proteins within the ER lumen until they are appropriately folded. Properly folded proteins are transported from the ER to the golgi in COPII (cytosolic coat protein II) vesicles.

A key class of folding enzyme in the ER are the oxidoreductases, and of these, protein disulphide isomerase (PDI) is the most abundant and well characterised. The highly oxidising environment in the ER lumen along with PDI promotes conversion of cysteine thiols into disulphide bridges which stabilise folded polypeptide structures. Prolyl peptidyl cis-trans isomerases

(PPIases) are also an important foldase class (Braakman & Hebert, 2013; Hebert & Molinari, 2007). These proteins catalyse the conversion of trans-prolines into cis-prolines. This is critical since all cis-prolines on proteins are isomerised into trans-prolines in order for them to have been inserted into the ER via the ribosome (Braakman & Hebert, 2013).

The highly specialised environment in the ER means it is acutely sensitive to changes in homeostasis. For example, inadequate levels of chaperones and foldases or cellular energy to meet protein folding demand, protein mutations that predispose to misfolding, and alterations in redox state or calcium concentrations, can all disrupt ER homeostasis and lead to an accumulation of mis/un-folded proteins which causes ER stress. The latter triggers a signalling response (the UPR) in an attempt to alleviate ER stress and thus restore ER homeostasis (Hetz, 2012). Collectively these signalling responses can be grouped into adaptive UPR responses.

1.3.2 The adaptive unfolded protein response (UPR):

Upon ER stress, the beta cell activates a series of signalling pathways collectively referred to as the UPR. These signalling pathways ultimately help alleviate ER stress and restore ER protein homeostasis through a variety of mechanisms including by transiently reducing nascent polypeptide influx into the ER, increasing ER folding capacity (by increasing the gene expression of protein chaperones) and by promoting the degradation of misfolded proteins. These signalling pathways can be grouped into three main arms each of which are controlled by the activation of three ER transmembrane proteins which act as ER stress sensors: inositol requiring enzyme 1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) (Hetz, 2012).

Studies support a model whereby in an unstressed state, these ER stress sensors are kept inactive through engagement with the protein chaperone BiP binding their luminal domain. Upon ER stress, demand for BiP to facilitate protein folding is increased and BiP bound to the ER stress sensors is

recruited. This allows the ER stress sensors to become activated, initiating UPR adaptive signalling (Figure 5).

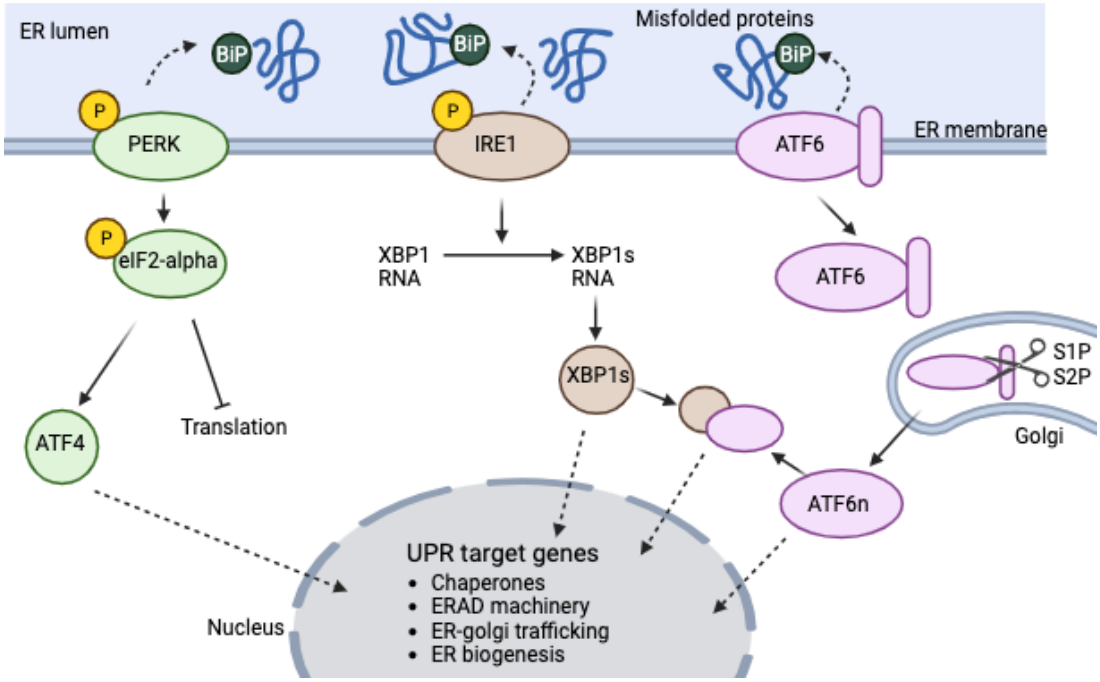


FIGURE 5: *The adaptive UPR:*

Schematic illustrating the adaptive UPR initiated under ER stress. Unfolded protein accumulation within the ER lumen causes BiP to dissociate from the three ER stress sensors (PERK, IRE1 and ATF6) allowing for their activation. Upon activation, PERK phosphorylates eIF2-alpha to attenuate global protein translation and permits the specific translation of ATF4 which acts as a transcription factor to drive the expression of genes involved in restoration of ER protein homeostasis. ATF6 liberation from BiP allows it to translocate to the Golgi where site 1 and site 2 proteases (S1P and S2P) remove its luminal and transmembrane anchor, this allows it to transit to the nucleus and regulate UPR target genes. XBP1s activation leads to activity of its endoribonuclease domain which cleaves XBP1 mRNA to XBP1s which is translated and whose protein acts as a transcription factor to regulate genes involved in recovery from ER stress. In addition, XBP1s and ATF6n can form heterodimers to regulate expression of genes involved in ER stress recovery. Image created with BioRender.com.

Dissociation of BiP from PERK allows its oligomerisation and subsequent trans-autophosphorylation of its cytosolic kinase domains thus activating serine/threonine kinase activity. Activated PERK then goes on to phosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 alpha (eIF2-alpha), an important component of the ternary complex which, together with the 40s ribosome and other initiation factors, initiates mRNA translation (Harding et al., 1999; Harding, Zhang, et al., 2000; Rabhi et al., 2014). Phosphorylation of eIF2-alpha inactivates the ternary complex preventing the initiation of mRNA translation. This has the effect of reducing nascent protein influx into the ER, which contributes to the reestablishment of ER homeostasis. eIF2-alpha phosphorylation also results in a preferential translation of mRNAs which harbour inhibitory upstream open reading frames (uPRFs); reduced ternary formation results in ribosome 'skipping' of the uPRF allowing translation. mRNAs harbouring uPRFs include ATF4, a basic leucine zipper (bZIP) transcription factor which induces the expression of genes to help restore ER protein homeostasis including protein chaperones genes (Harding, Novoa, et al., 2000).

IRE1 is also a kinase which upon release from BiP oligomerises and trans-auto-phosphorylates. Studies have shown that the luminal domain of IRE1, responsible for ER stress sensing, shares a high degree of structural similarity with PERK and therefore the mechanism for ER stress sensing (via BiP dissociation) is likely shared (Zhou et al., 2006). However, there is also evidence for IRE1 activation through the direct binding of misfolded proteins to its luminal domain (Credle et al., 2005; Gardner & Walter, 2011). This has led to the notion that misfolded protein binding and BiP dissociation may work together to regulate IRE1, with BiP binding stabilising its inactive state and misfolded protein binding promoting oligomerisation (Gardner et al., 2013). However, many of these studies were conducted in yeast and some have argued that the proposed misfolded protein binding groove at the luminal domain of human IRE1 is too narrow for protein binding (Zhou et al., 2006). Unlike PERK, IRE1 contains an endoribonuclease domain alongside its kinase domain and activation of this stimulates splicing of X-box binding protein 1 (XBP1) mRNA to form spliced XBP1 (sXBP1) mRNA (Calfon et al., 2002). The

latter encodes a transcription factor which promotes expression of protein chaperones and components of ERAD through binding to ER stress response elements (Yoshida et al., 2001). Non-specific activity of IRE1's endoribonuclease also acts to degrade mRNAs proximal to the ER, therefore reducing protein influx into the ER (Hollien & Weissman, 2006).

Unlike IRE1 and PERK, ATF6 is not a kinase but a bZIP transcription factor. Two isoforms of ATF6 exist, ATF6-alpha and ATF6-beta. It is believed that ATF6-alpha is exclusively responsible for ER stress responses whereas the function of ATF6-beta is less clear (Yamamoto et al., 2007). Dissociation of BiP from ATF6-alpha liberates it from the ER membrane and reveals Golgi-localisation sequences (GLS1 and GLS2) which drive its transit through COPII vesicles to the Golgi apparatus (Hillary & Fitzgerald, 2018). At the Golgi, the proteases S1P and S2P (site 1 and 2 proteases) remove the luminal and transmembrane anchor domain of ATF6-alpha allowing it to transit to the nucleus. In the nucleus the cleaved ATF6 (ATF6n) protein binds ER-stress response elements to upregulate the expression of a number of genes which collectively contribute to ER homeostasis restoration (Hillary & Fitzgerald, 2018). Knockout studies have shown that gene targets include protein chaperones (such as BiP) and proteins that promote misfolded protein degradation (Bobrovnikova-Marjon & Diehl, 2007; Wu et al., 2007). The ATF6 pathway also has a role in regulating the IRE1 pathway through upregulating gene expression of XBP1. Furthermore, XBP1s can form heterodimers with N-terminal ATF6-alpha to regulate genes involved in ER-associated degradation (ERAD) (Yamamoto et al., 2007).

1.3.3 The role of the adaptive UPR in beta cells:

Beta cells are particularly susceptible to ER stress owing to their role as professional secretory cells. Since ER stress limits the capacity for appropriate protein processing and folding within the ER, it is unsurprising that the adaptive UPR is important in the function of the beta cell. In addition to this, studies have also suggested that the adaptive UPR is important in beta cell survival as well as compensation in situations of increased beta cell demand.

Much evidence for the importance of the adaptive UPR in insulin biosynthesis and secretion comes from studies where components of the adaptive UPR were deleted or inhibited. For example, beta cell specific deletion of IRE1 in mice is associated with a reduction in pancreatic proinsulin and insulin content as well as a reduction in post-prandial insulin secretion, and this leads to glucose intolerance and elevated blood glucose concentrations (Hassler et al., 2015). Beta cell specific knockout of XBP1 (the mRNA of which is spliced downstream of IRE1) in mice also reduced beta cell insulin content and impaired glucose simulated insulin secretion, leading to elevations in blood glucose concentration in these mice (Lee et al., 2011). The ATF6 pathway also appears to be important in insulin biosynthesis and secretion since ATF6 deletion reduces insulin content in the pancreases of high fat fed mice and its overexpression improves insulin secretion in MIN6 cells treated with the ER stress inducer thapsigargin (Engin et al., 2013; Usui et al., 2012). Finally, inhibition of PERK in rat insulinoma (INS-1) cells and primary murine islets was found to impair glucose stimulated insulin secretion (Wang et al., 2013).

The adaptive UPR is also thought to play an important role in beta cell survival, with studies showing that knockdown of UPR components induces beta cell death. Global PERK knockdown in mice is associated with beta cell death and subsequent development of hyperglycaemia (Gao et al., 2012; Harding et al., 2001). XBP1 is an important component of the IRE1 pathway and its inhibition in mouse insulinoma-6 (MIN6) cells has also been found to exacerbate cell death in the presence of proinflammatory cytokines and palmitate (Chan et al., 2015). Supporting this, XBP1 inhibition in islets from both $Lep^{db/db}$ and NOD mice *in vitro* enhanced beta cell death (Chan et al., 2015). Although the role of the ATF6 pathway in beta cell survival is comparatively less well studied, knockdown in INS-1 cells results in increased beta cell death (Teodoro et al., 2012).

Beta cell proliferation occurs predominantly during development but can also occur as a compensatory response to increased insulin demand (for example resulting from insulin resistance). Several studies have suggested that the adaptive UPR is important in mediating beta cell proliferation and indeed it has

recently been found that beta cells with an active UPR are more likely to undergo proliferation (Sharma et al., 2015). The ATF6 pathway has received much attention in regard to this. Small molecule inhibition of ATF6 in primary mouse and human islets reduced beta cell proliferation, whereas the opposite effect was noted upon ATF6 overexpression (Sharma et al., 2015). In the same study, XBP1 inhibition was also associated with reduced beta cell proliferation in mouse and human islets *ex vivo*, however in contrast to ATF6, its overexpression also reduced beta cell proliferation suggesting that the IRE1 pathway can promote beta cell proliferation but within a narrow range. Further supporting a role for IRE1 in beta cell proliferation, diabetes caused by XBP1 deletion in mice is associated with a loss of beta cell mass in the absence of beta cell death (Lee et al., 2011). The picture is less clear for the involvement of the PERK pathway in beta cell proliferation, with studies conflicting one another. Loss of function PERK mutations in humans, which causes neonatal diabetes, is associated with a reduction in beta cell proliferation in the neonatal period causing a reduction in beta cell mass (Delepine et al., 2000). PERK knockdown in neonatal mice is also associated with reduced beta cell proliferation in the neonatal period (Zhang et al., 2006). However, it has also been found that heterozygous knockdown of PERK in mice increases beta cell proliferation and inhibition of PERK in human and murine islets *ex vivo* had no effect on proliferation (Sharma et al., 2015; Wang et al., 2014). Further study into PERK pathway involvement in beta cell proliferation is therefore required.

1.3.4 The maladaptive unfolded protein response (UPR):

Beta cells are continually exposed to conditions that result in mild ER stress. An example of this is the transient increase in insulin demand resulting from post prandial blood glucose spikes. In this situation, the UPR promotes beta cell survival through mitigating stress and restoring protein homeostasis as described previously (see section 1.3.3). However, when ER stress is robust and chronic, the adaptive UPR can no longer restore ER homeostasis and maladaptive UPR signalling (also termed terminal UPR signalling) is activated.

This maladaptive phenomenon in beta cells can result not only in functional decline but also in cell death, and thus has been implicated in beta cell failure

in many types of diabetes. All three of the UPR arms have pro-survival and pro-apoptotic outputs which are thought to be expressed concurrently during ER stress, the balance between these outputs ultimately determines life or death decisions (Figure 6) (Fonseca et al., 2012). However, the precise mechanisms that mediate the shift between adaptive and maladaptive UPR signalling are not fully understood. Here we will review what is known about maladaptive signalling through each of the UPR pathways.

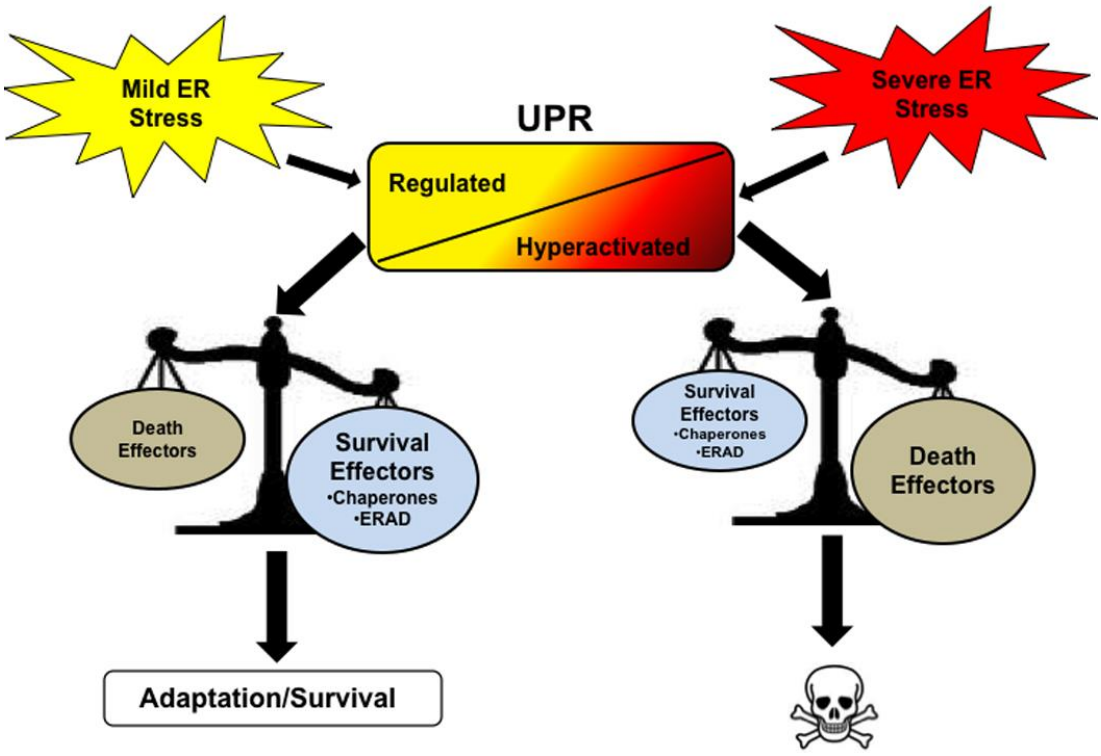


FIGURE 6: *The influence of the UPR on cell fate:*
The balance between adaptive and maladaptive signalling downstream of the three UPR pathways ultimately determines cellular fate. Mild and resolvable ER stress leads to cellular survival, whereas ER stress which is too robust and chronic to be resolved through adaptive mechanisms results in cellular failure (Fonseca et al., 2012).

PERK activation drives translational attenuation, reducing ER protein load and facilitating the restoration of ER protein homeostasis. However, this process also initiates the preferential translation of ATF4, a transcription factor

important in promoting C-/EBP homologous protein (CHOP) gene expression. It is predominately through enhancing levels of CHOP that PERK mediates its terminal signalling (Figure 7). Indeed, CHOP knockout in a beta cell ER stress-induced model of diabetes, the Akita mouse, reduces beta cell apoptosis and delays diabetes development (Oyadomari, Koizumi,et al., 2002). Moreover, CHOP deletion promotes beta cell survival in diet induced models of diabetes (Song et al., 2008). One of the main mechanisms by which CHOP induces apoptosis is through the mitochondrial-dependent pathway (Hu et al., 2019). CHOP enhances expression of pro-apoptotic Bcl-2 (B-cell lymphoma 2) family members and represses expression of pro-survival members to enhance the activation of Bax (Bcl-2 Associated X-protein) and Bak (Bcl-2 homologous antagonist killer) allowing their oligomerisation (Hu et al., 2019). Bak-Bax oligomers permeabilise the outer mitochondrial membrane, allowing release of cytochrome-c into the cytosol where it initiates a caspase cascade which ultimately causes apoptosis.

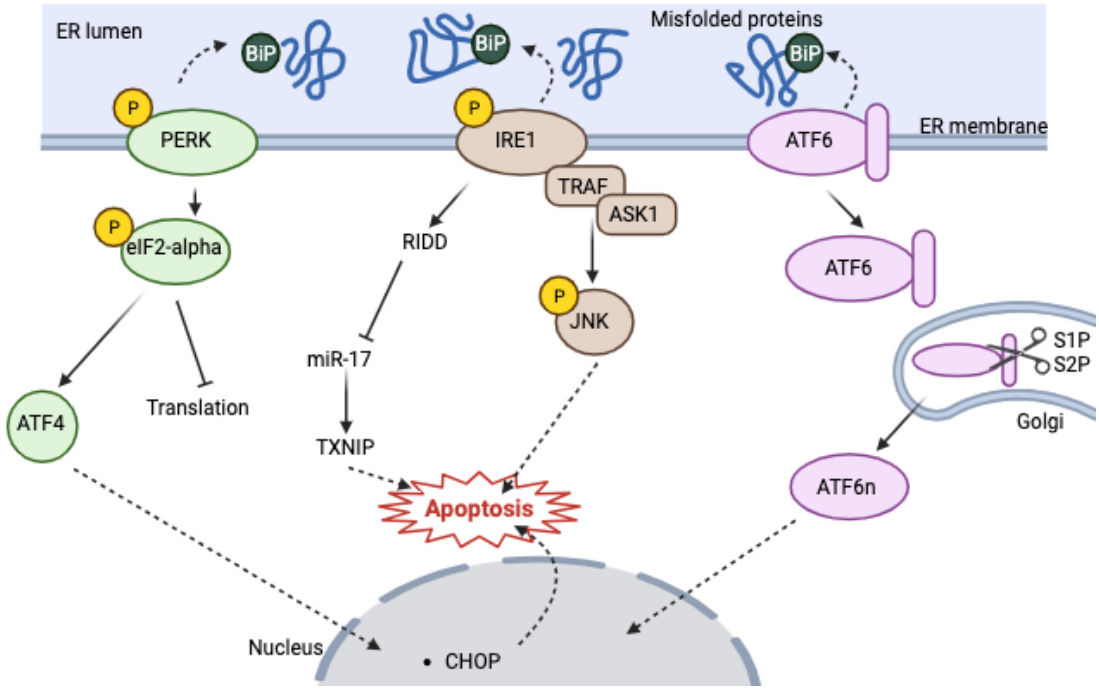


FIGURE 7: *The maladaptive UPR:*

Schematic illustrating the maladaptive UPR initiated under robust and unresolvable ER stress. Hyperactivation through PERK and ATF6 drives an upregulation CHOP which is proapoptotic. Hyperactivation of IRE1 leads to

JNK activation which is proapoptotic and leads to its endoribonuclease domain becoming less selective and degrading mRNAs critical for cell function and survival. Image created with BioRender.com.

CHOP also positively regulates gene expression of pseudokinase tribbles related 3 (TRB3) (Ohoka et al., 2005), death receptor 4 and 5 (DR4 and 5) (Yamaguchi & Wang, 2004) and ER oxidoreductase 1 alpha (ERO1-alpha) (Li et al., 2009), all of which have been associated with apoptosis (Hu et al., 2019). ERO1-alpha, for example, contributes to cell death by increasing reactive oxygen species (ROS) production and perturbing ER calcium stores by activating the inositol-1,4,5-trisphosphate receptor (IP_3R)(Li et al., 2009). ATF6, whose transcription factor activity is activated downstream of the ATF6 arm, can also promote CHOP gene expression. In line with this, hyperactivation of ATF6 results in impaired insulin expression and dysfunction in beta cells and exacerbates ER-stress induced apoptosis in endothelial cells (Huang et al., 2018). CHOP accumulation over time, resulting from chronic or robust ER stress that cannot be resolved, may tip the balance towards a cell death fate of the cell. CHOP also attenuates PERK adaptive signalling by promoting gene expression of the eIF2-alpha phosphatase GADD34 (growth arrest and DNA damage-inducible protein 34, which releases translational inhibition), which may further aid its ability to override adaptive signals (Marciniak et al., 2004).

The IRE1 pathway also plays a critical role in promoting apoptosis and cellular dysfunction upon its sustained activation and there are two proposed mechanisms for this (Iurlaro & Muñoz-Pinedo, 2016). The first is through its endoribonuclease activity. Sustained IRE1 activation may cause its RNase domain to lose its specificity for XBP1 mRNA by inducing its high-order oligomerisation (Han et al., 2009; Hollien et al., 2009). Whilst this may initially resolve ER stress by mass degrading mRNAs non-specifically and thus reducing ER protein load, prolonged mRNA degradation may cause cellular dedifferentiation as mRNAs encoding crucial beta cell proteins are depleted including insulin (Lipson et al., 2008). The latter is termed regulated IRE1 dependent decay of mRNAs (RIDD). RIDD may also lead to the degradation

of mRNAs crucial to cellular survival ultimately leading to cell death (Ghosh et al., 2019). Indeed, it is known that IRE1 can degrade microRNA-17 (miR-17) which is a repressor of the pro-apoptotic thioredoxin-interacting protein (TXNIP).

The second mechanism by which IRE1 induces apoptotic signalling is through phosphorylation of c-Jun N-terminal kinase (JNK) by forming a complex with TBF receptor associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) (Urano et al., 2000). Phosphorylated JNK can promote apoptosis by inhibiting anti-apoptotic proteins such as Bcl-XL (B-cell lymphoma-extra extra-large) and Mcl-1 (myeloid cell leukemia-1) (Iurlaro & Muñoz-Pinedo, 2016). Providing support for this, ASK1 knockout in primary neurons prevents ER-stress induced JNK activation and subsequent apoptosis (Nishitoh et al., 2002). Moreover, ASK1 knockout in Akita homozygote mice delayed the onset of diabetes through reducing beta cell apoptosis, implicating this pathway in the development of diabetes (Yamaguchi et al., 2013).

Alongside CHOP, IRE1/JNK signalling has also been implicated in tipping the balance between UPR pro-survival and pro-apoptotic outputs. Indeed, studies have shown that whilst IRE1 and XBP1s inhibition increased apoptosis, inhibition of JNK reduced apoptosis in beta cells subjected to ER stress through cytokine and fatty acid exposure (Chan et al., 2015). Additionally, Han *et al* have proposed that cell fate may be determined by the balance between IRE1 RNase outputs, after finding that cell fate decisions differed with the modulation of IRE1 RNase outputs with inhibitors (Han et al., 2009).

1.4 The role of beta cell ER stress in diabetes pathogenesis:

The adaptive UPR is critical in the maintenance of beta cell function and survival in the face of ER stress, as explored previously. Despite this, excessive ER stress that cannot be resolved through adaptive signalling can result in unfavourable consequences via the terminal UPR which is associated with cellular dysfunction and death. The latter has been associated with beta cell failure in the context of diabetes development and progression, although

the precise mechanisms are unknown. More recent evidence has also suggested that a failure of the adaptive UPR, rather than terminal UPR, is also associated with beta cell failure in diabetes. Here we will explore evidence supporting the notion that beta cell ER stress can drive beta cell failure before exploring evidence of this in monogenic forms of diabetes as well as type 1 and type 2 diabetes.

1.4.1 ER STRESS CAN DRIVE BETA CELL FAILURE:

Beta cell failure encompasses both a loss/decline in beta cell function as well as beta cell death which, as mentioned, drives a reduction in beta cell functional mass leading to diabetes development. Evidence supports involvement of the maladaptive UPR in both beta cell dysfunction and death (Figure 8).

One way in which excessive ER stress may drive beta cell dysfunction is through reducing insulin secretion and biosynthesis. Hyperactivation of IRE1 has been associated with enhanced proinsulin mRNA degradation (Kadowaki & Nishitoh, 2013). Indeed, high glucose mediated ER stress in beta cells *in vitro* stimulates proinsulin mRNA degradation, an effect found to be dependent on IRE1 since it is lost when IRE1 is expressed in its dominant negative form. Similarly, excessive signalling through PERK has also been associated with limiting insulin production with PERK overexpression in mice impairing glucose stimulated insulin secretion and reducing pancreatic insulin content (Gupta et al., 2010). This may be a result of sustained eIF2-alpha phosphorylation driving chronic mRNA translation attenuation.

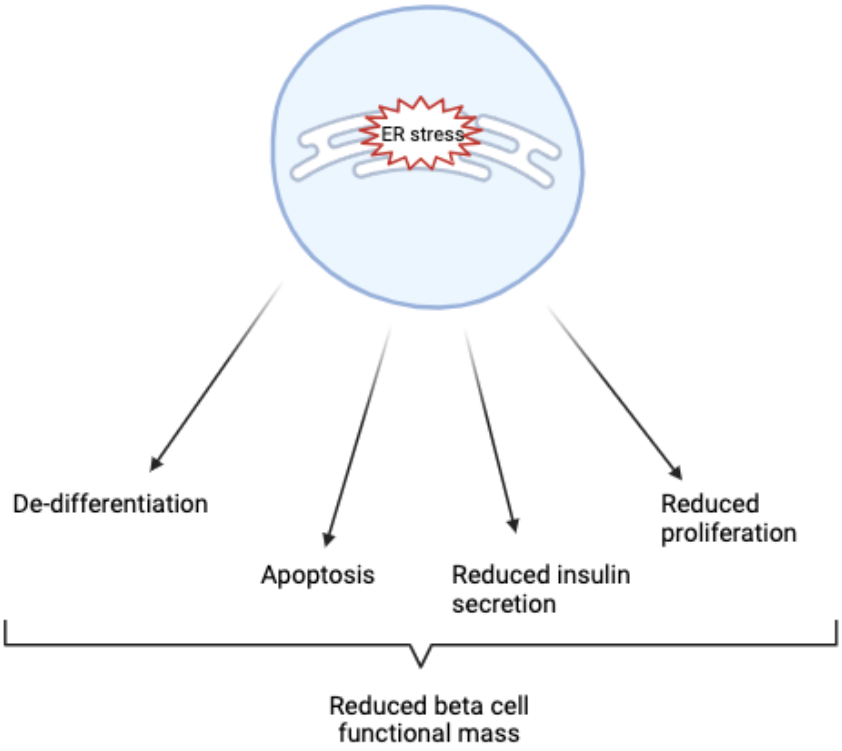


FIGURE 8: *Mechanisms by which ER stress drives beta cell failure: Schematic showing the different ways in which beta cell ER stress has been proposed to induce beta cell failure.*

There is also evidence that beta cell UPR activation can result in beta cell failure by driving a dedifferentiated state. Beta cell dedifferentiation leads to a loss of beta cell phenotype with a reduction in the expression of classical beta cell identity genes (such as *Ins*, *Pdx1* and *Nkx6.1*) and can therefore mediate a loss of beta cell functional mass. Excessive UPR signalling has been associated with the latter, with adenovirus mediated XBP1s overexpression in rat islets resulting in a reduction in the expression of beta cell specific genes, consistent with dedifferentiation (Allagnat et al., 2010). This overexpression also leads to increased beta cell apoptosis. ATF6 hyperactivation has likewise been associated with a reduction in beta cell specific genes including *Ins* (Seo et al., 2008).

Beta cell ER stress may also negatively regulate beta cell proliferation, which can contribute to an impaired beta cell compensatory response to stress driving a relative deficiency in insulin. As mentioned previously, although some

studies have found a positive association between ER stress and beta cell proliferation, with Sharma *et al.* finding that beta cell proliferation was upregulated in two models of diabetes (the Akita and the Lep$^{ob/ob}$ mouse), others have found this is downregulated (Sharma et al., 2015). Riahi *et al.* found that beta cell ER stress in the Akita mouse drove a reduction in beta cell proliferation in the early postnatal period due to its transient inhibition of mTORC1 (mammalian target of rapamycin complex 1) (Riahi et al., 2018). A separate study also found that insulin gene mutations (which drive ER stress) reduced proliferation of beta-like cells *in vitro* (Balboa et al., 2018). Further to this, in a mouse model of insulin gene reduction, beta cells were found to harbour reduced expression of ER stress markers and this correlated with a two-fold increase in beta cell proliferation compared to control mice harbouring all insulin gene copies (Szabat et al., 2016).

Finally, numerous studies support a role of pro-apoptotic UPR components in driving beta cell death. For example, inhibition of IRE1's RNase activity was found to protect against ER stress induced INS-1 cell apoptosis *in vitro* (Carlesso et al., 2019). This inhibition was also found to protect beta cells *in vivo*, preventing a reduction of beta cell mass in the Akita mouse and improving glycaemic control (Ghosh et al., 2014). Many studies have also found an important role for CHOP in driving beta cell ER stress induced death. CHOP expression is enhanced downstream of all three UPR pathways, although it is thought that most CHOP expression can be attributed to the PERK pathway (Sharma et al., 2021). The most compelling evidence of CHOP's role in beta cell death comes from *in vivo* studies where CHOP knockdown is associated with reduced beta cell loss and improvements in hyperglycaemia in animal models exhibiting beta cell ER stress, including the Akita mouse and the Lep$^{db/db}$ mouse (Oyadomari, Koizumi, et al., 2002; Song et al., 2008).

It has also recently been suggested that a failure of the UPR to be mounted under robust ER stress may drive beta cell failure, however the mechanisms underlying this have yet to be elucidated. This notion comes from studies showing a downregulation of UPR components (XBP1s and ATF6) in islets

from individuals with T1DM as well as in islets from the NOD mouse model of T1DM (Engin et al., 2013). A further study also found downregulation of these markers in the beta cells from Lep$^{ob/ob}$ and high fat fed mouse models of T2DM, as well as in beta cells of individuals with T2DM (Engin et al., 2014).

1.4.2 ER STRESS IN MONOGENIC FORMS OF DIABETES:

Some types of monogenic diabetes are driven by mutations in components of the UPR or misfolding mutations in insulin. Their existence provides compelling evidence that beta cell ER stress is sufficient to drive diabetes.

A major category of neonatal diabetes is caused by misfolding mutations in insulin, which result in beta cell failure as a result of unresolvable beta cell ER stress (Støy et al., 2007). One mutation, which causes a C96Y substitution in proinsulin2's B chain, is also the cause of diabetes in a mouse model called the Akita mouse (Yoshioka et al., 1997). This substitution perturbs the A7-B7 disulphide bridge in proinsulin which drives proinsulin misfolding within the ER. As misfolded proinsulin accumulates, beta cell ER stress occurs. The unpaired cysteines in the misfolded proinsulin also bind to cysteines from other mutant and WT proinsulin molecules, forming aggregates which prevent WT proinsulin export from the ER, and which further exacerbate ER stress (Hodish et al., 2010; Sun et al., 2020). Gene expression and protein studies have confirmed beta cell ER stress in the Akita mouse finding that UPR components including ATF6, XBP1s and CHOP are upregulated (Riahi et al., 2018).

The Akita model has been used to show that CHOP-mediated apoptosis is likely the primary mechanism by which beta cell ER stress drives diabetes development in individuals with the C96Y proinsulin substitution. Indeed, beta cell mass is reduced in this model but genetic ablation of CHOP limits this and improves glycemia (Oyadomari, Koizumi, et al., 2002). However, it should be noted that ER stress likely drives beta cell failure through other mechanisms as well since CHOP$^{-/-}$ Akita mice still develop diabetes. In line with this, recent evidence has suggested that the Akita mutation causes a reduction in beta cell proliferation at the pre-weaning stage which likely contributes to the loss of beta cell mass seen (Riahi et al., 2018).

Wolcott-Rallison syndrome (WRS) in humans is caused by loss of function mutations in the gene encoding PERK (*EIF2AK3*). This causes a loss of beta cell mass and the development of neonatal diabetes (Delepine et al., 2000). Modelling WRS in mice through PERK knockout has found that loss of function PERK removes translational attenuation of preproinsulin which results in excessive proinsulin synthesis (Harding et al., 2001; Zhang et al., 2006). The consequence of the latter is high levels of beta cell ER stress which cannot be resolved, and this is thought to result in impaired beta cell expansion during the neonatal period in these mice. Conditional knockdown of PERK in adult mice also induces diabetes through ER stress-induced beta cell death (Gao et al., 2012). Therefore, both beta cell death and impaired beta cell expansion in humans with this mutation likely drives diabetes.

1.4.3 Evidence for beta cell ER stress involvement in type 2 diabetes pathogenesis:

Evidence supporting the role of beta cell ER stress in T2DM pathogenesis mainly comes from the assessment of ER stress markers in the beta cells of individuals with T2DM and in animal models of T2DM, as well as GWAS studies. Further evidence comes from findings that factors known to contribute to T2DM pathogenesis are also known to induce beta cell ER stress, however this will be explored in a later section (see section 1.4.4).

T2DM pathogenesis is complex and thought to be the result of both environmental and genetic factors which collectively drive insulin resistance and beta cell failure. Indeed, more than 400 genes have been identified that are associated with an increased risk of T2DM development. Interestingly, this includes genes associated with the UPR, implicating beta cell ER stress in pathogenesis. For example, polymorphisms in the gene encoding ATF6 have been identified in individuals with prediabetes and T2DM (Chu et al., 2007). Moreover, IRE1 gene variants are associated with increased T2DM risk and XBP1 gene variants have been found to increase blood glucose concentrations (Liu et al., 2016; Shrestha et al., 2021).

More conclusive evidence for the role of ER stress in T2DM comes from studies investigating beta cell ER stress markers in individuals with T2DM. The results of these studies are somewhat conflicting, however. Laybutt *et al.* found that ER stress marker gene expression, including BiP and CHOP, were increased in pancreas sections from individuals with T2DM (Laybutt et al., 2007). Supporting this, a separate study found that beta cell ER volume was increased two-fold in individuals with T2DM, which is consistent with beta cell ER stress (Marchetti et al., 2007). Whilst this study did not find an increase in ER stress marker expression in isolated islets under basal conditions (5.5mM glucose), when stimulated with high glucose concentrations (11.1mM), expression of these markers increased only in islets from individuals with T2DM. Contrasting these findings, RNA sequencing of islets from individuals with T2DM found no increase in the expression of ER stress markers (Marselli et al., 2020). Why findings differ is unclear, however experimental methodology such as whether protein or gene expression was used to investigate upregulation of ER stress markers, and whether the analysis was carried out on isolated islets or fixed pancreatic sections, may play some role. Further investigations into beta cell ER stress in T2DM individuals is therefore warranted in future.

Intriguingly, a reduction in the expression of the UPR markers ATF6 and XBP1s in islets from individuals with T2DM has also been reported (Engin et al., 2014). Engin *et al.* proposed that a failure of the adaptive UPR to be mounted in the face of ER stress, rather than maladaptive UPR signalling, may contribute to beta cell failure in T2DM. This idea has also been suggested by Chan *et al.* who found that whilst an increased expression of ER stress markers was maintained over time in the beta cells from the leptin deficient obese but non-diabetic ($Lep^{ob/ob}$) mouse, expression of ER stress markers reduced over time in the leptin deficient diabetic ($Lep^{db/db}$) mouse (an obese model where failed beta cell compensation leads to diabetes) (Chan et al., 2013).

1.4.4 Mechanisms driving beta cell ER stress in type 2 diabetes:

As mentioned above, evidence exists implicating beta cell ER stress in T2D pathogenesis. Although some genetic factors have been associated with inducing ER stress in the context of T2DM, more research has focussed on the mechanisms by which environmental factors can induce beta cell ER stress. In fact, since many environmental factors implicated in T2DM also cause beta cell ER stress, it is possible that ER stress represents a common mechanism linking these factors to the development of diabetes. Here we will explore how environmental factors associated with T2DM can cause beta cell ER stress.

1.4.4.1 Insulin resistance:

Peripheral insulin resistance is a defining feature of T2DM and is often the result of increased adiposity. Insulin resistance increases insulin demand, and it is known that beta cells compensate for this by increasing insulin biosynthesis and secretion (Camastra et al., 2005). However, a chronic increase in insulin demand and thus chronic pressure on the beta cell to synthesise, fold and secrete a greater amount of insulin, decreases the protein folding capacity of the ER (Ghosh et al., 2019; Johnson & Kaufman,2021). Alongside the latter, increased insulin biosynthesis associated with insulin resistance and hyperglycaemia has been associated with an accumulation of misfolded proinsulin in the ER (Arunagiri et al., 2019). Both an increased folding demand and an increase in misfolded proinsulin within the ER likely result in beta cell ER stress. Supporting this concept, individuals with pre-diabetes and exhibiting insulin resistance show chronic beta cell UPR activation (Huang et al., 2007). It is possible that the strain that insulin resistance places on the beta cell ER, alongside environmental drivers of beta cell ER stress in T2DM (discussed below), may tip the balance between UPR adaptive signals and terminal UPR signals and drive beta cell failure (Ghosh et al., 2019). As mentioned previously, failure of the adaptive UPR under conditions of chronic ER stress may also drive beta cell failure.

1.4.4.2 Lipotoxicity:

Free fatty acids (FFAs) have been suggested to represent a major mechanistic link between obesity and T2DM development, as discussed previously (see

section 1.2.1). One of the reasons behind this is that obesity is associated with an increased level of circulating FFAs (due to dietary excess and adiposity) and chronic FFA exposure can impair glucose stimulated insulin secretion and induce beta cell apoptosis (effects known as lipotoxicity).

ER stress may represent a prominent mechanism by which FFAs induce beta cell failure (Han & Kaufman, 2016; Lytrivi et al., 2020). Beta cells exposed *in vitro* to palmitate show increased expression of ER stress markers and ultrastructural hallmarks of ER stress (including distended ER) (Laybutt et al., 2007). Moreover, attenuation of ER stress in palmitate treated beta cells reduces apoptosis (Tran et al., 2014). Although some *in vivo* FFA infusion studies both in humans and in animals have shown that FFAs induce beta cell dysfunction, other studies have found conflicting results (Carpentier et al., 2010; Giacca et al., 2011; Goh et al., 2007; Ivovic et al., 2017). Indeed, FFA infusion in rats has also been shown to have no effect on glucose stimulated insulin secretion whilst other studies have found FFA infusion to improve insulin secretion (Hagman et al., 2008; Magnan et al., 1999; Steil et al., 2001). Differences in findings may be attributed to differences in strain, treatment duration, sex or age of the rats (Lytrivi et al., 2020). The levels of beta cell ER stress in *in vivo* infusion models have not been investigated, however pre-treatment with the chemical chaperone PBA partially prevented beta cell dysfunction caused by 48-hour lipid infusion in non-diabetic overweight or obese men (Xiao et al., 2011). The latter implicates FFA in causing beta cell ER stress since PBA is known to reduce this. However, it is important to note that beta cell ER stress has not been directly investigated in any *in vivo* lipotoxicity studies. Furthermore, infusion studies tend to use FFA concentrations beyond physiological levels (Carpentier et al., 1999; Leung et al., 2004). Therefore, further study is required into the effects of physiological FFA on the expression of beta cell ER stress markers.

The mechanisms by which FFAs induce beta cell ER stress remain elusive, however *in vitro* studies have suggested these likely involve perturbation of ER calcium levels leading to aberrant chaperone activity, and blockade of protein transport from the ER to the Golgi leading to ER protein overload

(Gwiazda et al., 2009; Preston et al., 2009). In addition to this, FFAs may also induce beta cell ER stress indirectly through inducing oxidative stress and driving an accumulation of islet amyloid polypeptide (IAPP) both of which are thought to initiate ER stress (see sections 1.4.4.3 and 1.4.4.5) (Elsner et al., 2011; Qi et al., 2010). Moreover, FFAs can drive peripheral insulin resistance, and as mentioned previously (section 1.4.4.1) this results in an increased beta cell insulin demand and ER stress.

1.4.4.3 Islet amyloid polypeptide (IAPP):

Islet amyloid polypeptide (IAPP) has also been associated with beta cell failure in T2DM. It is co-secreted and co-packaged in secretory granules with insulin in response to beta cell secretagogues, such as glucose, and is proposed to have a role in controlling satiety, blood pressure, peripheral glucose uptake and more (Zhang et al., 2016). However, in pathological settings, IAPP can form fibrils (amyloid fibrils) which accumulate within beta cells and extracellularly. Intriguingly, 95% of T2DM patients show amyloid fibril formation within islets. Further supporting its role in T2DM pathogenesis, humans harbouring mutations in IAPP that increase its propensity to aggregate, develop early-onset T2DM (Cao et al., 2012).

The precise mechanisms by which IAPP aggregates induce beta cell cytotoxic action is unknown, however it has been proposed that ER stress is involved. Beta cell lines expressing human IAPP (hIAPP, which readily aggregates) and islets from hIAPP transgenic rodents show increased expression of ER stress markers which is associated with beta cell loss (Huang et al., 2007). Furthermore, inhibition of CHOP expression was found to reduce caspase-3 activity and apoptosis in beta cells expressing hIAPP (Huang et al., 2007). However, some studies have found no association with ER stress and IAPP. For example, Soty *et al.* found no induction of ER stress in INS-1 cells expressing hIAPP (Soty et al., 2011). Contrasting results may be due to differences in IAPP levels and further research is required in this area. One of the ways in which IAPP has been proposed to induce beta cell ER stress directly is through disruption of cellular lipid membranes and subsequent calcium perturbation. Indeed, IAPP oligomers have been shown to form pore-

like structures in lipid membranes *in vitro* (Anguiano et al., 2002). IAPP may also indirectly cause ER stress by promoting oxidative stress (Zraika et al., 2009).

1.4.4.4 Glucotoxicity:

Hyperglycaemia itself has been associated with beta cell failure, with exposure of beta cells to high glucose concentrations mediating gene expression changes associated with dedifferentiation and increasing beta cell apoptosis (Federici et al., 2001; Jonas et al., 1999). 'Glucotoxicity', the harmful effects of high glucose concentrations on the beta cell, has therefore been associated with diabetes progression.

Whilst the precise mechanisms by which high glucose levels mediates beta cell failure are unknown, evidence supports a role of ER stress in this. Indeed, acute, and chronic exposure of beta cells to high glucose concentrations increases the expression of the ER stress marker IRE1 *in vitro* and *in vivo* (Lipson et al., 2006). Whilst acute exposure was associated with enhanced proinsulin synthesis, chronic high glucose levels caused beta cell death and dysfunction. A further study found that chronic high glucose treatment of islets increases the expression of the pro-apoptotic protein CHOP (Elouil et al., 2007). It should be noted that other studies have found a reduction in ER stress markers in beta cells exposed to high glucose levels. Rat islets exposed to high glucose concentrations had reduced phosphorylation of eIF-2-alpha suggesting a reduction in PERK signalling. Similarly, eIF-2-alpha phosphorylation and PERK expression were reduced with high glucose treatment of islets from an alternate model of T2DM (*Psammomys obesus*) (Elouil et al., 2007; Leibowitz et al., 2010). The latter may lend support to the idea that chronic ER stress may lead to a failure of the adaptive UPR.

One way in which high glucose concentrations may induce ER stress is through proinsulin biosynthetic overload in a similar way as the response to insulin resistance (see section 1.4.4.1) (Johnson & Kaufman, 2021). High glucose levels stimulate the increased biosynthesis of proinsulin which limits ER folding capacity and increases proinsulin misfolding in the ER, which leads

to chronic beta cell ER stress (Arunagiri et al., 2019). It has also been postulated that chronic increases in insulin biosynthesis induces ER stress by interfering with ER calcium dynamics. The ER regulates calcium levels within a specific range to enable the optimal function of chaperones and foldases, however acute stimulation of beta cells with glucose causes ER calcium influx via SERCA pumps. Sustained influx of calcium into the ER in the context of hyperglycaemia may perturb calcium buffering, impairing the activity of ER folding machinery and therefore leading to ER stress as misfolded/unfolded proteins accumulate (Johnson & Kaufman, 2021). A high glucose concentration has also been proposed to induce ER stress through the generation of reactive oxygen species (ROS) caused by mitochondrial overload (Burgos-Morón et al., 2019).

1.4.4.5 Oxidative stress:

Oxidative stress occurs when there is an accumulation of ROS resulting from their inadequate disposal or increased production. At low levels, ROS are important for beta cell function, and this includes in regard to insulin secretion (Llanos et al., 2015). However, in pathological situations, such as high glucose or lipid concentrations, ROS accumulates as a result of the mitochondrial electron transport chain (ECT) being overloaded (Newsholme et al., 2019). ECT overload leads to increased leakage of electrons which react with oxygen to form the superoxide radical $O_2^{\bullet -}$, these can also be converted to the highly reactive hydroxide radical $OH^{\bullet -}$. ROS also accumulate as a result of alternative pathways that have been described elsewhere (Eguchi et al., 2021). ROS accumulation in beta cells is compounded by the fact that beta cells harbour relatively low levels of antioxidants. Accumulation of ROS causes beta cell oxidative stress, a state associated with beta cell failure in diabetes. Indeed, induction of oxidative stress through H_20_2 treatment in human islets impaired glucose stimulated insulin secretion, and beta cell oxidative stress is thought to be the main mechanism by which alloxan treatment induces diabetes in rodent models (Leenders et al., 2021; Lenzen, 2008).

Beta cell ER stress represents one of the mechanisms by which oxidative stress mediates beta cell failure. The mitochondria (a major site of ROS

generation) and the ER are directly linked by mitochondrial associated membranes (MAMs) and influx of ROS into the ER via these contact sites can disrupt ER redox state. Maintenance of a balanced redox state within the ER is critically important for the function of protein folding machinery which drives oxidative protein folding, and as such both reducing and oxidising agents have been found to promote protein misfolding causing ER stress (Cao & Kaufman, 2014; Malhotra & Kaufman, 2007). On the other hand, beta cell ER stress can drive oxidative stress since protein misfolding itself is associated with the production of ROS (Cao & Kaufman, 2014). Since oxidative and ER stress can exacerbate one another, it is possible that in the beta cell this sets up a vicious cycle which eventually leads to failure (Bhattarai et al., 2021; Cao & Kaufman, 2014).

1.4.5 Evidence for beta cell ER stress involvement in type 1 diabetes pathogenesis:

Although the role of beta cell ER stress in the development of T2DM has received more attention in the literature, an increasing body of evidence supports its role in the initiation and progression of T1DM. Some of this evidence comes from the assessment of ER stress markers in the beta cells of individuals with T1DM as well as in animal models.

A study by Marhfour *et al.* found that protein expression of CHOP was elevated in islets from individuals with autoimmune T1DM (Marhfour et al., 2012). BiP expression was also increased, however only in islets showing insulitis, but XBP1s levels were unchanged compared to non-diabetic individuals. In support of these findings, islets isolated from NOD mice (a mouse model of T1DM) show an age-dependent increase in the gene expression of ER stress markers, including CHOP, BiP and XBP1s, as well as ultrastructural signs of beta cell ER stress (Tersey et al., 2012). Intriguingly, this study found that beta cell ER stress marker expression preceded the development of diabetes in the NOD mice, suggesting that beta cell ER stress may play a role in diabetes initiation rather than occurring as a consequence of hyperglycaemia. It should be noted that a more recent study found that the ER stress markers ATF6 and XBP1s were reduced in islets from NOD mice and individuals with T1DM,

however the authors suggested this was the result of chronic ER stress impairing adaptive UPR signalling (Engin et al., 2013). Perhaps more compelling evidence that beta cell ER stress plays a direct role in T1DM comes from the finding that administration of the pharmacological chaperone, TUDCA (which reduces ER stress by facilitating appropriate protein folding), protects NOD and RIP-LCMV-GP (RIP lymphocytic choriomeningitis virus glycoprotein, a mouse model of insulin-dependent diabetes) models from T1D development (Engin et al., 2013).

In line with beta cell ER stress markers preceding diabetes development in NOD mice, it has been proposed that ER stress may be directly involved in the initiation of islet inflammation in T1DM. Common antigens described in T1DM are processed in the ER, and inappropriate folding of these as a result of ER stress may lead to the production of autoantigens (Mannering et al., 2005; Marré et al., 2016; McGinty et al., 2014). In agreement with this, studies have found that ER-stress can upregulate post translational modification enzymes which have been shown to contribute to the production of autoantigens including GAD65 and preproinsulin (Marré et al., 2016). It has been further postulated that physiological ER stress together with environmental factors known to induce ER stress (for example coxsackie virus B infection) may initiate the post translational modification of antigens to form autoantigens (Marré & Piganelli, 2017).

1.4.6 Mechanisms driving beta cell ER stress in type 1 diabetes:

1.4.6.1 Inflammation:

Autoimmune T1DM is primarily driven by the actions of autoreactive cytotoxic T cells which mediate beta cell death through binding to death receptors (FasLigand, membrane-bound TNF-alpha) and secreting perforin and granzyme. However, CD4 T cells and macrophages indirectly contribute to beta cell failure through the secretion of inflammatory cytokines, such as IL1-alpha (interleukin-1-alpha). One of the main mechanisms by which inflammatory cytokines can mediate beta cell death is through the generation of ER stress and subsequent activation of pro-apoptotic UPR signalling (Sahin

et al., 2021). Indeed, treatment of islets *ex vivo* with proinflammatory cytokines increases beta cell ER stress (Brozzi et al., 2015; Cardozo et al., 2005; Demine et al., 2020). It has been shown that cytokines mediate this effect through downregulating expression of the sarcoendoplasmic reticulum pump Ca^{2+} ATPase2b (SERCA2b), thus perturbing calcium levels within the ER and preventing protein folding activity of the calcium-dependent ER chaperone proteins (Cardozo et al., 2005). However, it should be noted that whilst more recent evidence has confirmed this mechanism of cytokine induced ER stress in rat beta cells, a different mechanism for this may exist in human and mouse beta cells and this requires further study (Brozzi et al., 2015). It is therefore plausible that the inflammatory milieu in islets in T1DM acts to increase beta cell ER stress which may then contribute to the progression of this disease by mediating beta cell failure.

1.4.6.2 Environmental factors preceding inflammation:

Beta cell ER stress mediated by proinflammatory cytokines likely play some role in diabetes progression after the initial autoimmune attack on beta cells has already been established. However, as stated above, beta cell ER stress has also been implicated in the initiation of this autoimmunity through faulty protein folding driving neoantigen generation (Sahin et al., 2021). Any inducer of beta cell ER stress preceding autoimmunity can therefore theoretically drive the production of beta cell neoantigens, initiating T1DM. Inducers of ER stress in this context may include viral infection, hyperglycaemia/insulin resistance and exposure to certain chemicals (Piganelli et al., 2021).

One viral inducer of beta cell ER stress that has received much attention in the literature is Coxsackie virus B (CVB) infection and this is because it has consistently been associated with the development of T1DM (as outlined in section 1.2.2). Interestingly CVB has been shown to activate ER stress upon entry into beta cells to facilitate its own replication; Colli *et al.* found that CVB enhances signalling through the IRE1 signalling arm of the UPR leading to increased JNK activation which is necessary for viral replication in beta cells (Colli et al., 2019). Supporting this, heightened CVB replication was reported in beta cells where ER stress had been induced prior to infection using

tunicamycin. In this context, viral induced beta cell ER stress may lead to the production of autoantigens initiating the autoimmune attack of the beta cells which defines pathogenesis in T1DM. Intriguingly, beta cell specific IRE1 deletion prior to insulitis in the NOD mouse prevents T1D development supporting a role of ER stress in initiating pathogenesis (Lee et al., 2020). It is important to note however, that this remains a controversial notion. Some have suggested that beta cell ER stress cannot alone mediate islet autoimmunity and this is supported by the fact that models and individuals with monogenic causes of beta cell ER stress do not harbour autoantibodies or insulitis (Sahin et al., 2021).

1.5 Animal models for studying beta cell ER stress in diabetes:

In the previous sections we have provided evidence for the involvement of beta cell ER stress not only in monogenic diabetes, but also in the more widespread T1DM and T2DM. Considering ER stress may represent a unifying mechanism of pathogenesis across different types of diabetes and that it underpins many known environmental factors associated with diabetes, it represents an attractive therapeutic target for the treatment of diabetes. However, how beta cell ER stress contributes to diabetes pathogenesis and understanding what factors manipulate beta cell ER stress requires further study.

Animal models exhibiting beta cell ER stress-induced diabetes as a result of single gene mutations are fundamental for investigating how ER stress contributes to beta cell failure and for testing novel ER-targeted diabetes therapeutics. Frequently used models and their pathogenesis and phenotypes shall be discussed here. It should be noted that this is not an exhaustive list, and other animal models of beta cell ER stress also exist (including those harbouring mutations in components of the UPR).

1.5.1 The Akita mouse

The Akita mouse is perhaps the most common model used to study beta cell ER stress in the context of diabetes. It harbours a mutation in *Ins2* which results in an amino acid substitution in preproinsulin (cysteine to a tyrosine at

position 96, C96Y) which disrupts formation of the A7-B7 disulphide bond (Figure 9) (Yoshioka et al., 1997). Mice homozygous for this mutation develop severe hyperglycaemia in the neonatal period and are emaciated, therefore mice heterozygous for this mutation are typically used. The Akita mutation is inherited in a dominant fashion and *Ins2*$^{+/C96Y}$ males show hyperglycaemia just after weaning whilst female *Ins2*$^{+/C96Y}$ mice only have a mild elevation in blood glucose levels (Yoshioka et al., 1997).

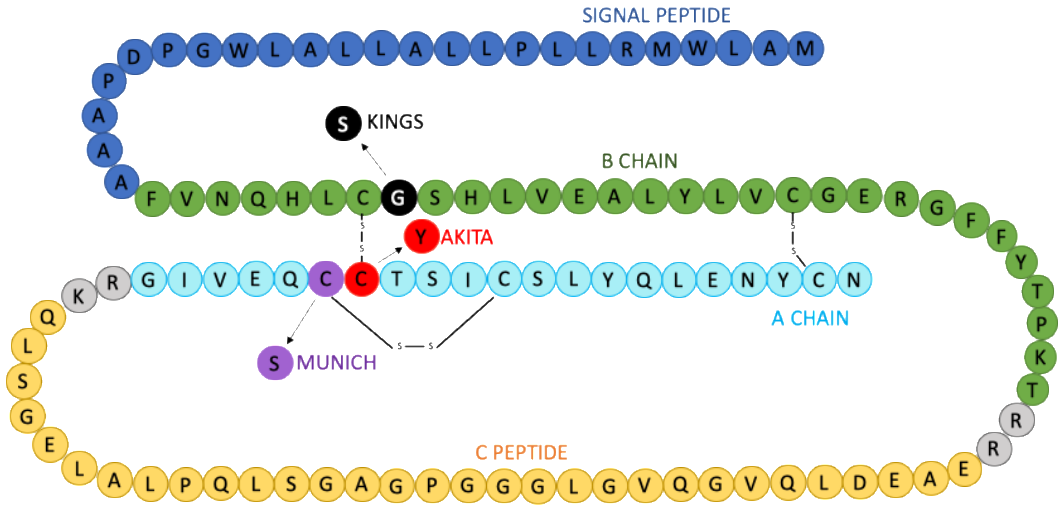


FIGURE 9: *Amino acid sequence of preproinsulin indicating the amino acid substitutions caused by different Ins2 mutations:*
Schematic indicating the sites of the amino acid substitutions in preproinsulin caused by different Ins2 gene mutations. The Akita mutation drives a cysteine to tyrosine substitution at position 96 of the A chain which perturbs formation of the A7-B7 disulphide bridge. The Munich mutation drives a cysteine to serine substitution of the A chain at position 95 which perturbs formation of the A6-A11 disulphide bridge. The KINGS mutation causes a glycine to serine substitution at position 32 of the B chain which is thought to disrupt the A7-B7 disulphide bridge. Image taken from (Austin et al., 2020).

Pathogenesis in the *Ins2*$^{+/C96Y}$ mouse is not driven by of a total loss of active insulin since these mice still have three functional insulin alleles (two nonmutated *Ins1* alleles, and the nonmutated *Ins2* allele), and even complete loss of both copies of *Ins2* or *Ins1* does not result in diabetes (Leroux et al.,

2001). On the contrary, the disruption of the A7-B7 disulphide bond in the mutated proinsulin is thought to drive its misfolding and thus retention in the ER, which causes beta cell ER stress as misfolded protein accumulates (Rajan et al., 2010). Indeed, beta cells from the Akita mice, as well as MIN6 cells induced to overexpress the Akita proinsulin, show increased expression of ER stress markers at the mRNA and protein level. This includes CHOP expression which is associated with terminal UPR (Oyadomari, Koizumi, et al., 2002; Riahi et al., 2018). Hallmark signs of ER stress have also been observed in Akita beta cells at the ultrastructural level through electron microscopy, which revealed ER swelling and mitochondrial distention with the absence of distinct cristae (Izumi et al., 2003).

One of the mechanisms by which ER stress in the beta cells of the Akita mice is thought to drive beta cell failure is through beta cell loss. This is because Akita mice show at least a 60% reduction in beta cell mass (Winnay et al., 2014). This has been in-part attributed to CHOP-mediated apoptosis, since CHOP knockout in Akita heterozygote male mice delays diabetes onset (Oyadomari, Koizumi, et al., 2002). However, it is important to note that CHOP knockout does not prevent diabetes and knockout in homozygous mice has no effect on glycemia. Therefore, other mechanisms likely play a role in pathogenesis, and this includes impaired beta cell insulin secretion. Reduced glucose stimulated insulin secretion and islet insulin content has been reported in these mice, and the extent of this is far greater than what is accounted for by a loss of a single WT *Ins* allele (Herlea-Pana et al., 2021). ER stress has been shown to reduce insulin secretion in *in vitro* studies, but it is also known that Akita proinsulin can form aggregates with WT proinsulin thus sequestering it in the ER and reducing insulin secretion (Liu et al., 2010). Proinsulin aggregates are also likely to exacerbate beta cell ER stress.

Other mechanisms of ER stress induced beta cell failure have also been reported in the Akita mice and this includes ER stress mediating a loss of beta cell maturation and proliferation in the neonatal period which leads to a reduced beta cell functional mass compared to wildtype in adulthood (Riahi et al., 2018). Izumi *et al.* has also suggested that misfolded proinsulin may drive

beta cell failure by impairing the early secretory pathway thus limiting the synthesis and folding of a variety of cellular proteins crucial for beta cell function and survival. Indeed, they showed that transit of a protein unrelated to proinsulin (secretory alkaline phosphatase) was reduced in Chinese hamster ovary (CHO) cells expressing Akita proinsulin (Izumi et al., 2003).

1.5.2 The Munich mouse:

The Munich mouse is less well characterised compared to the Akita mouse. It was generated through alkylating agent N-ethyl-N-nitrosourea (ENU) mutagenesis and harbours a cysteine to serine substitution at position 95 of the proinsulin molecule. This substitution prevents formation of the A6-A11 intrachain disulphide bridge during proinsulin folding within the ER (Herbach et al., 2007). Similar to the Akita mutation, the Munich mutation is autosomal dominant and since hyperglycaemia is severe and results in emaciation in homozygous mice, heterozygous mice are typically used. Both male and female *Ins2*$^{+/C96S}$ mice display stark glucose intolerance from 3 weeks and blood glucose concentrations are elevated compared to wildtype mice by 4 weeks of age. A sex difference is also seen in phenotype with males showing overt diabetes (blood glucose concentrations >16.7mM) whilst female blood glucose levels remain below this threshold. Fewer investigations have been made into the mechanisms behind diabetes development in this model, however it is likely that Munich proinsulin mis-folds within the ER generating ER stress (Balboa et al., 2018). Indeed, electron microscopy has revealed ultrastructural signs of ER stress in the beta cells of these mice including enlarged mitochondria and ER. Further study is required to establish whether markers of beta cell ER stress are elevated in this model, although unpublished work did not find BiP or CHOP protein to be increased in female or male mice (Kautz, 2010; Schuster, 2011). ER stress in this model is thought to drive both a reduction in insulin secretion as well as a loss of beta cell mass since islet insulin immunofluorescence and beta cell density are significantly reduced compared to wildtype mice which corresponds with lower plasma insulin levels (Herbach et al., 2007).

1.5.3 The KINGS mouse

The KINGS mouse is a novel model of diabetes harbouring a heterozygous mutation in *Ins2* which developed spontaneously within a C57BL/6J mouse colony at King's College London (Austin et al., 2020). The KINGS mutation drives a glycine to serine substitution at position 32 of preproinsulin's B chain which introduces a side chain resulting in a conformational change that perturbs the formation of the A7-B7 disulphide bond. This is thought to drive beta cell ER stress with ultrastructural signs of ER stress, including distended ER and distorted mitochondria, evident through electron microscopy (Austin et al., 2020). Similar to both the Akita and Munich mice, mice homozygous for the KINGS mutation are severely hyperglycaemic and emaciated from weaning and thus heterozygous mice have preferentially been used. Heterozygous KINGS mice also show stark differences in phenotype with male mice developing diabetes at 5-6 weeks of age whilst females remain under the threshold for overt diabetes (16.7mM) throughout life. Despite this, both sexes show glucose intolerance from 4 weeks of age when subjected to a glucose tolerance test. *Ex vivo* studies have shed light on the pathogenesis in this model, showing that glucose stimulated insulin secretion is impaired in islets from the KINGS mice from 10 weeks of age and islet insulin content is markedly reduced compared to wildtype islets from 4 weeks of age. In line with this, electron microscopy has revealed that beta cells from the KINGS mice are degranulated.

1.6 Sex differences in diabetes:

The Akita, Munich and KINGS models show stark sex differences in diabetes incidences, with females largely protected from hyperglycaemia. This female protection also extends to other models of diabetes as well as to humans. For example, type 1 diabetes represents the only autoimmune disease with a male predominance (for example, male predominance is 7:1 post puberty in Caucasian populations), and at the time of T1DM diagnosis women typically have superior residual beta cell function which is associated with reduced severity of disease (Gannon et al., 2018; Samuelsson et al., 2013). Incidence of T2DM is also lower in women prior to the menopause and furthermore men are classically diagnosed with diabetes at a lower body mass index (BMI) compared to women (Kautzky-Willer et al., 2023). It should be noted however

that post-menopausal women are at an increased risk of T2DM compared to age matched men. Physiological differences between the sexes which may drive differences in diabetes incidences can be attributed to differences in peripheral insulin sensitivity, differences in beta cell function or differences in the capacity of the beta cell to respond to stressors. The mechanisms driving the latter include the action of sex hormones as well as the action of sex chromosomes.

1.6.1 Sex differences in insulin sensitivity:

Impaired insulin sensitivity, as described previously, is thought to be a major contributor to beta cell failure especially in T2DM. Woman typically harbour increased fat mass, reduced skeletal muscle mass and have higher circulating levels of free fatty acids compared to men (Tramunt et al., 2020). Despite these factors being associated with the development of insulin resistance, studies have found that woman exhibit superior insulin sensitivity compared to men and this may play some role in mediating sex differences in diabetes incidences (Kautzky-Willer et al., 2012; Nuutila et al., 1995). For example, Nuutila *et al.* used positron emission tomography to show that women were more than 40% more insulin sensitive compared to BMI-matched men, a phenomenon that was attributed to enhanced glucose uptake by skeletal muscle. It is important to note that these studies were carried out in women prior to the menopause. Further studies have revealed that the menopause is associated with a reduction in insulin sensitivity to levels comparable to men, implicating oestrogen in the enhanced insulin sensitivity pre-menopause (Catalano et al., 2008; Greenhill, 2018).

One way in which oestrogen may mediate sex differences in insulin sensitivity is through its control of adipose distribution. Prior to the menopause, females preferentially accumulate fat in subcutaneous depots whereas men are more likely to accumulate visceral fat (Geer & Shen, 2009). The latter has been associated with both peripheral and hepatic insulin resistance in the context of normoglycemia and type 2 diabetes (Gastaldelli et al., 2007; Miyazaki et al., 2002). Interestingly, menopause is associated with a shift in fat storage to visceral sites which corresponds with a reduction in insulin sensitivity.

1.6.2 Sex differences in beta cell function:

Optimal beta cell function is crucial for blood glucose homeostasis and impairments in this underpins diabetes pathogenesis. Some studies have suggested that sex differences exist in beta cell function in a physiologically healthy context, with beta cell function believed to be superior in women. Indeed, at comparable blood glucose concentrations, women have been found to have enhanced C-peptide and preproinsulin levels compared to men and they also show a higher disposition index (Basu et al., 2006; Gannon et al., 2018; Mauvais-Jarvis, 2018). Hall *et al.* has also reported superior glucose stimulated insulin secretion *ex vivo* in islets isolated from women when directly compared to islets from men, indicative of beta cell functional differences (Hall et al., 2014). The latter also suggests that intrinsic sex differences exist in islets since differences prevail outside of the female/male environment. Interestingly, the authors suggested differences in insulin secretion could be attributed to differences in gene methylation since female islets showed increased methylation of genes associated with reduced insulin secretion including *Nkap* (encoding NF-kB). Supporting this, a separate large-scale gene expression analysis found that islets from male and female mice clustered separately, and genes involved in protein synthesis and processing as well as genes involved in the cellular response to ER stress were enriched in female islets (Brownrigg et al., 2023). This suggests that female beta cells may harbour an improved capacity to fold and process proteins (including insulin) which may explain findings of functional superiority.

1.6.3 Sex differences in beta cell susceptibility to failure:

The response of the beta cell to stressors, such as increased insulin demand, ultimately dictates whether glucose homeostasis will be maintained or whether beta cell failure will occur. Adaptive responses drive compensation in the face of stress, and this can include increasing insulin secretion, undergoing proliferation or resisting cell death. Maladaptive responses to stress on the other hand include functional failure and death and are therefore associated with diabetes development. Whilst response to stress is likely driven largely by the extent of the stress and type or stressor, differences in how male and

female beta cells respond to this may contribute to the differences seen in diabetes incidences. The fact that women have better residual beta cell function when diagnosed with T1DM compared to men supports this notion, however research into this area is currently limited (Samuelsson et al., 2013).

A recent study by Brownrigg *et al.* has shed light on sex differences in beta cell response to ER stress. In this study, islets isolated from female mice and treated with the ER stress inducer, thapsigargin, were found to maintain glucose stimulated insulin production and secretion better than thapsigargin treated male islets (Brownrigg et al., 2023). The study also found that protein expression after thapsigargin treatment differed between male and female islets; of the 47 proteins differentially expressed in female islets after treatment and 82 proteins differentially expressed in male islets, only 7 proteins were shared between the sexes. This supports the notion that response to stressors may differ between sexes, and indeed only male islets showed a downregulation of proteins involved in the insulin secretory pathway. This study and others have also shown that transcriptional differences exist between islets isolated from men and women with T2DM, implying that differences in beta cell responses to stressors extends to humans (Brownrigg et al., 2023; Drigo et al., 2019).

The latter studies were performed on islets *ex vivo* and are therefore suggestive of islet intrinsic sex differences which may be driven by the harbouring of different sex chromosomes. However, more study has been made into how the male/female *in vivo* environment can drive differential responses to stressors. In particular, the presence and actions of the sex hormone oestrogen has received much attention. Compelling evidence that oestrogen may drive sex differences in diabetes susceptibility comes from *in vitro* studies showing that oestroadiol treatment protects both rodent and human beta cells against a variety of stressors including high glucose, lipotoxicity, proinflammatory cytokines and oxidative stress (Kooptiwut et al., 2018; Le May et al., 2006; Xu et al., 2018). *In vivo* studies have further supported oestrogens protective capacity against stressors with male mice protected from streptozotocin (STZ) induced diabetes when treated with

oestradiol, and similarly oestradiol treatment protects against diabetes in other models including male hIAPP mice and male Zucker fat rats (Geisler et al., 2002; Paik et al., 1982; Tiano et al., 2011).

1.6.4 Sex hormone contribution to sex differences in diabetes:

The primary sex hormones that differ between men and women are oestrogen and progesterone, whose circulating levels are greater in females, and testosterone which has a higher circulating concentration in men. Although the principal functions of sex steroids are in sexual development and reproduction, they also play an important role in glucose homeostasis and as such have been proposed to contribute to sex differences seen in diabetes. Here we discuss the evidence for the role of these sex hormones in glucose homeostasis as well as evidence that they may be involved in driving sex differences in diabetes.

1.6.4.1 Oestrogen and its role in driving sex differences in diabetes:

Three forms of oestrogen exist: oestrone (E1), oestradiol (E2) and oestriol (E3). Since oestradiol is the main circulating oestrogen in pre-menopausal women and is thought to contribute more to glycaemic control than the other types, we will place focus on this hormone.

Oestradiol is primarily produced from the theca and granulosa cells of the ovary; however, it is also produced, albeit in much smaller quantities, in other tissues such as the testes, adrenal glands, skin and adipose tissue. In the ovary, cholesterol is converted to androstenedione and testosterone, and P450aromatase drives their aromatisation to oestrone (which can be subsequently converted to oestradiol) and oestradiol, respectively (Figure 10) (Xu et al., 2022). Although both males and females have circulating oestradiol, only 0.25% of testosterone in the testes is converted to oestradiol whereas 50% of the testosterone produced in the ovaries is converted to oestradiol (Merino & García-Arévalo, 2021). This results in considerably higher concentrations of oestrogen in pre-menopausal women compared to men.

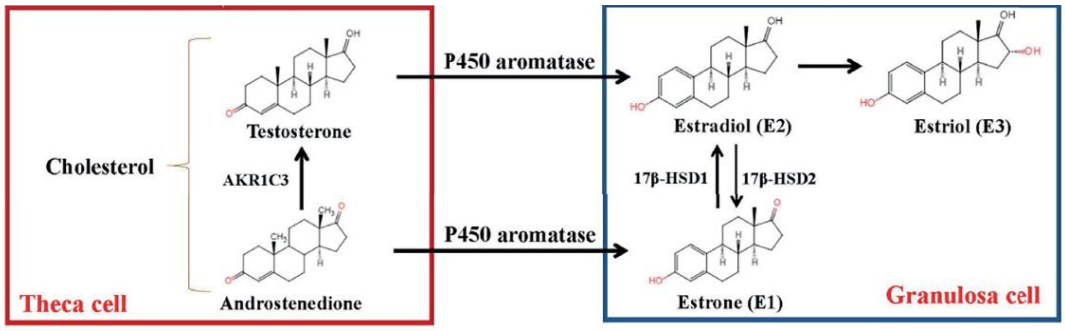


FIGURE 10: *Ovarian synthesis of oestradiol:*
Synthesis of oestradiol in the theca and granulosa cells of the ovary. Cholesterol is converted to androstenedione and testosterone in the theca cells. P450 aromatase drives the aromatisation of testosterone to form oestradiol in the granulosa cells and the aromatisation of androstenedione to form oestrone. Oestrone can be converted to oestradiol through the action of 17-beta-hydroxysteroid dehydrogenase 1 (17-beta-HSD1). Image taken and adapted from (Xu et al., 2022).

Oestradiol can signal via the classical oestrogen receptor, ER-alpha and beta, as well as the more recently described non-classical receptor G protein-coupled oestrogen receptor GPER (Merino & García-Arévalo, 2021). Intriguingly, all three receptors are present on beta cells from males and females suggesting a role of oestrogen signalling in beta cell function in both sexes. Much support for the latter comes from knockout studies. For example, beta cell specific ER-alpha knockout in mice impairs insulin secretion and increases beta cell apoptosis (Zhou et al., 2018). Zhou *et al.* recently suggested that activated ER-alpha may protect beta cells by reducing ER and mitochondrial stress through binding the promotors and repressing the expression of *CHOP* and *Oma1* (Zhou et al., 2018). CHOP mediates terminal UPR signalling, whilst Oma1 regulates mitochondrial stress signalling, and both are associated will cellular failure (Rivera-Mejías et al., 2023). Indeed, whilst ER-alpha knockdown in MIN6 cells was associated with increased Oma1 and CHOP expression and apoptosis susceptibility, ER-alpha overexpression in MIN6 cells saw a reduction in Oma1 and CHOP and

improved survival and insulin secretion. Many other mechanisms by which ER-alpha promotes beta cell function and survival have been suggested and are reviewed elsewhere and these include activating the transcription factor NeuroD1 which promotes insulin gene expression thereby increasing insulin synthesis (Merino & García-Arévalo, 2021; Wong et al., 2010).

Activation of ER-beta has consistently been associated with enhanced beta cell glucose stimulated insulin secretion. One way in which it does this through regulating closure of the ATP sensitive potassium channel activity to control beta cell membrane depolarisation which governs insulin secretion (Soriano et al., 2009). It has been proposed that E2 binding to ER-beta allows it to activate the guanylyl cyclase A (GC-A) receptor on the beta cell membrane which drives an increase in cyclic guanosine monophosphate (cGMP) and subsequent activation of the enzyme protein kinase G (PKG) which induces K_{ATP} channel closure. In line with this, whilst oestradiol treatment of WT murine beta cells inhibited K_{ATP} activity by 60%, this effect was lost when beta cells from ER-beta or GC-A knockout mice were used (Soriano et al., 2009). Oestradiol binding to the non-classical GPER has also been shown to have insulinotropic activity. Indeed, treatment of MIN6 cells and mouse islets with oestradiol or a specific GPER agonist (G-1) enhanced insulin secretion to a similar degree whilst co-treatment with a GPER antagonist (G15) abolished this effect (Sharma & Prossnitz, 2011). Several mechanisms by which GPER regulates insulin secretion have been suggested and this includes through inducing calcium release from intracellular stores as well as through activating ERK (extracellular signal-regulated kinase) which regulates insulin release in beta cells (Sharma & Prossnitz, 2011).

Although oestradiol is important for beta cell function and survival in both sexes through the mechanisms discussed, higher circulating oestradiol in women has been associated with driving sex differences in diabetes. Support for this comes from findings that diabetes incidence increases after the menopause which correlates with a decline in oestradiol levels (Muka et al., 2017). Moreover, menopausal women receiving hormone replacement therapy have a reduced risk of developing diabetes (Mauvais-Jarvis, Manson,

et al., 2017). It is important to note that oestradiol's influence on insulin sensitivity may be in-part responsible for these observed protective effects alongside changes in beta cell function and survival. The two effects are therefore difficult to distinguish from one another. Pre-clinical studies using ovariectomy and oestrogen to manipulate oestradiol levels in models of diabetes have provided more insight into this.

Generally speaking, ovariectomy in female pre-clinical models is associated with a loss of glycaemic control, whilst oestradiol treatment in males is protective against diabetes. The latter have been associated with direct effects of oestradiol on the beta cell. Santos *et al.* found that ovariectomy (ovx) in mice was associated with glucose intolerance and this could be in-part explained by a reduction in beta cell insulin secretion possibly caused by a downregulation of Syntaxin 1-A, a protein known to be important in insulin exocytosis (Santos et al., 2016). Treatment of ovx mice with exogenous oestradiol rescued this phenotype. Ovx mouse islets have also been shown to have reduced expression of SNARE complexes which are directly involved in insulin granule exocytosis (Santos et al., 2016).

Separate studies have also found that ovx is associated with a reduction in beta cell mass. In 90% pancreactomised rats, ovx caused a further reduction in beta cell mass whilst oestradiol treatment improved this through enhancing beta cell proliferation and reducing beta cell apoptosis (Choi et al., 2005). Improved beta cell proliferation with oestradiol treatment in this study was attributed to increased protein expression of IRS2 (insulin receptor substrate 2) and PDX1. Expression of both these proteins can be enhanced through the activity of the cAMP response element binding protein whose gene expression is positively regulated by oestradiol. Finally, ovx in a mouse model of beta cell ER stress, the Akita mouse, results in diabetes development in the otherwise protected female mice and treatment of male Akita mice with conjugated oestradiol reduced blood glucose concentrations (Xu et al., 2018). The authors proposed that oestradiol reduces beta cell ER stress by stabilising a component of the ER associated degradation machinery, thereby preventing ER stress induced beta cell apoptosis.

1.6.4.2 TESTOSTERONE AND ITS ROLE IN DRIVING SEX DIFFERENCES IN DIABETES:

Testosterone is the major male hormone. It is produced predominantly in the Leydig cells of the testes and is responsible for secondary sexual characteristics in males as well as reproductive function. Although circulating levels of testosterone are higher in men, testosterone is also important in female physiology with roles in reproductive function, cognition, and maintenance of bone density (Bianchi et al., 2021). Recognition that testosterone may play some role in blood glucose homeostasis initially came from findings that men deficient for this hormone, for example in men receiving androgen deprivation therapy (ADT) for prostate cancer, had higher incidences of diabetes. Keating *et al.* found that men diagnosed with prostate cancer and receiving ADT were 44% more likely to develop diabetes compared to men with prostate cancer but not treated with ADT (Keating et al., 2006). Supporting a protective role of testosterone in diabetes, other studies have found that men diagnosed with diabetes typically have lower levels of circulating testosterone (Elabbady et al., 2016; Yao et al., 2018). On the other hand, other studies have found no association between the two (Holmboe et al., 2016; Lakshman et al., 2010). Intriguingly, it has been proposed that testosterone may bi-directionally impact blood glucose homeostasis in males and females (Xu et al., 2019). Indeed, several studies have shown that women exhibiting androgen excess, including those diagnosed with polycystic ovary syndrome (PCOS), have a higher risk of developing T2DM, although again other studies have disputed this association (Ganie et al., 2016; Weerakiet et al., 2001; Zhang et al., 2018).

One of the ways in which testosterone may influence glucose homeostasis is through its effects on peripheral insulin sensitivity (Navarro et al., 2015). In men, low testosterone levels are associated with high visceral adiposity which in turn is associated with insulin resistance (Khaw & Barrett-Connor, 1992). Testosterone signals via the androgen receptor (AR) and supporting a direct role of testosterone in insulin sensitivity through effects on fat distribution, global AR knockout mice develop visceral obesity and insulin resistance (Fan et al., 2005; Lin et al., 2005). There is also evidence that testosterone acts to

enhance insulin sensitivity in the liver and skeletal muscle via the AR (Navarro et al., 2015). In contrast, elevated testosterone in females drives an increase in visceral adiposity which is associated with enhanced insulin resistance (Evans et al., 1988). Women with hyperandrogenism also show impaired insulin stimulated glucose uptake from skeletal muscle although the mechanism behind this is unknown (Diamond et al., 1998).

ARs are present on both male and female beta cells suggesting that testosterone also acts directly on the beta cell to impact regulation of blood glucose levels. Indeed, when the AR is specifically knocked down in the beta cells of male mice, glucose intolerance occurs (Navarro et al., 2016). This effect is exacerbated in mice fed a high fat diet, with AR knockout male mice exhibiting elevated blood glucose concentrations and worsened glucose tolerance compared to wildtypes. It should be noted however that no change in glycaemic phenotype is observed in female knockout mice even when fed a high fat diet. Navarro *et al.* has suggested that testosterone mediates its effects on insulin secretion by potentiating the cytosolic cAMP rise mediated by glucagon-like peptide-1 (GLP-1) binding to its receptor on the beta cell (Figure 11). Cytosolic cAMP drives PKA activation which enhances insulin secretion by driving the closure of potassium channels and releasing calcium from ER stores. Indeed, whilst testosterone improved glucose stimulated insulin secretion in islets, this effect was lost with GLP1 receptor antagonist treatment. This implicates testosterone in fine tuning insulin secretion. RNA sequencing of islets from beta cell-specific AR-KO male mice has provided more support that testosterone is important for beta cell function; 20% of dysregulated genes in the knockout mouse were associated with beta cell function which included genes involved in insulin exocytosis machinery and cellular stress (Xu et al., 2017).

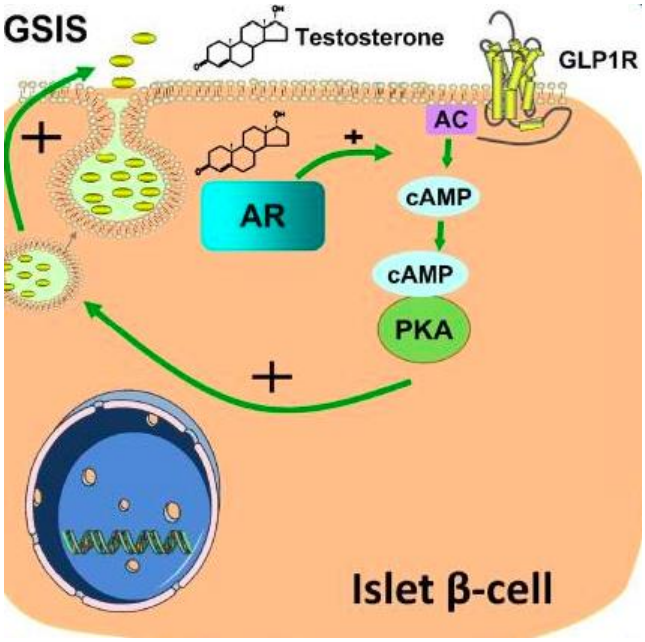


FIGURE 11: *Testosterone potentiates GLP-1 mediated insulin secretion: Testosterone acts via beta cell androgen receptors to potentiate GLP-1 mediated insulin secretion. It does this through amplifying cytosolic cAMP which leads to increased activation of PKA to enhance insulin secretion. Figure adapted from* (Navarro et al., 2016).

In contrast to the beneficial effects of testosterone on beta cell in males, high levels of testosterone in females drives beta cell dysfunction and this is believed to be mediated by hypersecretion of insulin driving beta cell exhaustion (Xu et al., 2019). Indeed, whilst testosterone treatment in female WT mice resulted in hyperinsulinemia and elevated blood glucose concentrations, beta cell specific AR knockout protected against this (Navarro et al., 2018). A separate study also found that dihydrotestosterone administration in female rats drove hyperinsulinemia, enhanced insulin secretion in response to glucose and increased beta cell *Ins1* gene expression (Mishra et al., 2018). In line with this, testosterone pre-treatment in female mice increases hyperglycaemia induced by STZ (Paik et al., 1982).

Although the above-mentioned evidence supports a differential role of testosterone in blood glucose homeostasis, it is important to point out that some studies contradict these findings. For example, removal of endogenous

testosterone in male mice through orchidectomy, or through use of anti-androgen, prior to STZ treatment protects against the development of diabetes (Maclaren et al., 1980; Paik et al., 1982). These findings implicate testosterone in driving sex differences in diabetes. Differences in findings may be explained by testosterone's effect on the beta cell being dependent upon the amount and nature of the stress that the beta cell is under, leading to testosterone being beneficial in some contexts whilst detrimental in others. For example, under healthy physiological conditions testosterone may be beneficial to beta cell functional mass in males, however in the context of oxidative damage (as is the case with STZ treatment) testosterone may contribute to beta cell failure. However, much more study is required in this area.

1.7 Research objectives:

Beta cell ER stress has been implicated in diabetes pathogenesis and indeed is a shared mechanism by which many beta cell stressors mediate beta cell failure. In addition to this, sex differences exist in diabetes incidences and severity in both humans and preclinical animal models, with females generally more protected. The mechanisms that drive this sex differences are elusive, as is whether sex differences exist in beta cell ER stress response or susceptibility. Gaining insight into the latter has the potential to reveal novel therapeutic avenues and guide sex-specific diabetes treatments.

The KINGS mouse is a novel model which harbours a heterozygous *Ins2* mutation thought to drive beta cell ER stress. Whilst the KINGS mutation drives diabetes development in male mice, female mice are protected. The aim of this study was to use the KINGS mouse as a model to gain further insight into sex differences in beta cell response/resilience to ER stress and how beta cell ER stress can be manipulated. To do this, we had 5 distinct research objectives:

- *Objective 1:* Re-characterise the phenotype of the KINGS mouse.

- *Objective 2:* Further characterise beta cell ER stress in the KINGS mice and investigate whether beta cell response to this differs between the sexes.

- *Objective 3:* Investigate the influence of the major female sex hormone, oestradiol, on beta cell ER stress and glycaemic control in the KINGS mice.

- *Objective 4:* Investigate whether beta cell ER stress can be exacerbated in female KINGS mice and determine whether this leads to a worsening of glycaemic control.

- *Objective 5:* Investigate whether diabetes development can be prevented in the KINGS mouse through treatment with therapeutics previously reported to reduce ER stress and through hormonal manipulation.

CHAPTER 2: METHODS

2.1 THE 3R'S, ANIMAL HUSBANDRY AND COLONY MAINTENANCE:

All experiments using animals were performed under a project licence in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 with 2012 amendments and were ethically approved by the institution's Animal Welfare and Ethical Review Board (AWERB). Although these legal obligations are critical, researchers should also be striving towards fulfilment of the recommendations set out by the 3Rs to ensure the highest possible animal welfare standards are met.

2.1.1 CONSIDERATION OF THE 3R'S:

The three R's: replacement, reduction, and refinement, forms an ethical framework which aims to improve the welfare of laboratory animals used for *in vivo* research. Replacement refers to methods which replace the need for the use of animals (for example, the use of cell lines). Partial replacement on the other hand involves the use of animal-derived tissue *ex vivo* or the use of alternative animals which are perceived to experience reduced pain (for example, using invertebrates in place of vertebrates). Reduction refers to approaches employed to reduce the number of animals used in a given experiment which may include maximising the amount of data that can be generated from an individual animal, for example. Finally, refinement includes any strategy that attempts to minimise stress, pain or suffering experienced by the animal and may include improvements to husbandry practices.

2.1.1.1 REPLACEMENT:

This project focussed on further characterising the KINGS mouse and thus the use of mice for this project could not be avoided. However, in experiments where glucose stimulated insulin secretion was investigated, this was assessed *in vitro* in isolated islets rather than *in vivo* where blood is sampled via the tail vein after the mouse is given a glucose bolus. This therefore represents a partial replacement of mice. The expression of beta cell ER stress

markers using western blot and immunohistochemistry also involved use of mouse-derived tissue *ex vivo* and thus also represents a partial replacement.

2.1.1.2 Reduction:

Colony breeding was planned using the Jackson Laboratory breeding colony size planning worksheet in order to reduce the number of surplus animals whilst also reaching our research needs (The Jackson Laboratory, n.d.). Where possible, mating trios rather than pairs were used to limit the number of male mice reserved for mating. For some experiments, pancreases from a single mouse were used both for harvesting islets (used for static insulin secretion assays) and for obtaining tissue for histology, thus maximising the use of individual mice, and reducing the need for more mice.

Finally, both male and female mice were used throughout this project reducing the number of surplus animals compared to if just one sex was used, something that is common practice in the literature. Typically, male mice have preferentially been used for *in vivo* diabetes research. Since a major aim of this project was to understand differences in beta cell response to stress, our findings may add to the body of research attempting to understand sex differences in glucose homeostasis which will likely lead to the greater incorporation of both sexes in future research studies.

2.1.1.3 Refinement:

Refinement measures reduce stress and pain experienced by animals and thus are important ethically. Moreover, since stress has been associated with alterations in multiple aspects of physiology, refinement measures are also important in obtaining reliable and physiologically relevant data. This is particularly pertinent for endpoints related to blood glucose homeostasis since studies have directly implicated stress hormones in elevating blood glucose concentrations (Jia et al., 2020; Zheng et al., 2018).

Refinement measures used in this project included those relating to husbandry. Enrichment was provided in cages in the form of nesting material and cardboard tubes. Since nest building is instinctive for mice, this allowed

them to carry out their natural behaviour as well as providing warmth. Cardboard tunnels could also be shredded by mice to build nests and offered a hiding space away from more aggressive littermates. When mice showed signs of aggression (which included seeing animals fighting or observing signs of fighting such as missing fur and scabbing), plastic housing was added to the cage to give additional hiding spaces and a gnawing block was also provided. In breeding cages, extra carboard tubes were added supplying more obscured spaces for mice to breed, deliver and feed pups. Finally, bedding and cardboard tunnels in cages were only replaced if they were heavily soiled, and even then, some bedding was still retained. The latter prevented complete removal of pheromones from cages which reduces stress and aggressive behaviour since pheromones are used to establish hierarchy in mice (Rasmussen et al., 2011; Zilkha et al., 2023). Breeding cages were changed and animals were handled less often when neonates were present to avoid the stress associated with this. The latter is important as stress can cause parents to cannibalise their litters.

Refinement measures specific to experiments included allowing 30 minutes for mice to acclimatise to procedure rooms before performing procedures (such as glucose tolerance testing) in an attempt to reduce stress. It was additionally decided to not perform glucose tolerance tests in mice where basal glucose concentrations exceeded 20mM. Fast lengths used for insulin tolerance tests were also reduced during the project to limit the number of animals experiencing hypoglycaemia. In addition, a smaller needle size (30G rather than 27G used previously by the laboratory) was used for needle pricks to the end of the tail in mice and for intraperitoneal injections. For the study where oestradiol was administered to mice continuously over several weeks, silastic capsules containing oestradiol were implanted rather than injecting mice with oestradiol on a daily basis (which can cause skin lesions and increases the amount of handling).

Refinement measures were also considered for the surgeries performed. This included providing food pellets at the base of the cage for several days following surgery, eliminating the need for animals to stretch to eat from the

food hopper which may cause pain especially around wound sites. Additionally, for all surgeries, mice were administered Carprofen as an analgesic rather than previously used buprenorphine which has been associated with respiratory depression. To reduce pain and reduce the risk of the mice biting suture knots at the wound site, Lidocaine was also injected around incision sites to provide short-term local anaesthetic immediately after surgery.

2.1.2 Animal husbandry:

Mice were housed in single sex groups of up to five in individually ventilated cages (GM500, Scanbur) and had *ad libitum* access to water and standard rodent diet 20 chow (Picolab UK). Mice were kept on a 7am-7pm light-dark cycle. Cages were typically changed on a weekly basis, however cages containing adult KINGS male mice were changed more often since polyuria in these mice resulted in bedding being soiled rapidly. Enrichment in cages included nesting material and cardboard tunnels. In cages where fighting was observed between mice, additional enrichment including plastic houses and wooden chewing blocks was added and mice were monitored for wounds. Any animals showing signs of distress, weight loss (>15%) or signs of pain or illness were culled using a schedule 1 method. Temperature in the animal facility was maintained within a narrow range (20-24ºC) as was humidity (45-64%) and this was checked at least once a day.

2.1.3 Colony maintenance:

The KINGS colony was maintained in-house and 6-20-week-old male/female KINGS were mated in duos or trios with wildtype 6-20-week-old male/female mice. Offspring heterozygous for the KINGS mutation were used as KINGS mutants and wildtype littermates were used as controls. Male breeder mice were mated continuously (not removed from cages after parturition) to take advantage of post-partum oestrous which lasts for a short period (~20 hours). Mice were weaned on average between 21 and 22 days of age. Any mouse that appeared runted and was noticeably smaller than littermates at 21-22 days of age were culled. At approximately 6 weeks of age, mice were monitored for malocclusion and any mouse exhibiting this was culled. On the

rare occasion that the mothers of neonatal (<21-22 days of age) mice were culled due to health concerns, foster mothers with their own pups within 1-3 days of age of the foster litter were used. To promote the fosters mother acceptance of the foster pups, bedding from the foster mother's cage was rubbed onto the pups before their transfer and foster pups were mixed in with the foster mother's birth pups. Foster pups were monitored for several days after the transfer to ensure they were being fed.

2.1.3.1 Refreshing the KINGS colony:

Overview: Owing to COVID restrictions starting in 2020, the KINGS colony was reduced to fewer than 20 individual mice. To mitigate risks of the founder effect causing genetic drift within the colony when colony size was subsequently increased, the KINGS colony was genetically refreshed. This is important because genetic drift over time can lead to a colony accumulating mutations which have potential to alter phenotype and impact experimental reproducibility.

Limitations: Complete genetic refreshment of a colony takes a significant amount of time, approximately 6-months.

Technique: KINGS mice were backcrossed with newly purchased C57BL/6J mice (strain code: 632, Charles River, UK) to ensure both male and female sex chromosomes and the mitochondrial genome was refreshed (Figure 12). To do this, female KINGS mice were crossed with newly purchased C57BL/6J male mice. KINGS male offspring from this mating were mated with newly purchased female C57BL/6J mice. The KINGS male offspring from this second mating were finally crossed with newly purchased female C57BL/6J mice to generate genetically refreshed offspring.

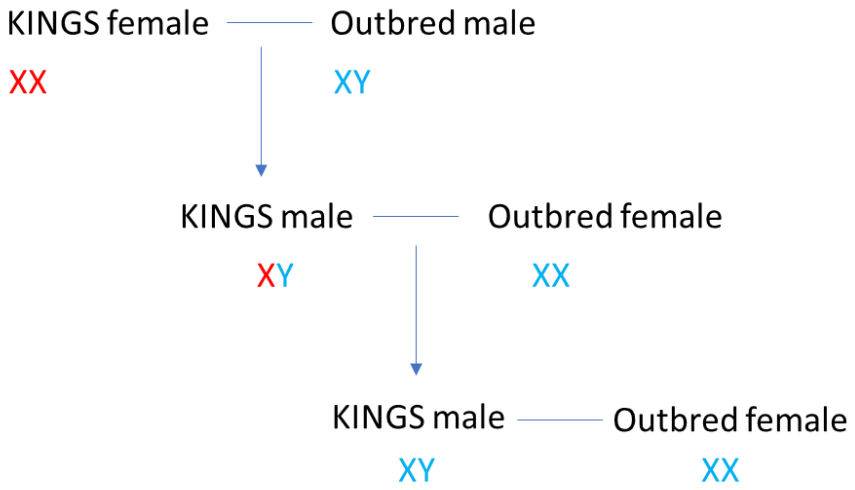


FIGURE 12: *Schematic showing backcrossing breeding used to genetically refresh the KINGS colony:*
The backcrossing scheme ensures that both sex chromosomes and the mitochondrial genome are refreshed.

2.1.3.2 GENOTYPING:

Overview: KASP (Kompetitive Allele specific PCR) genotyping was used to genotype mice from the KINGS colony based on the presence of the KINGS mutation.

Limitations: KASP genotyping is more expensive than the use of gel electrophoresis, however, is represents a quicker method.

Technique: Mice were genotyped based on the presence or absence of the KINGS single nucleotide polymorphism in *Ins2* using KASP (LGC, Hoddesdon, UK).

Ear clip digestion: Mice were scruffed and ear clippings were taken in restrained mice using an ear punch device. Ear clips were digested in DNA digestion buffer (50µL,
TABLE 1) overnight at 55ºC. Digested samples were heated for 20 minutes at 90ºC to inactivate the proteinase K and samples were stored at -20ºC until further PCR processing.

Component:	Volume (µL) for 1 sample:
Deionised water	43.25
10x Gitschier's buffer	5
20% Triton X-100	1.25
2-ß-Mercaptoethanol	0.1
Proteinase K solution (50µg/mL)	1
Total volume:	50

TABLE 1: *DNA digestion buffer used for genotyping.*

KASP genotyping: Approximately 50ng of DNA was combined with assay mix and Low-ROX KASP reaction mix. The assay mix contained two forward primers whose 3' end was either specific for the KINGS polymorphism in *Ins2* (GAAGGTCGGAGTCAACGGATTGCTTTTGTCAAGCAGCACCTTTGTA) or wildtype *Ins2* (GAAGGTGACCAAGTTCATGCTTTTGTCAAGCAGCACCTTTGTA). The tail sequence of each primer differed and was identical to oligonucleotides labelled with either FAM (wildtype forward primer) or HEX (KINGS forward primer) fluorophores. These fluorophores were quenched in an oligonucleotide cassette that contained primer tail-complementary oligonucleotides conjugated to a fluorophore quencher. The reaction mix contained the reporter cassettes as well as KASP Taq polymerase and dNTPs required for the PCR reaction.

The PCR mixture was subjected to thermocycling on the Roche Lightcycler 480 or a Roche Lightcycler 96 (Figure 13). The PCR mix was heated to 94ºC for 15 minutes to remove chemical inactivation of KASP Taq. The mix was then subjected to ten cycles of 20 seconds heating at 94ºC and 60 seconds at 61ºC. The temperature was reduced in 0.6ºC increments to 55ºC and then the PCR mix underwent 26 cycles of 20 second heating at 94ºC and 60 second heating at 55ºC. In brief, these heating cycles denatured the DNA template and allowed for annealing and extension of the two allele specific primers (which bound competitively to the DNA template) as well as annealing and extension of the common reverse primer. During the second round of PCR, a complement to the primer tail is generated allowing the FAM or HEX linked oligonucleotides from the reporter cassettes to bind, removing their quenching, and allowing fluorescence to be emitted. Cooling of the PCR mix to 37ºC

caused quenching of any FAM/HEX bound oligonucleotides that were not incorporated into the PCR product. Fluorescence emission from the sample which corresponded to HEX (523-568nm) or FAM (483-533nm) fluorophores was used to ascertain genotype.

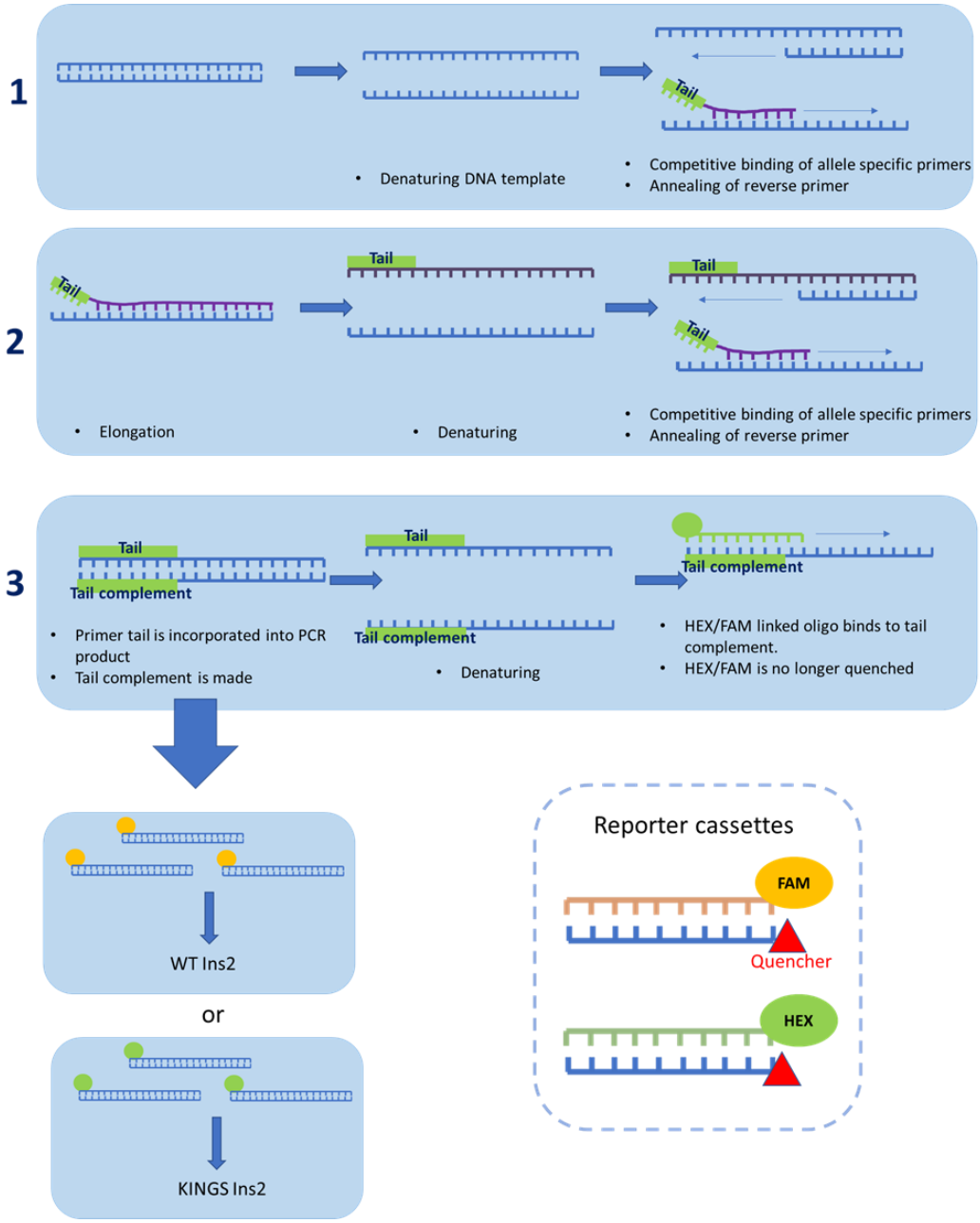


FIGURE 13: *KASP genotyping:*

KASP technology was used to genotype mice. (1) In the first PCR cycle DNA templates were denatured allowing for reverse primers corresponding to WT or KINGS Ins2 to competitively bind. Common reverse primers could also bind, and incorporation of dNTPs allowed for strand elongation. (2) PCR products

from the first PCR cycle are denatured allowing for allele specific primer binding, common reverse primer binding and strand elongation. (3) The tail region of the allele specific primer is incorporated into the PCR product and strand elongation results in a tail complement being formed. PCR products are denatured, and the HEX/FAM linked oligonucleotide from the reporter cassette can anneal to the tail complement releasing fluorophore quenching. An accumulation of either FAM or HEX linked oligonucleotides results in fluorescence emission of different wavelengths and detection of this is used to determine to presence of the KINGS Ins2 polymorphism.

2.2 Analysis of blood glucose homeostasis:

2.2.1 Non-fasted blood glucose measurements:

Overview: Measurement of non-fasted blood glucose concentration is relatively simple and does not require restraint of the animal. It was used in this thesis to investigate glycaemic control in experimental groups under fed (and thus physiologically normal) conditions.

Limitations: Blood glucose concentrations fluctuate constantly, and single blood glucose measurements do not capture this variability. Data can be influenced by time of day (likely a result of changes in food intake), although this can be accounted for by measuring blood glucose levels at the same time daily.

Technique: Non-fasted blood glucose concentrations were determined from a single blood droplet from a needle prick (30G needle) to the end of the tail in non-restrained mice using the Accu-Chek glucose meter and strips (Roche, UK). Weight and blood glucose readings were taken at the same time each day (8:00-9:00am) from weaning (at 21-22 days) to 42 days and every 2-3 days thereafter until 68-70 days, unless otherwise stated. The order in which non-fasted blood glucose concentrations were measured in experimental mice was randomised and different on different days.

2.2.2 Fasted blood glucose measurements:

Overview: Measurement of fasted blood glucose concentrations reduces the influence of food intake on glycaemic control and is therefore useful for studies where variation in food intake is suspected or if experimental groups are receiving diets of a different composition. This measurement was used to investigate glycaemic control in mice fed a high fat high sucrose diet.

Limitations: Prolonged fasting induces a state of starvation in mice and can lead to hypoglycaemia which has been associated with enhanced psychological stress.

Technique: Mice were fasted overnight for 16 hours after a cage change where bedding from the old cage was retained to reduce stress. Blood glucose concentrations were measured from a single blood droplet from a needle prick (30G needle) to the end of the tail using the Accu-Chek glucose meter and strips (Roche, UK) the following morning and feed was returned to the cage.

2.2.3 Glucose tolerance test:

Overview: Glucose tolerance tests were used to generate a state of enhanced beta cell stimulation to reveal impairments in glucose homeostasis that exist but may not be apparent under normal physiological fluctuations in blood glucose concentrations. This test was used to investigate glycaemic control under conditions of enhanced beta cell stimulation in experimental groups.

Limitations: Glucose tolerance tests alone do not provide information on the contribution of insulin resistance or impaired insulin secretion on glucose tolerance. Intraperitoneal glucose tolerance tests require fasting, restraint and intraperitoneal injection which can be stressful for the animal.

Technique: Mice were subjected to a morning 6-hour fast prior to the glucose tolerance tests (GTT) by transferring them to a new cage without food but keeping the bedding the same to limit stress. After the fast, mice were weighed to calculate glucose dose (2g/Kg, 30% sterile-filtered glucose solution, Sigma UK) and baseline blood glucose was taken from a single blood droplet from a tail prick using the Accuchek Performa glucometer and glucose strips (Roche,

UK). Glucose solution was subsequently injected intraperitoneally using a 1mL syringe and 30G needle in scruffed mice. Blood glucose concentrations were measured at 15, 30, 60, 90 and 120 minutes post-injection. Water was supplied *ad libitum* throughout, and mice were re-fed immediately after the experiment. Blood glucose readings that exceeded the Accucheck maximum (33.3mM) were re-measured using a StatXpress glucometer (Novabiomedical, USA).

Any mouse that did not experience a >5mM increase in blood glucose concentrations at 15 or 30 minutes following the intraperitoneal glucose injection was excluded from downstream analysis as it was assumed to be a technical failure. This exclusion criteria was based on previous studies from the laboratory that showed that the stress associated IP injection and restraint causes ~3mM blood glucose concentration increase in WT mice and ~5mM blood glucose concentration increase in KINGS mice. Less than 5% of data was excluded on this basis and in nearly all cases and where possible, the experiment was successfully repeated within a few days.

2.2.4 Insulin tolerance test:

Overview: Insulin tolerance tests were used to investigate whether insulin resistance contributed to lack of glycaemic control in the KINGS mice and whether changes in insulin resistance contributed to the effects of hormone or treatment intervention on glycaemic control in the KINGS mice.

Limitations: The insulin tolerance test is susceptible to stress-related increases in blood glucose concentrations, especially in the first 15-30 minutes of the test. On the other hand, at later time points mice can become hypoglycaemic (blood glucose concentrations <2.5mM) which causes hunching and lethargy and a risk of death if untreated.

Troubleshooting: In the initial insulin tolerance tests undertaken in this project, blood glucose concentrations did not fall as expected after injection with insulin. Upon troubleshooting it was found that filter sterilisation of the insulin solution was responsible for this and so for further experiments insulin

solutions were not filtered. It was additionally found that a 6-hour fast length was associated with many mice experiencing signs of hypoglycaemia and therefore fast length was reduced to 2 hours.

Technique: Insulin tolerance tests were performed prior to a 6-hour morning fast or 2h afternoon fast where mice were transferred with their bedding to a clean cage without food. Baseline blood glucose measurements was taken from a single blood droplet from a tail prick using the Accuchek Performa glucometer and glucose strips and mice were weighed to calculate insulin dose (0.75IU/kg, Sigma, UK). Insulin was injected intraperitoneally in scruffed mice using a 1mL syringe and 30G needle, and blood glucose concentration measurements were taken at 15, 30, 45 and 60 minutes post-injection. Mice were continuously monitored for signs of hypoglycaemia and any mice with blood glucose <2mM were injected with 2g/kg glucose solution. Data from mice injected with glucose solution to rescue them from hypoglycaemia were excluded from the final analysis and this occurred in <5% of mice. Mice not experiencing >1mM reduction in blood glucose concentrations at 15 or 30 minutes following the insulin injection were excluded from downstream analysis on assumption of technical failure. This accounted for <20% of experiments and in nearly all cases and where possible, the experiment was successfully repeated within a few days.

2.3 Manipulation of sex hormones in the KINGS mice:

2.3.1 Long-term oestradiol delivery via implantation of silastic capsules:

Overview: Silastic tubing (dimethylpolysiloxane, Dow Corning Corporation) is a type of silicone tubing frequently used for medical devices which has been shown to allow for the slow diffusion of steroids *in vivo* (Cohen & Milligan, 1993). Silastic capsules containing oestradiol provide sustained and consistent oestradiol delivery which is more physiologically relevant compared to commercially available oestradiol pellets. Silastic capsules also represent a more ethical method compared to daily injection of oestradiol which has been associated with the development of skin lesions. Subcutaneous implantation of oestradiol capsules was used to investigate whether oestradiol could protect

male KINGS mice from developing diabetes, and therefore whether it is involved in generating sex differences seen in diabetes phenotype.

Limitations: Implantation of silastic capsules require mice to undergo anaesthesia and surgery which can be psychologically stressful. As the oestradiol in the silastic capsules is depleted over time, oestradiol delivery reduces over time.

Technique:

2.3.1.1 Construction of oestradiol containing silastic capsules:

Silastic capsules containing 36µg/mL of 17-beta-oestradiol (Sigma, UK) were constructed as previously described (Ström et al., 2012). 2cm lengths of Silastic tubing (ID/OD: 1.575/3.175mm, VWR international Ltd, UK) were cut and sealed at one end using 3mm wooden applicator sticks (Figure 14). The entire length of tubing was then filled with 36µg/mL oestradiol (Sigma, UK) dissolved in sesame oil or sesame oil alone (vehicle) using a 1mL syringe and 27G needle. 3mm wooden applicator sticks were then used to close off the silastic tubing. Capsules were kept in sesame oil or 36µg/mL oestradiol solution overnight at room temperature to remove any air bubbles.

2.3.1.2 Implantation of silastic capsules:

On the day of weaning (21-22 days old), male KINGS and WT mice were anesthetised using 5% isoflurane, 95% oxygen in an induction chamber and 2-3% isoflurane supplied through a nose cone was used to maintain this. Once anaesthetised, mice were placed in a prone position on a heated mat and an eye lubricant (Viscotears) was applied to the eyes to maintain lubrication. A 2x2cm area was shaved at the dorsal aspect of the neck and surgical scrub (Hibiscrub) followed by 70% ethanol was used to disinfect the area. The animal was covered with a surgical sheet which was cut to expose the shaved site only. A 1cm incision was made using a scalpel and the skin was bluntly dissected to create a subcutaneous pocket into which the silastic capsule was inserted (Figure 14). The incision was then closed with suture (VICRYL 5-0, AstonPharma, UK) ensuring that the capsule was not putting strain on the skin

or incision site. Lidocaine (2mg/kg) was injected subcutaneously and proximal to the wound and Carprofen (4mg/kg) was injected intraperitoneally to minimise pain post-operation. Mice were transferred to a heated cabinet and monitored until fully recovered. Food was placed at the base of the cage for several days post-surgery to limit the need for mice to stretch to reach the food hopper which may have caused pain and placed strain on the wound site. Incision sites were re-sutured a maximum of once if the wound site opened as a result of the sutures coming undone and only if there were no signs of infection.

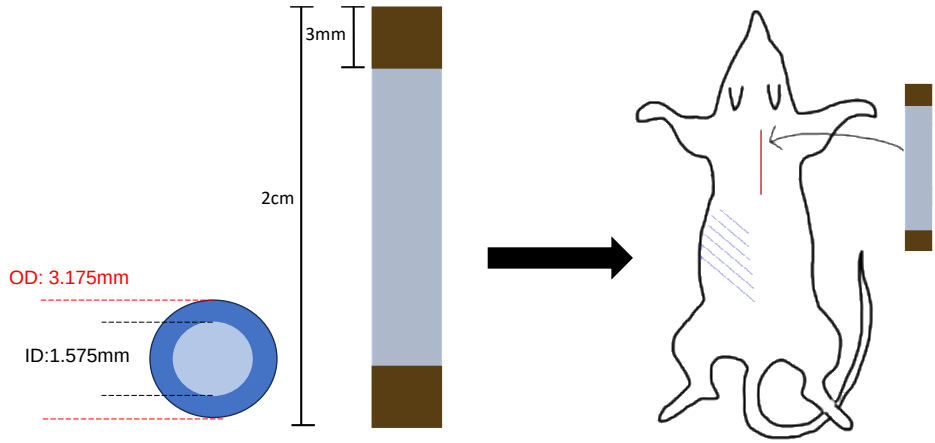


FIGURE 14: *Silastic capsule construction and location of subcutaneous implantation into mice:*
Silastic (ID/OD:3.175/1.575mm) tubing was cut to 2cm, filled with sesame oil or oestradiol solution and capped at both ends with 3mm wooden applicator sticks. An incision was made to the dorsal aspect of the animal's neck (red line), a subcutaneous pocket was bluntly dissected (blue line) and the silastic capsule was placed within this pocket.

2.3.2 OVARIECTOMY:

Overview: Ovariectomy represents the simplest method to remove endogenous oestradiol in female mice. It involves a ~15-20-minute surgical procedure which has a rapid recovery rate and had a 100% success rate in our hands. Ovariectomy was used to remove endogenous oestradiol from pre-

and post-puberty KINGS mice to investigate the contribution of the activational and organisational effects of this hormone in female protection from diabetes.

Limitations: The ovaries are responsible for the production of other hormones besides oestradiol, including progesterone, which may contribute to phenotype in ovariectomised mice. Oestradiol can be produced, albeit in lower amounts, at other sites including adipose tissue. This technique involves a relatively invasive surgical procedure which can be psychologically stressful for the animals. Finally, the ovaries in pre-pubertal mice are small and have a reduced blood supply making then harder to locate and so extending surgical time.

Technique: Mice were anaesthetised using 5% isoflurane, 95% oxygen in an induction chamber. The lower back of the mouse was shaved and cleaned using surgical scrub (Hibiscrub) and 70% ethanol. Anaesthesia was maintained using ~2-3% isoflurane supplied through a nose cone, mice were placed on a heated pad and lubricant (Viscotears) was applied to the eyes. A 2-3cm midline incision was made to the lower back and the skin was bluntly dissected away from the underlying fascia. Incisions (<0.5cm) were made in the fascia, left and right of the initial incision and just above the location of both ovaries. The ovaries were located using forceps and either suture was used to ligate the uterine horn and vessel (post-puberty mice), or a cautery pen was used to cauterise the blood vessel supplying the ovary (pre-puberty mice). The ovaries were subsequently cut and removed using a scalpel. Suture (VICRYL 5-0, AstonPharma, UK) was used to close the incisions and mice were administered Carprofen (4mg/kg) intraperitoneally and Lidocaine (2mg/kg) subcutaneously proximal to the incision site. Mice were transferred to a heated cabinet and monitored until fully recovered. For several days after surgery, food pellets were placed at the bottom of the cage to allow mice to feed without having to stretch up to food hopper which could cause pain and place a strain of the wound site. Any mice where the wound site opened-up were re-sutured once and only if there was no sign of infection.

2.3.3 Orchidectomy:

Overview: Orchidectomy is a simple method to remove endogenous testosterone from male mice which involves a 15-20-minute surgical procedure. This technique was used to investigate the influence of endogenous testosterone on diabetes development in male KINGS mice.

Limitations: The testes are responsible for producing other factors, including anti-Müllerian hormone, which may influence phenotype in orchiectomised mice. Testosterone is produced at sites other than the testes, albeit at smaller quantities. This technique involves invasive surgery which can be psychologically stressful to the animals.

Technique: Mice were anaesthetised using 5% isoflurane, 95% oxygen in an induction chamber. The lower abdomen of the mouse was shaved and cleaned using surgical scrub and 70% ethanol. Anaesthesia was maintained using ~2-3% isoflurane, mice were placed on a heated pad and lubricant (Viscotears) was applied to the eyes. A 0.5-1cm incision was made vertically down the abdomen, 0.5cm above the penis, and the skin was bluntly dissected away from the underlying fascia. An incision was made in the fascia underneath the initial incision site to access the abdominal cavity and forceps were used to locate the gonadal fat pads. Once located, the fat pads were gently pulled from the abdominal cavity along with the testes. A cautery pen was used to cauterise the blood vessel supplying the testes and these were removed using a scalpel. Suture (VICRYL 5-0, AstonPharma, UK) was used to close both wounds. Mice were administered Carprofen (4mg/kg) intraperitoneally and Lidocaine (2mg/kg) subcutaneously and recovered in a heated chamber. Mice were monitored for appropriate recovery. Food was placed at the base of the cage for several days post-surgery, limiting the need for mice to reach up towards the food hopper which may have caused pain or placed tension on the wound site. Incision sites were re-sutured once if the wound site opened and only if there were no signs of infection.

2.4 Administration of altered animal diet and drugs:

2.4.1 High fat high sucrose feeding:

Overview: High fat high sucrose (HFHS) feed mimics a western diet in humans and has been used frequently in the literature to model pre-diabetes and type 2 diabetes. We used HFHS feeding in an attempt to exacerbate beta cell ER stress in female KINGS mice, to investigate whether this induced a more male-like glycaemic phenotype. HFHS feed was used rather than high-fat feed which typically contains more fat (60%), this was because we hypothesised that elevating beta cell demand both directly (via elevated dietary sucrose) and indirectly (via elevated fat inducing insulin resistance) would prove more robust in exacerbating beta cell ER stress compared to just inducing insulin resistance (through a 60% high fat diet). Additionally, a HFHS diet is more translatable to human physiology since it contains a similar fat content to a western diet (Speakman, 2019).

Limitations: Mice tended to shred the HFHS feed, leading to food wastage, an inability to monitor food intake and the animals' fur becoming greasy.

Technique: Mice were fed irradiated HFHS feed (45kcal% fat, 30kcal% sucrose, ID: D09112601. Research Diets Inc, USA) or irradiated normal chow (NC: 13.2kcal% fat, 3.18kcal% sucrose, Rodent diet 20, PicoLab, UK) from weaning at 3 weeks of age until 20 weeks of age (female KINGS mice) or 10 weeks of age (male KINGS mice). Whilst both feeds were available *ad libitum*, halfway through the experiment, a smaller amount of HFHS pellets were placed at the bottom of the cage daily rather than in the food hopper in an attempt to limit the amount of feed shredding. Any feed that was soiled or shredded the following day was removed, and fresh pellets were added.

2.4.2 Drug administration

Overview: Two FDA approved drugs, Liraglutide (approved for T2DM treatment) and TUDCA (approved for liver disease), which have previously been associated with reducing beta cell ER stress, were administered to KINGS male mice to investigate whether this could protect them from developing diabetes. We also investigated whether Liraglutide treatment could alleviate established diabetes in KINGS males.

Limitations: Administration of both TUDCA and Liraglutide requires frequent injections which is likely to be stressful for the animals. Both TUDCA and liraglutide have been associated with pleotropic effects which may contribute to any phenotype seen.

Technique:

2.4.2.1 LIRAGLUTIDE ADMINISTRATION:

Liraglutide is a GLP-1 receptor agonist and was chosen instead of exenatide because of its longer half-life, requiring it only to be administered once daily. Although there are other GLP-1 receptor agonists that have a longer half-life than liraglutide (including dulaglutide and semaglutide), since previous studies have not investigated the effects of these of beta cell ER stress, liraglutide was used (Nauck et al., 2021). We also chose to administer liraglutide through subcutaneous injection since this is least invasive injection method and mimics how liraglutide is administered in humans.

Briefly, a 30G needle and 1mL syringe was used to inject liraglutide solution (200µg/kg/day) at the scruff of the mouse. Mice were injected once daily (9am) from 3 weeks of age until 6 weeks of age, and then again from 8 to 10 weeks of age. Control mice received PBS injections. A dose of 200µg/mL daily was chosen, as other studies in the Lep$^{db/db}$ and Akita mice have found an effect on beta cell ER stress with a similar dose (188-200µg/kg/day) and found that this was well tolerated with no welfare issues noted (Shimoda et al., 2011; Zhao et al., 2013). Despite this, we still undertook a pilot study whereby a KINGS male mouse with established diabetes was injected with the chosen dose and monitored hourly for a day for signs of ill-health and hypoglycaemia.

2.4.2.2 TUDCA ADMINISTRATION:

TUDCA was administered through intraperitoneal injection, twice a day at 9am and 6pm. The site of injection was alternated between left and right in an attempt to reduce irritation and pain for the animal. A daily dose of 500mg/kg (250mg/kg per injection) TUDCA was chosen as previous studies have found that this dose is sufficient to reduce ER stress and protect from diabetes

development in mice with no adverse health effects reported (Engin et al., 2013; Özcan et al., 2006). Other administration routes for TUDCA have been used in previous studies, commonly delivery in the drinking water. However, as it is difficult to control dosage when a drug is administered in this way, particularly since hyperglycaemia causes polydipsia, the intraperitoneal route was chosen.

2.5 Studies of isolated islets:

2.5.1 Islet isolation:

Overview: Islet isolation through perfusion of the pancreas with collagenase solution via the common bile duct is an established technique for the isolation of murine islets. This was used to isolate islets for downstream functional analysis (static insulin secretion assays) and for investigations in the expression of beta cell ER stress markers through western blotting.

Limitations: Histopaque, required in this technique to separate islets from exocrine tissue, can be toxic to islets and stress from the isolation process could potentially confound data. We therefore cultured islets overnight prior to any experiments to allow for recovery.

Technique: Mice were culled by schedule 1 and the abdominal cavity exposed. The ampulla of Vater was clamped and ~2.5mL of ice-cold collagenase solution (Sigma, UK; 1mg/mL in MEM, minimal Essential Medium, Sigma) was injected into the pancreas via the common bile duct (Figure 15). Perfused pancreases were excised and kept on ice before digestion in a water bath at 37ºC for 10 minutes. The digestion process was stopped by washing the tissue with ice-cold MEM or RPMI 1640 supplemented with 10% new-born calf serum (NCS) three times, spinning at 1400rpm for 1.15 minutes each time. After the third wash, the supernatant was discarded, and the tissue was resuspended in ice-cold MEM/RPMI+NCS and passed through a sieve to remove the exocrine tissue. Islets were purified by separation from the remaining exocrine tissue through histopaque (density of 1.077g/ml, Sigma, UK) gradient centrifugation. 15mL of histopaque was added to falcon tubes containing 1-3 pancreas digests and vortexed. 10mL of MEM/RPMI+NCS was then slowly

added to the falcon and gradient separation was achieved by centrifugation at 1900xg for 24 minutes (10ºC). Islets were removed from the interphase between the histopaque and MEM/RPMI+NCS and washed three times in fresh MEM/RPMI + NCS. Islets were handpicked into 100mm petri dishes containing RPMI (Sigma, UK) supplemented with 10% foetal bovine serum (FBS), 1000U/mL penicillin + 0.1mg/mL streptomycin and 1% l-glutamine.

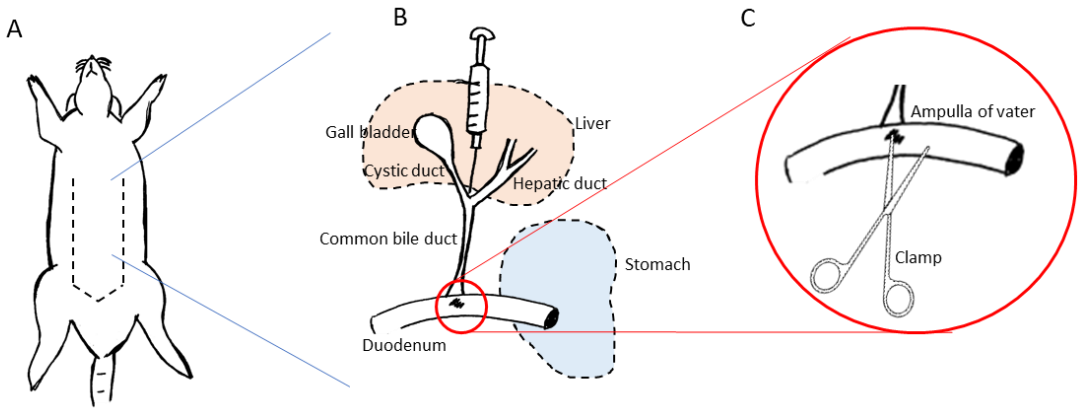


FIGURE 15: *Collagenase injection via the common bile duct for murine islet isolation:*

(A) Incisions are made to expose the abdominal cavity. (B) After clamping at the ampulla of Vater, pancreases were perfused with ice-cold collagenase solution (~2.5mL) by injection into the common bile duct. (C) The ampulla is characterised as a milky-white triangular area on the surface of the duodenum.

2.5.2 Islet culture:

Islets were picked in groups of ~200-250 into 100mm untreated petri dishes in RPMI (Sigma, UK) supplemented with 10% fetal bovine serum (FBS) and 1000U/mL penicillin + 0.1mg/mL streptomycin and cultured in a humidified incubator (37ºC, 95% air/5% CO_2). The culture media had a glucose concentration of 11.1mM.

2.5.3 Static insulin secretion assay:

Overview: Static insulin secretion assays allow for *ex vivo* analysis of beta cell insulin secretory function in response to glucose. This technique was used to

investigate whether sex hormone manipulation in the KINGS mice resulted in changes in glucose stimulated insulin secretion.

Limitations: Insulin secretion is low in KINGS islets are therefore more islets were required for this assay (10 islets per replicate compared to 3-5 typically used). KINGS mice harbour mutated insulin, which is predicted to misfold, it is not known whether the insulin antibody used for the radioimmunoassay is able to detect KINGS insulin.

Technique:

2.5.3.1 Static secretion assay:

Islets were cultured overnight in RPMI (Sigma, UK) supplemented with 10% FBS, 1000U/mL penicillin + 0.1mg/mL streptomycin (37ºC, 95% air/5%CO_2). Islets were then washed twice using 2mM glucose Gey and Gey buffer (5mM KCl, 111mM NaCl, 27mM $NaHCO_3$, 220uM $CaCl_2$, 1mM $MgCl_2 6H_2 0$, 0.5mg/mL BSA, pH 7.4 using $C0_2$). Islets were cultured in 2mM Gey and Gey for 1 hour at 37ºC, 95% air/5%CO_2. After this incubation, islets were washed once in 2mM glucose Gey and Gey buffer and picked in groups of ten in 10µL of Gey and Gey into 1.5mL Eppendorf tubes. 500µL of 2mM Gey and Gey or 20mM Gey and Gey was added to the islets to simulate basal and stimulatory glucose concentrations. The Eppendorf tubes containing islets were placed in a water bath at 37ºC for 1 hour and subsequently centrifuged at 1000rpm for 1 minute. The supernatants were removed and transferred to new Eppendorf tubes for measurement of insulin secretion. Samples were stored at -20ºC until measurement of insulin using radioimmunoassay.

2.5.3.2 Radioimmunoassay (RIA):

RIA was used to quantify insulin in supernatants from the static secretion assay. This technique relies on antibody-antigen interactions whereby the insulin present in the sample competes with iodine-125 radiolabelled insulin (tracer) for binding to an added insulin antibody. The amount of antibody bound to the tracer is negatively correlated with insulin concentration in the sample and this is used alongside samples with known insulin concentrations to calculate unknown insulin concentrations.

An insulin standard curve (ranging from 0.04-10ng/mL) was set up in triplicate through serial dilution of rat insulin (10ng/mL) in borate buffer. Unknown samples were either diluted in borate buffer or used neat to ensure that the insulin concentration measurements resided in the linear part of the standard curve. 2mM glucose secretion samples from KINGS islets were used neat and 20mM glucose samples were diluted 1:2, and 2mM glucose secretion samples from WT islets were diluted 1:2 and 20mM glucose samples were diluted 1:5. Samples were assayed in duplicate.

The tracer was diluted in borate buffer to give approximately 10000 radioactive counts per minute (CPM) and 50µL of diluted tracer was added to the sample and standard tubes. 50µL of guinea pig anti-insulin antibody (1µg/mL, in-house) was diluted 1:10 in borate buffer (giving a final dilution of 1:60000) and 50µL of this was also added to the standard and sample tubes. Several reference tubes were also prepared; maximum binding reference to measure binding of the tracer in the absence of unlabelled insulin (100µL borate buffer, 100µL insulin antibody, 100µL tracer), non-specific binding reference to measure the binding of the tracer in the absence of an antibody (200µL borate buffer, 100µL tracer), and total binding reference to measure the CPM of the tracer alone (100µL tracer). After addition of the tracer and antibody to the sample and standard tubes, tubes were left to equilibrate for 48 hours at 4ºC.

1mL of precipitant (15% polyethylene glycol, 1% γ-globulin in PBS, 0.5% Tween-20, Sigma, UK) was subsequently added to each of the tubes (excluding the total binding reference tube) to precipitate the antibody-insulin and antibody-tracer complexes and tubes were centrifuged for 15 minutes (3000rpm, 4ºC). The supernatants were then aspirated to remove any unbound insulin and radioactivity (in counts per minute per mL, CPM/mL) of the pellet was measured using a Packard Cobra II γ counter. CPM/mL of the standards was plotted against the log of their known concentration to generate a standard curve against which the concentration of insulin in the samples could be determined.

2.5.4 Western blotting:

Overview: Western blotting allows semi-quantitative analysis of the expression of proteins in cell lysates. This technique was used to analyse the expression of ER stress and UPR markers in islets isolated from the KINGS mice. Many studies use qPCR to investigate the latter, however many UPR components are regulated post-transcriptionally and thus we felt that analysis at the protein level was more appropriate.

Limitations: Whole islet lysates were used for western blotting and although beta cells make up the majority of the cells within the islet, other cell types are present. A large number of islets (minimum 200) are required to achieve enough protein for western blotting.

Technique:

2.5.4.1 Protein extraction:

After overnight culture, islets were pelleted and washed in phosphate buffered saline (Sigma, UK) before being lysed by incubating with RIPA (radioimmunoprecipitation assay) buffer supplemented with a protease and phosphatase inhibitor cocktail (Sigma UK, 6 islets per 1µL RIPA buffer) for 30 minutes on ice with gentle agitation. Following this, samples were spun at 40000 rpm for 30 minutes at 4ºC. The pellet was discarded, and protein lysates were frozen and stored at -70ºC.

2.5.4.2 Protein quantification:

Protein concentration in cell lysates was quantified using the bicinchoninic acid method (BCA) as per the manufacturer's instructions (ThermoFisher, UK). A serial dilution of bovine serum albumin (0mg/mL to 2mg/mL) in PBS was set up and dilutions were used as standards. Protein lysates were diluted 1:10 and were added alongside the standards to a 96-well plate in duplicate. 200µL BCA working solution was added to each well and the plate was incubated in darkness at 37ºC for 20 minutes. Absorbance at 560nm was measured using a PHERAstar plate reader (BMG Labtech, Aylesbury, UK) and a standard

curve was generated based on the standard wells and used to calculate protein concentration in samples.

2.5.4.3 Sodium Dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

Protein lysates were diluted in 1.5% Laemmli sodium dodecyl sulfate sample buffer (SDS, Fisher Scientific, UK) and Dithiothreitol (DTT, Fisher Scientific, UK) and boiled at 100ºC for 5 minutes. 10µg of protein per sample was loaded alongside a pre-stained molecular weight marker onto precast 12% gels (Fisher Scientific, UK) and the gel was allowed to electrophorese for 60-90 minutes (or until the dye front reached the bottom of the gel) in SDS-PAGE running buffer at 150V in a mini-cell electrophoresis chamber (**Table 2**).

Reagent	Components
SDS-PAGE running buffer	1M MOPS 1M Tris base 69.3mM SDS 20mM EDTA
Transfer buffer	25mM Tris base 192mM Glycine 20% Methanol
Blocking buffer (milk and BSA)	5% milk in TBST 5% BSA in TBST
TBST	137mM NaCl 2.7mM KCl 19mM Tris base 0.1% Tween-20

TABLE 2: *Reagents used for western blot and SDS-PAGE and their components.*

2.5.4.4 Western blotting:

SDS-PAGE separated proteins were transferred to a methanol soaked polyvinylidene fluoride (PVDF) membrane (Bio-rad, UK) by sandwiching the gel between the membrane and filter paper (FIGURE 16) in a cassette which was submerged in transfer buffer within a transfer tank (**TABLE 2**). Proteins were transferred at 90V for 2 hours. Complete protein transfer was confirmed through Ponceau S staining. Membranes were blocked for 1 hour in blocking

buffer (5% milk in Tris Buffered Saline-tween, TBST, Table 2) and incubated with primary antibodies diluted in blocking buffer (5% BSA for phosphorylated proteins, otherwise 5% milk, TABLE 3) overnight at 4ºC with gentle agitation. Primary antibody was removed by washing in TBST (10x for 10 minutes) and the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) for 1 hour at room temperature (either 1:1000 anti-rabbit HRP or anti-mouse HRP, Santa Cruz Biotechnology (TABLE 3). Membranes were washed in TBST (10x for 10 minutes) before incubation for 1 minute with the Amersham ECL chemiluminescent detection reagent (Cytiva, UK) which allowed for protein signal capture on a chemiluminescent film. Beta actin served as a loading control. Membranes were sequentially stained for antibodies and were washed in TBST between incubations with different primary antibodies but without membrane stripping.

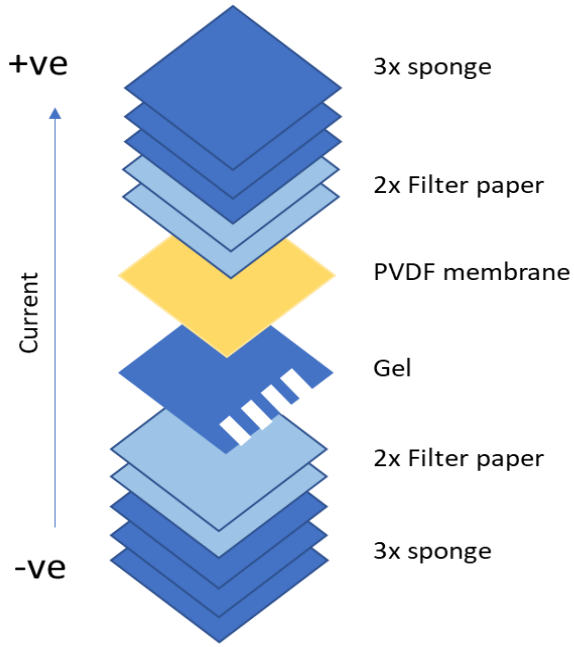


FIGURE 16: *Set up for protein transfer to a PVDF membrane:*
Proteins were transferred to a PVDF membrane by sandwiching the gel between the membrane, filter paper and sponges.

Antibody	Supplier	Specificity	Dilution	Diluent
Monoclonal mouse Beta-actin	Santa Cruz Biotechnology	Primary Ab	1:2000	5% milk
Polyclonal rabbit Bip	Cell signalling	Primary Ab	1:1000	5% milk
Polyclonal rabbit Phospho-eIF2-alpha	Cell signalling	Primary Ab	1:1000	5% BSA
Polyclonal rabbit spliced XBP1	Cell signalling	Primary Ab	1:1000	5% milk
Polyclonal mouse CHOP	Cell signalling	Primary Ab	1:100	5% milk
Monoclonal mouse ATF6	BioTechne	Primary Ab	1:500	5% milk
Anti-mouse HRP	Santa Cruz Biotechnology	Secondary Ab	1:25000	5% milk
Anti-rabbit HRP	Santa Cruz Biotechnology	Secondary Ab	1:25000	5% milk

TABLE 3: *Details of antibodies used for western blotting.*

2.5.4.5 Data analysis for western blot:

Images were analysed in FijiImageJ using the analyse gel tool which plots integrated density of each western blot band from which area under the curve can then be measured (Schindelin et al., 2012). Area under the curve for each band was expressed as a proportion of total area under the curve for all bands and this value was divided by the beta actin loading control proportion to account for differences in gel loading across the samples.

2.6 Histological studies of islets:

2.6.1 Pancreas removal, fixation, wax embedding and sectioning:

Overview: Removal of the whole pancreas and its fixation allows for the study of islets in the context of the intact pancreas. In this project, this technique was used to analyse islet size, beta cell mass, beta cell proliferation, beta cell apoptosis and expression of the ER stress marker, BiP.

Limitations: Fixation of pancreatic tissue prohibits the analysis of islet function and can interfere with the structure of some antigens impairing or preventing immunofluorescent antibody binding.

Technique:

2.6.1.1 Pancreas removal:

Immediately after mice were culled, the abdominal cavity was exposed, and the pancreas was removed using scissors and forceps as gently and as quickly as possible so not to damage the tissue.

2.6.1.2 Fixation:

Excised pancreases were immersed in 3.5% phosphate buffered formaldehyde at a volume >4x the volume of the tissue (Sigma, UK) for 48 hours at room temperature. Pancreases were then washed in 70% ethanol and immersed in 70% ethanol until further processing.

2.6.1.3 Wax embedding:

Fixed pancreases were placed in plastic tissue cassettes and were paraffin wax embedded using a Leica TP1020 embedding machine which incubates tissues in increasing ethanol concentrations before perfusing the tissue with xylene and wax embedding (Figure 17). After embedding, the tissues were taken out of the tissue cassettes and placed in metal moulds to which 60ºC molten wax was added using the Leica EG1150 Modular Tissue Embedding Center and allowed to solidify.

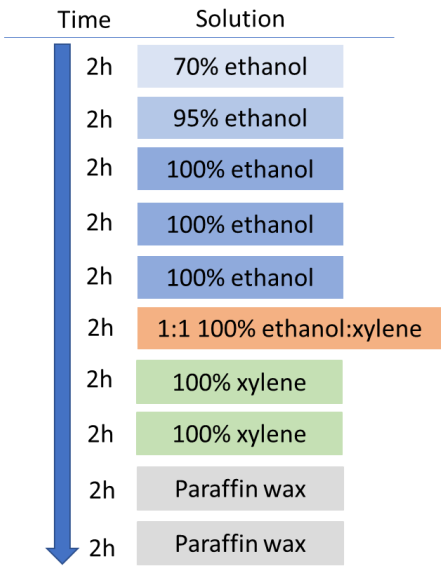


FIGURE 17: *Steps for paraffin wax embedding tissue: Incubation steps used with the Leica TP1020 embedding machine to paraffin wax embed tissues.*

2.6.1.4 Tissue sectioning:

A Leitza Wetzer microtome and MX35 Ultra microtome blades (ThermoFisher, UK) were used to cut entire pancreases into 5µm sections. Ribbons of sections were flattened in a 40ºC water bath, mounted on glass slides (Superfrost plus, VWR) and allowed to dry on a slide dryer overnight.

2.6.2 Immunofluorescent staining:

2.6.2.1 Islet area and mass:

Overview: Insulin and DAPI staining were used in this project to investigate islet size and islet area as a proportion of total pancreatic area (a proxy for beta cell mass) in KINGS mice.

Limitations: KINGS mice harbour mutated insulin, which is predicted to misfold, it is not known whether the insulin antibody used is able to detect KINGS insulin.

Technique: Tissue sections were de-waxed by heating on a slide dryer and incubation in 100% xylene or histoclear (2x 7 minutes). Sections were rehydrated by incubation in decreasing concentrations of ethanol (5 minutes:

100% ethanol, 5 minutes: 95% ethanol, 5 minutes: 75% ethanol). A pap pen was used to draw with wax around the sections and blocking buffer was directly applied, sections were blocked for 30 minutes at room temperature in a humidifying chamber (**Table 4**). Sections were then incubated overnight in 1:500 dilution of guinea pig anti-insulin (DAKO) or 1:200 dilution of guinea pig anti-insulin (Abcam) in blocking buffer. Slides were washed in Tris Buffered Saline (TBS, **Table 4**) for 3x 5 minutes and incubated for 1 hour in humidifying chambers with 1:200 dilution of anti- guinea pig Alexa fluor 488 (Jackson Laboratoies) and 1:500 dilution of DAPI (Sigma, UK). Following this, slides were washed in TBS (3x 5 minutes) and mounted using Fluoromount aqueous mounting medium (Sigma, UK) and cover slips. Individual islets were visualised using a Nikon Eclipse TE 2000-U at 10X or 20x magnification and whole sections were imaged using a Hamamatsu NanoZoomer S60 digital slide scanner at 20x magnification.

Reagent	Components
Citrate Buffer	10mM Citric acid (Sigma, UK) 0.05% Tween-20 (Sigma, Uk) pH 6.0 (using NaOH)
Tris buffered saline (TBS)	50mM Tris-Cl (Sigma, UK) 150mM NaCl (Sigma, UK) pH 7.6 (using NaOH)
BSA blocking buffer	1% bovine serum albumin (Sigma, UK) 5% goat serum (Sigma, UK) 0.1% Triton-X-100 Phosphate buffered saline

TABLE 4: *Reagents used for histology.*

2.6.2.2 BETA CELL PROLIFERATION:

Overview: Ki67 is a widely used nuclear marker of cellular proliferation since it can be detected during the cell cycle phases (G1, S, G2, M) but is downregulated in quiescent cells (G0). Immunofluorescent staining for Ki67 was used in this project to investigate beta cell proliferation in KINGS mice.

Limitations: Ki67 staining has been shown to be sensitive to differences in tissue fixation including in regard to cold ischaemia time, for example. Recent evidence has suggested that Ki67 expression is not binary and can vary

depending on cell proliferative history. Moreover, cells in a post-mitotic phase may show elevated Ki67 as it degrades over time. Finally, beta cells have low proliferation rates making proliferation analysis difficult.

Technique: Tissue sections were de-waxed and rehydrated as previously described (section 2.6.2.1). For antigen retrieval, sections were subsequently pressure cooked in 10mM pH 6.0 citric acid for 2.5 minutes and allowed to cool for 20 minutes (**Table 4**). A pap pen was used to draw with wax around the sections and blocking buffer was directly applied, sections were blocked for 30 minutes at room temperature in a humidifying chamber. Sections were subsequently incubated overnight in 1:500 dilution of guinea pig anti-insulin (DAKO) and 1:100 rabbit ani-Ki67 (Abcam) in blocking buffer. Slides were washed in TBS for 3x 5 minutes and incubated for 1 hour in humidifying chambers with 1:200 dilution of anti- guinea pig Alexa fluor 488 (Jackson Laboratories), 1:200 dilution of anti-rabbit Alexa fluor 594 (Jackson Laboratories) and 1:500 dilution of DAPI (Sigma, UK). Slides were washed again in TBS (3x 5 minutes) and mounted using Fluoromount aqueous mounting medium (Sigma, UK) and cover slips. Individual islets were visualised using a Nikon Eclipse TE 2000-U at 10X or 20x magnification. Spleen was used as a positive control to confirm specificity of the Ki67 staining.

2.6.2.3 Beta cell apoptosis:

Overview: Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) is a routinely used technique for the identification of apoptotic cells based on the principle that apoptotic cells have DNA double strand breaks. TUNEL staining was used in this project to investigate levels of beta cell apoptosis in the KINGS mice.

Limitations: Apoptosis rates in beta cells are low making it difficult to measure this accurately. It has been suggested that TUNEL staining can label non-apoptotic cells, including proliferating cells where DNA repair rates are high.

Technique: TUNEL staining was performed using the ApopTag Fluorescein In situ Apoptosis Detection Kit from Merck as per the manufacturers guidelines. Slides with wax embedded pancreatic sections were deparaffinised through incubation in two changes of xylene solution, each for 7 minutes. Tissue sections were then rehydrated in decreasing concentrations of ethanol (100%, 95%, 75%) for 5 minutes for each ethanol change and then washed in PBS for 5 minutes. 20mg/mL of proteinase K was added directly to the tissue and left for 15 minutes for antigen retrieval. Slides were subsequently washed in 2 changes of PBS for 2 minutes each and equilibration buffer was applied to the sections. The sections were then incubated with a terminal deoxynuceleotidyl transferase solution for 1 hour at 37ºC in a humidifying chamber after which the action of this enzyme was stopped by incubating sections in stop buffer. Terminal deoxynucleotidyl transferase mediates the binding of DNA strand breaks with free nucleotide triphosphates in the reaction solution which are conjugated to dioxigenin. Sections were washed in PBS before being incubated anti-digoxigenin antibody conjugated to Apoptag fluorescein for 30 minutes in a humidifying chamber at room temperature. This conjugated antibody binds to the dioxigenin, thus indirectly labelling DNA strand breaks (Figure 18). For the identification of beta cells, slides were subsequently incubated with a primary antibody towards insulin (guinea pig anti-insulin, Abcam, 1:200) overnight at four degrees in a humidifying chamber, and then with a secondary anti-guinea pig Alexa Fluor-594 (1:200) antibody and DAPI (1:500) for 1 hour at room temperature. Slides were mounted with Fluoromount aqueous mounting medium (Sigma, UK) and imaged at 10x or 20x magnification on a Nikon Eclipse TE 2000-U microscope. Positive control tissues were used to confirm specificity of the TUNEL staining and included spleen and foetal mouse tissue.

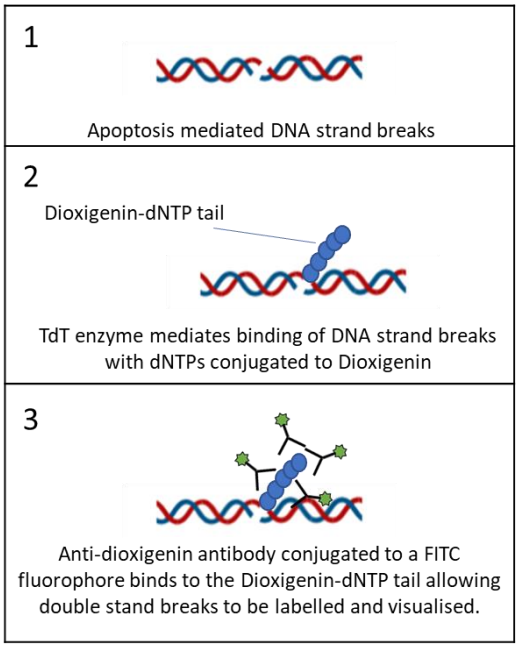


FIGURE 18: *Process by which the apoptag fluorescein in situ apoptosis detection kit labels apoptotic nuclei:*
(1) Apoptosis causes single and double DNA strand breaks. (2) A reaction solution containing TdT and free dNTPs conjugated with Dioxigenin is applied to the tissue section and TdT mediated dNTP- Dioxigenin binding occurs resulting in a dNTP- Dioxigenin tail bound to the DNA break site. (3) A solution containing Anti-dioxigenin antibody conjugated to a FITC fluorophore is applied to the tissue section, indirectly labelling any DNA strand breaks by binding to Dioxigenin. This can be visualised with a fluorescent microscope.

2.6.2.4 BETA CELL ER STRESS:

Overview: BiP is a general ER stress marker whose protein expression is increased upon enhanced ER stress. BiP immunofluorescent staining was used to investigate beta cell ER stress in the KINGS mice.

Limitations: ER stress signalling pathways are highly dynamic and therefore having more than one marker of beta cell ER stress is advantageous.

Troubleshooting: Antibodies for a variety of ER stress and UPR markers were tested for immunofluorescent use, including CHOP, XBP1s and p-eIF2-alpha, however we were unable to detect these proteins using the antibodies in a positive control (Akita islets and thapsigargin-treated islets). Optimisation was

attempted and this included ordering antibodies from different suppliers, changing primary antibody dilutions, changing secondary antibody dilutions and trialling different antigen retrieval methods. These were unsuccessful, however.

Technique: Tissue sections were de-waxed and rehydrated as previously described (section 2.6.2.1). Sections were boiled in 10mM citric acid in a pressure cooker for 2.5 minutes for antigen retrieval and allowed to cool for 20 minutes. A pap pen was then used to draw with wax around the sections and blocking buffer was directly applied, sections were blocked for 30 minutes at room temperature in a humidifying chamber. Sections were subsequently incubated overnight in 1:200 dilution of guinea pig anti-insulin (Abcam) and 1:100 rabbit anti-BiP (Cell Signalling Technology) in blocking buffer. Slides were washed in TBS for 3x 5 minutes and incubated for 1 hour in humidifying chambers with 1:200 dilution of anti- guinea pig Alexa Fluor 488 (Jackson Laboratories), 1:200 dilution of anti-rabbit Alexa Fluor 594 (Jackson Laboratories) and 1:500 dilution of DAPI (Sigma, UK). Slides were washed again in TBS (3x 5 minutes) and mounted using Fluoromount aqueous mounting medium (Sigma, UK) and cover slips. Individual islets were visualised using a Nikon Eclipse TE 2000-U at 10X or 20x magnification and whole sections were imaged using a Hamamatsu NanoZoomer S60 digital slide scanner at 20x magnification.

2.6.3 Haematoxylin and eosin staining:

Overview: Haematoxylin and eosin (H&E) staining is a common histological staining technique where haematoxylin alongside aluminium salt (which acts as a mordant) binds nucleic acids, staining the nucleus purple, and eosin binds basic structures pink including the cytoplasm. H&E staining of pancreatic sections allows clear visualisation of the islets distinct from the surrounding exocrine. It was used in the current study to investigate islet size in the KINGS mice and to determine whether use of insulin immunofluorescent staining to determine islet size is appropriate.

Limitations: H&E staining can only be used to gain morphological insight into islets.

Technique: Slides with mounted paraffin-embedded tissue sections were heated on a slide dryer to melt the wax before being immersed in two changes of xylene for 7 minutes each time. Tissue sections were then rehydrated in decreasing concentrations of ethanol (100%, 95%, 75%) for 5 minutes for each ethanol change. After rehydration, slides were submerged in haematoxylin for 5 minutes and washed in running water for 5 minutes. Slides were dipped thrice in acidic alcohol (0.5% HCl in 70% ethanol) to remove excess staining and washed again in running water for 2 minutes. To stain with eosin, slides were dipped in eosin solution five times and then washed in running water (2 minutes), this was followed by dehydration in increasing concentrations of ethanol (100%, 95%, 75%, 5 minutes in each ethanol change). DPX mounting media (Sigma Aldrich) and glass coverslips were used to mount the slides. Stained pancreas sections were imaged using a Zeiss Axiophot microscope, 20x objective.

2.6.4 Imaging and image analysis:

Individual islets were either imaged using a Nikon Eclipse TE 2000-U at 10X or 20x magnification or whole sections were imaged using a Hamamatsu NanoZoomer S60 digital slide scanner at 20x magnification (fluorescently stained sections) or were imaged using a Zeiss Axiophot microscope, 20x objective (H&E-stained sections). Where individual islets were imaged, all the islets within a section were imaged.

All analysis was carried out using FijiImageJ and in a blinded fashion by attributing numbers to animals and file names (Schindelin et al., 2012).

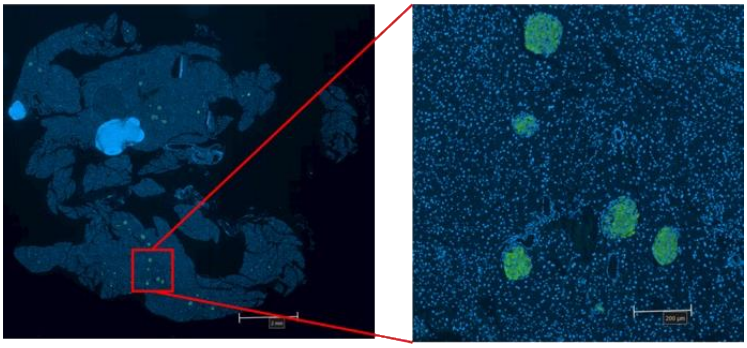
2.6.4.1 Islet area:

Islet size was investigated by manually drawing around islets using the polygon selection tool to generate a region of interest (ROI), the measure function was then used to calculate area of the ROI.

2.6.4.2 Beta cell area/total pancreatic area:

Beta cell area as a proportion of total section pancreatic area was calculated semi-automatically. A macro was developed in ImageJ to automatically select insulin positive area in images of whole pancreatic sections (Figure 19) (Schindelin et al., 2012). This macro encoded instructions for the following analysis pipeline; the insulin channel was filtered using a Gaussian blur and the image was made into a binary image. The system was then told to select any areas of insulin positive signal that was within a given size range and circularity range. These automatic selections were then manually checked to ensure only insulin positive areas were selected and that any insulin positive areas not detected using the analysis pipeline were selected. The total pancreatic area semi-automatic analysis used the same analysis pipeline as for the insulin positive area, however different thresholding limits were used when the image was made binary so that auto-fluorescent signal (pancreatic tissue) as well as insulin signal could be detected. This again was checked manually to ensure that adipose tissue, blood vessels and splenic tissue was not selected.

A



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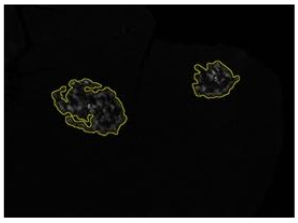

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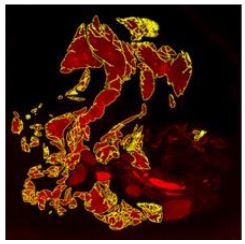

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run (“Gaussian Blur…”, “sigma=5”);
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//run(“Threshold…”);
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//setThreshold (14, 255);
setOption(“BlackBackground”, true);
run(“convert to Mask”);
run(“Analyze Particles…”, “size=4000-Infinity display exclude summarize add”);
```

FIGURE 19: *Macro for investigating insulin positive area/total pancreatic area:*

(A) Pancreatic sections were immunofluorescently stained for insulin (green) and DAPI (blue). Whole pancreatic sections were imaged using a NanoZoomer S60 fluorescence and brightfield slide scanner using a 20x objective. Scale bars represent 2mm (left image) and 200µm (right image). (C) Islet area was analysed semi-automatically in ImageJ such that regions of insulin signal were selected using the analysis pipeline indicated. (C) Whole pancreatic area was also analysed semiautomatically in ImageJ generating selections around the entire section using the analysis pipeline indicated. These selections were then manually checked to ensure accurate selection.

2.6.4.3 Proliferation and apoptosis:

Rates of beta cell proliferation and apoptosis were measured manually using the cell counter tool to count total beta cell nuclei in an islet and number of TUNEL or Ki67 positive beta cell nuclei.

2.6.4.4 BiP expression:

In images of individual islets, an ROI was manually drawn around the islet using the polygon selection tool and mean fluorescence in the 594nm channel was measured using the measure function. In images of whole pancreatic sections, the macro described previously (Figure 19) was used to generate ROI's around insulin positive areas, which were manually checked and mean fluorescence within each ROI was measured using the measure function.

2.7 Statistical analysis:

All graphs were constructed using GraphPad Prism 8.0 of 9.0 software (GraphPad Software, San Diego, CA) and statistical analysis was carried out in SigmaPlot 14.0 (Systat software Inc, San Jose, California). Graphs show mean ± standard error of the mean (SEM) unless otherwise stated. Comparisons between two groups were carried out using an unpaired two-tailed student's T-test. For comparison between more than two groups where there was one independent variable, a one-way ANOVA with Holm-Sidak's post hoc was used. For comparison between more than two groups where there were two independent variables, a two-way ANOVA with Holm-Sidak's post hoc was used. For comparisons between more than two groups where

there were two independent variables and one of these was time (repeated measures), a two-way repeated measures ANOVA with a Bonferroni post hoc test was used. Results were considered statistically significant if $p<0.05$.

CHAPTER 3: CHARACTERISING THE KINGS MOUSE PHENOTYPE

Chapter snapshot:

The KINGS mouse is a newly established model of diabetes first discovered in 2016 and maintained through sibling mating. Inbred colonies are subject to genetic drift which has the potential to alter phenotype.

The aim of this study was to re-characterise the KINGS phenotype and to establish whether this has changed from the previous characterisation study performed in 2016-2018.

3.1 INTRODUCTION:

In 2016, at King's College London, male mice from a C57Bl/6J mouse colony harbouring a double gene knockout in TRPA1 (transient receptor potential ankyrin 1) and TRPV1 (transient receptor potential cation channel subfamily V member 1) developed polydipsia and polyuria, hallmark indicators of hyperglycaemia. Measurement of blood glucose concentrations in these mice confirmed this, with blood glucose concentrations exceeding 30mM in some male mice. In an attempt to understand the cause of hyperglycaemia, the *Ins2* gene in these mice was sequenced revealing a single nucleotide polymorphism (chromosome 7, position 142679440) which causes a glycine to serine amino acid substitution at position 32 of preproinsulin 2 (Figure 9) (Austin et al., 2020). Interestingly, this mutation exists in humans and causes a rare form of neonatal diabetes (Edghill et al., 2008; Fu et al., 2020).

Male mice displaying hyperglycaemia were backcrossed using wildtype C57Bl/6J mice for several generations to ensure that the *Ins2* mutation was present on a wildtype C57B/6J background (without the TRPA1 and TRPV1 knockout). These mice were subsequently named the KINGS mice, reflecting the institution of their discovery (**K**ing's College London), their mutated gene

(***In***s*2*), and the amino acid substitution in preproinsulin (**G**32**S**). This mutation was found to be dominantly inherited. Male and female mice homozygous for this mutation showed severe hyperglycaemia from weaning (>30mM), were emaciated, and thus rapidly reached ethical endpoints requiring them to be culled. Therefore, only mice heterozygous for the KINGS mutation were used in the previous and current study (Austin et al., 2020). Other mouse models harbouring *Ins2* mutations (Munich and Akita mice) are also mainly used as heterozygotes owing to the severity of phenotype of the homozygous mice.

Ins2$^{+/G32S}$ male mice become overtly diabetic (>16.7mM) from 5 weeks of age, whereas female mice remain normoglycemic although blood glucose concentrations are still significantly elevated compared to wildtype littermates (Austin et al., 2020). Intriguingly, both male and female KINGS mice showed significant glucose intolerance when subjected to intraperitoneal glucose injection (Austin et al., 2020).

The KINGS mutation exists on a C56Bl/6J genetic background which has been maintained through sibling mating from 2016. Inbred colonies maintained in this way are subject to genetic drift which is caused by spontaneous mutations randomly arising and becoming fixed in the population (Casellas, 2011; Zeldovich, 2017). The latter occurs when a random mutation, commonly occurring during DNA replication or repair, arises in the germline and so can be propagated to the offspring. The rate at which genetic drift occurs increases as colony size decreases (Daniels Gatward et al., 2021). Although genetic drift is inevitable, some mutations that become fixed can go unnoticed but carry the potential to influence phenotype. Therefore, it is important to establish whether this has impacted the phenotype of interest (glycaemic control) in the KINGS mouse before their further use in future experiments.

To re-characterise the KINGS phenotype and determine whether this has changed as a possible result of genetic drift in the colony, long term random blood glucose concentrations of male and female KINGS mice were monitored and compared to historic data (Austin et al., 2020). In addition, glucose tolerance tests in these mice were performed. We also wanted to determine

whether insulin resistance contributed to glycaemic control, something that has not been determined previously. Insulin sensitivity was therefore investigated through intraperitoneal insulin tolerance tests at different ages which encompass different points in the disease course in males (4 weeks: pre-diabetes, 10 weeks: established diabetes).

3.2 Aims:

- Re-characterise the KINGS phenotype and determine whether this has changed compared to the original characterisation study.
- Determine whether insulin resistance contributes to the KINGS phenotype.

3.3 Methods:

3.3.1 Experimental outline:

Glycaemic control was assessed in KINGS mice and WT littermates by measuring non-fasted blood glucose concentrations over time from weaning at 3-weeks and by carrying out glucose tolerance testing at 3 and 4 weeks (prior to the onset of diabetes in the males) and at 10 weeks of age (after the onset of diabetes). To assess whether insulin resistance contributes to a loss of glycaemic control in the KINGS mice, insulin tolerance tests were carried out at 4 and 10 weeks of age. Data from the present study (2019) was compared to data obtained from the previous study (2016-2018).

3.3.2 Animals:

Mice were housed in single sex groups of up to five in individually ventilated cages with *ad libitum* access to water and standard rodent diet 20 chow (Picolab, UK). Animals were kept on a 7am-7pm light-dark cycle. Heterozygous KINGS and wildtype mice were maintained through in-house conventional breeding where wildtype/KINGS 6-8-week female mice were mated singly or in duos with wildtype/KINGS male mice. Heterozygous KINGS and wildtype offspring were used for experiments. All *in vivo* procedures were approved by our institution's ethics committee and performed under a project license in accordance with the U.K. Home Office Animals (Scientific Procedures) Act 1986 with 2012 amendments. The KINGS mice can be found

on the Mouse genome informatics (MGI) database: ID: 6449740 (https://www.informatics.jax.org/strain/MGI:6449740).

3.3.3 Non-fasted blood glucose concentration and weight monitoring:

Non-fasted blood glucose concentrations and mouse weight was measured daily or weekly between 9-10:00. Blood glucose concentrations were measured using a glucometer (Performa glucometer, Roche UK) and glucometer strips (Informa II, Roche UK), and blood droplets were obtained from the mice through a needle prick to the tip of the tail using a 30G needle and gently massaging of the tail from the base upwards.

3.3.4 Glucose tolerance tests:

Mice were fasted for 6 hours prior to glucose tolerance tests by removing food from the cages and any residue food at the bottom of the cages rather than changing the cage. Mice were acclimatised to the room in which the test was carried out for 30 minutes to minimise stress and after this time basal blood glucose concentrations were measured immediately before administration of 2g/Kg glucose (30% filtered glucose solution) through intraperitoneal injection. Blood glucose concentrations were subsequently measured at 15, 30, 60, 90 and 120 minutes after glucose injection.

3.3.5 Insulin tolerance tests:

Mice were fasted for 6 hours prior to insulin tolerance tests by removing food from the cages and any residue food at the bottom of the cages rather than changing the cage. Mice were acclimatised to the room in which the test was carried out for 30 minutes to minimise stress, after this time basal blood glucose concentrations were measured immediately before administration of 0.75IU/kg bovine insulin solution (130 mIU/ml) through intraperitoneal injection. Blood glucose concentrations were subsequently measured at 15, 30, 45 and 60 minutes after glucose injection.

3.3.6 Statistical analysis:

GraphPad Prism software 8.0 or 9.0 (GraphPad Software, San Diego, CA) was used to construct graphs which show mean±SEM and SigmaPlot 14.0

(Systat software Inc, San Jose, California) was used for all statistical analysis. For comparisons between multiple groups where there were two independent variables, a two-way ANOVA with Holm-Sidak post hoc test was used and for comparisons between multiple groups where repeated measures were taken over time, a two-way repeated measures ANOVA with Bonferroni post hoc test was used. A P value <0.05 was considered statistically significant.

3.4 Results:

3.4.1 Non-fasted blood glucose monitoring in the KINGS mice:

Non-fasted blood glucose concentrations of wildtype and KINGS male and female mice were monitored daily from weaning (22 days) until 6 weeks, and weekly thereafter until 10 weeks of age. Both male and female wildtype mice showed normoglycemia throughout as expected (Figure 20). KINGS male blood glucose levels became consistently significantly elevated compared to wildtype from 31 days of age and overt hyperglycaemia (blood glucose concentrations >16.7mM) developed at day 39 on average (Figure 20A). Blood glucose concentrations progressively worsened in the male KINGS mice over time, with glucose levels at 8 weeks of age (56 days) significantly higher than all previous measurements. Female KINGS mice did not develop overt diabetes at any point and whilst blood glucose concentrations in individual mice did occasionally exceed 16.7mM, the maximum average blood glucose concentration was 12.6±0.7mM (Figure 20B). Blood glucose concentrations were still significantly elevated compared to wildtype females from 23 days of age at most of the timepoints measured but there was no progressive worsening in blood glucose concentrations over time.

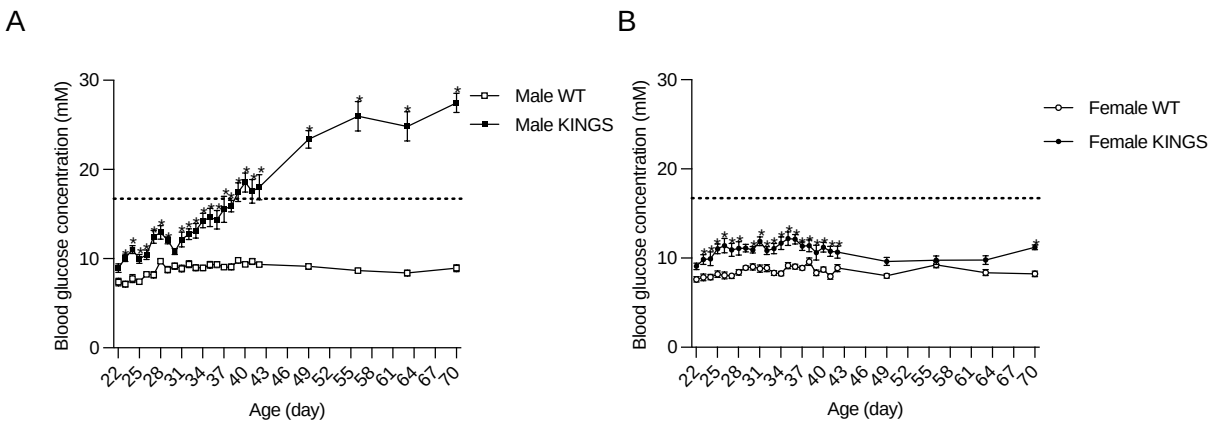


FIGURE 20: *Non-fasted blood glucose monitoring in the KINGS mice: (A-B) Random blood glucose monitoring in KINGS and WT males (A) and females (B) daily from 22 days to 42 days and weekly from 42 days to 70 days of age. Data represents the mean±SEM, two-way repeated measures ANOVA with Bonferroni post hoc, *p<0.05, n=7-13.*

Male KINGS blood glucose concentrations in the current study were significantly lower compared to the previous blood glucose monitoring studies at 38, 41, 49, 56 and 63 days of age but not at any other points measured (Figure 21A). However, by 10 weeks of age blood glucose concentrations were not significantly different and age of diabetes onset between the previous and current study was similar, 37 and 39 days respectively. The blood glucose concentrations in KINGS female mice were largely similar between the previous and current study, only differing significantly at 31 and 35 days of age where mice from the current study showed higher blood glucose concentrations by a maximum of 2.6mM (Figure 21B). This was also true for WT males, where blood glucose concentrations were only significantly increased compared to the previous study at 28 and 30 days of age, and the WT females where blood glucose concentrations were only increased compared to the previous study at 25 and 38 days of age (Figure 21C-D).

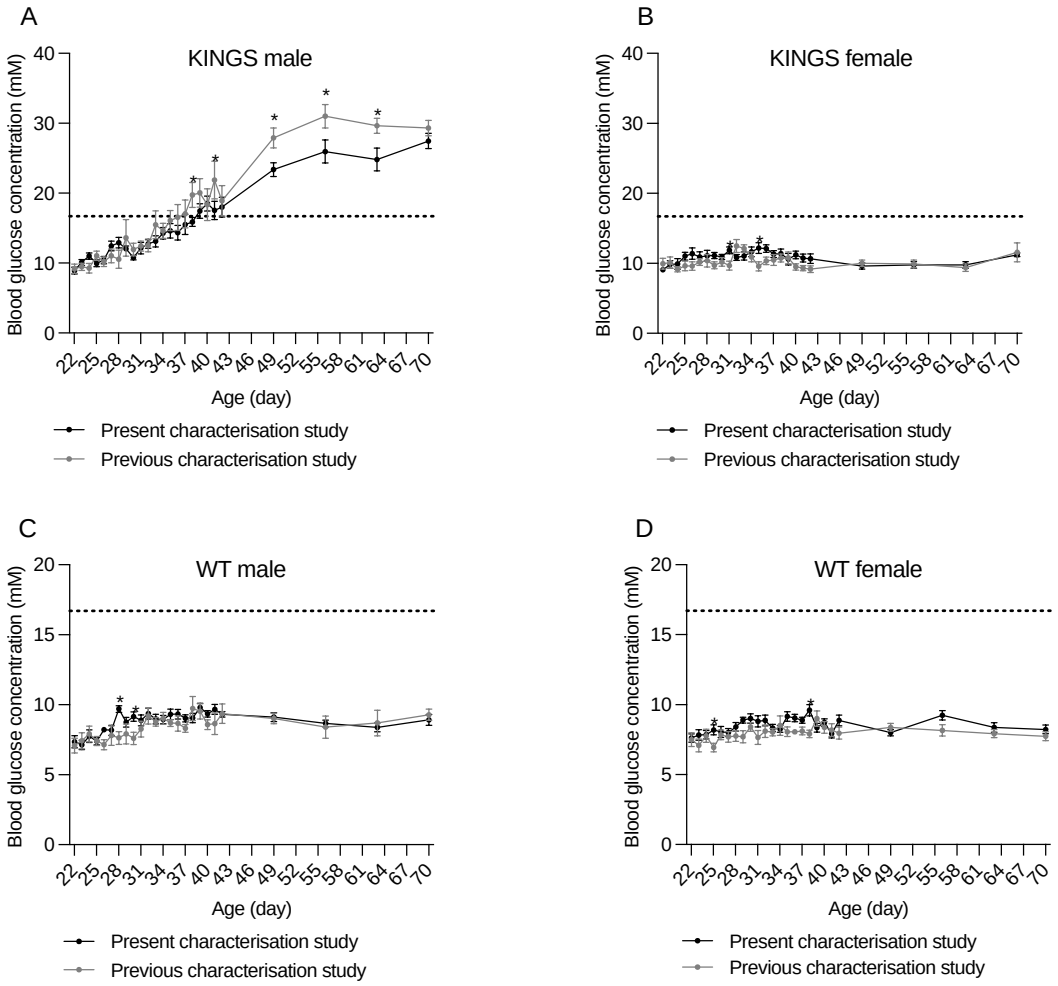


FIGURE 21: *Comparison of non-fasted blood glucose concentrations between the present and previous characterisation studies:*
Non-fasted blood glucose concentration monitoring in (A) KINGS male, (B) KINGS female, (C) WT male (C) and (D) WT female mice conducted in the present study (2019-2020, black line) and previous study (2016-2018, grey line). Blood glucose concentrations were monitored daily from 22 (weaning)-42 days and weekly from 6-10 weeks. Data represents the mean±SEM, two-way repeated measures ANOVA with Bonferroni post hoc, n=6-15.

3.4.2 Weight monitoring in the KINGS mice:

Body weights of wildtype and KINGS male and female mice were monitored daily from 22 days at weaning until 42 days and monitored weekly thereafter until the mice were 10 weeks of age. As expected, weight was gained rapidly from weaning up until puberty (~5 weeks of age) in all groups and stabilised

thereafter (Figure 22). We found no differences in weight between wildtype and KINGS mice of the same sex at any age.

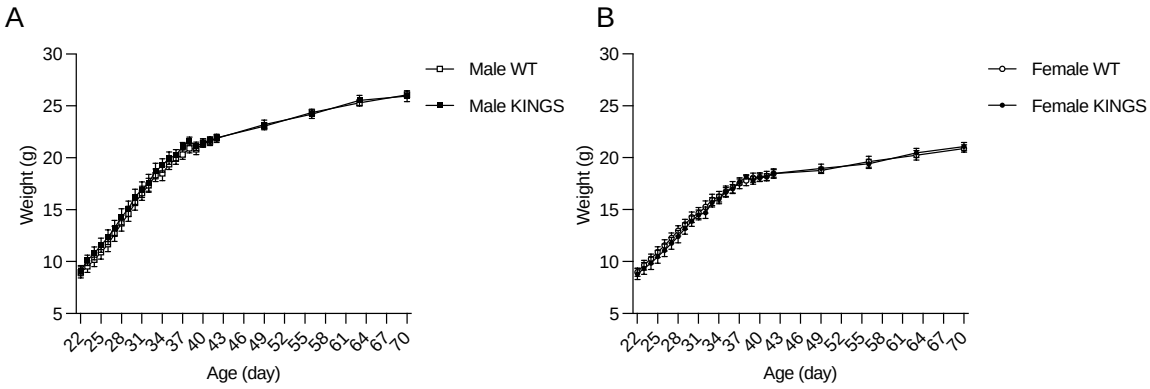


FIGURE 22: *Weight monitoring in the KINGS mice:*
*(A-B) Weight monitoring in KINGS and WT males (A) and females (B) daily from 22 days to 42 days and weekly from 42 days to 70 days. Data represents the mean±SEM, two-way repeated measures ANOVA with Bonferroni post hoc, *p<0.05, n=7-13.*

Previous weight monitoring from 2016-2018 found that KINGS males had reduced weight gain compared to WT littermates from 42 days of age, however we did not find this. On top of this, although we found no difference in weight in the KINGS females between the previous and present study (Figure 23B), male KINGS mice in the present study were heavier compared to the previous study at 7, 8, 9 and 10 weeks of age (Figure 23A). This reached a maximum difference of 2.8g at 10 weeks of age.

No differences were observed in weights at any time in the WT female mice between the previous and current study (Figure 23D). This was largely true for the male WT mice, however, at 4 weeks of age weight was 1.85g lower compared to the previous study (Figure 23C).

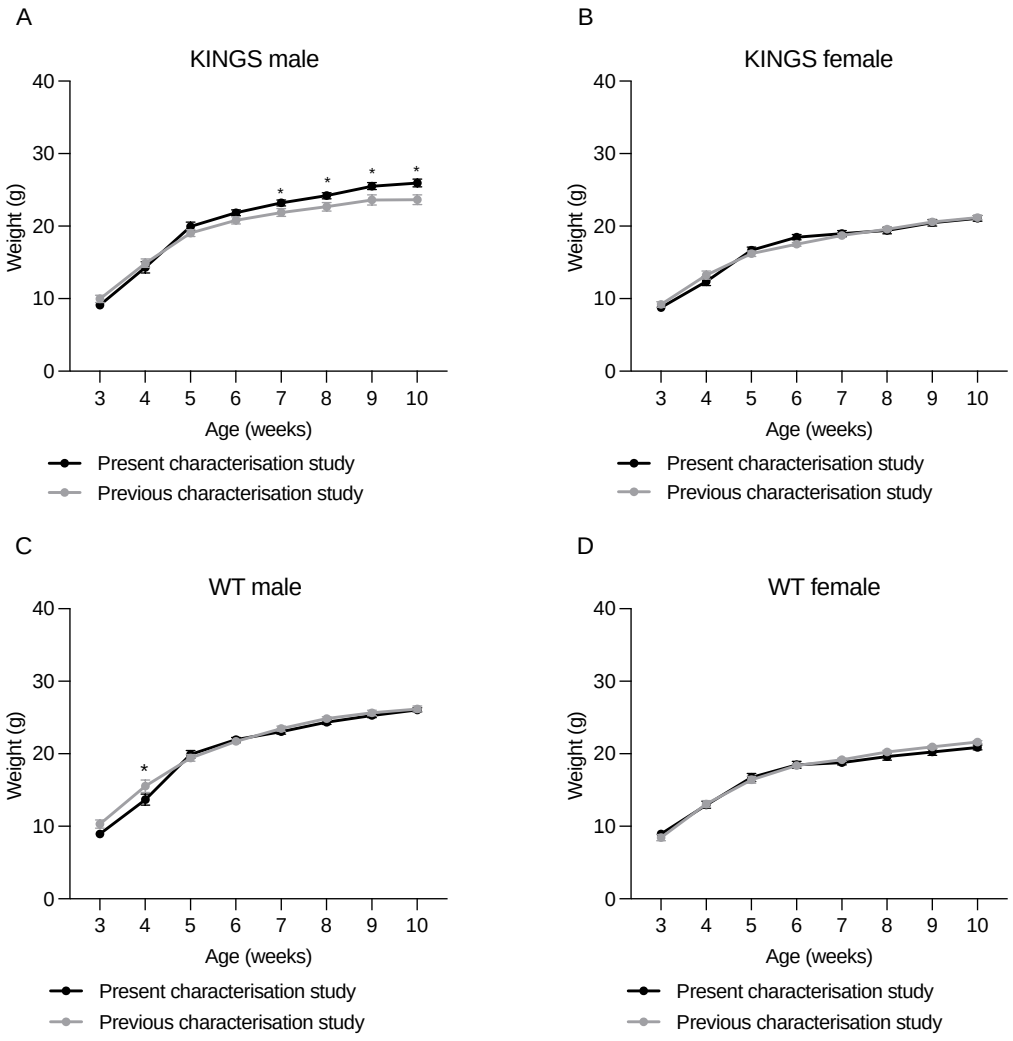


FIGURE 23: *Comparison of mouse weights between the previous and present characterisation studies:*
*Weight monitoring in (A) KINGS male, (B) KINGS female, (C) WT male (C) and (D) WT female mice conducted in the present study (black line) and in the previous study (grey line). Data represents the mean±SEM, two-way repeated measures ANOVA with Bonferroni post hoc, *p<0.05, n=6-15.*

3.4.3 KINGS MICE ARE GLUCOSE INTOLERANT FROM 3 WEEKS OF AGE:

Glucose tolerance in the KINGS mice was investigated at 3, 4 and 10 weeks of age by subjecting mice to an intraperitoneal glucose injection. Male KINGS mice showed significantly impaired glucose tolerance compared to WT littermates from 3 weeks of age, with area under the curve values (AUC) for the glucose tolerance tests higher in the KINGS males compared to WT males (Figure 24). In contrast, AUC values were not significantly different between

KINGS and WT female mice at 3 weeks but became significantly higher in female KINGS mice from 4 weeks of age.

A profound deterioration in glucose tolerance was seen in both male and female KINGS mice between 3 and 4 weeks of age. At 3 weeks, AUC values for the glucose tolerance test were approximately 1.3-fold greater in KINGS males compared to WT littermates, however this increased to 1.7-fold by 4 weeks. Similarly, in the female KINGS mice AUC values were 1.3-fold that of WT mice at 3 weeks and this increased to 2.1-fold at 4 weeks. Whilst glucose tolerance stabilised for the KINGS females after 4 weeks, with AUC values similar between 4 and 10 weeks of age (3044mM/120 min at 4 weeks vs. 3014 mM/120min at 10 weeks), this progressively worsened in the male KINGS mice. AUC values in the KINGS males increased from 2906mM/120min (1.7-fold that of WT males) to 3671mM/120min (2.1-fold that of WT males) between 4 and 10 weeks. In line with a deterioration in glucose tolerance only in the KINGS male and not female mice, male KINGS mice showed higher AUC values for the GTT compared to female KINGS mice at 10 weeks only. Interestingly, at 10 weeks, WT male mice also had significantly higher AUC values compared to WT female mice.

Historic glucose tolerance tests conducted at 4 and 10 weeks of age in these mice similarly showed that glucose tolerance is impaired by 4 weeks in both males and female KINGS mice. They also showed that this deteriorates only in male KINGS mice thereafter. Importantly, datasets cannot be directly compared because of different fasting periods used (6h in the present study vs. 16h in the previous study).

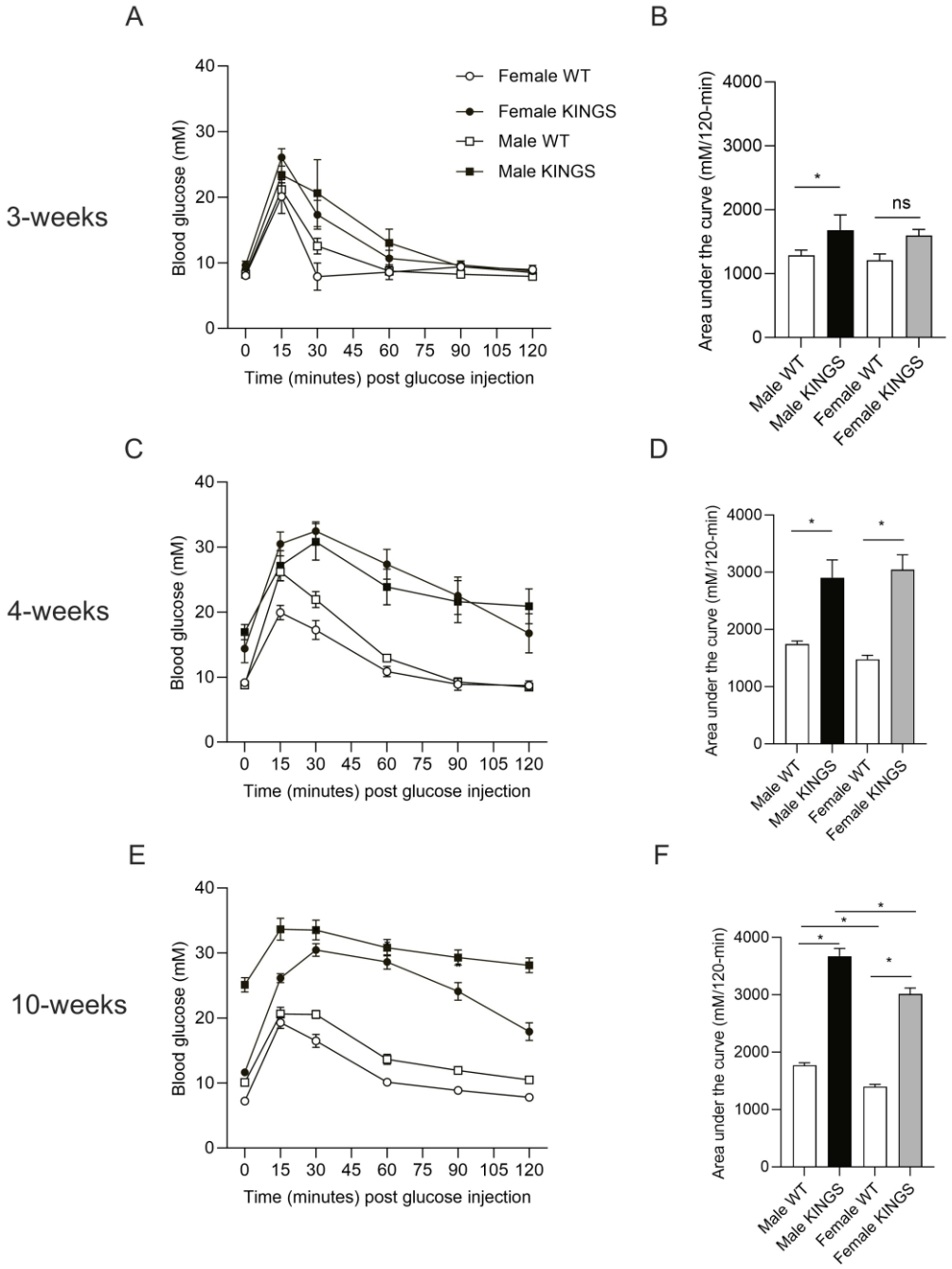


FIGURE 24: *Glucose tolerance in the KINGS mice:*

*(A, C, E) Glucose tolerance tests performed in (A) 3-week, (C) 4-week and (E) 10-week KINGS male (closed square), KINGS female (closed circle), WT male (open square) and WT female (open circle) mice. Data represents the mean±SEM, n=3-15. (B, D, F) Area under the curve (AUC) values for the glucose tolerance tests performed at (B) 3 weeks, (D) 4 weeks and (F) 10 weeks. Data represents the mean±SEM, two-way ANOVA with Holm-Sidak post hoc, *p<0.05, n=3-15.*

3.4.4 KINGS MICE DO NOT SHOW OVERT INSULIN RESISTANCE:

To investigate the contribution of insulin resistance to a lack of glycaemic control in the KINGS mice, intraperitoneal insulin tolerance tests were performed at 4 and 10 weeks of age. Although both WT and KINGS mice showed a reduction in blood glucose concentrations in response to insulin administration (Figure 25A-B), comparisons of the raw data between groups for the insulin tolerance tests was difficult owing to KINGS male mice exhibiting a higher baseline blood glucose measurement. Therefore, blood glucose values after insulin injection were expressed as percentage of baseline blood glucose measurements and area above the curve (AAC) was calculated so that groups could be directly compared (Figure 25C-D).

AAC values were significantly higher in male KINGS mice at 4 weeks of age compared to WT male mice (Figure 25). However, this did not differ significantly from female KINGS mice. By 10 weeks, AAC values were comparable between the male KINGS and WT mice.

At 4 weeks of age AAC values for the insulin tolerance test did not differ between WT and KINGS female mice. However, at 10 weeks, AAC values were significantly higher in the KINGS female mice. Importantly, values did not differ significantly between KINGS females and KINGS and WT males, but WT female values were significantly lower compared to WT male mice.

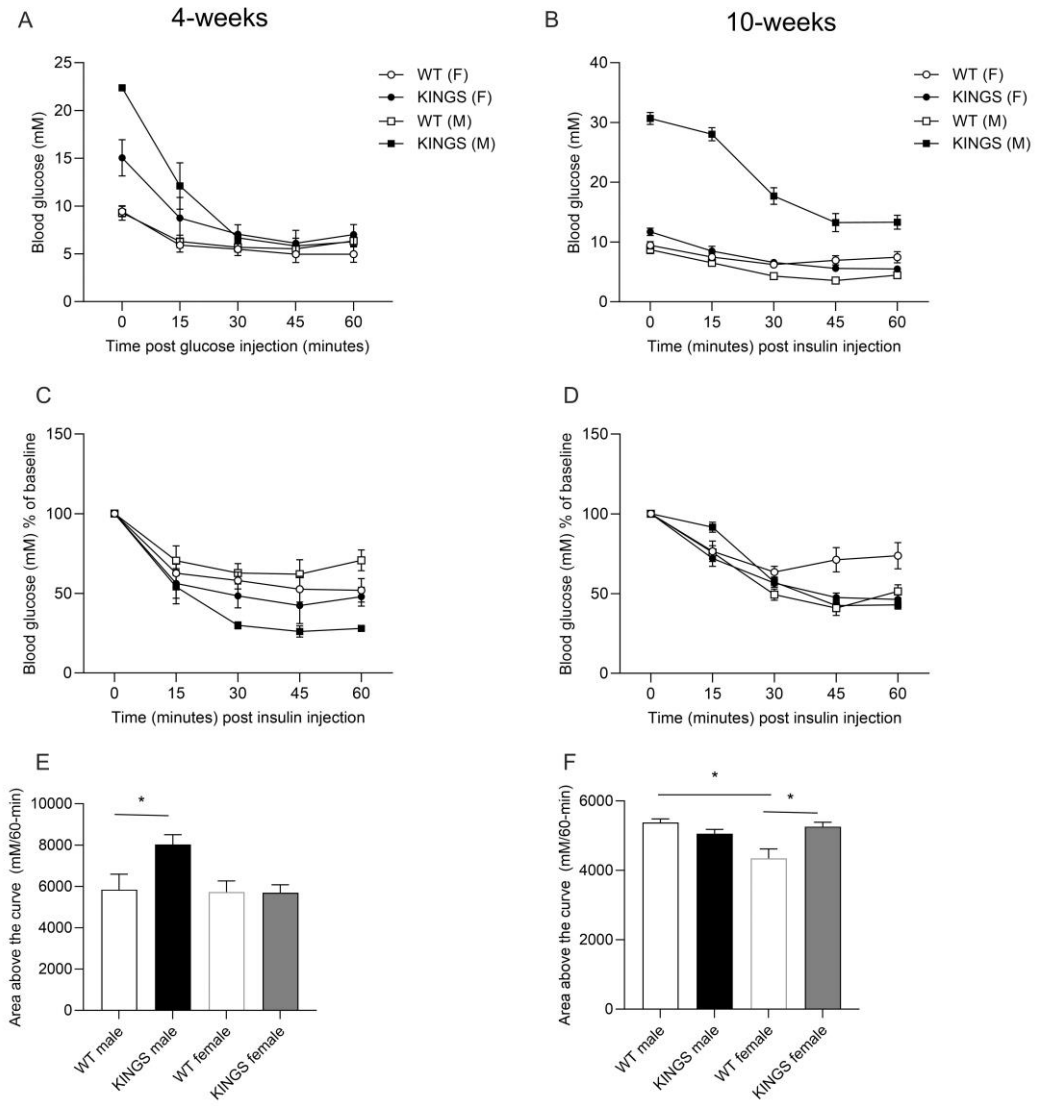


FIGURE 25: *Insulin tolerance testing in the KINGS mice:*

*Raw blood glucose data for insulin tolerance tests performed in (A) 4-week and (B) 10-week-old KINGS male (closed squares), WT male (open squares), KINGS female (closed circles) and WT female mice (open circles). (C-D) Blood glucose measurements for the insulin tolerance test expressed as a percentage of baseline blood glucose concentrations in (C) 4-week and (D) 10-week mice. (E-F) Area above the curve (AAC) data values for insulin tolerance tests performed at (E) 4 weeks and (F) 10 weeks. Data represents the mean±SEM, two-way ANOVA with Holm-Sidak post hoc, *p<0.05, n=3-8.*

3.5 DISCUSSION:

The KINGS mouse harbours a single polymorphism in *Ins2* which causes a glycine to serine substitution in preproinsulin2 thought to drive beta cell ER stress. It has been 3-years since the KINGS mouse was first characterised, and since it is maintained through sibling mating it is subject to genetic drift over time (Austin et al., 2020). Genetic drift has the potential to change a model's phenotype and therefore it is important to re-establish the phenotype before using the model for any further experiments (Casellas, 2011; Zeldovich, 2017). Another important factor to consider that can lead to variations in phenotype over time are seemingly minor changes within the animal environment and this includes with reference to noise, bacterial and viral infections as well as diet. In this study, we confirm whether the phenotype of interest in the KINGS mice, a lack of glycaemic control and the development of diabetes, was changed from the previous study.

KINGS male mice from both studies showed a progressive deterioration in glycaemic control over time. However, KINGS male blood glucose concentrations in the current study were significantly lower than in the previous study at 5 out of the 24 time points measured. This indicates that phenotypic changes have taken place in this model, with the current KINGS males displaying milder hyperglycaemia. Nevertheless, it is important to note that time of diabetes onset was very similar between the two studies (39 vs. 37 days of age) emphasising only a subtle change. KINGS male mice in the present study also showed improved weight maintenance compared to the previous findings; in the present study weight was no different to WT mice but was significantly higher than KINGS mice from the previous study from 7 weeks of age. Better maintenance of weight may suggest the KINGS males from the present study are healthier, possibly a consequence of a slightly milder hyperglycaemia. Indeed, prolonged and uncontrolled hyperglycaemia is associated with weight loss (Mouri & Badireddy, 2023).

Although these observed phenotypic differences between the two studies may be indicative of mild genetic drift, it is important to consider that seemingly inconsequential environmental changes can impact phenotype. This includes differences in ambient noise, which may have differed between the two studies

as a result of construction works, for example. Greater ambient noise can act as a stressor which is known to enhance blood glucose concentrations with corticosterone directly implicated in this (Jia et al., 2020; Zheng et al., 2018). Although WT blood glucoses concentrations were largely unchanged between the previous and present study, diabetic KINGS mice may be more susceptible to increases in blood glucose concentrations in response to stress. In line with this, previous work from our laboratory has suggested that male KINGS mice are more susceptible than WT mice to increases in blood glucose concentrations resulting from the stress associated with intraperitoneal injections (Austin, 2018).

Phenotypic differences may also be the result of a change in animal diet. Between the previous and present study, the normal chow used by the animal facility changed from RM1 (Special Diets Services) to Rodent Diet 20 (PicoLab). Pilot studies run by the animal facility staff found that the current diet was associated with a better maintenance of weight throughout life, and this may go some way to explaining weight differences seen. Although it is worth pointing out that no increase in weight was seen in the other groups monitored. The composition of the two diets differs and this includes with respect to protein composition which is higher in the PicoLab diet (20% vs. 17.5%) and in regard to fat content which is lower in the PicoLab diet (4.5% vs. 7.5%) (McNeilly et al., 2016). Diets with higher protein and lower fat content have consistently been shown to be associated with better glycaemic control in humans, and rodents fed higher fat diets are often used to model insulin resistance in type 2 diabetes (King, 2012; Kirk et al., 2008; Malaeb et al., 2019). A higher fat content and lower protein content may have therefore exacerbated hyperglycaemia in the KINGS male mice from the previous study.

Glucose tolerance tests in the KINGS mice were also performed in this study to investigate whether glycaemic control in the context of increased insulin demand differed from previous findings. Our results cannot be directly compared to the previous study as different fasting lengths were used (16 hours vs 6 hours). Fasting mice prior to a glucose tolerance test is common practice since it reduces the impact of variations in food intake between mice

which may lead to differences in glucose homeostasis (Kennard et al., 2022). Historically, 16-hour overnight fasts have been carried out and this was used in the previous study, however recent evidence suggests that this causes cardiovascular changes, hypothermia, significant weight loss as well as severe hypoglycaemia. Indeed, continuous blood glucose monitoring has been used to show that 16-hour fasting causes a non-physiological drop in blood glucose levels to lower than 2.8mM in some mice (Kennard et al., 2022). Studies have also shown that corticosterone levels increase only after a 16-hour fast, suggesting it induces a stress response which is known to influence glycaemic control (Jensen et al., 2013; Jia et al., 2020). Therefore, 16-hour fasting is not representative of a normal physiological state and raises significant welfare questions. With this in mind, a 6-hour fast was used in the present study since this length of fast has been associated with no deviation from the normoglycemic range for the duration of the fast (Kennard et al., 2022).

We found impaired glucose tolerance in KINGS male and female mice which was consistent with the previous findings; glucose tolerance was impaired from 3 weeks in male and from 4 weeks in female KINGS mice, and this only deteriorated further in male KINGS mice by 10 weeks. Of note, baseline blood glucose concentrations in all groups were lower in the previous study which used a longer fasting period, and this is in line with findings that a 16-hour fast causes hypoglycaemia. At 10 weeks, baseline blood glucose concentrations in the male KINGS mice were over 20mM compared to approximately 18mM after a 16-hour fast. Since a fasting blood glucose concentration as high as this clearly shows a lack of glucose tolerance, and since therefore further injection of glucose is not ethical, for future glucose tolerance testing in this thesis we decided to only inject a bolus of glucose if baseline fasted blood glucose concentrations were less than 20mM.

An additional glucose tolerance test was carried out at 3 weeks of age, and this found that whilst glucose tolerance is still impaired in KINGS males compared to the wildtype littermates, this was mild and much improved compared to at 4 weeks. Furthermore, glucose tolerance was not significantly different between KINGS and WT female mice at 3 weeks. A deterioration in

glucose tolerance between 3 and 4 weeks of age may be explained by the onset to puberty and related growth and increase in fat mass. Puberty occurs between the age of 4-5 weeks in C57BL/6J mice and although a recent study found that it was only associated with insulin resistance in female mice, it has been associated with insulin resistance in both pubescent men and women (Kelsey & Zeitler, 2016). Although the mechanisms behind transient pubertal insulin resistance is unclear, it has been attributed to increased levels of growth hormone (GH) (Kelsey & Zeitler, 2016; Moran et al., 2002). Indeed, the greatest increase in weight in the KINGS mice occurred between 3 and 4 weeks of age which correlates with the drastic deterioration in glucose tolerance. In future, it would be worthwhile to carry out insulin tolerance tests at 3 weeks of age to determine to what extent changes in insulin sensitivity drives the latter.

The impairment in glucose tolerance may also be the result of a dietary switch (from maternal milk to solid food) or differences in stress levels in response to handling. The 3-week-old mice were recently weaned and thus had a mixed diet of maternal milk and solid chow, whereas the 4-week-old mice would have had a diet of solid chow alone for a week. The latter may influence their response to glucose during a glucose tolerance test. 3-week mice will have also been handled significantly less compared to the 4-week-old mice and likely experience an exaggerated stress response. In line with this, our laboratory has previously reported a ‘first GTT phenomenon’ whereby mice appear significantly more glucose intolerant in their first GTT compared to later GTTs conducted, indicative of a stress response which diminishes with increased habituation to handling and the GTT procedure (Kennard et al., 2021). This stress response may have masked differences between WT and KINGS glycaemic control.

Insulin tolerance tests were also performed but results are difficult to interpret owing to differences in baseline blood glucose concentrations. Importantly however, KINGS mice of both sexes were able to respond to insulin. When blood glucose concentrations were expressed as a percentage of baseline values, male KINGS mice had greater area over the curve values compared

to WT males at 4 weeks whereas values were not different between the two genotypes at 10 weeks. By 10 weeks of age male KINGS mice are overtly diabetic, thus the fact that insulin sensitivity does not differ from WT males at this age suggests that insulin resistance does not contribute to a loss of glycaemic control. It is unclear why at 4 weeks male KINGS mice exhibited a larger decrease in blood glucose concentrations expressed as percentage of baseline. Whilst this is suggestive that peripheral insulin sensitivity is superior in KINGS males at this age, it is more likely to be attributed to the fact that baseline blood glucose levels are higher in the KINGS males. This is because blood glucose concentration decrease in response to insulin injection is limited by glucose counterregulatory responses which works to enhance hepatic glucose output and prevent hypoglycaemia (Sprague & Arbeláez, 2011). The blood glucose level at which these counterregulatory responses are initiated are likely similar between the genotypes, however when expressed as a percentage reduction from baseline blood glucose concentration, this is greater for KINGS mice. Indeed, raw blood glucose concentration values fall to a similar level (5-6mM) in KINGS and WT mice. Supporting this idea, Jacobson *et al.* suggested that counterregulatory responses to insulin in mice are activated when blood glucose levels fall to a similar level (~4.4mM) (Jacobson et al., 2006). However, a similar effect was not seen at 10 weeks despite baseline blood glucose levels being higher than WT. This may be because insulin dosage was not sufficient to reduce blood glucose levels to the level seen at 4 weeks owing to a higher basal blood glucose concentration (22.4mM at 4 weeks *vs.* 30.7mM at 10 weeks). It is worth noting that insulin tolerance tests may not be sensitive enough to detect subtle but biologically relevant differences in insulin sensitivity. Hyperinsulinaemic-euglycaemic clamping in these mice in the future would offer a more sensitive approach to measure insulin resistance (Bowe et al., 2014; Kennard et al., 2021).

Area over the curve values for the insulin tolerance test conducted in 4-week WT and KINGS female mice were not different, again suggesting that insulin resistance does not contribute to the loss of glycaemic control associated with the KINGS mutation. A difference was detected at 10 weeks however where WT females showed lower area above the curve values whereas values

between all other groups measured were similar. This was unexpected given that female mice have been found to be more insulin sensitive compared to males in the literature (Macotela et al., 2009). This was largely the result of higher blood glucose concentrations in the female WT mice at 45 and 60 minutes post insulin injection since blood glucose levels are similar at 0, 15 and 30 minutes. This may be suggestive of differences in the glucose counterregulatory response in this group. Of note however, variability in glucose concentrations at 45 and 60 minutes was much greater in this group compared to the other groups. This may indicate that sampling error or technical error (such incorrect injection site) may be responsible for the differences seen.

High blood glucose levels have been shown to drive an increase in peripheral insulin resistance in animal models and in human studies, and this is thought to be mediated by a number of mechanisms including through ceramide production and oxidative stress (Tomás et al., 2006). Indeed, 48-hour glucose infusion in non-diabetic individuals increased hepatic insulin resistance and whilst hyperglycaemia induced through partial pancreatectomy in rats also causes peripheral insulin resistance, normalisation of blood glucose levels in this model using phlorizin restores this (Rossetti et al., 1987; Tripathy et al., 2019). In future, it would therefore be of interest to investigate insulin resistance in older KINGS mice that have experienced chronic hyperglycaemia (>20 weeks of age) to investigate whether this contributes to the progressive deterioration in glucose tolerance and hyperglycaemia previously reported (Austin et al., 2020).

A pilot insulin tolerance test was performed in 30-week-old KINGS mice (Figure 26). These mice showed no response to the typical 0.75IU/kg insulin dose, however when dosage was increased ten-fold (7.5IU/kg) blood glucose levels did decrease to 8.7mM. This pilot data suggests that chronic hyperglycaemia may impair insulin sensitivity, however further study is required.

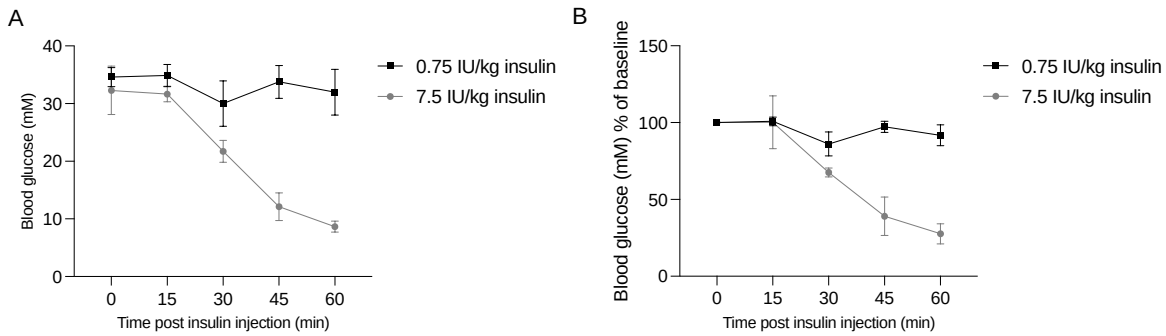


FIGURE 26: *Insulin tolerance testing in older KINGS males:*
(A) *Raw blood glucose concentrations after administration of 0.75IU/kg (black line) and 7.5IU/kg (grey line) insulin to 30-week-old KINGS male mice. (B) Blood glucose concentration measurements for the insulin tolerance test expressed as a percentage of baseline blood glucose concentrations. Data represents the mean±SEM, n=2-3.*

Overall, we have shown that the glycaemic control in the KINGS mice is largely consistent with the previous study conducted between 2016-2018, however male KINGS mice may show a slightly milder diabetic phenotype possibly due to genetic drift and/or changes in the environment within the animal department. Despite this, day of diabetes onset is similar to previous findings further emphasising predictability of diabetes onset in this model. A better maintenance of weight and slightly milder hyperglycaemia in the KINGS male mice from the present study may indicate a healthier phenotype despite clear hyperglycaemia making the model more attractive ethically and practically. We have also shown that insulin resistance does not contribute to a lack of glycaemic control in the female KINGS mice and does not contribute to the development of diabetes in the male KINGS mice. However, further study is warranted into whether insulin resistance contributes to the deterioration in glycaemic control seen in the male KINGS mice after 10 weeks of age. Our results emphasise the importance of regularly checking phenotype of interest and highlights the importance of using appropriate controls for each experimental question rather than relying on historic controls

CHAPTER 4: HOW DO BETA CELLS RESPOND TO ER STRESS IN THE KINGS MOUSE?

Chapter snapshot:

The KINGS mouse harbours a heterozygous mutation in *Ins2* which results in a glycine to serine substitution in preproinsulin2. Structural studies have suggested that this causes a misfolding of the proinsulin molecule in the ER and electron microscopy experiments have implicated it in inducing beta cell ER stress.

The aim of this study was to establish whether KINGS mice exhibit beta cell ER stress and subsequent activation of the UPR, whether this precedes the onset of diabetes in male mice, and whether this differs between male and female KINGS mice. Since ER stress and the UPR can impact beta cell survival and proliferation, we also investigated whether beta cell apoptosis and proliferation were changed in the KINGS mice.

4.1 INTRODUCTION:

Beta cell functional impairment and death, collectively referred to as beta cell failure, underpin the pathogenesis of diabetes. Beta cell endoplasmic reticulum (ER) stress is believed to be an important driver of this and indeed is a common feature of many types of diabetes including T2DM (Eizirik et al., 2008; Shrestha et al., 2021). The ER of the beta cell is highly specialised to cope with a constant and fluctuating demand for insulin, however such a large metabolic burden makes the beta cell particularly susceptible to ER stress. Under physiologically normal conditions, ER stress activates the adaptive unfolded protein response (UPR) which encompasses three parallel signalling arms which are initiated by the phylogenetically conserved transmembrane proteins PERK, IRE1-alpha and ATF6-alpha (Ron & Walter, 2007). The latter collectively attempt to restore ER protein homeostasis through various mechanisms including through increasing ER associated degradation (ERAD)

of misfolded proteins (whereby mis-folded proteins are retro-translocated to the cytosol and undergo proteasomal degradation), reducing mRNA translation, expanding the ER membrane through enhanced lipid synthesis and activating the transcription of genes associated with protein folding including those encoding protein chaperones (Ron & Walter, 2007).

Insulin synthesis fluctuates between 10% of total beta cell protein production under low-levels of stimulation, and 50% under high levels (Miranda et al., 2021). Confounding this burden on the beta cell, it is thought that up to 20% of proinsulin misfolds, failing to reach its mature conformation (Sun et al., 2015). Adaptive UPR signalling is crucial in preserving protein synthesis homeostasis in spite of this metabolic burden and is therefore necessary for the maintenance of a functional beta cell mass (Lenghel et al., 2021; Rabhi et al., 2014). Indeed, Xin *et al* used single cell RNA sequencing to show that human beta cells oscillate between periods of high insulin biosynthesis (and low UPR expression) and a recovery state where insulin biosynthesis is low and UPR expression is high (Xin et al., 2018). Recent evidence has also suggested that the adaptive UPR is important in beta cell adaptation. For example, several studies have shown that beta cells with an active UPR are more likely to proliferate (Sharma et al., 2015; Xin et al., 2018). Sharma *et al* found that beta cell proliferation was dependent on the ATF6-alpha pathway since its overexpression in cultured primary mouse islets enhanced beta cell proliferation whilst its inhibition and knockdown reduced this. It should also be noted that whilst IRE1 knockdown did reduce proliferation, its overexpression had the same effect suggesting that whilst it is involved in beta cell proliferation in response to stress, it is not sufficient to drive this. UPR signalling has also been implicated in protection from apoptosis and heightened insulin secretory function (Lee et al., 2011, 2022; Sharma et al., 2015, 2021). Supporting an important role of the UPR in the survival and function of beta cells, failure of the adaptive UPR to be mounted has been associated with beta cell demise. Indeed, beta cell specific deletion of components of the adaptive UPR in mouse models and humans causes diabetes. For example, loss of function in the PERK pathway in individuals with Wolcott-Rallison syndrome (WRS)

drives beta cell loss and diabetes emphasising the importance of optimal UPR signalling (Delepine et al., 2000; Hassler et al., 2015; Zhang et al., 2002).

The UPR, however, represents a double-edged sword; when ER stress is too chronic or robust and cannot be resolved through adaptive UPR signalling, then maladaptive UPR signalling ensues and this can lead to beta cell death (Ghosh et al., 2019; Shrestha et al., 2021). In the context of the whole organism, this could be viewed as advantageous since it prevents the sustenance of cells that produce misfolded, potentially harmful, proteins possibly deleterious to the whole organism. However, since beta cells have a low replicative capacity (excluding during the neonatal period or during pregnancy), non-resolvable ER stress which could lead to cell dysfunction and death becomes problematic.

Chronic PERK signalling drives activation of proapoptotic C/EBP homologous protein (CHOP) as well as a global inhibition of cellular translation which can lead to dedifferentiation (Sharma et al., 2021). Chronic signalling through IRE1 leads to the formation of IRE1 multimers (thought to have heightened activity compared to IRE1 dimers). The latter acts as a scaffold to drive recruitment of TBF receptor associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) and subsequent phosphorylation and activation of the pro-apoptotic protein c-Jun N-terminal kinase (JNK). Sustained signalling through IRE1 also mediates global mRNA degradation through RIDD which can lead to a loss of cellular identity and apoptosis if maintained. In situations of prolonged ER stress, ATF6 can also lead to beta cell death through transcriptional upregulation of CHOP.

Maladaptive UPR signalling resulting from irremediable ER stress has been associated with the development of diabetes in animal models and in humans (Eizirik et al., 2008; Shrestha et al., 2021). For example, the Akita mouse harbours a heterozygous mutation in *Ins2* which drives proinsulin misfolding in the ER, this has been associated with the upregulation of UPR components and ultimately CHOP-mediated beta cell death leading to hyperglycaemia (Yoshioka et al., 1997). Beta cell activation of UPR components has also been

evidenced in individuals with type 2 diabetes, for example increased immunostaining for ATF4 and CHOP UPR markers among others have been reported in islets from patients with T2DM (Huang et al., 2007; Laybutt et al., 2007). It should be noted however that other studies have found conflicting results. Notably, Engin *et al.* found that XBP1s and ATF6 expression was reduced in islets of individuals with T2DM arguing that a failure of the UPR is associated with diabetes (Engin et al., 2014). Additionally, the largest RNA sequencing study on human islets carried out so far did not find any significant differences between UPR marker expression in islets from T2D patients and control patients. Difference in studies may be attributed to the use of different techniques to study beta cell ER stress, for example use of pancreatic sections *vs*. use of isolated islets or analysis of mRNA *vs*. protein expression. Moreover, the UPR can be activated by factors distinct from ER stress, such as viral infection as well as sex hormones, and this may go some way to explaining differences (Cirone, 2021; Shapiro et al., 2016). Clearly more work is required to establish the importance of ER stress in diabetes development (Marselli et al., 2020; Shrestha et al., 2021). Much of the literature focusses on beta cell death as the main maladaptive response to ER stress and commonly used models of beta cell ER stress display mass beta cell loss. However as mentioned, the ER stress has also been associated with a loss of beta cell function and identity, and this may play some role in beta cell failure associated with diabetes, although the extent of its role is unknown.

The KINGS mouse harbours a heterozygous single nucleotide polymorphism in *Ins2* which causes a glycine to serine substitution proximal to the A7-B7 disulphide bridge in preproinsulin. This glycine residue is conserved across insulin and insulin-like growth factor sequences, implying it is important either to the structure or function of the polypeptide (Støy et al., 2007). Glycine uniquely lacks a side chain and thus it has been hypothesised that its replacement with another amino acid, and hence introduction of a side chain, causes a conformational change in the polypeptide chain that impairs disulphide bond formation between the local cysteines (A7-B7). Indeed, Støy *et al* used Insight graphic environment software to predict the conformational change in the insulin molecule resulting from a G32S substitution (Figure 27)

which clearly shows that it causes a stark conformational change in the molecule which no longer allows an interaction between the B7-cysteine and A7-cysteine (Støy et al., 2007). Interestingly, the same paper identified the G32S mutation in several individuals from separate families (or genetic pedigrees), suggesting that this position may represent a 'hot spot' for mutation in humans.

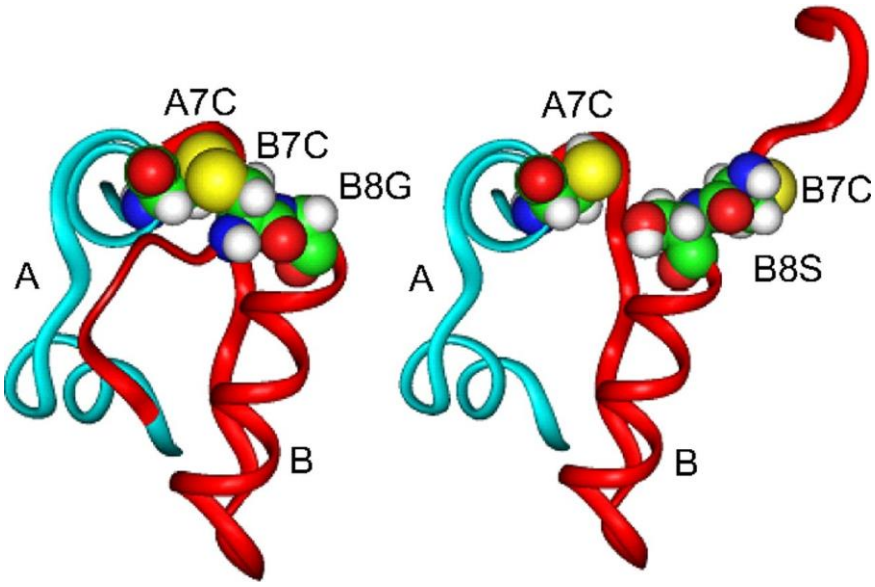


FIGURE 27: *Predicted structure of the G32S mutated insulin: (Left) Structure of the wildtype mature insulin molecule. (Right) Predicted structure of the insulin molecule harbouring a G32S substitution. Image taken from Støy et al. 2007* (Støy et al., 2007)

The Akita mutation similarly impacts the A7-B7 disulphide bridge and has been shown to result in beta cell ER stress and the development of diabetes in mice (Herbach et al., 2007; Yoshioka et al., 1997). Numerous studies have shown increased expression of components of the UPR in this model (Oyadomari, Koizumi, et al., 2002; Wang et al., 1999). Additionally, *in vitro* studies whereby MIN6 cells were induced to express the Akita mutation, as well as the KINGS mutation, found that mutant proinsulin molecules were retained within the ER and induced ER stress analysed through CHOP expression (Rajan et al., 2010).

The characterisation of novel animal models harbouring insulin mutations and beta cell ER stress will aid our understanding of what causes beta cell ER

stress and how this is associated with beta cell failure and diabetes, as well as provide a model whereby factors that exacerbate and alleviate beta cell ER stress can be tested. This is not only important to individuals with mutant INS-gene-induced Diabetes of Youth (MIDY), but also those living with other forms of diabetes since ER stress has frequently been associated with these, as well as poor graft survival and function in islet transplantation (Eizirik et al., 2008; Negi et al., 2012).

Previous electron microscopy imaging of the beta cells from the KINGS mice (both male and female mice) has shown they exhibit hallmark signs of ER stress including enlarged ER and distorted mitochondria (Figure 28), however a pronounced increase in the gene expression of ER stress markers in KINGS islets was not observed (Austin et al., 2020). In the present study, we aimed to further establish whether the KINGS mouse represents a model of beta cell ER stress-induced diabetes by investigating whether protein markers of ER stress and the UPR are upregulated in the beta cells of the KINGS mice. Since we also saw a sex difference in the KINGS phenotype in the previous chapter, here we investigate whether ER stress and activation of the UPR differs between male and female mice. Considerable sex differences exist in diabetes, with T2DM being more common in men compared to pre-menopausal women and a male predominance is also seen in many animal models. Investigating ER stress and beta cell response to this in this model may provide insight into why these sex differences exist in humans. Finally, we also investigated whether beta cell ER stress in the KINGS mice had any effect on beta cell mass and turnover.

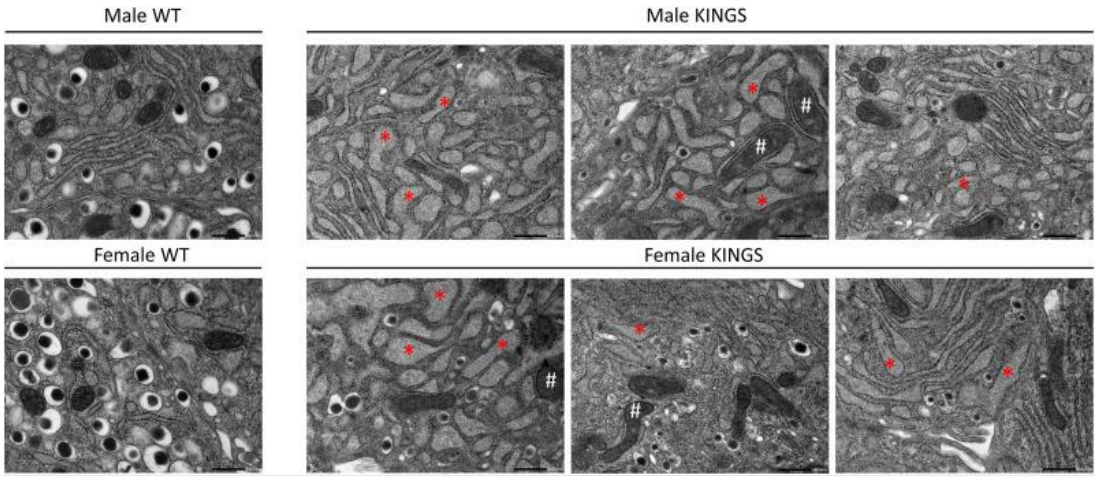


FIGURE 28: *Ultrastructural signs of beta cell ER stress in THE KINGS mice: Transmission electron microscopy images of beta cells from male WT (top left), female WT (bottom left), KINGS male (top right) and KINGS female (bottom right) mice. Swollen ER is indicated by the red asterisks and distorted mitochondria are indicated by the white hash signs. Image taken from* (Austin et al., 2020).

4.2 AIMS:

- Determine whether protein markers of ER stress and the UPR are upregulated in KINGS islets and whether this occurs prior to or after the onset of overt diabetes and differs between sexes.
- Determine whether beta cell ER stress drives changes in beta cell turnover (proliferation and apoptosis).
- Determine whether beta cell ER stress drives a reduction in beta cell mass in the KINGS mouse.

4.3 METHODS:

4.3.1 EXPERIMENTAL OUTLINE:

Investigating islet ER stress and activation of the UPR in the KINGS mice:

Pancreases were excised from KINGS mice at different ages encompassing the disease course in males; 4 weeks (before the onset of overt diabetes), 10 weeks (established diabetes) and at 20 weeks of age (chronic hyperglycaemia). Pancreatic sections were stained for the general ER stress marker, BiP, through immunofluorescent staining to investigate islet ER stress.

Western blotting was used on isolated islet protein lysates to investigate the expression of markers of each arm of the UPR (PERK, IRE1 and ATF6) in the KINGS mice at 4 weeks and 10 weeks of age which represent a periods before diabetes onset and when diabetes is established in the KINGS male mice, respectively.

Investigating beta cell turnover and beta cell mass in the KINGS mice:
Pancreases were excised from KINGS mice at different ages encompassing the disease course in males; 4 weeks (before diabetes onset), 10 weeks (established diabetes) and at 20 weeks of age (chronic hyperglycaemia). Ki67 immunofluorescent staining was used to investigate beta cell proliferation, TUNEL immunofluorescent staining was used to investigate beta cell apoptosis and insulin immunofluorescent staining was used to investigate islet area and beta cell area as a proportion of total pancreatic area (a proxy for beta cell mass) in pancreatic sections from the KINGS mice.

4.3.2 Animals:

Mice were housed in single sex groups of up to five in individually ventilated cages with *ad libitum* access to water and standard rodent diet 20 chow (Picolab, UK). Animals were kept on a 7am-7pm light-dark cycle. Heterozygous KINGS and wildtype mice were maintained through in-house conventional breeding where wildtype/KINGS 6-8-week female mice were mated singly or in duos with wildtype/KINGS male mice. Heterozygous KINGS and wildtype offspring were used for experiments.

4.3.3 Genotyping:

DNA was extracted from animal ear clips through digestion with DNA lysis buffer at 55°C (10% 10× Gitschier buffer, 0.5% Triton X-100, 1% β-mercaptoethanol, 2% 50 µg/mL proteinase K). Kompetitive allele specific PCR (KASP; LGC, Hoddesdon, UK) was used to determine the presence of the KINGS mutation as previously described (section 2.1.3.2). Forward primers harbouring fluorescent tags corresponding to the wildtype *Ins2* (GAAGGTGACCAAGTTCATGCTTTTGTCAAGCAGCACCTTTGTG, FAM fluorophore) and KINGS *Ins2*

(GAAGGTCGGAGTCAACGGATTGCTTTTGTCAAGCAGCACCTTTGTA, HEX fluorophore), with a common reverse primer for *Ins2* (AGAGCCTCCACCAGGTGGGAA), were used. PCR was carried out using a LightCycler480 or LightCycler96 (Roch, Switzerland) and genotype was determined through the fluorescent signal of the sample.

4.3.4 Islet isolation:

Mouse pancreatic islets were isolated through collagenase digestion after mice were culled via cervical dislocation; pancreases were perfused with ~2.5mL of 1mg/mL collagenase solution via bile duct injection, excised and incubated in a water bath at 37°C for 10 minutes. Digested pancreases were washed in MEM or RPMI supplemented with 10% foetal bovine serum before Histopaque-1077 was added to generate a density gradient from which the islets were collected. Islets were washed and then cultured at 37°C in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin.

4.3.5 Western blotting:

Chilled RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail was added to islets (1µL per 6 islets, >200 islets in total per n) and islets were left on ice to lyse for 30 minutes before being spun at 13000rpm for 30 minutes. Supernatants were stored at -70°C. A BCA assay was used to quantify protein content and 10µg of protein per sample was subjected to SDS PAGE for 2 hours and then transferred to a PVDF membrane. The membrane was sequentially incubated with antibodies towards ER stress markers and beta-actin (loading control): rabbit anti-BiP (1:1000, Cell signalling technology), mouse anti-beta-actin (1:5000, Santa Cruz Biotechnology), rabbit anti-XBP1s (1:1000, Cell signalling technology), rabbit anti-p-eIF2-alpha (1:1000, Cell Signalling Technology), mouse anti-CHOP (1:100, Santa Cruz Biotechnology) and mouse anti-ATF6 (1:500, BioTechne). Bands were detected by subsequent incubation with the relevant anti-mouse and anti-rabbit HRP conjugated antibodies (1:2500, Santa Cruz Biotechnology) and membranes were washed between incubations with different primary antibodies but without stripping. Densitometry analysis was performed in ImageJ and peak percentages were normalised to the beta actin loading

control (Schindelin et al., 2012). The experimental unit was pooled islets from 2-3 mice.

4.3.6 Histology:

4.3.6.1 Tissue fixation, paraffin embedding and sectioning:

Mice were culled by cervical dislocation and their pancreases were immediately excised and placed in 3.5% phosphate buffered formalin for 48 hours. Pancreases were subsequently washed with phosphate buffered saline and stored in 70% ethanol until paraffin embedding as previously described. A Leica Wetzer microtome and MX35 Ultra microtome blades (ThermoFisher, UK) were used to cut 5µm tissue sections from paraffin blocks which were mounted on glass slides.

4.3.6.2 Beta cell apoptosis:

TUNEL staining was performed using the ApopTag Fluorescein In situ Apoptosis Detection Kit from Merck as per the manufacturers guidelines and as previous described (section 2.6.2.3). One section mid-way through the pancreas was stained per mouse. Sections were deparaffinised through heating and incubation in two changes of xylene solution. Tissue sections were rehydrated through incubation in decreasing concentrations of ethanol before 20µg/mL of proteinase K was added directly to the tissue for antigen retrieval. Slides were washed in PBS, incubated with equilibration buffer and sections were then incubated with a terminal deoxynuceleotidyl transferase solution for 1 hour at 37°C. Sections were washed in stop buffer, washed in PBS and the incubated in anti-digoxigenin antibody conjugated to Apoptag fluorescein for 30 minutes. Sections were subsequently stained with for insulin and DAPI as described above. Slides were mounted with Fluoromount aqueous mounting medium (Sigma, UK) and imaged at 10 or 20x magnification on a Nikon Eclipse TE 2000-U microscope. All image analysis was carried out manually in ImageJ and in a blinded fashion as described (section 2.6.4.3)(Schindelin et al., 2012).

4.3.6.3 Beta cell proliferation and islet area:

1-2 pancreas section per mouse approximately mid-way through the wax block was stained for insulin, Ki67 and DAPI as previous outlined (section 2.6.2.2). In brief, sections were de-waxed by heating and incubation in xylene. Slides were rehydrated through incubation in decreasing concentrations of ethanol before 10mM citric acid antigen retrieval. Sections were incubated overnight with guinea-pig anti-insulin antibody (Dako, 1:500) and rabbit anti-Ki-67 antibody at 1:100 (Abcam, UK). Sections were subsequently incubated with a secondary anti-guinea pig antibody (Alexa Fluor 488, 1:200; Jackson ImmunoResearch), anti-rabbit antibody (Alexa Fluor 594, 1:200, Jackson ImmunoResearch) and DAPI (1:500) for 1 hour at room temperature. Fluoromount aqueous mounting solution (Sigma Aldrich) was applied to the tissue and a glass coverslip was placed over the sections. Islets were imaged at 10-30x magnification on a Nikon Eclipse TE 2000-U microscope. All islets from 1-2 pancreatic sections were imaged in total. All image analysis was carried out manually and in a blinded fashion in ImageJ as described (section 2.6.4) (Schindelin et al., 2012).

Previous electron microscopy of beta cells from the KINGS mice show that they are degranulated. Therefore, it is possible that islet area could be underestimated using insulin immunostaining. Therefore, haematoxylin and eosin staining was also used to measure islet size in the same groups. One pancreas section per mouse approximately mid-way through the wax block was stained with haematoxylin and eosin as previously described (section 2.6.3). Briefly, wax was removed through heating and incubating slides in xylene. The tissue was then rehydrated by incubation in decreasing concentrations of ethanol and subsequently submerged in haematoxylin and eosin solution. Tissue was dehydrated by submersion in increasing ethanol concentrations, DPX mounting media was applied, and a glass coverslip was placed over the section. Stained pancreas sections were imaged using a Zeiss Axiophot microscope with a 20x objective and all islets from one pancreatic section per mouse were imaged. Islet images were analysed blindly in FijiImageJ where the polygon selection tool was used to manually draw around islets as previously described (section 2.6.4) (Schindelin et al., 2012).

4.3.6.4 Beta cell mass:

Insulin positive area as a proportion of total section pancreatic area was used as a proxy for beta cell mass. 3-4 pancreatic sections spanning the entire pancreas and ~250µM apart were stained for insulin and with DAPI as previously described (section 2.6.2.1). Wax was removed from the tissue by heating the slides and incubating them in xylene. Sections were blocked and subsequently incubated with a guinea-pig anti-insulin antibody at 1:200 diluted in blocking buffer overnight at 4°C (abcam, UK). After washing in TBS, sections were incubated with a secondary anti- guinea pig antibody (Alexa Fluor 488, 1:200; Jackson ImmunoResearch) and DAPI (1:500) for 1 hour at room temperature in a humidifying chamber. Whole pancreatic sections were imaged using a NanoZoomer S60 fluorescence and brightfield slide scanner (Hamamatsu) with a 20x objective. Beta cell area and whole pancreatic area were analysed semi-automatically in FijiImageJ as previously described (section 2.6.4) (Schindelin et al., 2012).

4.3.6.5 Beta cell ER stress:

Immunoflourescence staining for the general ER stress marker, BiP, was used to investigate ER stress in KINGS islets. One section per animal was stained for BiP, insulin and DAPI as previously outlined in section 2.6.2.4. In short, sections were de-waxed by heating treatment with xylene. Slides were rehydrated through incubation in decreasing concentrations of ethanol before antigen retrieval by pressure cooking in 10mM citric acid. Sections were incubated overnight with guinea-pig anti-insulin antibody (Dako, 1:500) and rabbit anti-BiP (1:100, Cell Signalling Technology). Sections were subsequently incubated with a secondary anti-guinea pig antibody (Alexa Fluor 488, 1:200; Jackson ImmunoResearch), anti-rabbit antibody (Alexa Fluor 594, 1:200, Jackson ImmunoResearch) and DAPI (1:500) for 1 hour at room temperature. Fluoromount aqueous mounting solution (Sigma Aldrich) was applied to the tissue and a glass coverslip was placed over the sections. Islets were imaged at 10-20x magnification on a Nikon Eclipse TE 2000-U microscope. All islets from 1 pancreatic section were imaged in total. All image analysis was carried out manually and in a blinded fashion in ImageJ as described (section 2.6.4) (Schindelin et al., 2012).

4.3.7 Statistical analysis:

All graphs were constructed using GraphPad Prism 8.0 or 9.0 software (GraphPad Software, San Diego, CA) and statistical analysis was carried out in SigmaPlot 14.0 (Systat software Inc, San Jose, California). For comparisons between multiple groups, ANOVAs with Holm-Sidak post hoc was used. All data represent the mean ± standard error of the mean (SEM). $P<0.05$ was considered statistically significant.

4.4 Results:

4.4.1 ER stress and UPR activation in KINGS islets:

Activation of the maladaptive UPR and a failure of the adaptive UPR to be mounted have both been proposed to link ER stress with beta cell failure in diabetes. Previous investigations using electron microscopy have suggested that the KINGS mutation drives ER stress. To investigate this further, immunofluorescent staining of pancreatic islets for the general ER stress marker, BiP, was used.

Mean fluorescent intensity of BiP was increased by at least 1.5-fold that of the sex-matched wildtype in both male and female KINGS mice from 4 weeks of age onwards (Figure 29A-C) consistent with increased beta cell ER stress. Sex differences were seen in BiP expression in wildtype mice with females showing elevated BiP expression compared to males at 4 and 10 weeks, however this difference was lost by 20 weeks of age. A sex difference was also seen in BiP levels in the KINGS mice at 20 weeks, with males showing a 1.5-fold increase compared to KINGS females (Figure 29C). Interestingly, the range in mean fluorescent intensity was frequently higher in the male KINGS mice from 10 weeks of age, suggesting increased inter-islet heterogeneity in BiP expression in this group.

BiP expression was also variable within islets, with individual beta cells showing heterogeneity in BiP mean intensity levels. Therefore, maximum islet BiP intensity was also investigated (Figure 29D-F). These findings largely reflected the mean fluorescent intensity findings, however at 4 weeks of age a

sex difference was observed in both KINGS and WT mice, with females showing higher maximum islet BiP intensity compared to genotype-matched males. In the wildtype mice this sex difference is lost from 10 weeks, and from 10 weeks in the KINGS mice maximal BiP intensity levels are higher in the male mice.

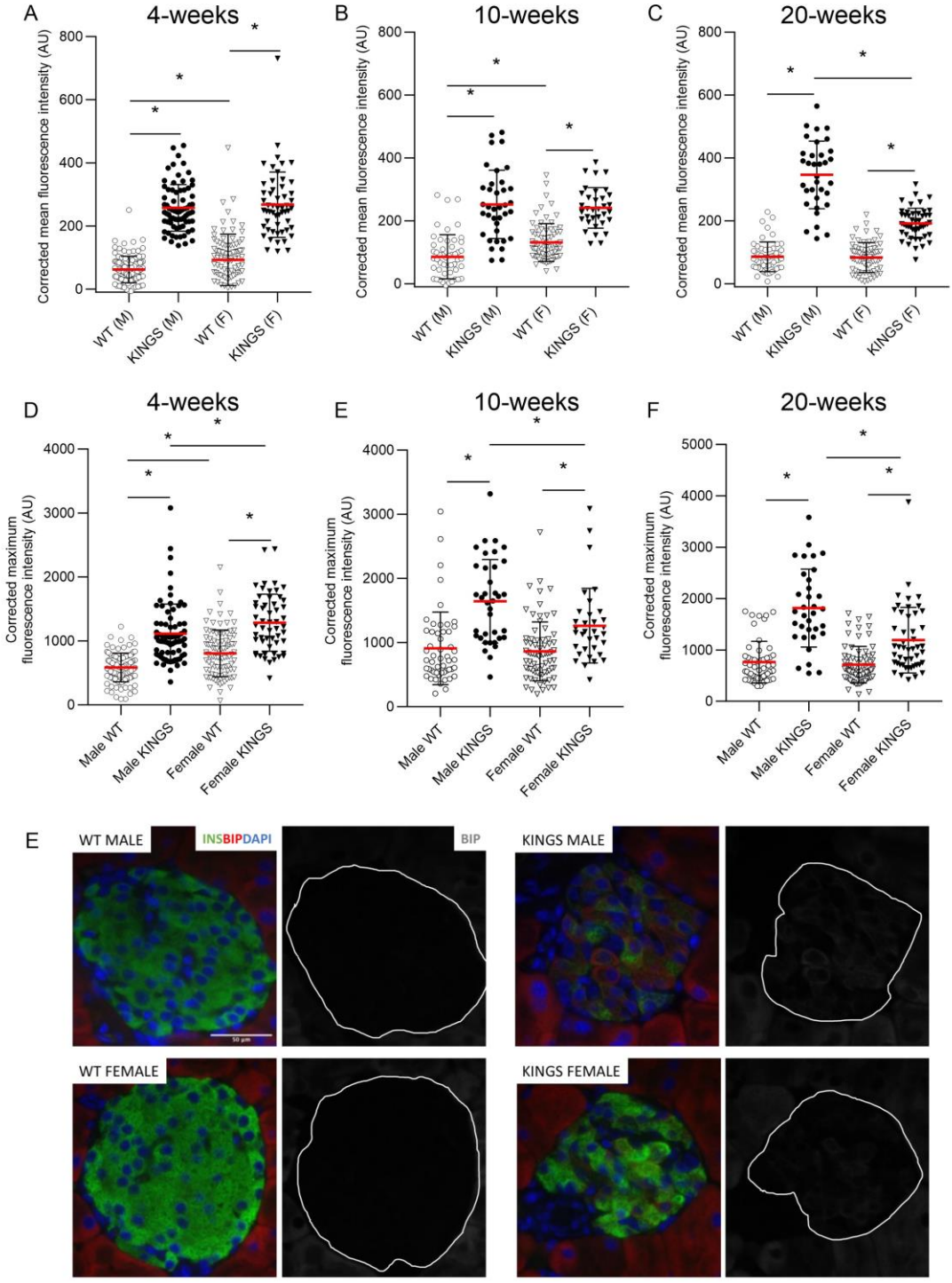


FIGURE 29: *BIP expression in KINGS islets:*

*(A-C) Mean fluorescent intensity of BiP in islets from pancreata of WT and KINGS, male and female mice. (D-F) Maximal BiP fluorescent intensity in islets from pancreata of WT and KINGS, male and female mice. AU, arbitrary unit. Two-way ANOVA with Holm-Sidak post hoc, *p<0.05, data represents individual islets from n=34-105 islets from 3 mice per group. (E) Representative images of islets from 10-week-old male and female, WT and KINGS mice stained for insulin (green), BiP (red and grey) and DAPI (blue). The BiP channel alone is also shown where the islet is outlined in white. The scale bar indicated on the top left image represents 50µm.*

Increased BiP was also confirmed in islets of male KINGS mice through western blotting, which showed that BiP was increased from 4 weeks of age (Figure 30A and Figure 31A). Islet BiP showed increased expression in the female KINGS mice as well, but this only reached significance at 4 weeks of age.

Western blotting was also used to investigate markers of the UPR (Figure 30B-D, Figure 31B-E). Full length ATF6 indicates activation of the ATF6 arm, XBP1s indicates activation of the IRE1 arm and phosphorylated-eIF2-alpha and indicates activation of the PERK arm. Male KINGS islets showed increased protein levels of ATF6 and phosphorylated eIF2-alpha from 4 weeks (Figure 30C-D, Figure 31C-D), and XBP1s was also increased at 10 weeks compared to wildtype male islets (Figure 31B). This pattern of increased expression of these markers in KINGS islets was also noted in female mice, however this only reached significance for ATF6 at 4 and 10 weeks (Figure 30C, Figure 31C). 10-week CHOP expression tended to be variable in KINGS islets with some replicates showing increased expression compared to wildtype, whereas for others it was comparable, however overall, the mean expression did not significantly differ from wildtype expression (Figure 31E). When investigating CHOP expression at 4 weeks, we only detected bands in 2 out of the 5 replicates and therefore could not undertake statistical analysis, however in these two replicates CHOP expression appears elevated in KINGS males (Figure 30F).

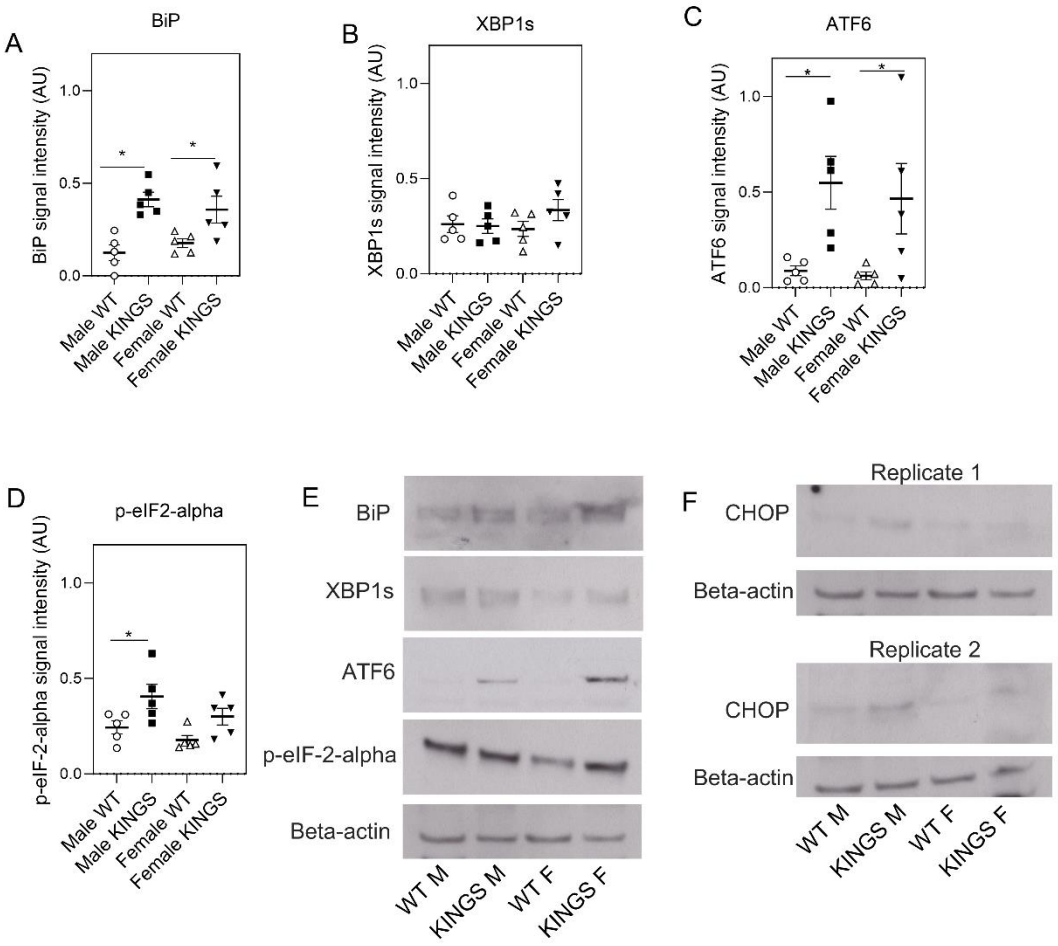


FIGURE 30: *Protein expression of ER stress and UPR markers in 4-week-old KINGS mice:*

*(A-D) Levels of ER stress markers were investigated by western blot in islets isolated from 4-week-old KINGS mice. n=5, two-way ANOVA with Holm-Sidak's post hoc, *p<0.05 KINGS vs wildtype. (E) Representative western blots: wildtype male (lane 1), KINGS male (lane 2), wildtype female (lane 3), KINGS female (lane 4). (F) Western blots for CHOP from two replicates: wildtype male (lane 1), KINGS male (lane 2), wildtype female (lane 3), KINGS female (lane 4).*

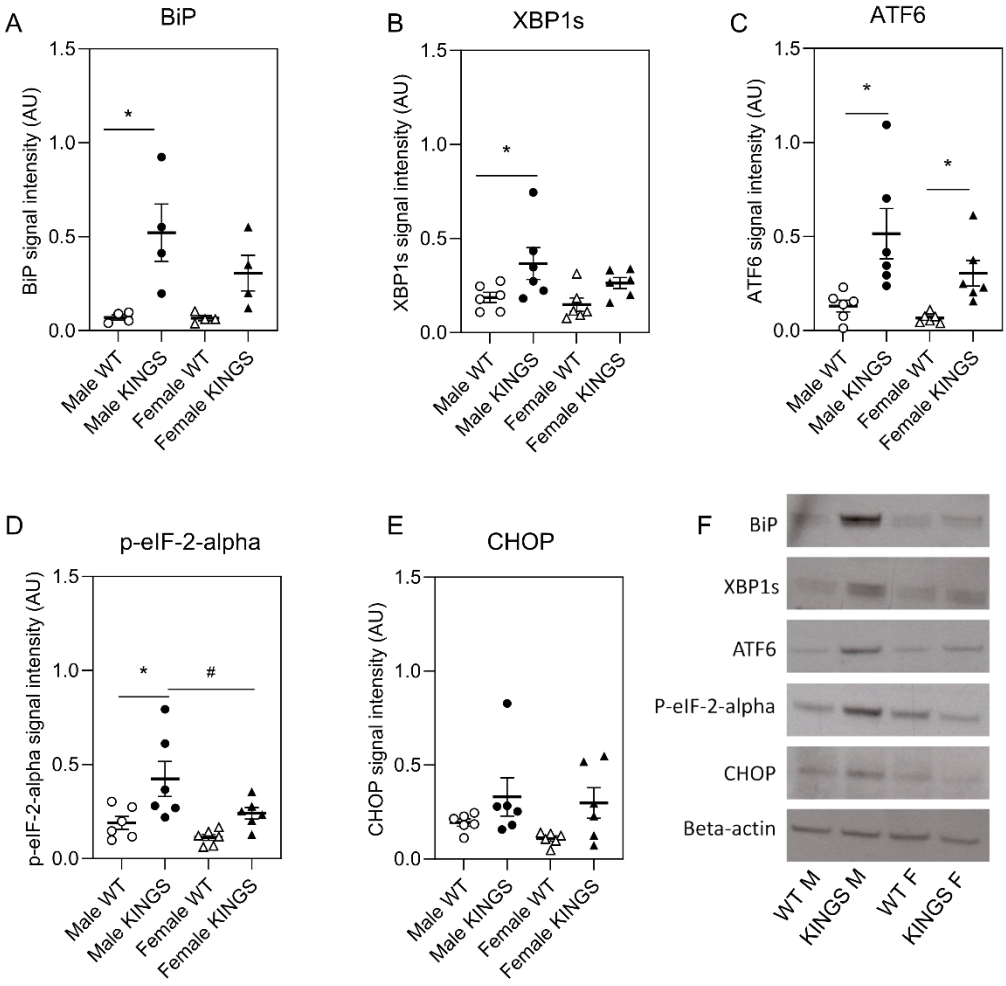


FIGURE 31: *Protein expression of ER stress and UPR markers in 10-week-old KINGS mice:*

*(A-E) Levels of ER stress markers were investigated by western blot in islets isolated from 10-week-old KINGS mice. (F) Representative western blots: wildtype male (lane 1), KINGS male (lane 2), wildtype female (lane 3), KINGS female (lane 4). N=4-6, two-way ANOVA with Holm-Sidak's post hoc, *=p<0.05 KINGS vs wildtype, #=p<0.05 KINGS male vs KINGS female.*

4.4.2 Beta cell apoptosis in the KINGS mice:

Activation of the maladaptive UPR can drive beta cell death, therefore beta cell apoptosis was investigated in islets from 4-, 10- and 20-week-old KINGS mice. Beta cell apoptosis was investigated in pancreatic sections

immunostained for TUNEL (for identification of cells undergoing apoptosis) and insulin (for identification of beta cells).

Beta cell apoptosis levels were very low in all groups at 4 and 10 weeks of age with many groups having no detectable beta cells undergoing apoptosis (Figure 32). Use of spleen and foetal mouse tissue as a positive control confirmed that this was not due to technical error, since many apoptotic cells could be identified in the controls. This low rate was also observed in wildtype mice and KINGS female mice at 20 weeks of age. However, at 20 weeks male KINGS mice showed an increase in beta cell apoptosis which was an average 12-fold that of wildtype males. Apoptosis rate at this age in male KINGS mice was also significantly higher than at 4 and 10 weeks of age.

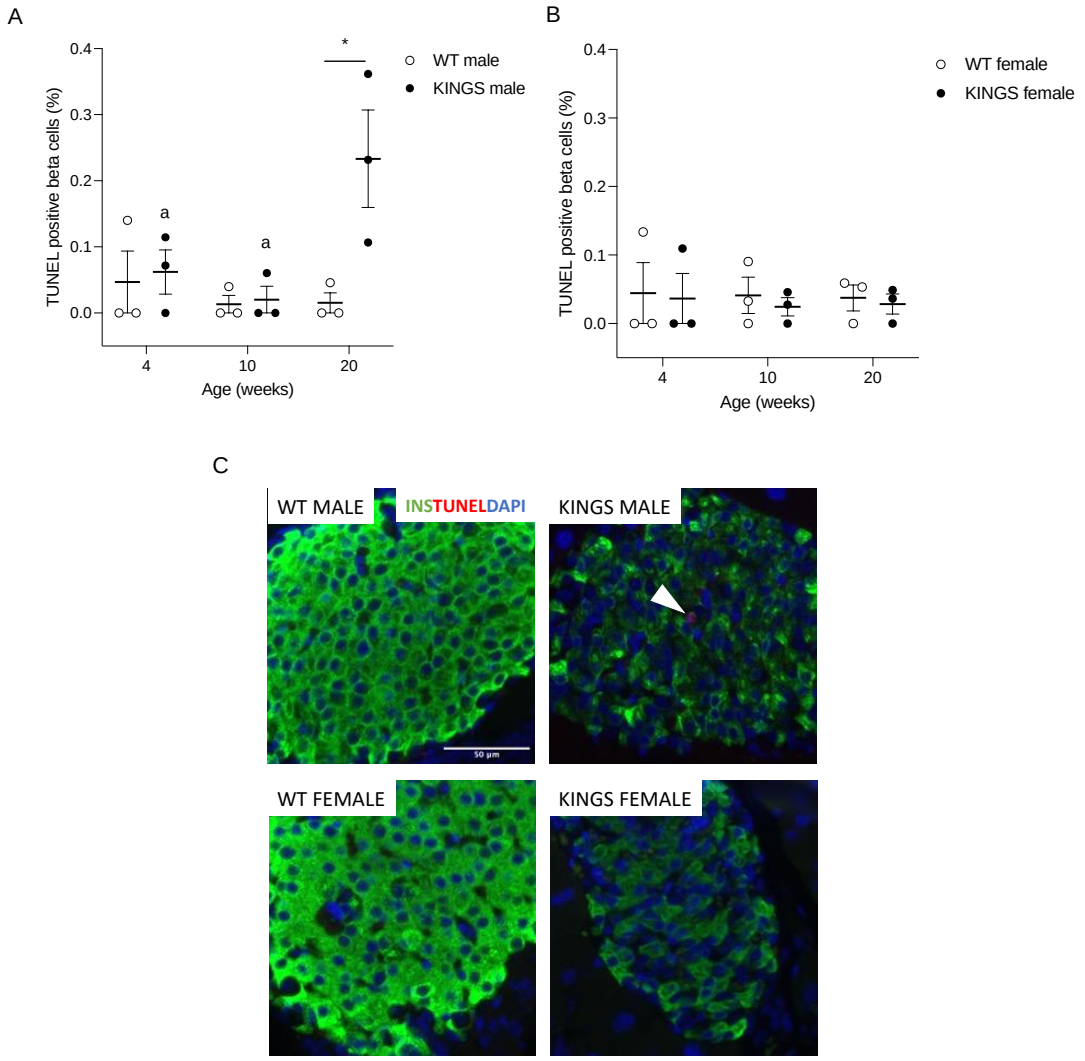


FIGURE 32: *Beta cell apoptosis in the KINGS mouse:*

*Percent total analysed beta cells that were positive for TUNEL staining in (A) male KINGS and WT mice at 4, 10 and 20 weeks of age (B) female KINGS and WT mice at 4, 10, and 20 weeks of age. Two-way ANOVA with Holm-Sidak post hoc, *= WT vs. KINGS p<0.05, a=vs KINGS 20-week p<0.05. n=3, 3286-8082 beta cells analysed per group. (C) Representative 10x magnification images of islets from 20-week-old WT and KINGS, male and female mice stained for insulin (green), TUNEL (red) and DAPI (blue). The scale bar in the top left image indicates 50µm and a white arrow indicates a TUNEL positive beta cell in the KINGS male islet.*

4.4.3 Beta cell proliferation in the KINGS mice:

Adaptive UPR signalling has been associated with increased beta cell proliferation, whilst maladaptive signalling has been associated with reduced proliferation. Beta cell proliferation was investigated at in the KINGS mice at 4, 10 and 20 weeks of age through immunostaining pancreatic sections with the proliferation marker Ki67, insulin to indicate beta cells, and DAPI stain to identify cellular nuclei. Any Ki67 signal that colocalised with DAPI staining was deemed a replicating cell.

Beta cell proliferation was highest in all groups at 4 weeks of age and was substantially lower from 10 weeks of age onwards (Figure 33). There was no difference in beta cell proliferation between wildtype female and KINGS female mice at any age. However, although this was also seen in the males at 10 and 20 weeks, at 4 weeks of age KINGS male mice had reduced beta cell proliferation compared to wildtype mice.

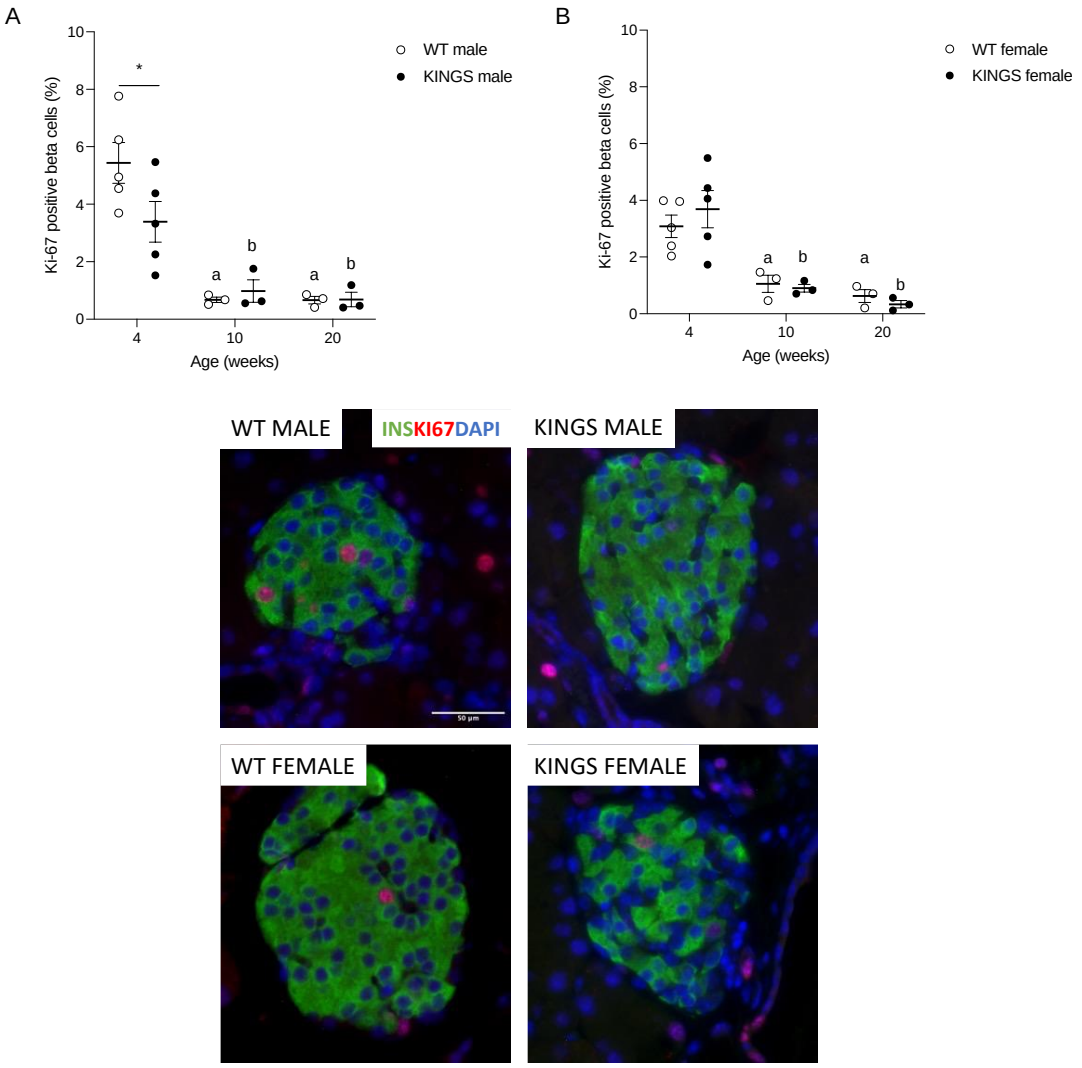


FIGURE 33: *Beta cell proliferation in the KINGS mice:*

*Percent of Ki67 positive beta cells in (A) male KINGS and WT mice at 4, 10 and 20 weeks of age (B) female KINGS and WT mice at 4, 10, and 20 weeks of age. n=3-5, 3985-7460 beta cells analysed per group. Two-way ANOVA with Holm-Sidak's post hoc, *= WT vs. KINGS p<0.05, a= vs. WT 4-week p<0.05, b=vs. KINGS 4-week p<0.05. (C) Representative 20x magnification images of islets from 4-week-old WT and KINGS, male and female mice stained for insulin (green), Ki67 (red) and DAPI (blue). The scale bar in the top left image indicates 50µm.*

4.4.4 Islet area in the KINGS mouse:

It is known that beta cell proliferation and apoptosis rates are typically low in mice thus differences in these outputs can be difficult to detect. However, small changes in beta cell turnover may be biologically relevant and impact beta cell

mass. For this reason, islet area was measure by immunostaining pancreatic sections for insulin and staining the nuclei with DAPI (Figure 34A-B). Islet area significantly increased with age in all groups with islet size at 20 weeks at least 1.9-fold that of islet size at 4 weeks in all groups. For example, islet area in WT females increased from an average of 4114µm^2 to 8081µm^2. No differences between wildtype and KINGS islet size were observed in male or female mice.

Haematoxylin and eosin staining was also used to investigate islet size in the same groups. Results were similar to the results obtained with immunofluorescence insulin staining, with islet size increasing with age in all groups and encouragingly mean islet area values tended to be similar (Figure 34C-D). There was no difference in islet size between WT female and KINGS female mice, however a significant decrease in islet size was observed in male KINGS mice compared to wildtype at 10 weeks of age only.

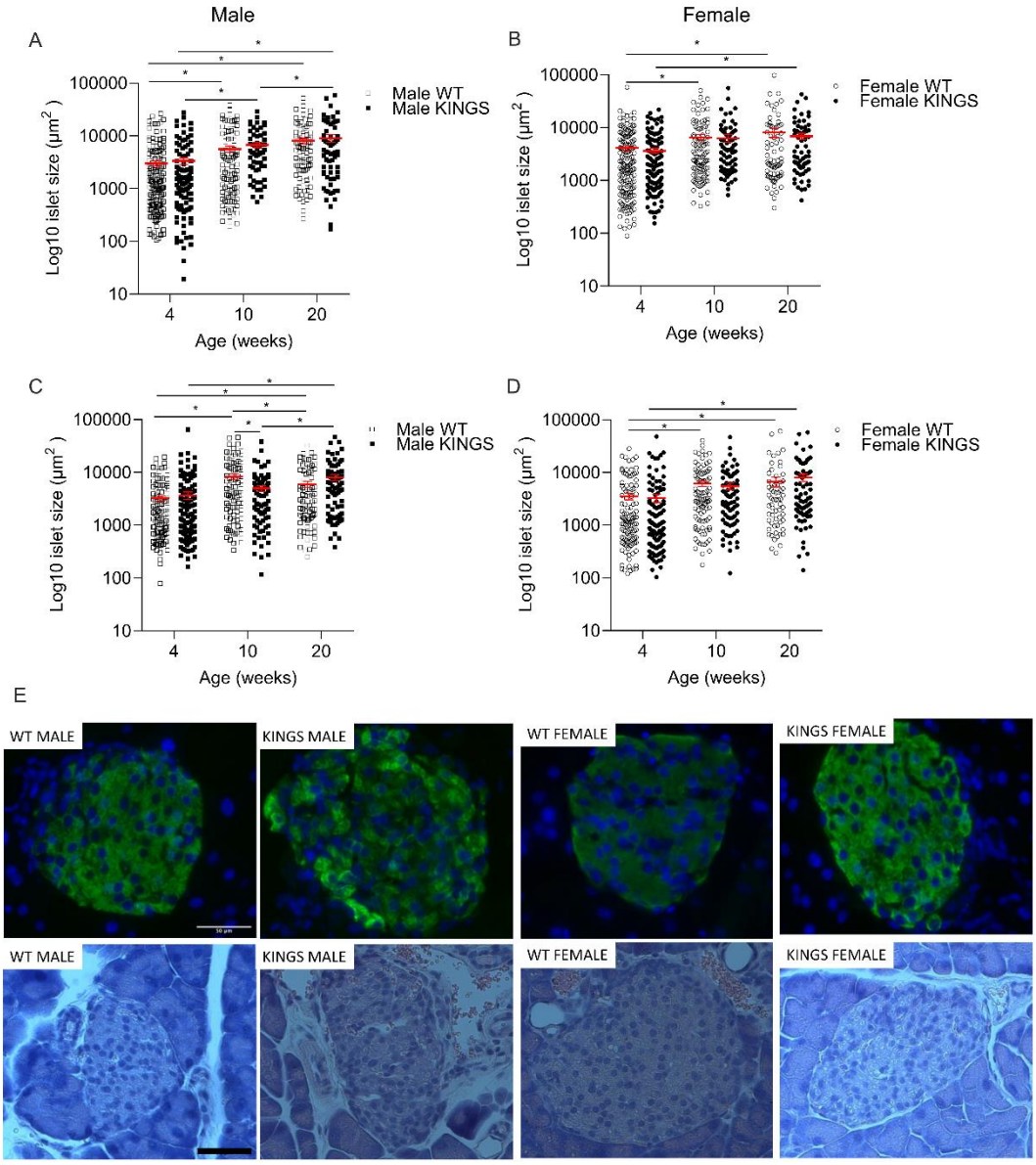


FIGURE 34: *Islet area in the KINGS mice:*

*(A-B) Islet area in male and female, KINGS and WT pancreata measured by insulin immunofluorescent staining. Two-way ANOVA with Holm-Sidak's post hoc, *p<0.05, islets pooled from n=3-5 mice (59-161 islets per group). (C-D) Islet area in male and female, KINGS and WT pancreata measured by hemotoxylin and eosin staining. Two-way ANOVA with Holm-Sidak's post hoc, *p<0.05, islets pooled from n=3 mice (63-137 islets per group). (E) Representative immunofluorescent stained islets with insulin (green) and DAPI (blue) (top panel) and haematoxylin and eosin-stained islets (bottom panel) from KINGS and WT, male and female mice at 10 weeks of age. Scale bars represent 50µm and are indicated in the first image of both panels.*

4.4.5 Beta cell mass in the KINGS mice:

Beta cell mass can differ even if no differences in islet size are detected as a result of differences in the number of islets. Therefore, beta cell area as a proportion of total pancreatic area was investigated by immunostaining pancreatic sections for insulin and staining with DAPI and imaging whole sections using a Hamamatsu slide scanner. Insulin proportion of total pancreatic area was measured in KINGS mice at 4, 10 and 20 weeks of age. In line with no changes seen in islet size, no differences were observed in insulin proportion between wildtype and KINGS mice at any age (Figure 35). A slight reduction in insulin proportion was seen in the KINGS males at 20 weeks compared to wildtype males, however this did not reach significance.

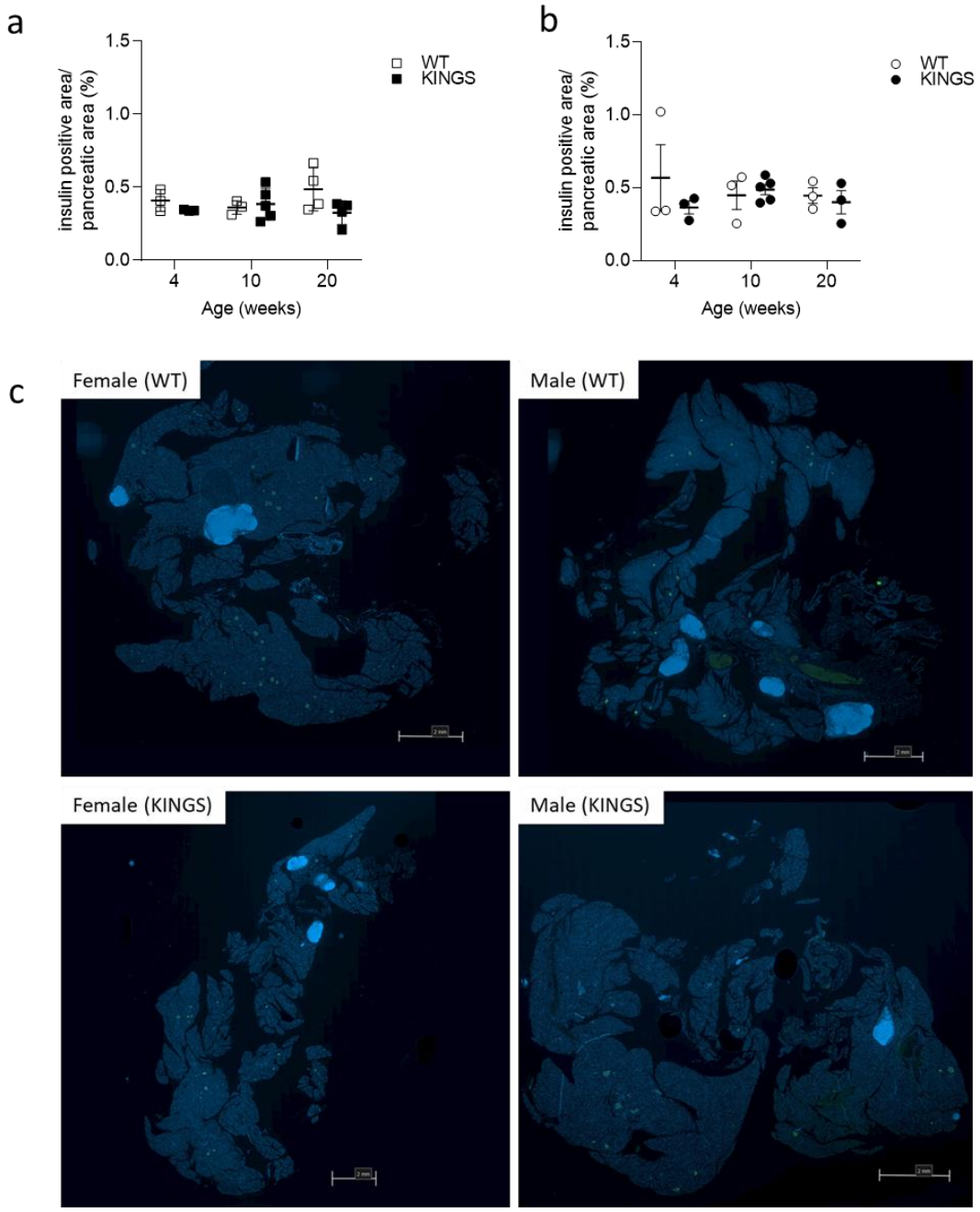


FIGURE 35: *Percentage insulin positive area of total pancreatic area in the KINGS mice:*

Percentage insulin positive area of total pancreatic area was measured in (a) male KINGS and wildtype mice and (b) female KINGS and wildtype mice. Two-way ANOVA with Holm-Sidak post hoc test, p>0.05, n=3-5. (c) Representative images of whole pancreatic sections from 10-week-old mice stained for insulin (green) and DAPI (blue) at 20x magnification. Scale bars in each image represent 2mm. Areas of bright DAPI fluorescence are splenic tissue and

areas of these, as well as any adipose tissue and large blood vessels, were excluded from the whole pancreatic area measurement.

4.6 Discussion:

Beta cell ER stress has been implicated in the pathogenesis of many types of diabetes, including type 1 and type 2 diabetes. Findings support the idea that activation of the maladaptive UPR, as a result of robust ER stress that cannot be resolved, can induce beta cell death and this is proposed to be the mechanism of pathogenesis in the Akita mouse model. On the other hand, failure of the adaptive UPR to mount has also been suggested to link ER stress with beta cell failure. In this chapter we firstly investigated whether the KINGS mutation was associated with an increase in beta cell ER stress and activation of the UPR, and whether this differed between male and female KINGS mice. We also investigated the impact that the KINGS mutation has on beta cell mass and turnover.

Beta cell ER stress in the KINGS male mice was confirmed through elevated levels of the general ER stress marker, BiP, from 4 weeks of age. Importantly, elevated BiP expression in the KINGS males precedes the onset of overt diabetes (which occurs between 5-6 weeks of age) and occurs when blood glucose concentrations are still within a normoglycemic range. Hyperglycaemia is known to induce ER stress (discussed in more detail below) and therefore our results suggest that ER stress is likely to contribute to a loss of glycaemic control rather than merely being a result of it. A similar pattern was observed in female KINGS mice with BiP expression observed through immunofluorescence and western blot significantly increased compared to wildtype females. These findings are also in line with previous experiments showing that KINGS beta cells harbour ultrastructural signs of ER stress including distended mitochondria and swollen ER (Austin et al., 2020). However, it goes against previous findings that BiP mRNA levels were only increased in female KINGS mice compared to wildtype and not in male KINGS mice. Differences in findings may be explained by the fact that BiP is highly regulated post-transcriptionally, and thus measurements at the gene level may not be representative of protein expression (Gulow et al., 2002). It should be

noted however that studies have shown increased BiP in the Akita mouse (a similar model of ER stress induced diabetes to the KINGS mouse) both at the protein and at the mRNA level (Oyadomari, Araki, et al., 2002). Expression of KINGS and Akita mutated insulin in MIN6 cells in a previous study found that whilst both the KINGS and Akita mutations were associated with an increase expression of the pro-apoptotic UPR marker CHOP (suggesting the activation of ER stress and the UPR), this was higher with the Akita mutation. The latter may suggest that ER stress is lower in the KINGS mice which may explain the disparity between BiP expression at the protein and gene level in the two models.

Although no difference in BiP expression was found between the sexes using western blot, when BiP expression was analysed by immunofluorescent staining we found that male KINGS mice exhibited increased maximal BiP expression from 10 weeks and increased mean BiP expression at 20 weeks of age compared to female KINGS mice. This may imply an increased level of beta cell ER stress in KINGS male *vs.* female mice. At these ages the male KINGS mice have established diabetes, and since hyperglycaemia is known to stimulate ER stress, this likely plays some role in the sex differences seen. Intriguingly our immunofluorescent BiP expression data showed that female wildtype mice showed increased islet BiP expression at 4 and 10 weeks, although this difference was lost at 20 weeks of age, it suggests at earlier ages female beta cells may exhibit enhanced beta cell stress. Rossetti *et al* observed a similar sex difference in mouse hepatocytes; pre-pubertal female mice showed an increase in the BiP, XBP1s and IRE1-alpha gene expression, and this difference was lost in adult mice- an effect suggested to be dependent on testosterone (Rossetti et al., 2019). Oestradiol has also been shown to activate an ‘anticipatory’ UPR response in cancer cells which promotes survival and proliferation by enhancing cellular protein folding capacity through elevating BiP expression (Andruska et al., 2015). This phenomenon may also explain why higher BiP protein levels were seen in WT female mice.

Upon ER stress, cells activate the UPR which has adaptive functions in restoring ER protein homeostasis. Not only has maladaptive UPR signalling

been associated with beta cell dysfunction and death, but a failure of the adaptive UPR to be mounted has also been associated with this. To investigate whether ER stress in the KINGS beta cells activates the UPR, markers of the three UPR arms (PERK, IRE1 and ATF6) were investigated through western blotting using islet protein lysates. From 4 weeks of age there is activation of the UPR in both the male KINGS islets, which show an upregulation of ATF6 and phosphorylated-eIF2-alpha, and in female KINGS islets which show a significant upregulation of ATF6. These results suggest that ER stress and either a partial or full activation of the UPR precedes the onset of overt diabetes in the male KINGS mice.

From 10 weeks of age, male KINGS islets showed activation of all three UPR arms with XBP1s (indicative of IRE1 signalling), full length ATF6 (indicative of ATF6 pathway activation) and phosphorylated e-IF2-alpha (indicative of PERK pathway activation) all increased compared to WT. At this age, male KINGS mice exhibit hyperglycaemia and therefore there is a failure of the UPR to adequately resolve ER stress and prevent the development of diabetes. Female KINGS islets showed a similar pattern of UPR marker upregulation at 10 weeks; however, this was only significant for ATF6. Female KINGS mice are glucose intolerant at 10 weeks but do not show overt diabetes indicating that the female beta cells can adapt to the ER stress, or that the ER stress is less than in males. In line with this, expression of UPR markers and BiP were typically lower in the female islets, however, this was only significant for phosphorylation of e-IF2-alpha at 10 weeks of age.

The effects of hyperglycaemia may partially explain why 10-week-old male islets showed the highest expression of UPR markers detected through western blotting. At 10 weeks, blood glucose concentrations are between 25-30mM in the male KINGS mice and high glucose has been shown to cause beta cell ER stress *in vitro* and *in vivo*. This is thought to be because high glucose concentrations place a greater metabolic demand on the beta cell to produce insulin which overwhelms the beta cell ER protein folding capacity. Additionally, glucotoxicity has been associated with increased beta cell reactive oxygen species (ROS) which in turn can also cause ER stress.

However, it is relevant to note that islets used to investigate UPR marker expression in this study were cultured overnight in 11.1mM glucose media, providing an environment where male KINGS islets may have partially recovered from the effects of hyperglycaemia. Additionally, in a previous study where wildtype islet transplantation was used to alleviate hyperglycaemia in male KINGS mice, endogenous male KINGS islets were still found to have an 80% reduction in insulin content compared to wildtype islets (Austin et al., 2020). This suggests that beta cell dysfunction and ER stress is not exclusively mediated by glucotoxicity in this model.

The UPR has also been found to be upregulated in the Akita and Munich mice models which harbour amino acid substitutions in preproinsulin but unlike the KINGS substitution these substitutions directly interfere with the disulphide bonds. In similarity with the KINGS mouse, UPR markers in Akita islets are upregulated at from as early as 2 weeks of age which is before the onset of diabetes in this model (Riahi et al., 2018; Sharma et al., 2015). The Akita mutation also drives an ER-stress mediated upregulation of the proapoptotic protein, CHOP. Studies suggest that CHOP drives beta cell loss and thus diabetes development in the Akita mouse. Indeed, CHOP knockout in these mice reduces beta cell apoptosis and delays the development of diabetes (Oyadomari, Koizumi, et al., 2002). In contrast, CHOP expression was variable in the KINGS islets of males and female mice, and we did not detect any significant differences compared to wildtype or between the sexes. Supporting this finding, as mentioned previously, earlier *in vitro* studies have shown that introduction of the Akita mutation into MIN6 cells results in higher CHOP protein expression compared to introduction of the KINGS mutation. UPR activation can not only drive apoptosis by enhancing the expression of CHOP downstream of the PERK and ATF6 pathways, but also through JNK activation downstream of the IRE1 pathway. However, we did not detect an increased in beta cell apoptosis in the KINGS mice at 4 or 10 weeks, suggesting that beta cell apoptosis does not drive diabetes in this model. Increased beta cell apoptosis was observed in 20-week-old male KINGS mice which may be the result of glucotoxicity, and this has previously been associated with ER-stress and apoptosis in other model systems.

The UPR can also manipulate beta cell turnover through its effects on proliferation. Mild ER stress has been shown to promote beta cell proliferation through the ATF6 arm of the UPR. However, despite activation of the ATF6 pathway, when beta cell proliferation was investigated in the KINGS mice, we found that males had reduced beta cell proliferation compared to wildtype at 4 weeks of age. This difference was lost by 10 weeks. Interestingly, reduced beta cell proliferation in neonatal Akita mice has also been observed and this has been proposed to contribute to the reduced beta cell mass seen in these mice at later ages despite beta proliferation rates reaching wildtype levels by 2-3 months of age (Riahi et al., 2018). Riahi *et al.* proposed that impaired beta cell proliferation in Akita neonates was a result of ER stress reducing expression of mTORC1 which is required for neonatal beta cell expansion and growth. However, although restoration of mTORC1 expression in Akita mice improved beta cell expansion in the neonatal period, it did not prevent the development of diabetes and therefore reduced beta cell proliferation cannot be the sole cause of diabetes. The same effect of ER stress on beta cell proliferation may exist in humans; Balboa *et al*. showed that induced pluripotent stem cells taken from individuals with insulin gene mutations and differentiated into beta cells had increased ER stress which was associated with reduced proliferation rates whilst apoptosis was unchanged (Balboa et al., 2018). Similar defects in neonatal beta cell proliferation have been reported in mice deficient for PERK (Zhang et al., 2006). Intriguingly, these results and the results from the present study contradict findings in a study by Sharma *et al* in which mild ER stress was associated with increased beta cell proliferation in the neonatal period in Akita mice, in the pre-diabetic stage in $Lep^{db/db}$ mice and in human islets treated *ex vivo* with UPR-inducing agents (Sharma et al., 2015). It is not clear as to the cause of the differences in findings between the studies, however it may be due to the inherent difficulties of measuring a relatively rare event. In the future it would be worthwhile administering bromodeoxyuridine (BrdU) *in vivo* to the KINGS mice to ascertain proliferation over a longer period. Since BrdU remains incorporated by cells, it is possible to measure the active proliferating beta cells as well as any beta cells that have proliferated since BrdU administration.

Similar to beta cell proliferation, beta cell apoptosis rates are also low and so any changes in these which may have biological significance, can be hard to detect. Indeed, some studies have been unable to observe an increase in beta cell apoptosis in the Akita mouse, whilst others have. For this reason, in this study we also investigated islet size and insulin area/pancreatic area in the KINGS mice. No significant differences between KINGS and wildtype mice for either islet size or insulin area/pancreatic area was detected when pancreas sections were stained for insulin, and only a mild reduction in islet size in 10-week-old male KINGS mice was detected when sections were stained with haematoxylin and eosin. We saw a trend for reduced beta cell mass at 4 and at 20 weeks in KINGS male mice, and although this did not reach significance, these results are in line with our findings of reduced beta cell proliferation at 4 weeks of age and increase beta cell apoptosis at 20 weeks in this group. Therefore, it is possible that there is a subtle reduction of beta cell mass in this group. This contrasts to the previous findings in the Akita mouse where at 2-3 months of age a ≥60% reduction in beta cell mass is observed (Riahi et al., 2018; Winnay et al., 2014). This may suggest that diabetes in the KINGS mice is primarily driven by beta cell dysfunction rather that beta cell death. In support of this, we have previously shown reduced insulin content and impaired glucose stimulated insulin secretion in KINGS mice which at 10 weeks in the male KINGS mice is reduced by ≥70% of wildtype. A reduction is also seen in the KINGS females relative to wildtype, but this is not as severe as in the KINGS males and may go some way to explaining sex differences in the model.

ER stress and activation of the UPR can reduce insulin secretion in several ways including through reducing the mRNA translation and gene expression of factors involved in insulin biosynthesis, processing and secretion in an effort to limit protein folding demand placed on the cell (Brusco et al., 2023; Chen et al., 2022). However, it is important to consider that mutant G32S insulin may have alternative pathogenic mechanisms distinct from inducing beta cell ER stress. Indeed, it has been shown that the Akita mutant insulin engages in complexes with wildtype insulin preventing wildtype insulin secretion (Hodish

et al., 2010; Liu et al., 2007). Expression of the green fluorescent protein (GFP)-tagged G32S (KINGS) insulin in MIN6 cells in a separate study showed that wildtype insulin secretion was reduced ~50% compared to cells expressing wildtype insulin only (Rajan et al., 2010). These results imply that the KINGS mutant insulin may sequester wildtype insulin inside the beta cell thus contributing to a reduction in insulin secretion seen in the KINGS mouse. It would be interesting to investigate this *in vivo* in the KINGS mouse through tagging wildtype insulin with GFP and measuring GFP secreted insulin in response to a glucose tolerance test, for example, and comparing this with wildtype mice. It is worth noting that *in vitro* studies have shown that KINGS mutated insulin is partially processed and the majority (70%) is trafficked to secretory granules and secreted from MIN6 cells in a glucose dependent manner (Rajan et al., 2010). In fact, 5% of total KINGS mutant insulin was found to be secreted from MIN6 cells under glucose stimulation compared to 12% of WT insulin in MIN6 cells expressing WT insulin. This contrasts the Akita mutated insulin which was completely retained in the ER in MIN6 cells. It would therefore be interesting in future to investigate the activity of the KINGS mutant insulin and whether it is able to stimulate glucose uptake as effectively as wildtype insulin.

Overall, in this chapter we have shown that the KINGS mouse harbours beta cell ER stress which precedes the development of diabetes in this model. This adds to the growing evidence in the literature suggesting that beta cell ER stress can drive the development of diabetes. We found differences in the level of beta cell ER stress and expression of UPR markers between male and female KINGS mice, with male mice expressing higher levels of BiP suggesting enhanced ER stress levels from 10 weeks and increased expression of more UPR markers compared to wildtype than females. The latter may go some way to explaining the sex difference we see in phenotype. Since we found no difference in beta cell mass between the KINGS mice and wildtype mice, it is likely that diabetes in this model is predominantly driven by ER stress-mediated beta cell dysfunction rather than beta cell loss (either through increased beta cell apoptosis or reduced proliferation). The KINGS mice may therefore provide a model of beta cell ER stress where maladaptive

UPR signalling drives the development of diabetes in the absence of mass beta cell mass loss. This model may be relevant for studying the role of ER stress in type 2 diabetes in humans, where beta cell dysfunction rather than loss initiates a loss of glycaemic control in the earlier stages of disease.

CHAPTER 5: THE ROLE OF OESTRADIOL IN MEDIATING SEX DIFFERENCES IN THE KINGS PHENOTYPE AND ITS IMPACT ON BETA CELL ER STRESS

Chapter snapshot:

Sex differences in diabetes exist in humans and in animal models. Understanding what mediates this may reveal novel therapeutics and could lead to more personalised diabetes therapy. Although 17-beta-oestradiol (oestradiol, E2) has been implicated in this, how it mediates its protective effects warrants further study, especially in the context of beta cell ER stress.

The aim of this study was to investigate to what extent the effects of oestradiol (both organisational and activational) are involved in generating sex differences in glycaemic phenotype in the KINGS mice and whether this is caused by influencing beta cell ER stress.

5.1 INTRODUCTION:

Sex differences exist in energy homeostasis and metabolism, and this has a profound impact on the pathogenesis and incidence of many metabolic disorders, including diabetes (Mauvais-Jarvis, Arnold, et al., 2017). In most of the world, diabetes incidence (including those of T1DM and T2DM) is higher in men and this disparity is most stark in middle-aged populations (Wild et al., 2004). Indeed, the International Diabetes Federation suggested that in 2017, 12.3 million more men than woman were living with diabetes (Cho et al., 2018). Men are also often diagnosed with diabetes at a lower BMI compared to women (Logue et al., 2011; Pettersson et al., 2012). In line with these observations in humans, many genetically induced animal models of diabetes show an increased incidence and severity of phenotype in males, although some exceptions such as the NOD mouse do exist (Mauvais-Jarvis, Arnold, et

al., 2017). Additionally, female animals are typically more resistant to dietary induced metabolic disorders.

Much of the protection seen in women and in female animals from diabetes has been historically attributed to the effects of the oestrogen, 17-beta-oestradiol (oestradiol, E2). This stemmed from findings that the menopause, which sees circulating E2 levels decline, is associated with impaired glucose tolerance and increased diabetes incidence in women (Mauvais-Jarvis, Manson, et al., 2017). In line with this, premature menopause and ovarian insufficiency in women is also associated with an increase in risk of T2DM development (Anagnostis et al., 2019; Muka et al., 2017).

It should be noted that two other main forms of oestrogen exist; estrone (E1) and estriol (E3). E1 and E3 represent the least potent oestrogens and are important after the menopause and during pregnancy, respectively (Cui et al., 2013). In physiologically healthy pre-menopausal women, E2 is the main circulating oestrogen. It can act on distant tissues and is primarily produced in the ovaries by the theca and granulosa cells following the aromatisation of androstenedione to E1 and E1 conversion to E2 (Figure 10) (Cui et al., 2013). In contrast, in men and in post-menopausal women circulating E2 is primarily synthesised at extra-gonadal sites (adipose and skin). Although E2 is important in both sexes for a variety of physiological processes, the most prominent being reproductive function and secondary sexual characteristics, circulating levels of this are significantly higher in pre-menopausal women.

E2 impacts whole organism metabolism in a myriad of complex ways. These can be categorised into specific effects on the beta cell or peripheral effects (including on peripheral insulin sensitivity and hepatic glucose production). The latter are often difficult to distinguish from one another, making investigations into single effects difficult. The effect of E2 can be further broadly divided into organisational and activational. Organisational effects are permanent, occur at an early stage in development and persist even in a future absence of circulating E2. An example of the organisational effects of E2 unrelated to the beta cell is vaginal opening in mice. On the other hand,

activational effects are transient, requiring the presence of E2 and occur throughout life. An example of this is murine oestrous cycling which resembles the menstrual cycle in women with regards to hormone fluctuations, although important differences such as length (4-5 days in mice vs. ~28 days in humans) exist. Importantly, although rodents do experience age-related disruption in oestrous cycling, they do not experience a human-like menopause in that 17-beta-estradiol levels can remain moderately high despite ovarian dysfunction in older age (Diaz Brinton, 2012). Ovarian in-tact mice are consequently not typically used for modelling the human menopause.

E2 exerts its effects through binding to the classical oestrogen receptors ER-alpha (or ERS1) and ER-beta (ERS2) which belong to a family of nuclear steroid hormone receptors. Upon binding to E2 these receptors are liberated from HSP90, a protein chaperone that sequesters the receptors, and dimerise (homodimers of the same ER can form, or heterodimers of ER-alpha and beta can form) (Merino & García-Arévalo, 2021). ER dimers can exert their effects through the classical pathway whereby they translocate to the nucleus and bind at estrogen response elements (ERE) at gene promotors to regulate gene expression, or through the non-classical pathway whereby the regulation of genes is carried out through ER-dimer interaction with other transcription factors (Gregorio et al., 2021). Many effects of ER-alpha and beta signalling however are too rapid to rely on genomic signalling cascades, and therefore they have also been associated with non-genomic signalling, which is currently not as well understood but involves interaction of membrane-localised ER -alpha and beta with downstream signalling components (Mauvais-Jarvis et al., 2022). Some non-genomic effects of E2 have also been attributed to the more recently discovered G protein coupled estrogen receptor 1 (GPER) which resides at cellular membranes (Sharma & Prossnitz, 2021). All three of these receptors have been identified at peripheral sites important in glucose homeostasis (including adipose and skeletal muscle) as well as on beta cells and therefore E2 has been associated with a complex array of islet and extra-islet effects to regulate glucose homeostasis.

One way in which oestradiol has been shown to impact glucose homeostasis is through its effects on peripheral insulin resistance (De Paoli et al., 2021). Indeed, the menopause is associated with reduced insulin sensitivity and E2 hormone replacement therapy (HRT) in post-menopausal women improved this, although it should be noted that other studies have disputed this, and this may be the result of differences in choice of insulin sensitivity test and cohort characteristics (Bitoska et al., 2016; Godsland et al., 1993; Lindheim et al., 1993; Salpeter et al., 2006). There seems to be a fine balance in E2 level in regard to insulin resistance since higher levels of E2, including those induced through oral contraceptive therapy, has been associated reduced insulin sensitivity (Godsland, 2005). Therefore, differences in HRT findings in post-menopausal women may also be attributed to differing E2 dosing regimens, and indeed Lindheim *et al* found only a moderate but not high E2 dosing was associated with improved insulin sensitivity in postmenopausal women (Lindheim et al., 1993). Murine ovariectomy and knockout of the aromatase enzyme (which converts androgens to oestrogen) in mice is associated with insulin resistance, an effect that is reversed through E2 treatment (Riant et al., 2009; Van Sinderen et al., 2017). ER-alpha signalling has been associated with this since mutations in this receptor in humans and its knockdown in mice results in insulin resistance as well as obesity (Heine et al., 2000). One way by which ER-alpha mediates this effect is through upregulating the GLUT4 receptor, responsible for insulin-dependent glucose uptake, in peripheral tissues. In line with this, GLUT4 expression is decreased in ER-alpha knockout mice resulting in enhanced insulin resistance and adiposity (Heine et al., 2000). Intriguingly, ER-beta is thought to have the opposite effect and downregulates GLUT4 expression; indeed, ER-beta knockout in mice is associated with an increase in GLUT4 expression at peripheral sites (Barros et al., 2009). It is important to note that the ratio of ER-alpha to ER-beta in individual cells seems to play a large role in the biological effect of E2, with ER-beta abundance higher than that of ER-alpha in muscle, and ER-alpha higher in adipose tissue (Ahmed et al., 2022). E2 can also impact glucose homeostasis through its effects on hepatic gluconeogenesis. ER-alpha signalling has been shown to supress the transcription factor Foxo1, which is responsible for increasing hepatic gluconeogenesis by increasing gene

expression of glucose-6-phosphatase. Yan *et al* found that whilst oestradiol treatment in ovariectomised female and male wildtype mice was associated with reduced gluconeogenesis and improved insulin sensitivity, this effect was lost in liver-specific *Foxo1* knockout mice (L-F1KO) (Yan et al., 2019).

As mentioned, oestradiol can also act directly on the beta cell to regulate glucose homeostasis and animal models with beta cell-specific knockout of oestrogen receptors have been fundamental in understanding this (Merino & García-Arévalo, 2021). Indeed, beta cell knockdown of all three receptors has been associated with reduced beta cell functional mass and impaired glucose homeostasis (Merino & García-Arévalo, 2021). One anti-diabetogenic effect of oestradiol is increased insulin secretion in response to high glucose, and this has been largely attributed to ER-beta and GPER signalling. For example, Soriano *et al* showed that E2 exerts inhibitory activity on the K_{ATP} channel of beta cells, enhancing cytoplasmic calcium influx by enhancing membrane depolarisation, but this effect was lost in islets from ER-beta knockout mice (Soriano et al., 2009). Further knockdown of the membrane guanylate cyclase A receptor in mice showed that ER-beta mediates a reduction in K_{ATP} channel activity via this receptor. E2 treatment of pancreatic islets has also been shown to increase insulin biosynthesis and this has been attributed to non-classical ER-alpha signalling (Wong et al., 2010). ER-alpha signalling facilitates neurogenic differentiation factor 1 (NeuroD1) binding to the insulin gene promotor to enhance expression. E2 has also been implicated in beta cell proliferation and hypertrophy, as well as beta cell resistance to and survival in the context of cellular stress (Merino & García-Arévalo, 2021).

ER stress, whereby protein folding requirements exceed the protein folding capacity of the cell, represents a major beta cell stressor that has been implicated in the development of many types of diabetes. ER stress initially activates the adaptive UPR which encompasses signalling pathways that collectively aim to restore protein homeostasis. However, if protein homeostasis cannot be restored then maladaptive UPR signalling can drive beta cell dysfunction and apoptosis. Numerous studies have shown that oestradiol can protect against beta cell functional impairment and death

following insult from beta cell stressors known to initiate beta cell ER stress, including pro-inflammatory cytokines, high glucose, oxidative stress and lipotoxicity (Kooptiwut et al., 2014, 2018; Le May et al., 2006; Xu et al., 2018). In line with this, E2 treatment of INS-1 cells is protective against high glucose induced cell death and is associated with a downregulation of ER stress markers (Kooptiwut et al., 2014). More recently Xu *et al.* showed that E2 treatment of male Akita mice was able to prevent the development of diabetes whilst ovariectomy in Akita females caused overt diabetes (Xu et al., 2018). Further *in vitro* investigation also showed that conjugated oestrogen treatment reduced ER stress in *Ins2*$^{+/Akita}$ INS-1 cells and female Akita islets.

In this study, we investigate whether oestradiol drives sex differences in glucose handling in a distinct model of beta cell ER stress, the KINGS mice. We also investigate whether oestradiol is capable of reducing ER stress *in vivo*. Previous studies suggesting oestradiol reduces beta cell ER stress have all been conducted *in vitro*, however this does not take into consideration the fact that oestradiol levels fluctuate *in vivo* in line with oestrous cycling, and beta cell ER stress levels are also known to fluctuate as a result of changes in insulin demand. These factors may impact the effects of oestradiol on beta cell ER stress and therefore we aimed to fill this gap in our current knowledge. In addition to this, there is currently a lack of research into the organisational *vs.* activational effects of oestradiol on glucose homeostasis. Here, we used the KINGS mouse to investigate the role of organisational and activational effects of oestradiol in the protection of female KINGS mice from developing overt diabetes. Insight into the relative impacts of the organisational and activational effects of oestradiol on glycaemic control and the beta cell is important in understanding the broader picture of why sex differences exist in diabetes. Additionally, if we can understand what mediates sex differences, it may reveal avenues for novel therapeutic development and may lead to more sex-tailored diabetes therapies.

5.2 Aims:

- Determine whether removal of the organisational or activational effects of oestradiol in female KINGS mice induces the development of overt

diabetes and whether this is associated with changes in beta cell ER stress and beta cell function.

- Determine whether oestradiol treatment in male KINGS mice protects them from the development of overt diabetes and whether this is associated with changes in beta cell ER stress and beta cell function.

5.3 METHODS:

5.3.1 EXPERIMENTAL OUTLINE:

Removal of the organisation and activational effects of E2 (Figure 36): Female mice were ovariectomised (ovx) pre-puberty (4 weeks of age) or post-puberty (10 weeks of age) to assess the effects of endogenous oestradiol removal on glycaemic control. Non-fasted blood glucose concentrations and weight measurements were taken weekly. At 10 weeks (pre-puberty ovariectomy study) and 20 weeks of age (post-puberty ovariectomy study) glucose tolerance and insulin tolerance tests were carried out and mice were subsequently culled. Islets were isolated and pancreatic tissue fixed from post-puberty ovx mice to assess glucose stimulated insulin secretion and BiP expression. Islets and pancreatic tissue for histology were obtained from the same mice by clamping off the splenic region of the pancreas (which was removed using a scalpel and fixed in formalin) and perfusing the remaining portion of the pancreas with collagenase for islet isolation.

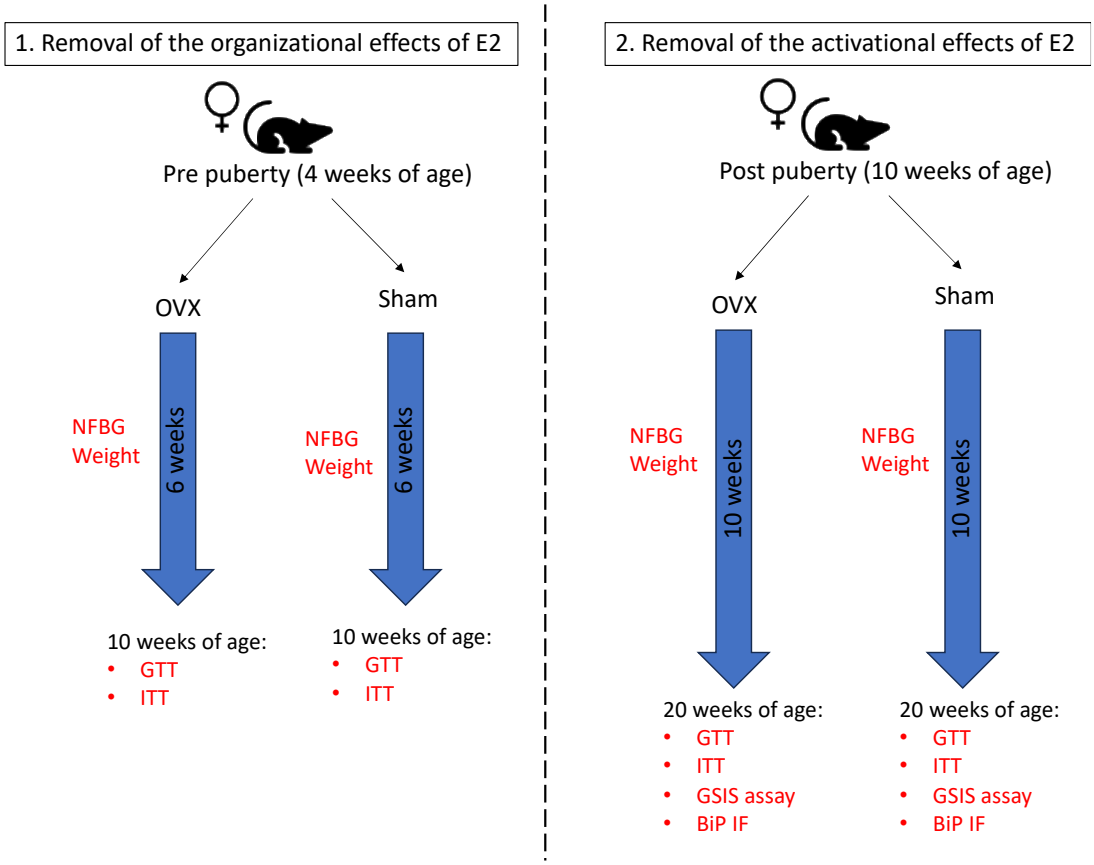


FIGURE 36: *Schematic indicating the experimental outlines for investigating the effects of oestradiol removal on the female KINGS phenotype: Experimental outlines to 1) investigate the organisational effects of E2 in generating sex differences in diabetes in the KINGS mice and 2) investigate the activational effects of E2 in generating sex differences in diabetes in the KINGS mice. Mice were either ovariectomised (ovx) or sham operated at 4 (pre-puberty) or 10 (post-puberty) weeks of age. Mice were either culled at 10 weeks of age (1) or 20 weeks of age (2). Experimental readouts are in red: non-fasted blood glucose measurements (NFBG), glucose tolerance test (GTT), insulin tolerance test (ITT), glucose stimulated insulin secretion (GSIS), BiP immunofluorescent staining (BiP IF).*

Exogenous E2 administration (Figure 37): Male mice were implanted with silastic capsules containing oestradiol or vehicle from 3 weeks of age to assess the effects of exogenous oestradiol delivery on glycaemic control. Non-fasted blood glucose concentrations and weight measurements were taken daily until 7 weeks of age. In a subset of mice, blood glucose measurements

were also taken thrice weekly from 7 weeks until 10 weeks of age. At 7 weeks of age glucose tolerance and insulin tolerance tests were carried out and mice were culled. In the subset of mice where blood glucose was monitored until 10 weeks of age, these mice were culled at 10 weeks. Islets were isolated and pancreatic tissue fixed to assess glucose stimulated insulin secretion and BiP expression. Islets and pancreatic tissue for histology were obtained from the same mice through the same technique as described above.

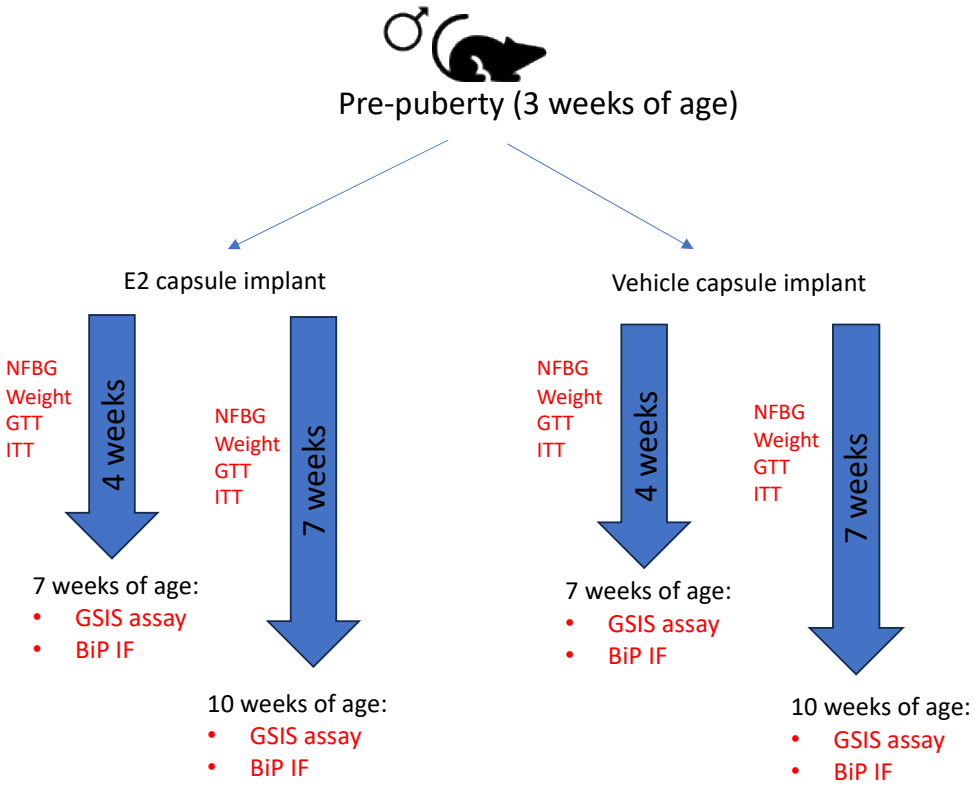


FIGURE 37: *Schematic showing the experimental outline to investigate whether exogenous administration of E2 in male KINGS mice can prevent diabetes:*

Mice were implanted with E2-containing or vehicle-containing silastic capsules at 3 weeks of age (pre-puberty). Mice were culled at either 7 weeks of age or 10 weeks of age. Experimental readouts are in red: non-fasted blood glucose measurements (NFBG), glucose tolerance test (GTT), insulin tolerance test (ITT), glucose stimulated insulin secretion (GSIS), BiP immunofluorescent staining (BiP IF).

Exogenous E2 and replacement study (Figure 38): E2 was assumed to be depleted from silastic capsules from 5-6 weeks after implantation. To test this hypothesis, a pilot experiment was carried out whereby male KINGS mice were implanted with an oestradiol capsule at 3 weeks of age and at 6 weeks of age this was either 1) not replaced, 2) replaced with a vehicle-containing capsule, or 3) replaced with a new E2-containing capsule. The three groups thus represented 1) a natural depletion of E2 from silastic capsules, 2) forced depletion of E2 3) constant E2 delivery. Non-fasted blood glucose concentrations were monitored thrice weekly.

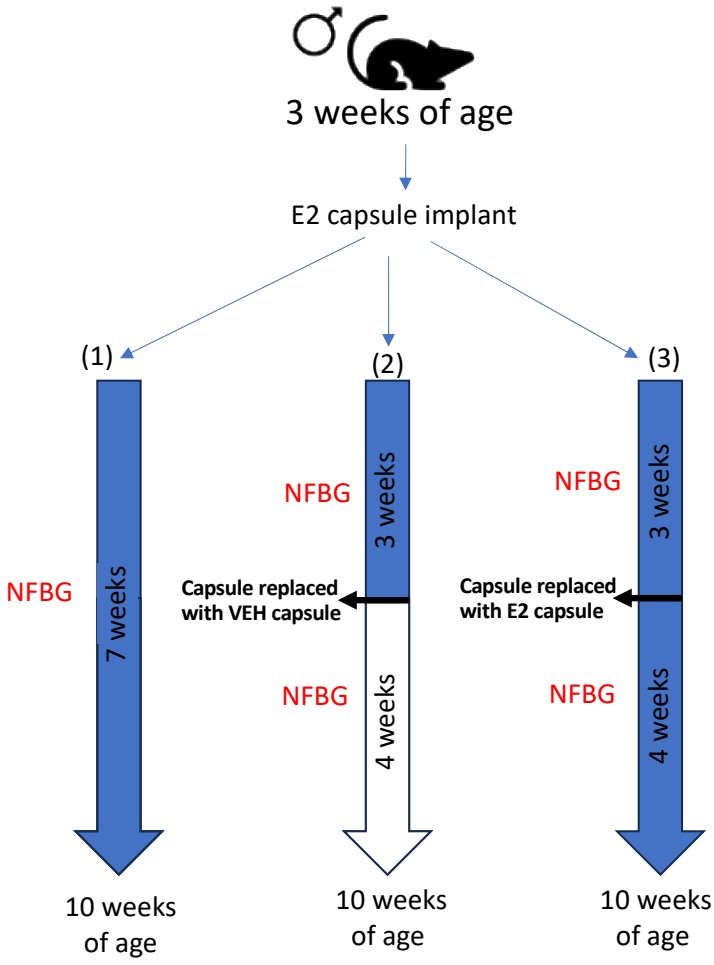


FIGURE 38: *Schematic showing the experimental outline for the exogenous E2 and replacement study:*

Mice were implanted with E2 containing capsules at 3 weeks of age (pre-puberty). After 3 weeks (when mice were 6 weeks of age) the capsule was either (1) not removed to allow natural depletion of E2, (2) replaced with a vehicle containing capsule to force E2 depletion or (3) replaced with a E2

containing capsule so that circulating E2 levels remained elevated. Mice were culled at 10 weeks of age. Experimental readouts are in red: non-fasted blood glucose measurements (NFBG).

5.3.2 Ovariectomy:

Ovariectomy was performed in pre-pubertal (~4 weeks of age) mice and post-puberty mice (~10 weeks of age) to determine the effect of E2 removal on glycaemic control as previously described (section 2.3.2, Figure 39). In brief, mice were anaesthetised using 5% isoflurane, 95% oxygen and anaesthesia was maintained at ~2-3% isoflurane. Mice were placed on a heated mat for the surgery and viscotears was applied to the eyes. A 2-3cm midline incision was made to the lower back which had been shaved and cleaned using Hibiscrub and ethanol. After blunt dissection of the skin either side of the incision from the underlying fascia, an incision was made in the fascia left of the initial incision site and just above the predicted location of the left ovary. The ovarian fat pad was located and gently pulled out of the abdominal cavity along with the ovary. Suture was used to ligate the uterine horn and vessel (10-week-old mice), or a cauterising pen was used to cauterise the vessel and the ovary was removed. The process was repeated for the right ovary and suture was used to close the wounds. Mice were administered Carprofen (4mg/kg) intraperitoneally and Lidocaine (2mg/kg) subcutaneously proximal to the incision site. In sham-operated mice, ovaries were removed and returned to the abdominal cavity. Mice recovered in a heated chamber and were monitored for appropriate recovery.

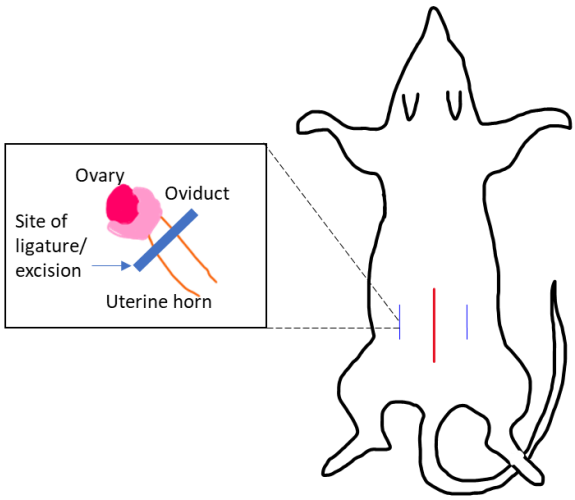


FIGURE 39: *Incision sites and site of uterine horn ligature/excision for ovariectomy surgery:*
Ovariectomy surgery was performed by making an incision in the skin (red line) followed by two incisions (blue lines) in the fascia through to the abdominal cavity either side of the initial incision. Both ovaries were removed and either a suture was used to ligate the uterine horn and vessel, or a cautery pen was used to cauterise the uterine horn or vessel and the ovary was removed using a scalpel.

5.3.3 Assessment of puberty and oestrous cycling in female mice:

To determine the success of ovariectomy in mice ovariectomised prior to puberty, mice were assessed for vaginal opening and uterine horn development at the time of death. Vaginal opening is one of the first secondary sexual characteristics to occur at the onset of puberty in female mice and oestradiol is required for uterine horn growth and development which initiates at puberty (Hoffmann, 2018). Successful ovariectomy prior to puberty therefore results in the absence of vaginal opening and limited uterine horn development. To assess vaginal opening, the base of the tail was held and with the free fingers supporting the hips, the tail was lifted, and the vulva was examined for vaginal opening (Figure 40). At the time of death and after removal of the pancreas from the mice, the uterine horns were observed for signs of growth and development. Ovariectomy was associated with small and

underdeveloped uterine horns, whereas sham operation was associated with larger uterine horns (Figure 40).

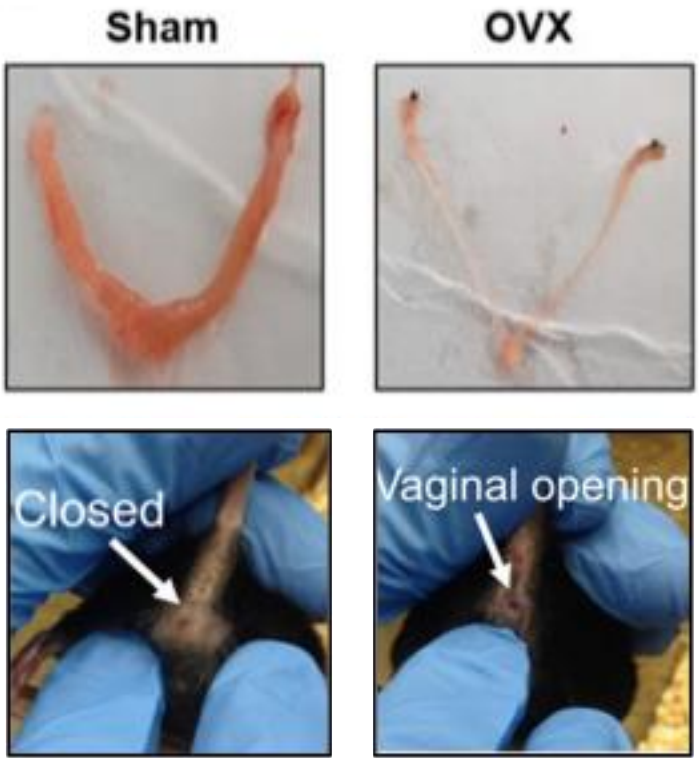


FIGURE 40: *Representative images of uterine horn development and vaginal opening in ovariectomised and sham-operated mice:*
(Top panel) Images of uterine horns from sham-operated and ovariectomised (ovx) mice. Development of the uterine horns is limited in the ovx mice. Image taken and adapted from (Park et al., 2020)*. (Bottom panel) Assessment of vaginal opening as an external sign of puberty onset in female mice; no vaginal opening indicates no onset of puberty whilst vaginal opening indicates puberty has occurred. Image taken and adapted from* (Hoffmann, 2018).

The absence of oestrous cycling (a process dependent on oestradiol) was used to determine successful ovariectomy in mice ovariectomised after puberty. Vaginal swabs were taken for 5 consecutive days one week following surgery (to allow for convalescence) and smeared onto glass microscope slides. Vaginal smears were stained for 20 minutes in methylene blue dye and washed in water before being examined under a light microscope. In mice undergoing oestrous cycling, smears show a cycling through different cell

types which is associated with the different stages of oestrous (Figure 41). This cycling is absent in successfully ovariectomised mice.

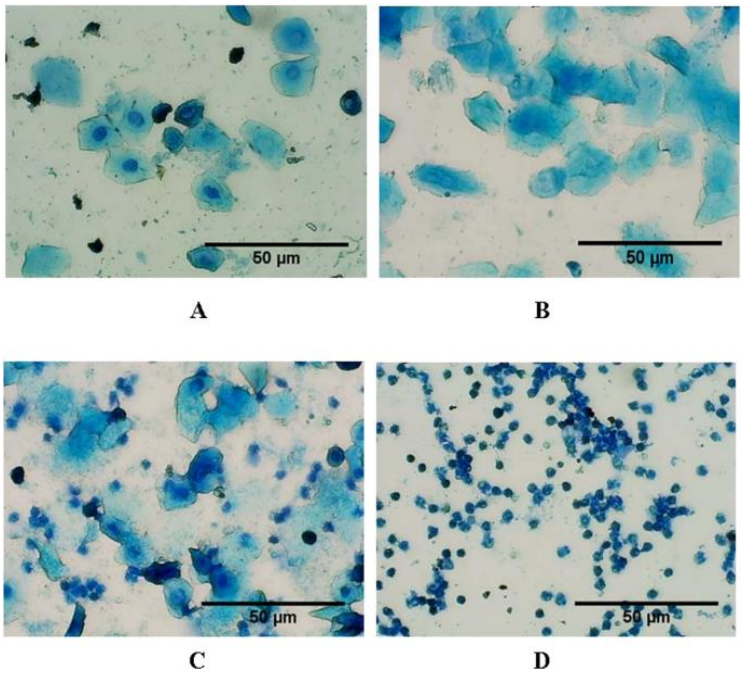


FIGURE 41: *Vaginal smears taken from a mouse undergoing oestrous cycling taken on consecutive days and stained with methylene blue:*
(A) Proestrus stage, consisting of mainly nucleated epithelial cells, (B) Oestrous stage, consisting of mainly non-nucleated cornified cells, (C) Metestrous stage, consisting of nucleated epithelial cells, non-nucleated cornified cells and leukocytes, (D) Diestrous stage, consisting of mainly leukocytes. Image taken from (Shaw et al., 2020).

5.3.4 Oestradiol administration:

Subcutaneously implanted silastic oestradiol capsules were used to determine the effect of oestradiol administration on glycaemic control in male mice. Silastic capsules containing 1µg oestradiol (Sigma, UK) or vehicle (sesame oil) were made by filling 2cm lengths of silastic tubing (ID/OD: 1.575mm/3.175, VWR international Ltd, UK) with 36µg/mL oestradiol in sesame oil and capping the ends with 3mm wooden applicator sticks. Capsules were then incubated at room temperature in 36µg/mL oestradiol or sesame oil overnight to remove any air pockets.

Male mice heterozygous for the KINGS mutation and wildtype littermates were anaesthetised using 5% isoflurane, 95% oxygen and anaesthesia was maintained at ~2-3% isoflurane. Mice were placed on a heated mat, viscotears was applied to the eyes and a razor was used to remove fur on the neck and upper back to expose the skin which was cleaned using Hibiscrub and ethanol. An incision was made to the dorsal aspect of the animal's neck and the skin was dissected away from the underlying fascia to generate a subcutaneous pocket ~2.5cm in length (Figure 42). Silastic capsules containing oestradiol or vehicle were placed into this pocket and suture was used to close the incision. Mice were administered Carprofen (4mg/kg) intraperitoneally and Lidocaine (2mg/kg) subcutaneously proximal to the incision site. Mice recovered in a heated chamber and were monitored for appropriate recovery.

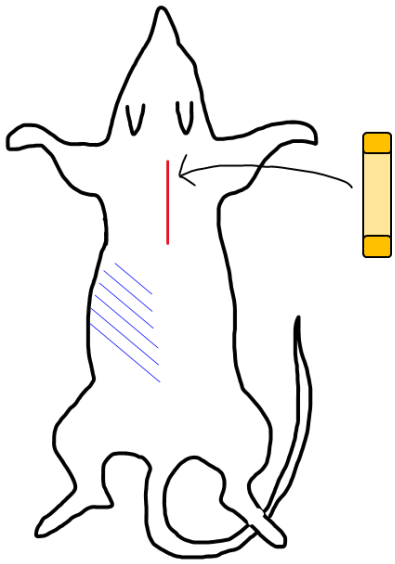


FIGURE 42: *Subcutaneous silastic capsule implantation into mice: Location of incision (red line) and blunt dissection of the skin from the underlying fascia to generate a subcutaneous pocket (blue lines) for silastic capsule implantation.*

5.3.5 Removal and replacement of silastic capsules:

The effect of oestradiol capsule implantation in KINGS mice was found to be lost 5-6 weeks post-surgery. To determine whether this was a result of oestradiol depletion from the capsule, silastic capsules were removed in some mice 3 weeks after initial implantation and replaced with a new silastic capsule

containing either oestradiol or vehicle alone. These mice were anaesthetised using the same anaesthetic procedure as described above. A razor was used to remove fur on the neck and upper back, and the shaved area was cleaned using Hibriscub and ethanol. The mice were placed on a heated pad and a lubricant (Viscotears) was applied to the eyes. Anaesthesia was maintained using 3% isoflurane and an incision was made using a scalpel to dorsal aspect of the animal's neck. The silastic capsule was located and dissected out of the subcutaneous pocket. Silastic capsules were replaced with new silastic capsules containing either 1µg oestradiol or vehicle in the same subcutaneous pocket. Suture was used to close the incision and mice were administered Carprofen (4mg/kg) intraperitoneally and Lidocaine (2mg/kg) subcutaneously proximal to the incision site. Mice recovered in a heated chamber and were monitored for appropriate recovery.

5.3.6 Non-fasted blood glucose concentration monitoring:

Non-fasted blood glucose concentrations measurements were used to investigate glycaemic control in a normal physiological context. Blood glucose concentrations were measured at 9-10am using an Accu-Chek glucose meter after generating a droplet of blood through pricking the end of the tail with a 30-gauge needle.

5.3.7 Glucose tolerance test:

To investigate glycaemic control under glucose stimulatory conditions, mice were subjected to a glucose tolerance test. Mice were fasted for 6 hours by changing the cage and removing any food but used bedding was retained to minimise stress. Baseline blood glucose concentration was measured, and 2g/kg was injected intraperitoneally. Blood glucose concentrations were subsequently monitored 15, 30, 60, 90 and 120 minutes after the glucose bolus.

5.3.8 Insulin tolerance test:

To investigate the effect of hormonal intervention on insulin sensitivity mice were subjected to an insulin tolerance test. Mice were fasted for 2 hours by changing the cage, removing any food but whilst retaining used bedding to

minimise stress. Baseline blood glucose concentration was measured, and 0.75IU/kg was injected intraperitoneally. Blood glucose concentrations were subsequently monitored 15, 30, 45 and 60 minutes after insulin administration.

5.3.9 Measurement of food intake:

To investigate whether endogenous oestradiol removal impacted food intake, female mice ovariectomised post-puberty were singly housed at 20 weeks of age to monitor food intake over 24 hours. The food hopper was removed and weighed at 9am. At 9am the following day, the food hopper was re-weighed, and the base of the cage was checked for any food pellets whose weight was also added. The difference between the food hopper weight over the 24-hour period was taken as an estimate of food intake per mouse. Mice were re-housed with their original cage mates afterwards.

5.3.9 Pancreas processing for both in-tact tissue and islet isolation:

Single pancreases from mice that received hormonal intervention were used both to isolate islets (to investigate glucose stimulated insulin secretion) and for fixation in formaldehyde (to investigated BiP expression through immunofluorescent staining). After the mice were culled, the abdominal cavity was exposed, the tail end of the pancreas was clamped using a small bulldog clamp and a scalpel was used to excise the tail of the pancreas which was placed in 3.5% phosphate buffered formaldehyde for 48 hours before further histological processing. With the bulldog clamp still in place, another bulldog clamp was used to clamp the ampulla of Vater and ~2.5mL of cold collagenase solution was injected into the remaining pancreas portion via the common bile duct. Perfused pancreases were processed for islet isolation as previously described (see section 2.5.1).

5.3.10 Histological processing and immunofluorescent staining:

For immunofluorescent staining, sections were first de-waxed by heating on a slide dryer and immersion in two changes of histoclear. Sections were then rehydrated through incubation in decreasing concentrations of ethanol. Heat mediated citric acid antigen retrieval was then carried out whereby sections were pressure cooked in 10mM citric acid. Sections were blocked in blocking

buffer before incubation with primary antibodies against rabbit anti-BiP (1:100 dilution, Cell signalling Technology) and guinea pig anti-insulin (1:200 dilution, Abcam) overnight at 4°C. Sections were subsequently washed in three changes of TBS and then incubated with secondary anti-rabbit (Alexa Fluor 594, 1:200 dilution, Jackson ImmunoResearch) and anti-guinea pig 488 (Alexa Fluor 488, 1:200 dilution; Jackson ImmunoResearch) antibodies, as well as DAPI (1:500 dilution), for 1 hour at room temperature.

Whole pancreatic sections were imaged using a NanoZoomer S60 fluorescence and brightfield slide scanner (Hamamatsu) with a 20x objective. FijiImageJ was used to semi-automatically generate selections around every islet within the section based on insulin staining (Schindelin et al., 2012). Mean fluorescent intensity in the BiP channel within these selections was then measured as previously described (see section 2.6.4.4).

5.3.11 Static insulin secretion assays:

Islets were cultured overnight to allow for recovery after the isolation process. Islets were washed twice in 2mM Gey and Gey buffer before being cultured for 1 hour in 2mM fresh Gey and Gey buffer at 37°C. Islets were subsequently subjected to incubation in 2mM or 20mM Gey and Gey for 1 hour at 37°C before the supernatants were removed and stored at -20°C until further downstream processing. An in-house RIA was used to determine insulin concentrations in static secretion assay samples as previously described (see section 2.5.3.2).

5.3.12 Statistical analysis:

All graphs were constructed using GraphPad Prism 8.0 or 9.0 software (GraphPad Software, San Diego, CA) and statistical analysis was carried out in SigmaPlot 14.0 (Systat software Inc, San Jose, California). For comparison between multiple groups where there were two independent variables, a two-way ANOVA with Holm-Sidak post hoc was used and where repeated measures over time were taken, a two-way repeated measure ANOVA with Bonferroni post hoc was used. To assess significant differences between two

groups, unpaired T-tests were used. All data represent the mean ± standard error of the mean (SEM). P<0.05 was considered statistically significant.

5.4 Results:

5.4.1 Ovariectomising adult KINGS females does not cause development of overt diabetes:

The activational effects of oestradiol were investigated by ovariectomising KINGS female mice at 10 weeks age, which is around 5 weeks after the onset of puberty. Successful ovariectomy was confirmed though imaging of vaginal swabs taken from these mice for 5 consecutive days. Smears from sham-operated mice showed a predictable cycling of cell types (nucleated epithelial cells, anucleated cornified cells and leukocytes) representing cycling through the different oestrous stages, however smears from ovariectomised mice did not show any cycling (Figure 43).

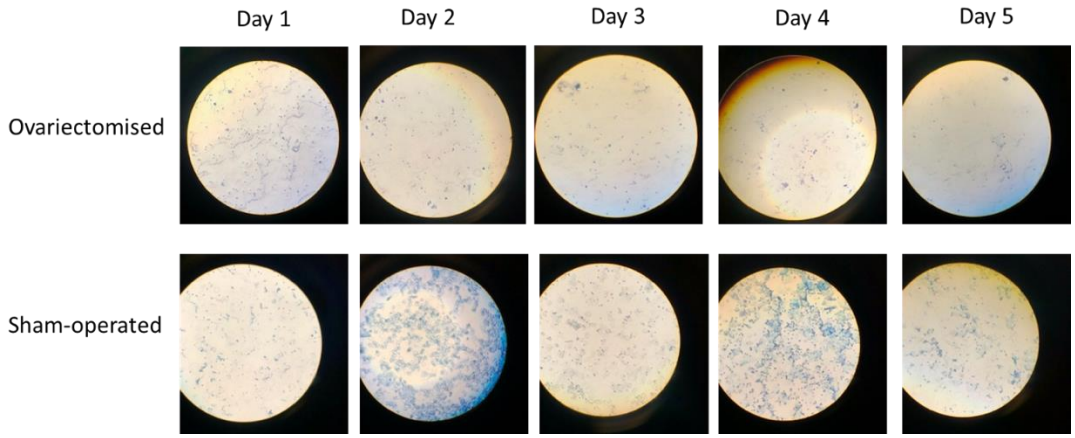


FIGURE 43: *Methylene blue stained vaginal swabs from sham-operated and ovariectomised mice:*

Representative methylene blue staining and imaging of vaginal swabs taken from female mice that were either ovariectomised (top panel) or sham-operated (bottom panel). Swabs from ovariectomised mice do not show the classical cycling through cell types that defines the oestrous stages, whereas classical cycling can be seen in the sham-operated mouse.

Raw body weights and weight gain of ovariectomised WT mice were significantly increased compared to sham-operated controls from 3 weeks

post-surgery (Figure 44A,C). Although raw weight values were not significantly increased in ovariectomised KINGS mice compared to sham-operated KINGS mice (Figure 44B), weight gain was increased and reached significance at 3, 4 and 10 weeks post-surgery (Figure 44D). Despite increases in weight gain in these mice, we found no difference in food intake at 20 weeks of age (Figure 45).

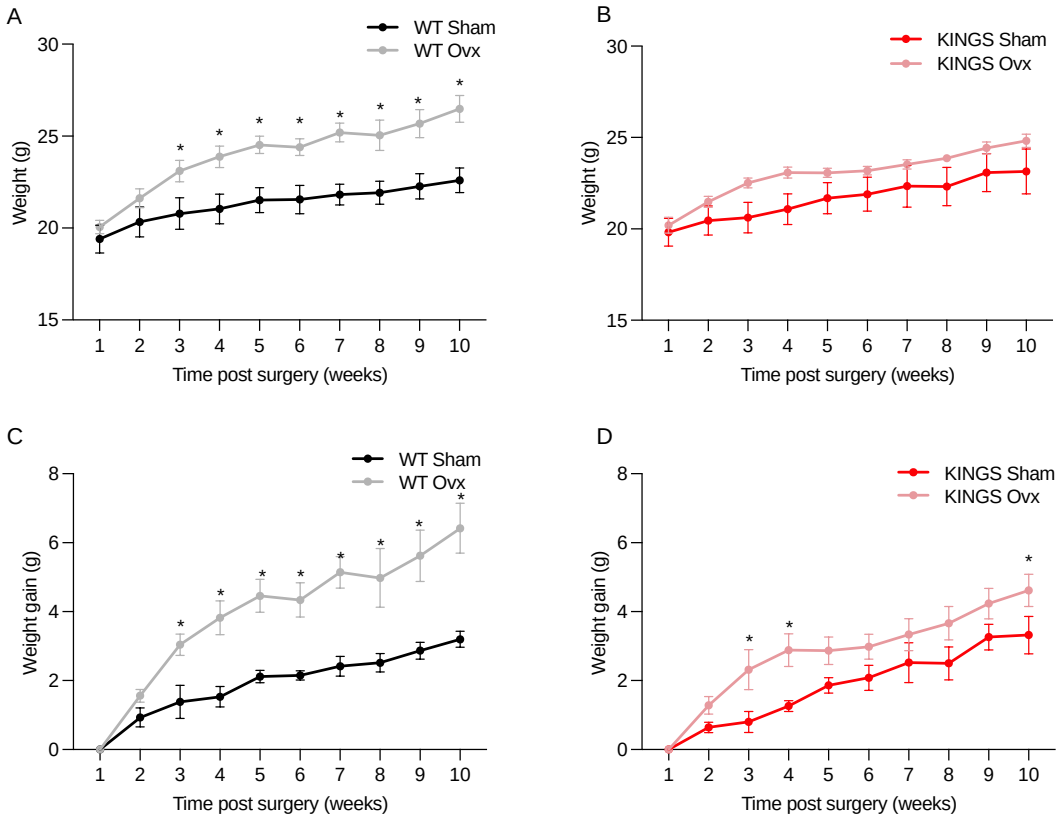


FIGURE 44: *Weight and weight gain in sham and post-puberty ovariectomised mice:*

*Raw body weight (A-B) and weight gain (C-D) after sham surgery or ovariectomy (ovx) at 10 weeks (post-puberty) in (A,C) WT mice and (B,D) KINGS mice. Data represents the mean±SEM, n=5-6, *p<0.05, two-way repeated measures ANOVA with Bonferroni post-hoc.*

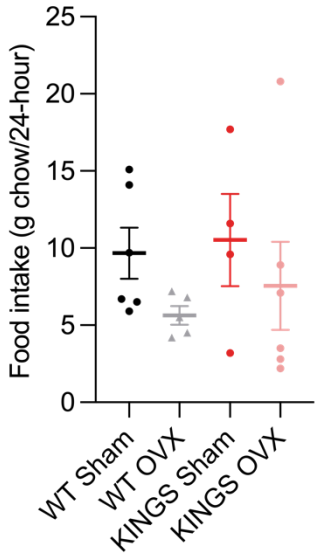


FIGURE 45: *Food intake in sham-operated and post-puberty ovariectomised mice*

Food intake over 24 hours in 20-week-old wildtype and KINGS female mice ovariectomised (ovx) or sham-operated at 10 weeks of age (post-puberty). n= 4-6, two-way ANOVA, p>0.05.

Non-fasted blood glucose concentrations were monitored for 10 weeks post-surgery until mice were 20 weeks of age to investigate whether ovariectomy had any effect on glycaemic control under physiological conditions. Ovariectomy had no effect on non-fasted blood glucose concentration in wildtype female mice (Figure 46A). Ovariectomised female KINGS mice on the other hand showed a trend for increased non-fasted blood glucose concentrations compared to sham-operated KINGS mice from 2 weeks after surgery, and this became significant from 7 weeks post-surgery onwards (Figure 46B). By 20 weeks of age, blood glucose concentrations were approximately 5mM higher in the ovariectomised KINGS mice compared to sham-operated KINGS mice. Despite this increase in blood glucose concentrations, mean values never exceeded the 16.7mM threshold for overt diabetes. It should be noted however, that two ovariectomised KINGS mice (out of six) had a blood glucose exceeding 16.7mM on three occasions (ranging from 18.1-22.4mM). Blood glucose concentrations in these particular mice were below the threshold for overt diabetes (9.1-16.1mM and 7.7-15mM) at all other time points measured.

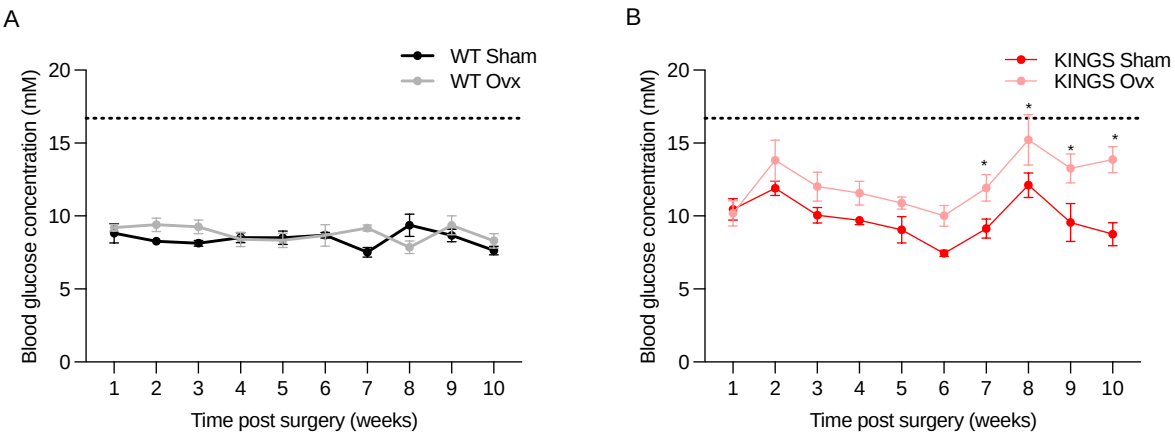


FIGURE 46: *Non-fasted blood glucose concentrations in sham-operated and post-puberty ovariectomised mice:*
*Non-fasted blood glucose measurements after sham surgery or ovariectomy (ovx) at 10 weeks (post-puberty) in (A) WT female mice and (B) KINGS female mice. Data represents the mean±SEM. *p<0.05, two-way repeated measures ANOVA with Bonferroni post-hoc, n=5-6.*

Glucose tolerance tests were carried out at 20 weeks of age (10 weeks following surgery) to investigate the activational effect of oestradiol on glucose handling under conditions of increased beta cell demand. Ovariectomy had no significant effect on glucose tolerance in female WT mice, in line with no differences seen in non-fasted blood glucose concentrations (Figure 47). Similarly, no difference in glucose tolerance was seen between sham and ovariectomised KINGS mice.

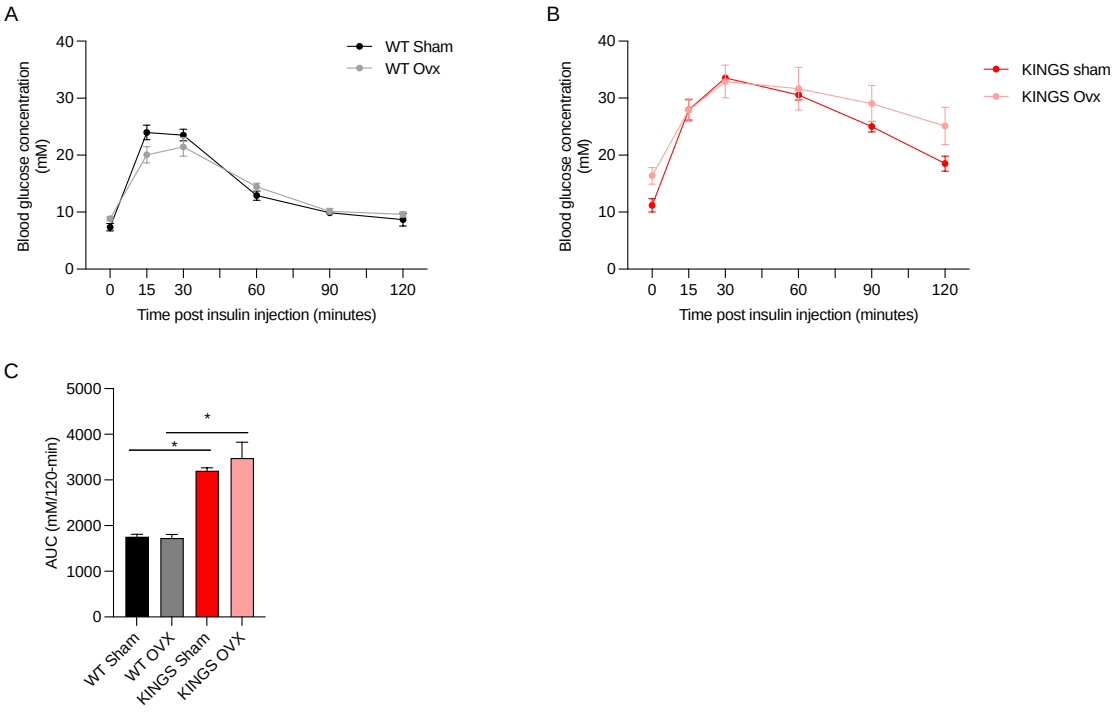


FIGURE 47: *Glucose tolerance in sham-operated and post-puberty ovariectomised mice:*

*Glucose tolerance tests carried out at 20 weeks of age after a 6-hour fast in (A) WT mice sham operated or ovariectomised (ovx) post-puberty and (B) KINGS mice sham operated or ovariectomised post-puberty. (C) Area under the curve (AUC) values for glucose tolerance tests carried out in WT and KINGS females sham operated or ovariectomised post-puberty. Data represents the mean±SEM. *p<0.05, two-way ANOVA with Holm-Sidak post hoc test, n=4-6,*

As previously mentioned, oestradiol can influence glucose homeostasis in numerous different ways including through mechanisms independent of the beta cell. One of the main ways in which it can do this is through influencing insulin resistance. To investigate whether the increases in non-fasted blood glucose concentrations seen in the ovariectomised KINGS mice were due to changes in peripheral insulin sensitivity, an insulin tolerance test was also carried out in these mice at 20 weeks of age (10 weeks after surgery). Baseline blood glucose concentrations were not comparable between the groups making it difficult to directly compare the insulin response between the groups (Figure 48A-B). For this reason, data was also presented as percentage of

baseline blood glucose concentrations (Figure 48C-D). We found no difference in area above the curve values for blood glucose concentrations during the ITT expressed as percentage baseline concentrations between any of the groups (Figure 48E).

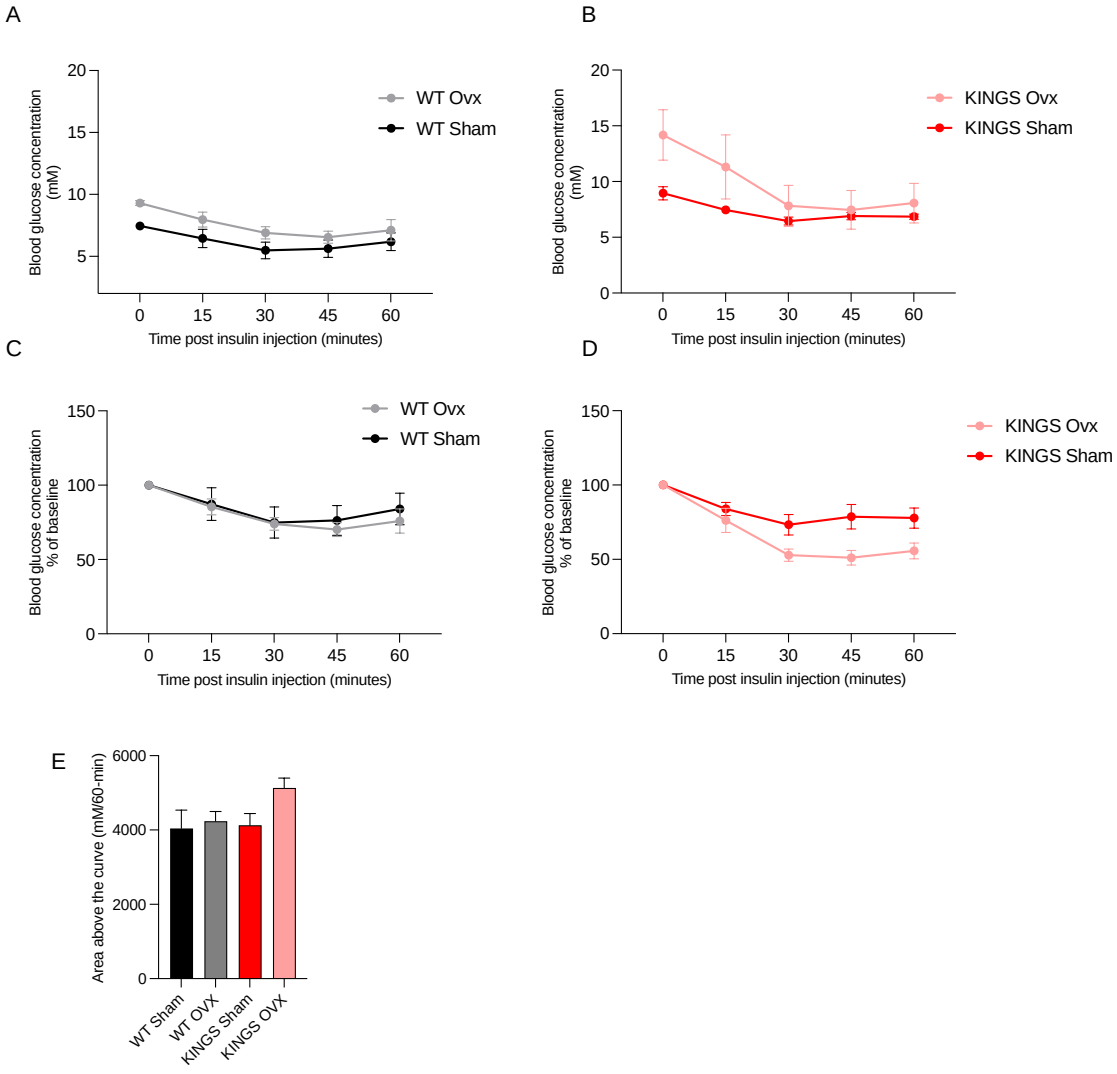


FIGURE 48: *Insulin tolerance in sham-operated and post-puberty ovariectomised mice:*

Insulin tolerance tests carried out at 20 weeks of age after a 2-hour fast in (A) WT mice sham operated or ovariectomised (ovx) post-puberty and (B) KINGS mice sham operated or ovariectomised post-puberty. Insulin tolerance test data expressed as percentage of basal glucose concentrations in (C) WT sham and ovariectomised mice and (D) KINGS sham and ovariectomised mice. (E) Area above the curve (AAC) values for insulin tolerance tests carried out in WT and KINGS females sham operated or ovariectomised post-puberty.

Data represents the mean±SEM, two-way ANOVA with Holm-Sidak post hoc, n= 4-6.

Other mechanisms by which oestradiol may reduce non-fasted blood glucose concentrations are through direct effects on beta cell function. Therefore, islets were isolated from mice that had been ovariectomised or received sham-surgery 10 weeks prior, cultured overnight and then subjected to a static secretion assay. All groups showed an enhanced insulin secretory response when exposed to 20mM glucose compared to 2mM (Figure 49). Interestingly however, whilst ovariectomy in wildtype females was associated with a significant increase in islet insulin secretion when exposed to 20mM glucose compared to sham operated controls (Figure 49A), ovariectomy in the KINGS mice was associated with a significant reduction in 20mM glucose insulin secretion compared to controls (Figure 49B). No differences between sham and ovariectomy groups were found with 2mM basal glucose.

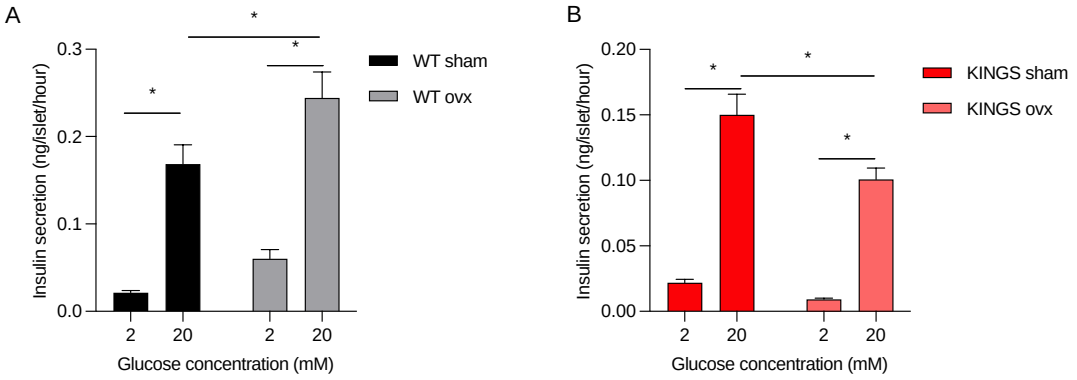
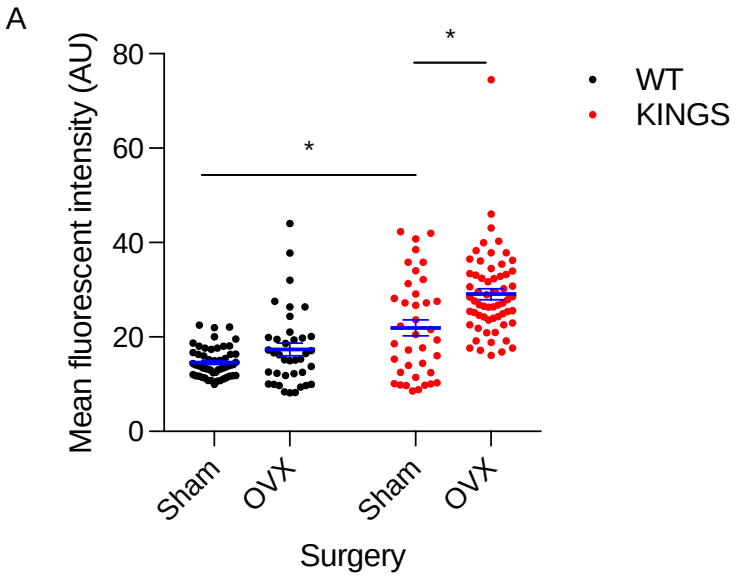


FIGURE 49: *Glucose stimulated insulin secretion in islets from sham and post-puberty ovariectomised mice:*
*Glucose stimulated insulin secretion in islets isolated from 20-week-old post-puberty ovariectomised (ovx) and sham-operated wildtype (A) and KINGS (B) female mice. Data represents the mean±SEM. *p<0.05, two-way ANOVA with Holm-Sidak post hoc test, n=11-16 replicates from 3-4 animals.*

The reduction in islet insulin secretion and increase in non-fasted blood glucose concentrations observed in ovariectomised KINGS female mice may be the result of changes in levels of beta cell ER stress. We tested this through

immunofluorescent staining of pancreas sections for BiP, a general ER stress marker. BiP expression was increased in islets from KINGS sham-operated mice compared to WT sham-operated mice as expected and consistent with our previous findings (Figure 50, section 4.4.1). Ovariectomising KINGS females was associated with an even greater increase in islet BiP levels which were significantly higher than those in the KINGS sham-operated control mice. This effect of ovariectomy on BiP levels was not seen in wildtype mice however, although it should be noted that the range of islet BiP expression was greater in ovariectomised WT mice with some islets showing more than double average BiP expression of the WT shams, possibly suggesting a subtle increase in islet ER stress in this group.



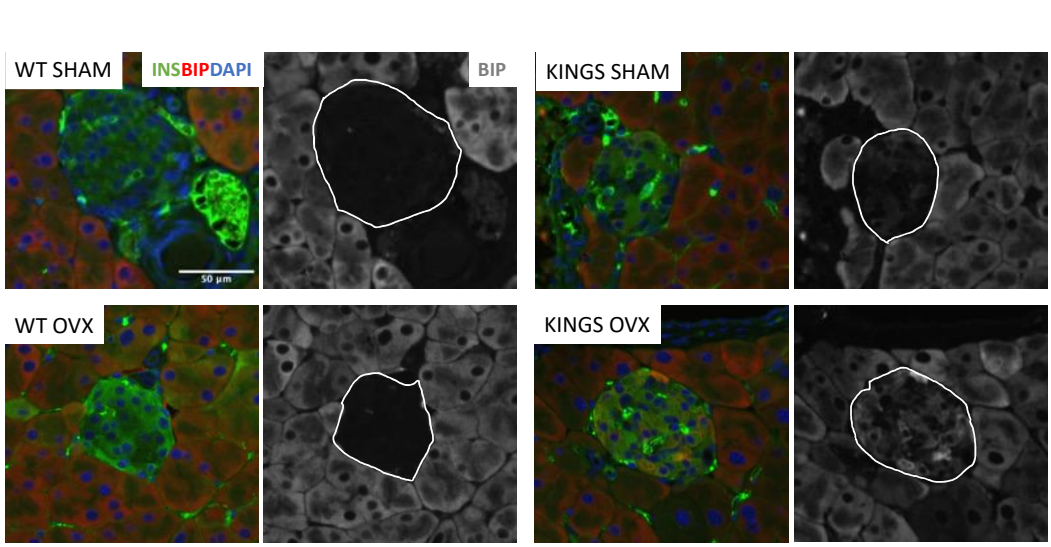


FIGURE 50: *BiP expression in islets from sham and post-puberty ovariectomised mice:*

*(A) BiP mean fluorescence intensity in islets from 20-week-old WT (black) and KINGS (red) mice ovariectomised (ovx) or sham operated post-puberty. Data represents individual islets from n=38-61 islets from 5-6 mice and mean±SEM is indicated in blue. *P<0.05, two-way ANOVA with Holm-Sidak post hoc. (B) Representative islets from WT and KINGS ovariectomised and sham-operated mice stained for insulin (green), BiP (red) and DAPI (blue). BiP staining alone (grey) is also shown and the islet area is outlined by the white line. The scale bar in the top left image represents 50mm.*

5.4.2 Ovariectomising pre-pubescent KINGS females does not cause development of overt diabetes:

The effects of oestradiol can also be organisational (and some of these known organisational effects include developmental changes associated with puberty) and are permanent. To test whether organisational effects of E2 contribute to the sex differences in diabetes phenotype in the KINGS mice, female KINGS mice were ovariectomised before puberty, at ~4 weeks of age. Successful ovariectomy was confirmed through determining vaginal opening (an indicator of puberty) and assessment of uterine horn size at the time of death.

Contrasting our previous findings that post-puberty ovariectomy caused an increase in weight gain, we found that ovariectomy did not significantly alter either raw body weight or weight gain in the WT mice (Figure 51C,E). Consistent with this, there were no changes in weight gain with ovariectomy in the KINGS mice, although raw weight values were significantly higher at 4 and 5 weeks of age suggestive of a mild impact of pre-puberty ovariectomy on weight in this group (Figure 51D,F).

In line with the post-puberty ovariectomy findings, pre-puberty ovariectomy was associated with an increase in non-fasted blood glucose concentrations in KINGS mice from 3 weeks post-surgery and this was significant at 4 and 5 weeks post-surgery (Figure 51B). Interestingly this was the same age at which

raw body weight was elevated in this group. It is important to note that this increase was not as stark as when ovariectomy was performed post-puberty, with 2.7mM being the maximum average increase compared to sham *vs.* >5mM in the post-puberty ovariectomised mice. Intriguingly, we also found that sham operated KINGS mice showed a higher blood glucose concentration at 1-week post-surgery with an average blood glucose concentration of 14.3mM, however this did reduce from 2 weeks and was maintained between 8.8-10.5mM thereafter. Non-fasted blood glucose concentrations in WT ovariectomised mice were similar to WT sham-operated control mice (Figure 51A).

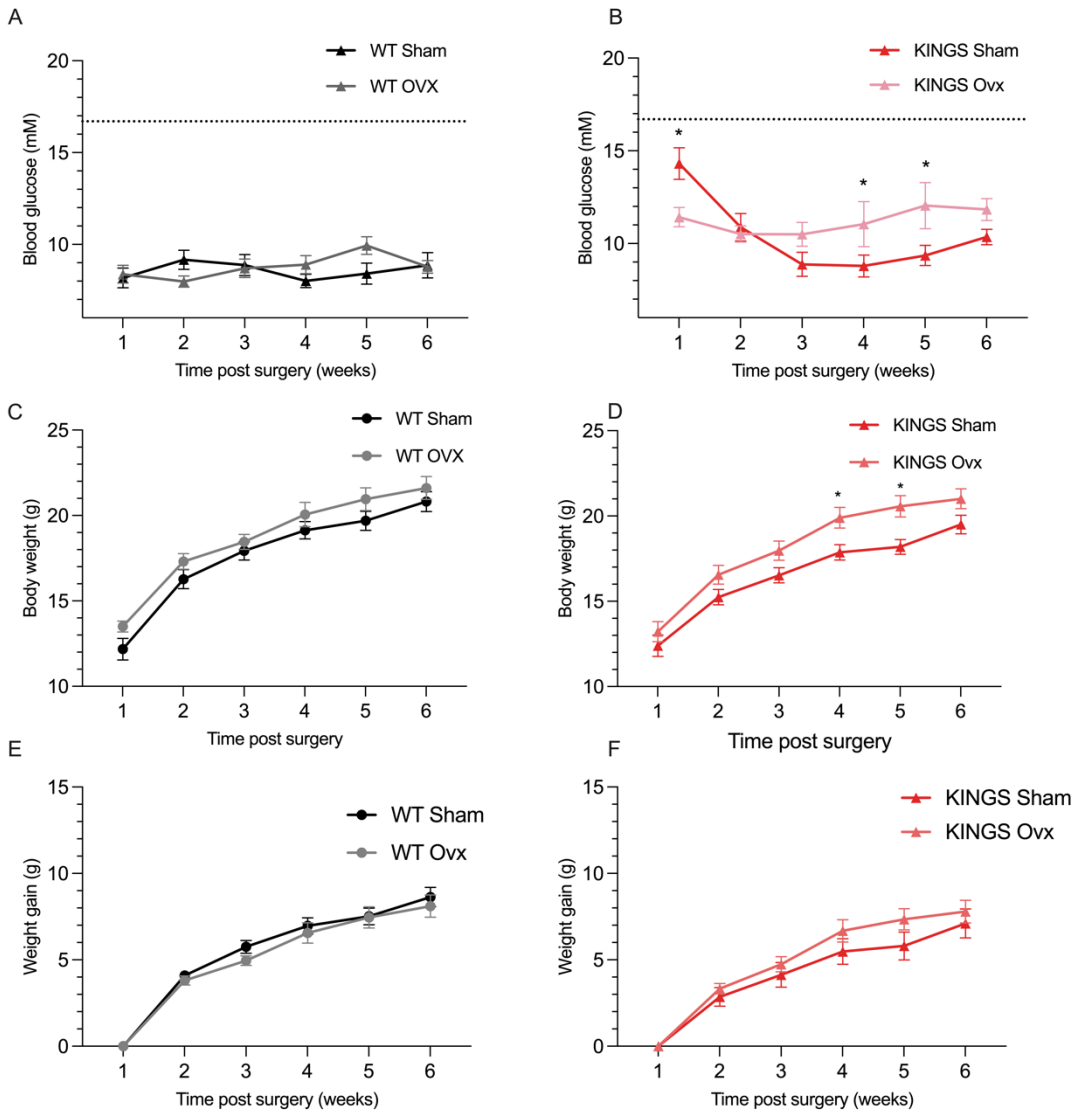


FIGURE 51: *Non-fasted blood glucose concentrations, weight, and weight gain in sham and pre-puberty ovariectomised mice:*

*Non-fasted blood glucose measurements after sham surgery or ovariectomy (ovx) in (A) WT mice and (B) KINGS mice. Body weight and weight gain in (C, E) WT ovariectomised or sham-operated mice and (D, F) KINGS ovariectomised or sham-operated mice. Data represents the mean±SEM. *p<0.05, two-way repeated measures ANOVA with Bonferroni post-hoc, n=8-9.*

To investigate the organisational effect of oestradiol on glucose handling under conditions of increased beta cell demand, glucose tolerance tests were carried out in pre-puberty ovariectomised and sham operated mice at 10 weeks of age. We observed no significant effect of ovariectomy on glucose tolerance in either the WT or KINGS mice (Figure 52).

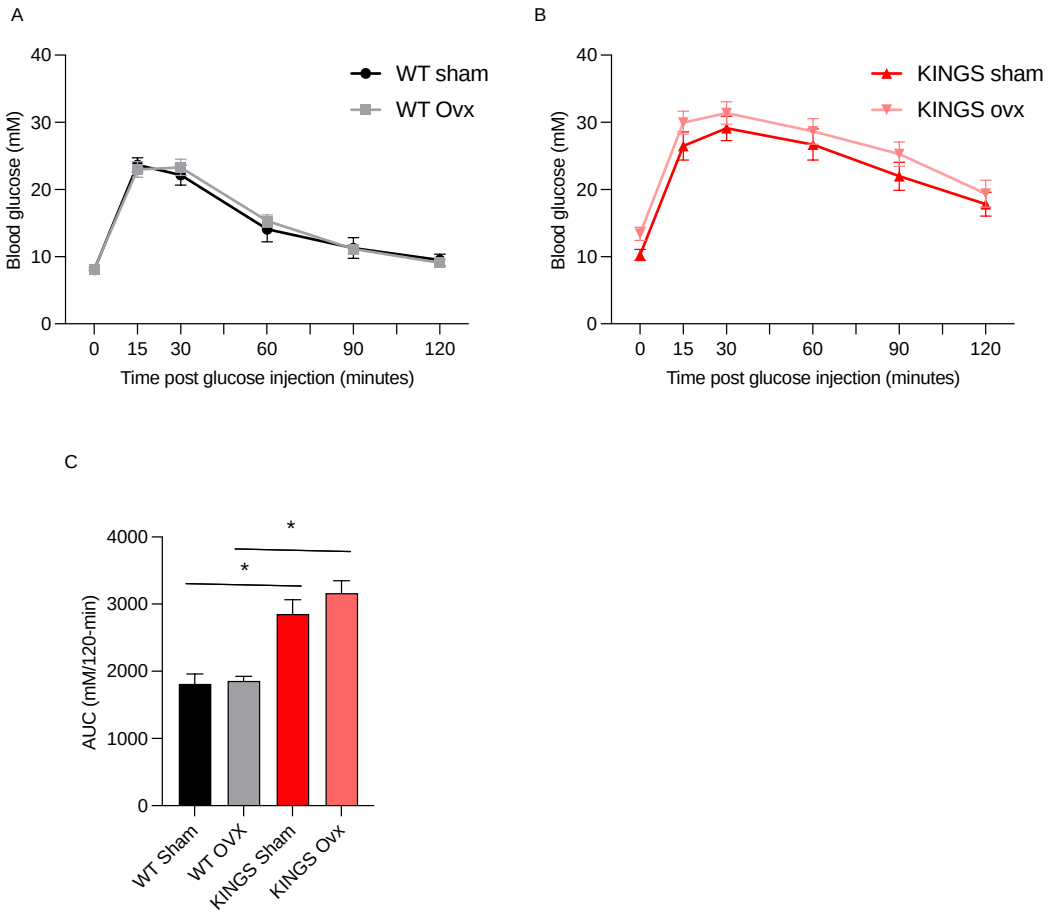


FIGURE 52: *Glucose tolerance in sham and pre-puberty ovariectomised mice: Glucose tolerance tests carried out at 10 weeks of age after a 6-hour fast in (A) WT mice sham operated or ovariectomised (ovx) pre-puberty and (B)*

*KINGS mice sham operated or ovariectomised pre-puberty. Data represents the mean±SEM, n=8-9. (C) Area under the curve (AUC) values for glucose tolerance tests carried out in WT and KINGS females sham operated or ovariectomised prior to puberty. Data represents the mean±SEM. *P<0.05, two-way ANOVA with Holm-Sidak post hoc, n= 8-9.*

Insulin tolerance tests were also carried out to determine whether the effect of pre-puberty ovariectomy on non-fasted blood glucose concentrations in the KINGS mice was the result of changes to peripheral insulin sensitivity. Basal 2-hour fasting blood glucose measurements were slightly higher ovariectomised KINGS and WT mice compared to sham controls therefore data was expressed as % of baseline blood glucose concentrations to make comparisons between groups easier (Figure 53). No differences were observed in area above the curve values for the insulin tolerance tests where data was presented as percentage of baseline (Figure 53E).

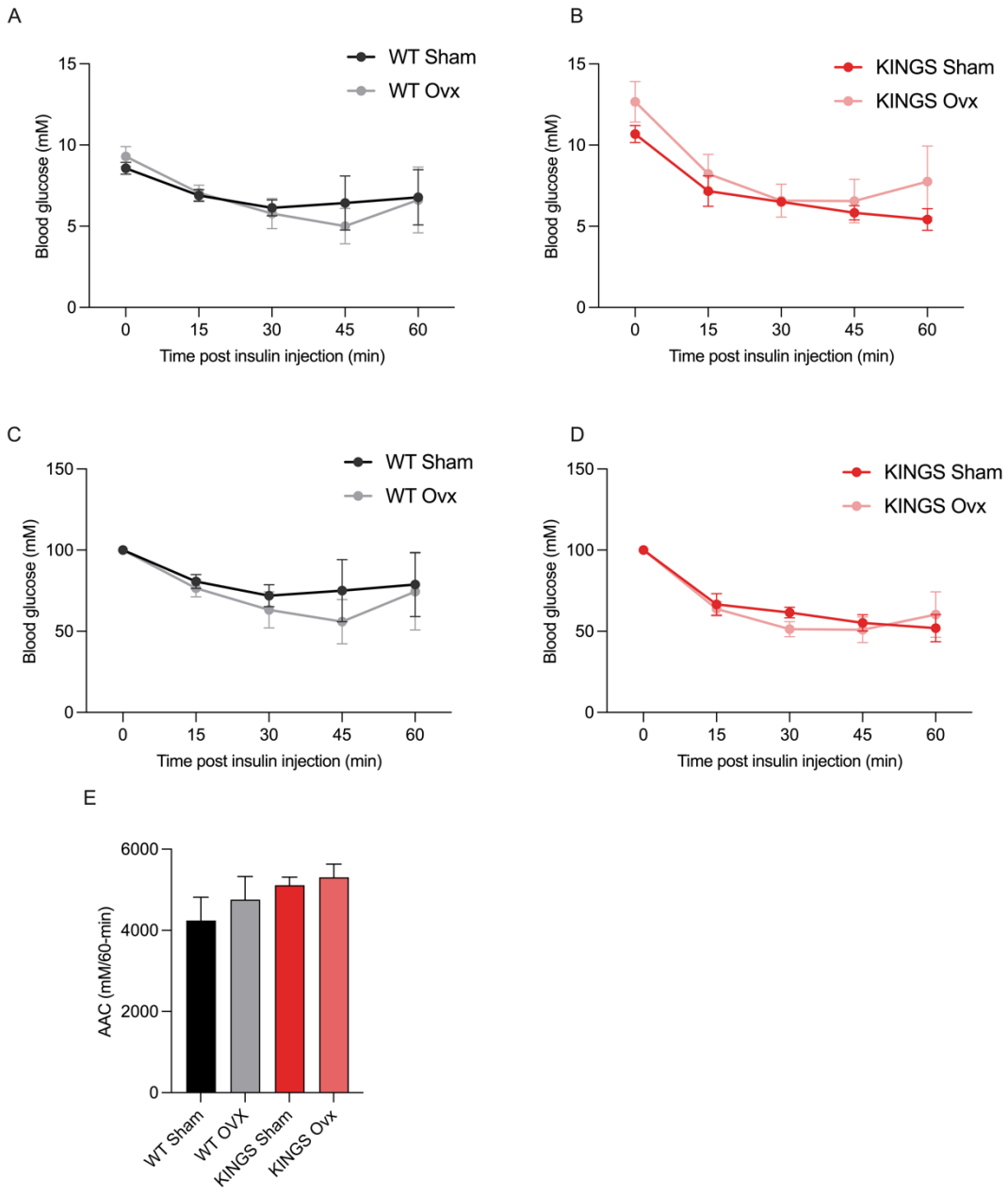


FIGURE 53: *Insulin tolerance tests in sham and pre-puberty ovariectomised mice:*

Insulin tolerance tests carried out at 10 weeks of age after a 2-hour fast in (A) WT mice sham operated or ovariectomised (ovx) pre-puberty and (B) KINGS mice sham operated or ovariectomised pre-puberty. Insulin tolerance test data expressed as percentage of basal glucose concentrations in (C) WT sham and ovariectomised mice and (D) KINGS sham and ovariectomised mice. (E) Area above the curve (AAC) values for insulin tolerance tests carried out in WT and KINGS females sham operated or ovariectomised prior to puberty. Data

represents the mean±SEM, p>0.05, two-way ANOVA with Holm-Sidak post hoc, n= 4-6.

5.4.3 Oestrogen administration in male KINGS mice prevents the development of overt diabetes:

Ovariectomising females KINGS mice resulted in a subtle impairment in glucose homeostasis, although it did not result in overt diabetes. To investigate whether E2 could prevent male KINGS mice from developing overt diabetes, exogenous oestradiol was administered continuously to male KINGS mice starting before the onset of puberty (~3 weeks) which is also before the usual onset of overt diabetes at 5-6 weeks of age.

Although raw weight values were not changed, oestradiol administration was associated with a reduced weight gain in wildtype and KINGS male mice compared to vehicle controls from 13 days (WT) and 14 days (KINGS) after implantation of oestradiol capsules (Figure 54C-F). Oestradiol treatment also resulted in a reduction in non-fasted blood glucose concentrations in male wildtype mice and this reached significance at 7, 13, 14, and 17 days post-surgery (Figure 54A). In line with this, KINGS male mice that were administered oestradiol had lower blood glucose concentrations compared to vehicle controls and this was significant on most days from 15 days post-surgery (Figure 54B). Additionally, whilst glucose concentrations in KINGS-vehicle mice progressively increased, reaching the threshold for overt diabetes at 20 days post-surgery, KINGS mice treated with oestradiol never crossed this threshold. Blood glucose concentrations in KINGS mice treated with oestradiol remained between 8.1-11.6mM up until 28 days post-surgery. At this time blood glucose concentrations were on average 5.5mM less than the vehicle controls.

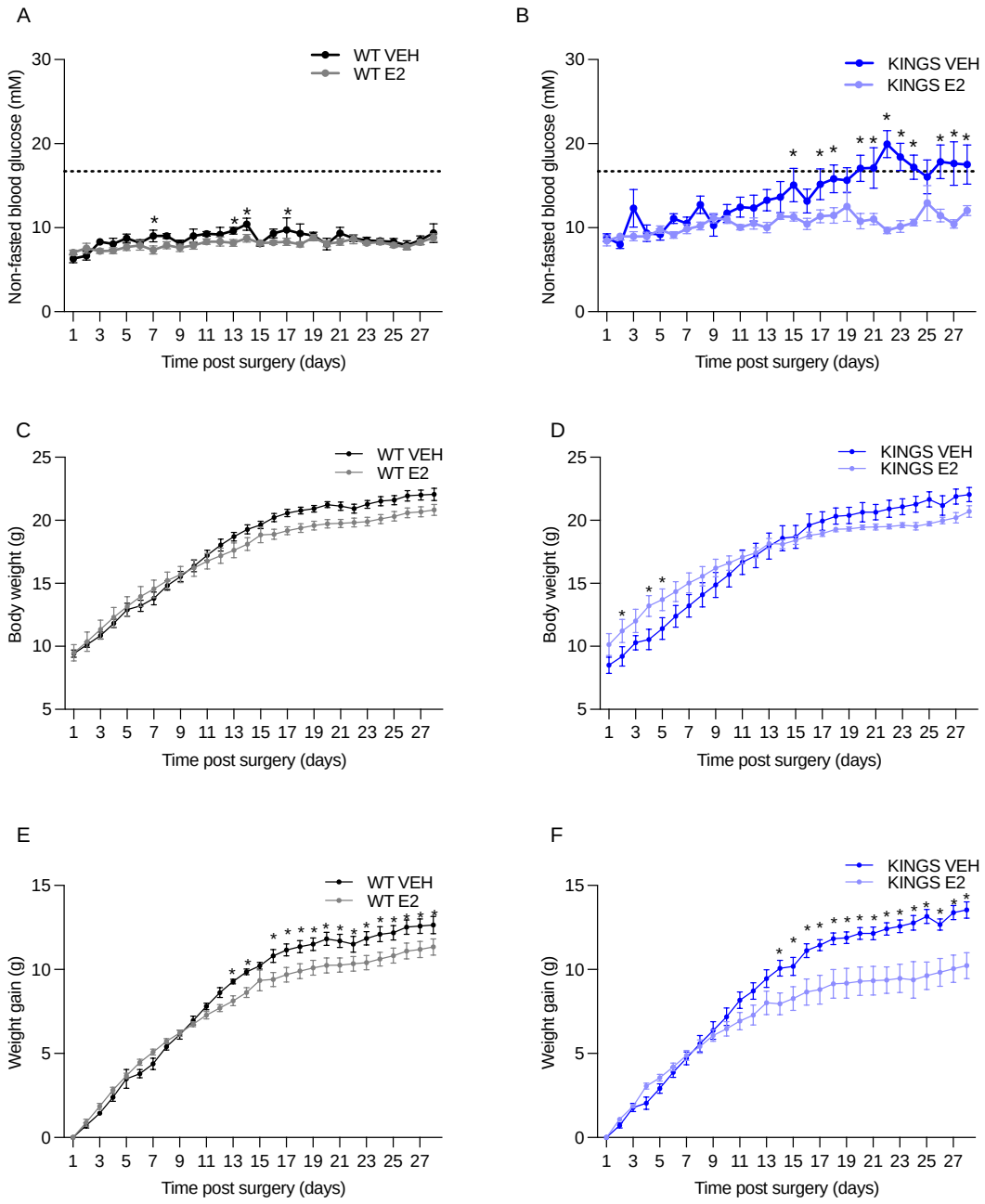


FIGURE 54: *Non-fasted blood glucose concentrations, weight, and weight gain in male mice administered E2 or vehicle pre-puberty:*

Non-fasted blood glucose concentrations in (A) WT and (B) KINGS male mice administered oestradiol continuously via implantation of silastic capsules from ~3 weeks of age. (C, E) Raw body weight and weight gain in male WT mice administered oestradiol or vehicle from 3 weeks of age (D, F) Raw weight and weight gain in male KINGS mice administered oestradiol or vehicle from 3

*weeks of age. *P<0.05, two-way repeated measures ANOVA with Bonferroni post-hoc, n=5-8.*

To determine whether there was also a difference in glucose homeostasis under stimulatory conditions, mice treated with oestradiol or vehicle were subjected to a glucose tolerance test 3 weeks post-surgery (at 6 weeks of age). Whilst area under the curve values for the glucose tolerance tests were significantly higher in KINGS mice compared to WT mice in both the vehicle and E2 treated groups, E2 had no effect on glucose tolerance with no difference in area under the curve values between vehicle treated mice and E2 treated mice of both genotypes (Figure 55).

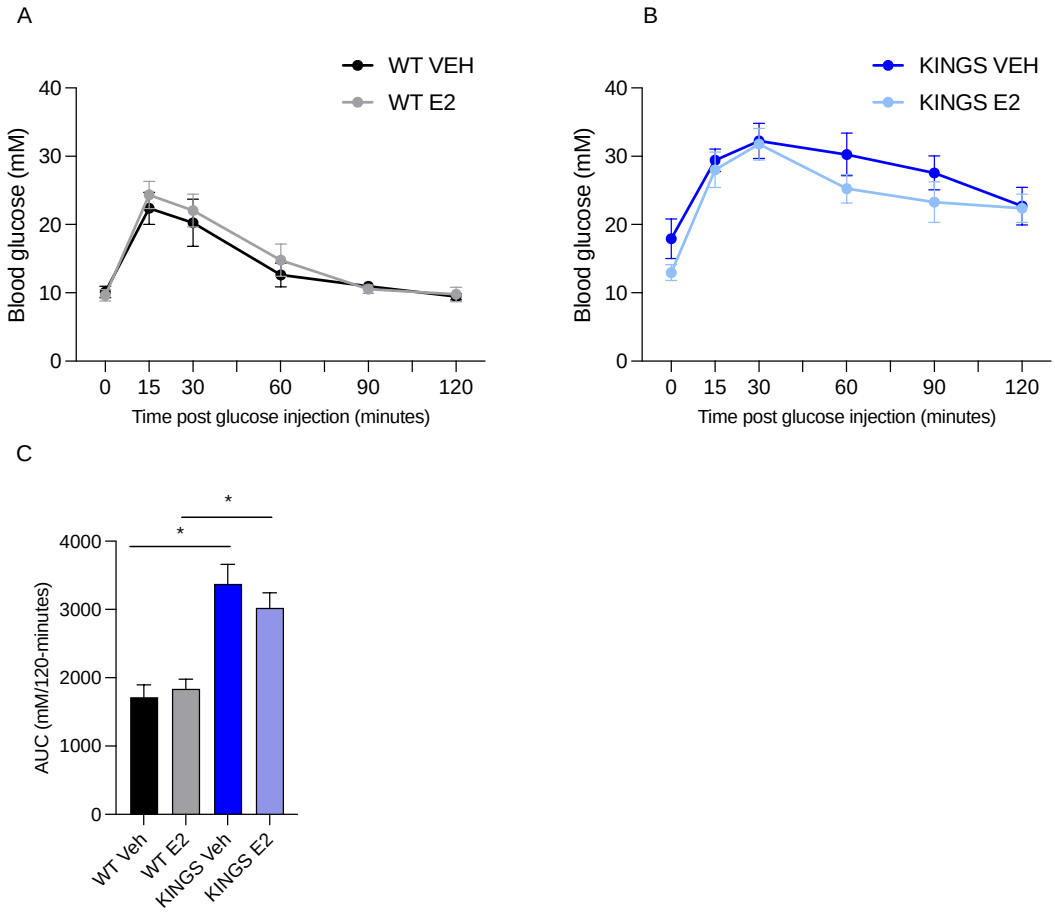


FIGURE 55: *Glucose tolerance in male mice administered E2 or vehicle pre-puberty:*

Glucose tolerance tests carried out at 6 weeks of age after a 6-hour fast in (A) WT male mice implanted with vehicle or E2-containg capsules and (B) KINGS male mice implanted with vehicle or E2-containg capsules. Data represents

*the mean±SEM, n=5-7. (C) Area under the curve (AUC) values for the glucose tolerance tests carried out in WT and KINGS males implanted with vehicle or E2-containing capsules. Data represents the mean±SEM. *P<0.05, two-way ANOVA with Holm-Sidak post hoc, n= 5-7.*

A subset of male KINGS mice (n=3) treated with vehicle or E2 were followed until 10 weeks of age (6 weeks post-surgery) to establish whether effects of E2 on blood glucose levels remain after E2 diminishes. E2-treated mice became overtly diabetic by 40 days post-surgery and blood glucose concentrations were not significantly different from vehicle treated mice from 42 days post-surgery (Figure 56A). To determine whether this increase in blood glucose levels were indeed the result of silastic capsule depletion, or whether E2 is no longer sufficient to protect males with advancing age, a pilot oestradiol replacement study was carried out. Male KINGS mice were implanted with a silastic E2-containing capsule at 3 weeks of age and after 3 weeks this was either not replaced, replaced with a new capsule containing E2 or replaced with a capsule containing vehicle.

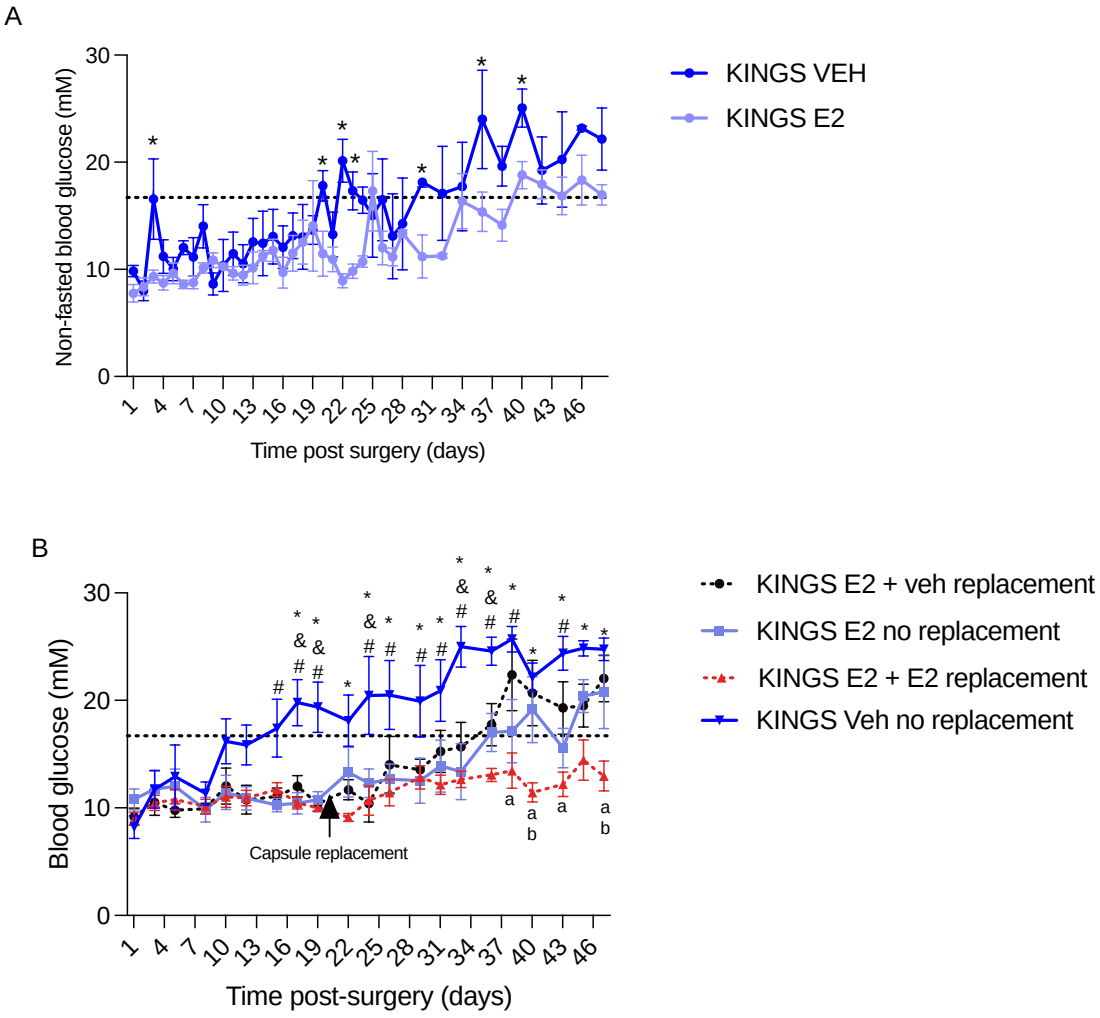


FIGURE 56: *The effect of diminishing E2 administration on blood glucose concentrations in the KINGS male mice:*

*(A) Non-fasted blood glucose concentrations in KINGS male mice implanted with silastic capsules containing E2 or vehicle from 3 weeks of age. n=3, two-way ANOVA with Bonferroni post-hoc, *p<0.05. (B) Non-fasted blood glucose concentrations in KINGS male mice implanted with silastic capsules containing vehicle (veh) or E2 which were then not replaced (KINGS E2 no replacement), replaced with silastic capsules containing vehicle (KINGS E2 + veh replacement) or replaced with silastic capsules containing E2 (KINGS E2 + E2 replacement). n=5-7, two-way ANOVA with Bonferroni post-hoc,* *******=p<0.05 KINGS veh no replacement vs. KINGS E2 + E2 replacement,* ***&****=p<0.05 KINGS veh no replacement vs KINGS E2 + veh replacement, #=p<0.05 KINGS veh no replacement vs KINGS E2 no replacement,* ***a****=p<0.05*

KINGS E2 + E2 replacement vs KINGS E2 + vehicle replacement, ***b****= p<0.05 KINGS E2 + E2 replacement vs KINGS E2 no replacement.*

As expected, and in line with previous findings, male KINGS mice implanted with a silastic capsule containing vehicle showed a higher blood glucose concentration compared to all other groups by day 17 and crossed the threshold for overt diabetes at day 15 post-surgery (approximately 5 weeks of age, Figure 56B). Male KINGS mice implanted with E2 capsules that were not replaced had a delayed onset of diabetes compared to the vehicle treated group, but still crossed the threshold for overt diabetes at 37 days post-surgery and blood glucose concentrations were not significantly different from the vehicle treated group from 45 days. Mice implanted with oestradiol capsules which were then replaced at 21 days with vehicle capsules showed a similar development of diabetes, crossing the 16.7mM threshold at the same time and blood glucose concentrations did not differ from KINGS vehicle mice from 38 days post initial surgery. The similar blood glucose profiles between these groups suggests that it is the depletion of oestradiol that drives an increase in blood glucose concentrations. In line with this, KINGS mice implanted with an E2 capsule that was then replaced with another E2 capsule (so did not experience E2 depletion) did not develop overt diabetes, remaining below the 16.7mM threshold for the duration of the experiment and had significantly lower blood glucose concentrations compared to vehicle treated mice from 17 days post-implantation surgery until the end of the study. Blood glucose concentrations in the KINGS E2 + E2 replacement group were also significantly lower compared KINGS E2 + vehicle replacement group at 38, 40, 43 and 47 days post initial surgery, and compared to the KINGS E2 no replacement group at 40 and 47 days.

To determine whether oestradiol administration was associated with changes in insulin sensitivity, which may contribute to changes in non-fasted blood glucose concentrations seen in the KINGS mice treated with E2, we attempted to perform insulin tolerance tests in mice from the previous study where mice either received silastic capsules containing E2 or vehicle. However, none of the groups showed any response to insulin suggesting a technical failure (data

not shown). We hypothesised that the stock insulin may have degraded over time and thus ordered new insulin. We used this to carry out insulin tolerance tests on a small subset of KINGS mice from the E2 replacement study at 6 weeks of age (prior to the second surgery). Basal 2-hour fasted blood glucose concentrations were considerably different (11.4mM) between oestradiol treated and non-treated groups, making the data difficult to interpret (Figure 57A). The data was therefore also presented as percentage of basal glucose concentration to overcome this issue (Figure 57B). Area above the curve values were significantly higher in the KINGS mice treated with E2 indicating that they are more insulin sensitive compared to the vehicle control group (Figure 57C).

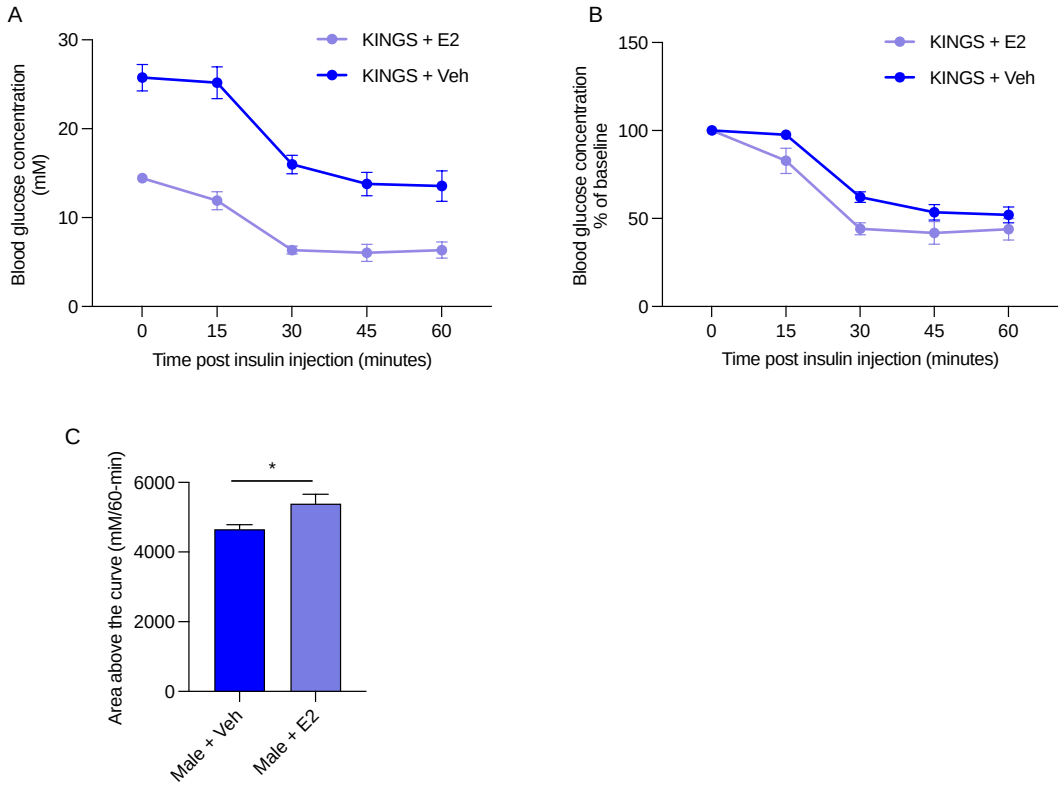


FIGURE 57: *Insulin tolerance tests in male KINGS mice administered E2 or vehicle:*

*(A) Raw values and (B) normalised blood glucose measurements for an insulin tolerance test carried out in KINGS mice treated with E2 or vehicle (veh) for 3 weeks from weaning. (C) Area above the curve (AAC) for normalised insulin tolerance test values. Unpaired T-test, *P<0.05, n=4.*

We subsequently wanted to investigate whether the protective effects of E2 on blood glucose concentrations may also be caused by direct effects on beta cell function. A static insulin secretion assay was performed on islets isolated from 7-week-old male KINGS and WT mice implanted with oestradiol/vehicle capsules from 3 weeks. Islets from the subset of male KINGS mice implanted once with either oestradiol or vehicle containing capsules but culled at 10 weeks of age were also included in this assay. The capsules were presumed to be depleted in these older mice, and so E2 or vehicle were no longer available.

E2 administration in male KINGS mice was associated with an improvement in insulin secretion when islets were exposed to 20mM glucose (Figure 58B). However, this was not the case for wildtype male islets where insulin secretion levels at 2mM and 20mm glucose were comparable between oestradiol and vehicle treated groups (Figure 58A). Intriguingly, E2-treated male KINGS mice that were followed for a longer time after implantation surgery and that became overtly diabetic, still showed a significant improvement in islet insulin secretion in 20mM glucose solution compared to vehicle-treated controls (Figure 58C). No differences were seen in insulin secretion with 2mM basal glucose with E2 treatment in any of the groups.

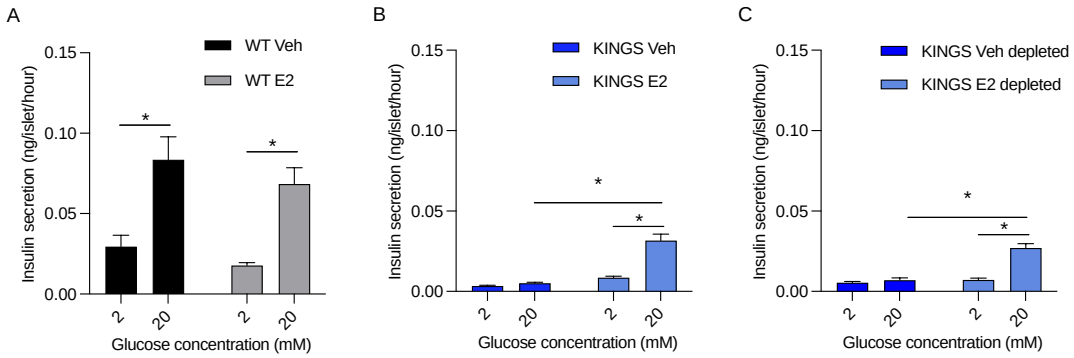


FIGURE 58: *Glucose stimulated insulin secretion in islets from male mice treated with E2 or vehicle:*

Glucose stimulated insulin secretion in islets isolated from (A) 7-week-old WT male mice implanted with vehicle (veh) or E2 containing silastic capsules at 3 weeks of age, (B) 7-week-old KINGS male mice implanted with vehicle or E2

*containing silastic capsules at 3 weeks of age and (C) 10-week-old KINGS male mice implanted with vehicle or E2 containing silastic capsules at 3 weeks of age which are presumed to have been depleted. Data represents the mean±SEM. *p<0.05, two-way ANOVA with Holm-Sidak post hoc test, n=6-19 replicates from 3-8 animals.*

To investigate whether E2 may be mediating protective effects on the beta cell by reducing ER stress, immunofluorescence was used to compare expression of the general ER stress marker, BiP, in beta cells from oestradiol-treated and vehicle-treated mice. As expected and consistent with previous findings, BiP expression was higher in KINGS islets compared to WT islets (Figure 59, section 4.4.1). E2 treatment was associated with a reduction in BiP expression in islets from both wildtype and 7-week KINGS male mice. In contrast, in male KINGS mice followed up for a longer time after E2 silastic capsule implantation that subsequently developed overt diabetes, although there was a trend for reduced BiP expression compared to vehicle controls this did not reach significance.

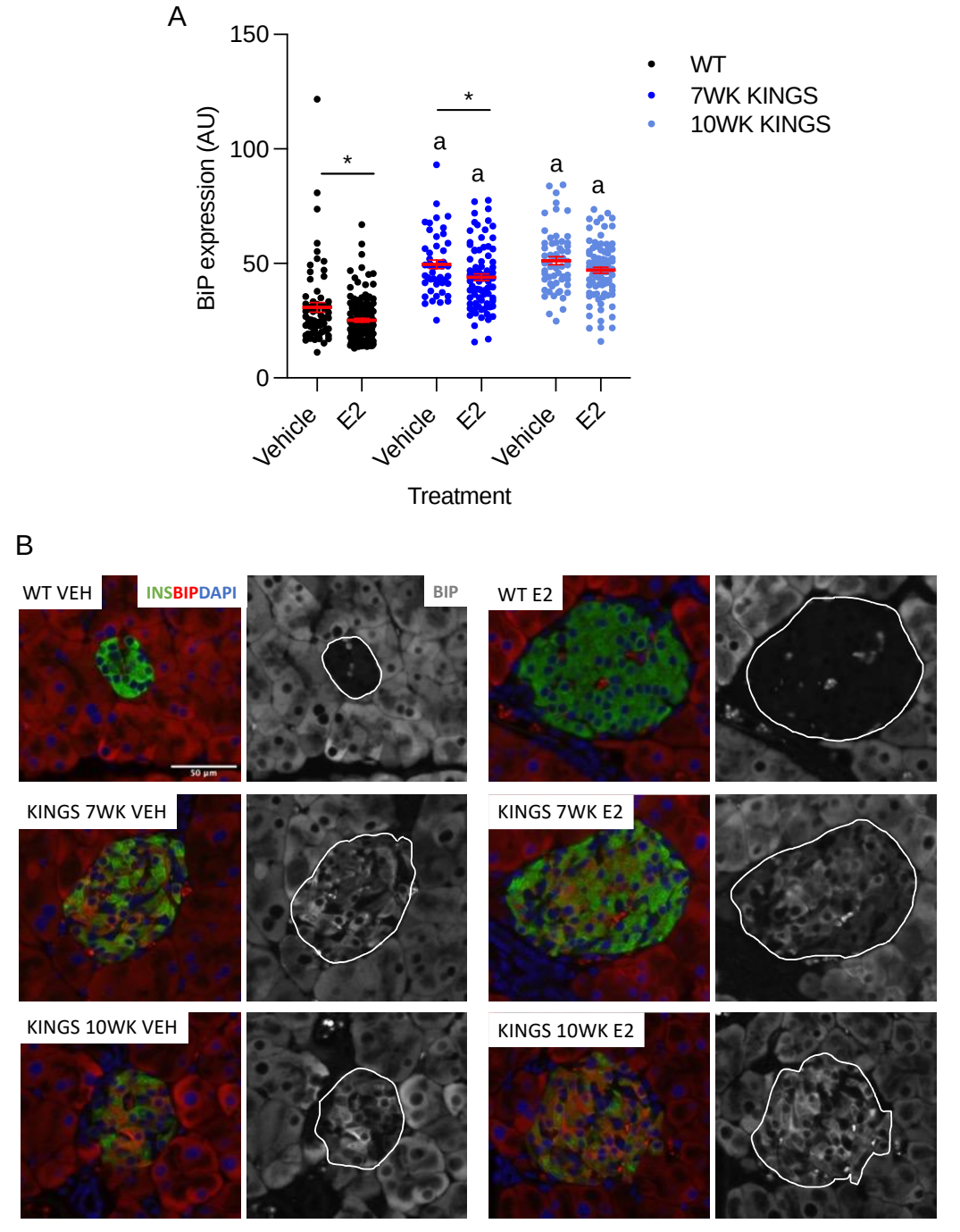


FIGURE 59: *BiP expression in islets from male mice treated pre-puberty with E2 or vehicle:*

*(A) BiP mean fluorescence intensity in islets from 7-week-old WT male mice implanted with vehicle (veh) or E2 containing silastic capsules at 3-weeks of age (black), 7-week-old KINGS male mice implanted with vehicle or E2 containing silastic capsules at 3 weeks of age (pale blue) and 10-week-old KINGS male mice implanted with vehicle or E2 containing silastic capsules at 3 weeks of age (dark blue). *= E2 vs. veh p<0.05, a=vs WT veh p<0.05, two-*

way ANOVA with Holm-Sidak post hoc, data represents n=59-131 individual islets from 3-5 animals and mean±SEM is indicated in red. (B) Representative islets from WT and KINGS mice treated with E2 or vehicle stained for insulin (green), BiP (red) and DAPI (blue). BiP is also represented alone in grey, and the islet outline is indicated by the white line. The scale bar in the top left image represents 50mm.

5.5 Discussion:

Sex differences in diabetes in humans as well as in animal models have long been recognised and E2 has consistently been implicated in driving this. The KINGS mouse model of beta cell ER stress also displays stark sex differences with male KINGS mice developing overt diabetes by 5-6 weeks of age whilst the female mice remain protected. Since beta cell ER stress has been associated with diabetes pathogenesis, the role of E2 in generating sex differences in the KINGS mouse was investigated as well as whether organisational and activational effects of E2 differentially contribute to generating this sex difference. Finally, we also investigated whether E2 mediates at least some of its effects by acting directly on the beta cell.

Endogenous E2 removal from female KINGS mice both pre- and post-puberty via ovariectomy resulted in a mild increase in non-fasted blood glucose concentrations (maximum 5mM increase) suggesting they may be somewhat protected by ovarian-derived E2. Despite this, ovariectomised female KINGS mice did not exhibit non-fasting blood glucose concentrations in the diabetic range (>16.7mM) and phenotype was not as severe compared to KINGS male mice. This suggests that oestradiol cannot completely explain the female protection from hyperglycaemia in this model.

These results are similar to findings by Xu *et al.* and De Paoli *et al.* who found that ovariectomising Akita mice and ApoE-/- Akita mice, respectively, increased blood glucose concentrations (De Paoli et al., 2022; Xu et al., 2018). However, this increase was greater in the Akita mice (up to a maximum increase of 8-9mM) compared to what was observed in the KINGS mice in this study (5mM) and occurred over a shorter timescale (3 days post-surgery

compared to 7 weeks post-surgery in the KINGS mice). Similarly, De Paoli *et al.* also found that ovariectomy in ApoE-/- Akita mice resulted in a greater increase in blood glucose levels (reaching 20mM) compared to the KINGS mice (De Paoli et al., 2022). The latter suggests that the impairment in glucose homeostasis caused by ovariectomy is less severe in the KINGS model. One possible reason for this is that glucose homeostasis impairment (irrespective of the ovariectomy) is less severe with the KINGS mutation, and indeed it has previously been found that KINGS mice show reduced non-fasted blood glucose concentrations when directly compared to Akita mice (Austin et al., 2020). Another study showed that ovariectomy in the Munich mice also resulted in an increase in non-fasted blood glucose concentrations by 5 days post-surgery which rose to approximately 20mM- again this phenotype is more severe than that seen in the KINGS ovariectomised mice (Schuster, 2011).

E2 levels rapidly rise during puberty (from around 30 days of age in mice, Figure 60) and therefore ovariectomising mice prior to this increase prevents the organisational as well as transient effects of this sex hormone, whilst ovariectomy after puberty only removes the transient effects. KINGS mice ovariectomised after sexual maturation showed a greater increase in non-fasted blood glucose concentrations compared to the pre-puberty ovariectomised mice; a 5mM maximal increase was seen in KINGS mice ovariectomised post-puberty whilst this increase was only 2.7mM in the pre-puberty ovariectomised KINGS mice. The activational effects of oestradiol (those that occur at any age, and which are transient) therefore appear to be more critical in glucose homeostasis in this model compared to the organisational effects of oestradiol (those that occur during developmental programming, and which are permanent). Xu *et al* also investigated the effects of both pre- and post-puberty ovariectomy and found that both were associated with elevated blood glucose concentrations (Xu et al., 2018). Although the two were not directly compared, ovariectomised mice in both studies had similar maximum blood glucose concentrations (~14-15mM) supporting the idea that activational E2 effects are more important for glucose homeostasis compared to organisational effects.

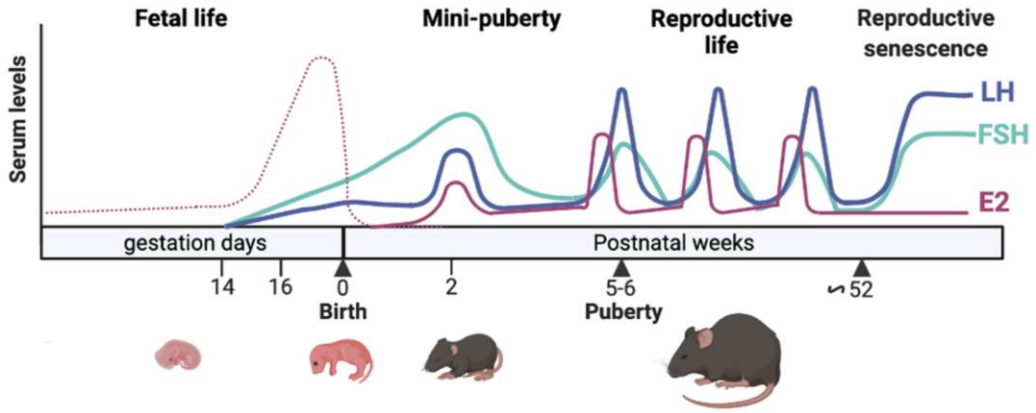


FIGURE 60: *Schematic showing levels of oestradiol (E2), luteinising hormone (LH) and follicle stimulating hormone (FSH) in female mice throughout life from gestation:*
The major sex-hormones related events are also indicated. The dotted pink line indicates maternal oestradiol. Image taken and adapted from (Devillers et al., 2022).

Surprisingly and in contrast to findings in female KINGS mice, exogenous E2 delivery to male KINGS mice completely prevented the development of overt diabetes and maintained blood glucose concentrations within a normoglycemic range. This suggests that whilst oestradiol is not the only factor mediating sex differences in the KINGS phenotype, it is sufficient to rescue KINGS males from diabetes. Xu *et al* made similar findings in the Akita mouse, although it is worth noting that in their study Akita-vehicle mice exhibited only mild hyperglycaemia (blood glucose concentrations ~16-17mM) which is much lower than what has previously been reported in this model and in the KINGS mice and this may have influenced the ability of E2 to rescue diabetes (Xu et al., 2018).

It is possible that the reason that E2's introduction/removal had differing effects on diabetes outcome in male and female KINGS mice was because circulating E2 concentrations differed. Male mice were implanted with silastic capsules containing 36µg/mL E2. Ingberg *et al* found that this resulted serum E2 concentrations just under 100pg/mL at day 7 post-implantation which slowly decreased to ~35pg/mL by day 28 (Ingberg et al., 2012). E2's

physiological range in female mice is thought to be between 5-35pg/mL and therefore it is likely that serum concentrations of E2 in the treated KINGS males exceeded physiological concentrations. Indeed, by 28 days post implantation blood glucose concentrations in the KINGS mice began to increase and approach the threshold for overt diabetes. This suggests that physiological concentrations of oestradiol may not be sufficient to prevent diabetes in the KINGS mice, supporting our findings that endogenous oestradiol removal from the KINGS mice does not induce diabetes, and suggesting that sex differences in the KINGS phenotype is not only driven by oestradiol. In future, more insight could be provided into this by lowering the dose of oestradiol administered to male KINGS mice in an endeavour to achieve more physiological E2 concentrations. It would also be beneficial to be able to measure plasma E2 concentrations. Unfortunately, the majority of enzyme-linked immunosorbent assays (ELISA's) available to investigate plasma E2 are not sensitive enough to detect E2 levels in mice and those that are have been found to be unreliable (Haisenleder et al., 2011). The one available ELISA that has shown reliability was discontinued, but a human E2 ELISA supplied by the same company was used in an attempt to measure E2 levels in serum samples since E2's structure is the same between humans and mice (Calbiotech, USA). However, a pilot study using this ELISA found no differences in plasma E2 levels between post-puberty sham and ovariectomised mice suggesting that it cannot be dependably used to measure E2 levels. It should be noted that successful ovariectomy had been determined in these mice by checking for oestrous cycling.

The finding that the protective influence of oestradiol is lost ~5 weeks post implantation suggests that the beneficial effects of oestradiol on glucose homeostasis are transient rather than permanent. This corresponds with our findings that pre-puberty ovariectomy in the KINGS female mice was not associated with a more severe impairment in glucose homeostasis compared to post-puberty ovariectomy and suggests that it is the activational effects of E2 which may be somewhat protecting female KINGS mice from developing overt diabetes. Supporting this, continued protection from diabetes was seen in KINGS male mice where the initial E2 capsule was replaced by a new E2

capsule midway through the study (providing a constant E2 supply). This was not the case when capsules were exchanged for vehicle containing capsules or not replaced. Diabetes prevention is more achievable than reversal, however the latter is more clinically relevant. Therefore, in future it would be interesting to investigate whether oestradiol is capable of reversing diabetes in the KINGS males after they have developed diabetes.

Despite our findings that endogenous E2 removal increases non-fasted blood glucose concentrations in female KINGS mice pre- and post-puberty, and E2 treatment prevents diabetes in male KINGS mice, we found no effect of oestradiol removal or treatment on glucose tolerance. This may suggest that oestradiol is not protective under conditions of increased beta cell stimulation and perhaps only under physiological fluctuations in blood glucose concentrations. Indeed, this correlates with the fact that female KINGS mice are glucose intolerant despite being able to maintain non-fasted blood glucose concentrations within a normoglycemic range. This contrasts findings in the Munich mice that post-puberty ovariectomy resulted in impaired glucose tolerance compared to sham-operated mice (Schuster, 2011). It is worth noting that oral glucose tolerance tests were carried out in the Munich mouse study, however, which may go some way to explaining the differences in findings. Indeed, studies have suggested that oestradiol may be involved in elevating the incretin response since women show an enhanced GLP-1 response following oral glucose tolerance testing (Færch et al., 2015). It is also worth noting that whilst numerous studies have mostly shown that ovariectomy in wildtype mice does not affect glucose tolerance (in line with our findings), when paired with a diabetogenic phenotype or environment it impairs glucose tolerance further compared to sham controls. For example, in one study, a high fat diet was only found to impair glucose tolerance when female mice were ovariectomised (Riant et al., 2009). Our findings are not in line with this. Differences may be explained by differences in the pathogenesis underlying metabolic dysfunction between the models. In the high fat fed mouse for example, beta cell dysfunction as well as insulin resistance contributes to glucose intolerance, whereas in the KINGS mouse, pathogenesis is beta-cell specific. Oestradiol can influence glucose homeostasis through actions on

endocrine and non-endocrine tissues, and since the underlying pathogenesis of the models differs, it is possible that so too does the effect of E2 removal on glucose tolerance.

Insulin sensitivity was improved in KINGS male mice administered E2 and this may contribute to the improvement in glucose homeostasis seen in this group. Contrasting this finding however, ovariectomising WT and KINGS females had no impact on insulin sensitivity. It should be noted that insulin tolerance was difficult to directly compare between groups as basal blood glucose concentrations varied (male-E2 KINGS mice had a lower basal glucose compared to vehicle treated mice, and female KINGS ovariectomised mice had a higher basal glucose level compared to sham operated mice). The current literature broadly suggests that ovariectomy has minimal effect on insulin sensitivity in physiologically healthy rodent models, but in the context of metabolic dysfunction (such as when placed on a high fat high diet) ovariectomy exacerbates impairments in insulin sensitivity. For example, Fahmy *et al.* found that ovariectomy only exacerbated insulin resistance when an impairment in insulin sensitivity was already present in high fat fed rats (Fahmy et al., 2018). In addition, in contrast to our findings, ovariectomy in the Munich mouse (a similar model of beta cell ER stress to the KINGS mouse) did impair insulin sensitivity investigated through insulin tolerance testing. However, it is notable that this impairment was subtle. In the ApoE-/- Akita mouse, ovariectomy was also found to impair insulin sensitivity, with HOMA-IR values increasing more than double that of sham-operated mice (Venegas-Pino et al., 2016). It is possible that subtle differences in insulin sensitivity between ovariectomised and sham-operated mice in the present study were not detected using insulin tolerance tests which may be the reason for differences in findings. A more sensitive approach to measuring insulin sensitivity using hyperinsulinemic-euglycemic clamping may be of benefit for future studies (Kennard et al., 2021).

Another factor which may go some way to explaining non-fasted blood glucose concentration differences with E2 removal/administration is changes in weight gain since a higher body weight is associated with an increased insulin

demand. Indeed, post-puberty endogenous E2 removal in the female KINGS mice was associated with an increase in weight gain whilst exogenous E2 administration in male KINGS mice was associated with a reduced weight gain. These results were not surprising since a large body of research has implicated oestradiol in regulating fat mass. Evidence includes findings that the menopause is associated with an increase in fat mass (Juppi et al., 2022). Studies in rodent models have additionally shown that ovariectomy results in an increase in weight gain whilst its exogenous replacement rescues this phenotype, although some studies have not found this (Mason et al., 2010; Nishio et al., 2019; Zhu et al., 2013). The effect of E2 on fat mass has been attributed to many factors including its effects of energy intake and expenditure and fat distribution. For example, E2 has been shown to influence adipocyte leptin expression, an important hormone in regulating energy homeostasis which exerts activity in the hypothalamus (where it influences the expression of neuropeptide Y and pro-opiomelanocortin among other neuropeptides responsible for food intake regulation). Ovariectomy in various animal models and the menopause in humans has been associated with reduced circulating leptin levels and this may go some way to explaining differences in weight gain seen in the KINGS mice (Dedeoğlu et al., 2009; Fungfuang et al., 2013). We only monitored food intake in the post-puberty ovariectomised mice in this study and found no differences between the groups, although in future it would be interesting to measure this through a more sophisticated method for example through using metabolic cages.

Although changes in weight may somewhat explain changes in glycaemic control seen in ovariectomised female and E2- treated male KINGS mice, it is important to note that ovariectomised female KINGS mice still reached the same weight as the male KINGS mice are when they become overtly diabetic. Therefore, differences in weight between male and female mice is unlikely to be the sole driver of sex differences in diabetic phenotype. It has previously been found that impaired insulin secretory function contributes to lack of glycaemic control in the KINGS mouse, and this is more severe in the male KINGS mice (Austin et al., 2020). To determine whether the latter and the observed effects of E2 removal/administration on non-fasted blood glucose

concentrations in the current study were due to E2's effect on glucose stimulated insulin secretion (GSIS), islets were isolated from ovariectomised female and E2-treated males and subjected to an insulin secretion assay. We broadly found that E2 was associated with improved GSIS in both male and female KINGS mice, which is in line with current literature that suggests that E2 improves insulin biosynthesis and secretion (Soriano et al., 2009; Wong et al., 2010). Whilst a similar result was noted for WT males administered E2, ovariectomy in WT females subtly enhanced GSIS. Veras *et al.* also found a subtle improvement in GSIS in islets derived from ovariectomised rats compared to sham controls however introduction of a high fat diet on top of ovariectomy impaired GSIS (Veras et al., 2014). This is in line with our findings that ovariectomy only impaired GSIS when paired with an additional metabolic stressor (the KINGS mutation). The small increase in GSIS that accompanies ovariectomy in WT mice may be a result of a subtle increase in weight gain. Intriguingly, although we largely found that exogenous and endogenous E2's effect on glycaemic control was transient, we found that improved GSIS associated with exogenous E2 delivery to male KINGS mice was maintained in mice where E2 is thought to have been depleted. This may suggest that some beneficial effects of E2 on islet function may be longer lasting. Indeed, for all the static secretion experiments, islets were cultured overnight in the absence of oestradiol but despite this, effects of oestradiol treatment were still observed.

One of the ways in which E2 has been associated with improving beta cell function is through reducing beta cell ER stress, although this mainly comes from evidence from *in vitro* studies. For example, Xu *et al* have recently shown that E2 signalling through ER-alpha helps to stabilise ER-associated degradation (ERAD) thereby promoting proteasomal degradation of misfolded proteins and alleviating beta cell ER stress (Xu et al., 2018). We investigated whether the presence of E2 was associated with a reduction in beta cell ER stress *in vivo* in the KINGS males and females. Islet BiP expression was increased in ovariectomised female KINGS mice, whilst E2 administration in male KINGS mice reduced BiP expression. This suggests that oestradiol reduces beta cell ER stress *in vivo* and that this may at least in-part contribute

to the augmentations to non-fasted blood glucose concentrations seen in ovariectomised KINGS females and E2 treated KINGS males, and possibly account in-part for sex differences seen in the KINGS phenotype. Interestingly, islets from E2-treated 10-week-old male KINGS mice showed a trend for a reduction in BiP expression compared to vehicle controls but this was not significant. At this time, E2 silastic capsules had been implanted for 7 weeks and were assumed to be depleted. Since the significant reduction in BiP is lost when E2 is no longer present, this further supports the notion that the protective effects of oestradiol are transient.

We believe that this is the first study to show that oestradiol can reduce beta cell ER stress *in vivo*. Previous studies, although have shown that oestradiol improves glucose homeostasis *in vivo*, have only used *in vitro* studies to suggest that this may involve augmentation of beta cell ER stress levels. For example, studies on MIN6 cells, *Ins2*$^{+/Akita}$ INS-1 cells and Akita mouse beta cells treated with ER stress inducers (including glucosamine and thapsigargin) have shown that E2 treatment aids in resolving ER stress, showing that it reduces the expression of ER stress markers including BiP, ATF4, CHOP and XBP1s (L. Kang et al., 2014; Xu et al., 2018). It is important to note that high glucose levels have also been associated with enhanced beta cell ER stress, complicating our finding that exogenous E2 administration in male mice reduced beta cell ER stress since E2 treated mice had lower blood glucose levels compared to the vehicle-treated mice. With this in mind however, E2 treatment still reduced beta cell ER stress in the male WT mice despite their blood glucose concentrations being within a similar (and normoglycemic) range to the vehicle treated WT males. This suggests that the effect of E2 on beta cell ER stress is direct and not an indirect result of reduced blood glucose levels. To remove this caveat in future experiments, it would be interesting to control for differences in blood glucose levels by treatment with phlorizin or endogenous insulin, or islet transplantation prior to or during E2 administration.

It is worth also bearing in mind that our study did not consider the transient endogenous E2 rise that occurs during the mini-puberty of infancy at from

around 2 weeks after birth in female mice and which is conserved across other mammals including humans (Figure 60) (Devillers et al., 2022). At this time, a surge in oestradiol is thought to drive differentiation and development of a variety of tissues including the mammary gland and uterus. It is possible that at this stage oestradiol may also drive sex specific changes at the beta cell level which may be important in female protection against the development of diabetes. However, to date this has not been investigated. In future it would be interesting to investigate the role of the mini puberty E2 surge in generating sex differences in the KINGS mice. It may be technically challenging to achieve removal of endogenous E2 in mice at such a young age, although since many beneficial effects of E2 on the beta cell are thought to involve signalling via the oestrogen receptors an option would be to use oestrogen receptor antagonists or use oestrogen receptor knockout mice.

To conclude, we have shown that whilst exogenous E2 administration in the KINGS male mice was able to prevent the development of diabetes through at least in-part reducing beta cell ER stress and improving beta cell insulin secretion, its endogenous removal through ovariectomy in female mice did not cause the development of overt diabetes and only led to a mild impairment in glycaemic control. This suggests that whilst oestradiol may play some role in generating sex differences in diabetes in the KINGS mouse, other factors must also be involved. Our results are consistent with those in the literature suggesting that E2 protects against diabetes in-part through reducing beta cell ER stress. In addition, we have also shown that the activational (transient) rather than the organisational (chronic) effects of oestradiol appear to be more important in driving sex differences in glucose homeostasis since pre-pubertal ovariectomy in KINGS females did not impair glycaemic control beyond that of the post-puberty ovariectomy. To our knowledge, this is the first study to focus on the different contributions of activational and organisational effects of E2 on glucose homeostasis. Together, these results shed more light on what mediates sex differences in diabetes.

Chapter 6: Can manipulation of beta cell ER stress in the KINGS mice abolish sex differences in diabetes phenotype?

Chapter snapshot:

Beta cell ER stress is implicated in diabetes pathogenesis in humans and drives diabetes development in the KINGS model. Although female KINGS mice exhibit beta cell ER stress, they do not develop diabetes suggesting female beta cells respond adaptively to this.

The aim of this study was to investigate whether female KINGS mice are still able to respond adaptively when ER stress is exacerbated through high fat high sucrose feeding, which mimics the dietary excess often associated with T2DM. This may give insight into the concept that beta cell ER stress beyond a critical threshold drives diabetes development and how female KINGS mice are protected from diabetes.

6.1 Introduction:

We have shown in previous chapters that a heterozygous KINGS mutation in *Ins2* results in beta cell ER stress in both male and female mice (see Chapter 4), however only male mice develop overt diabetes. It is unclear whether female KINGS mice respond adaptively and maintain normoglycemia because they have reduced beta cell ER stress levels or whether their beta cells respond adaptively to the ER stress. Much of the literature suggests that ER stress levels correlates with cellular fate; moderate and resolvable levels of ER stress induce an adaptive response (which inevitably restores ER protein homeostasis and thus promotes cellular survival), on the other hand, once a critical ER stress threshold is met this leads to unfavourable outcomes for the cell including a loss of identity and eventually death (Figure 61)(Ghosh et al., 2019; Lenghel et al., 2021).

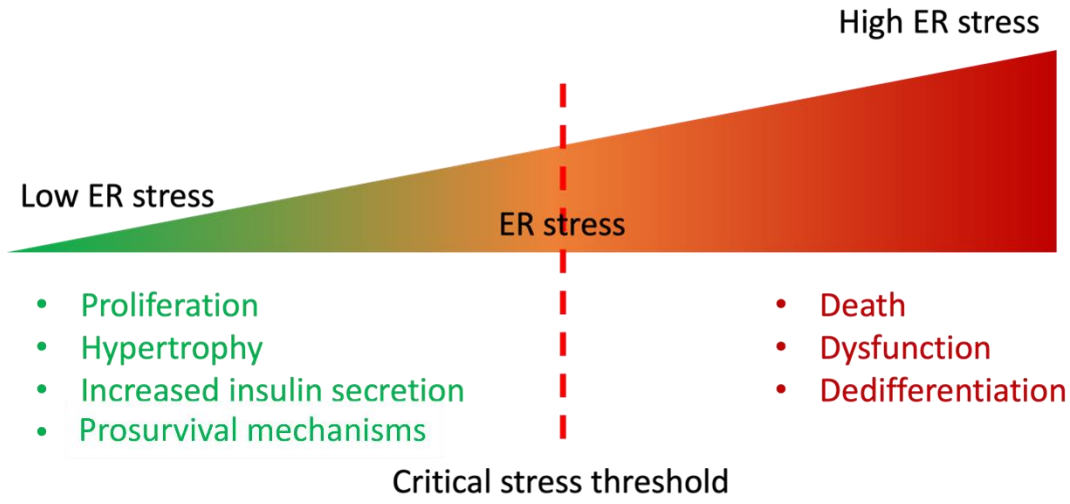


FIGURE 61: *Beta cell ER stress levels correlate with cellular fate: Low levels of ER stress which are resolved through UPR signalling induce adaptive beta cell responses such as beta cell proliferation and enhanced insulin secretion. However, when ER stress reaches a critical threshold where it is unable to be resolved, maladaptive UPR signalling ensues which drives beta cell death, dysfunction, and dedifferentiation.*

Studies have shown that this is true for activation of the ATF6 UPR pathway, for example. Sharma *et al.* showed that ATF6 was necessary for a beta cell proliferative response to ER stress both *in vitro* and *in vivo* in the Akita and $Lep^{db/db}$ mice. Additionally, ATF6-/- mice fed a high fat diet experience increased beta cell ER stress, impaired glucose tolerance and diminished insulin secretory capacity, and several ATF6 polymorphisms have been associated with T2DM in humans (Sharma et al., 2015; Usui et al., 2012). However, ATF6 overexpression has been shown to be harmful; overexpression in INS-1 cells reduces activity of the insulin gene promotor, reduces levels of beta cell-defining proteins (such as PDX1, MAFA, NEUROD-1), and abolishes glucose stimulated insulin secretion (Seo et al., 2008). Similarly, signalling through the IRE1-alpha arm of the UPR is adaptive under mild ER stress but enhanced signalling is associated with beta cell dysfunction and death. IRE1 deletion in mice induces hyperglycaemia and similarly deletion of XBP1 (a component of the IRE1 downstream signalling pathway) induces beta cell dedifferentiation *in vitro* and its knockdown in high fat fed and

Lep$^{ob/ob}$ mice reduces insulin secretory capacity causing impaired glycaemic control (Hassler et al., 2015; Lee et al., 2022). Conversely, robust IRE1 signalling in beta cells from rat islets has been shown to cause dedifferentiation, reduced glucose stimulated insulin secretion as well as a downregulation of *Mafa, Ins2, Pdx1* and *Glut2* (Allagnat et al., 2010). Finally, whilst PERK signalling is critical in maintaining beta cell function and survival, excessive signalling through this pathway promotes dysfunction and death. Global PERK knockdown in mice causes a progressive loss of beta cells and subsequent development of diabetes, and specific beta cell PERK knockdown also results in a loss of beta cell mass and diabetes (Gao et al., 2012; Zhang et al., 2002). In contrast, excessive signalling through PERK has also been associated with maladaptive outcomes for the beta cell with PERK overexpression in mice shown to impair beta cell glucose stimulated insulin secretion (Sharma et al., 2021).

As described, there is a delicate balance between adaptive and maladaptive signalling through the UPR, and optimal signalling is critical in the maintenance of a functional beta cell mass. Numerous factors have been implicated in manipulating this balance, causing the threshold for maladaptive UPR signalling to be met. This includes gene mutations, such as the KINGS and Akita mutations, but also environmental factors. Obesity is one factor that is of particular interest because rates of obesity have almost tripled from 1975 and an estimated 39% of adults worldwide are currently classed as overweight (World Health Organisation, 2021). Obesity is the leading risk factor for the development of T2DM, and one of the pathological drivers behind this is believed to be excessive ER stress. Indeed, nutrient excess (the main driver of obesity) has been linked to enhanced adipose ER stress and the development of insulin resistance in many studies. For example, adipose ER stress marker expression is increased in high fat fed mice and in obese individuals (Boden et al., 2008; Kawasaki et al., 2012).

Evidence also exists suggesting that excess nutrient intake induces beta cell ER stress, although this is less well characterised. Yi *et al.* recently found that mice fed a high fat diet showed increased beta cell expression of the UPR

markers ATF6 and phosphorylated e-IF2-alpha (Yi et al., 2020). Excess nutrient consumption may induce ER stress through enhancing levels of free fatty acids, and indeed *in vitro* beta cell exposure to palmitate has been shown to enhance expression of ER stress markers including XBP1, ATF4, phosphorylated-eIF2-alpha and ATF6 (Karaskov et al., 2006; Yi et al., 2020). However, since excessive nutrient intake also induces peripheral insulin resistance, enhanced insulin demand also likely plays a role in increasing beta cell ER stress since the requirement to synthesise and fold more insulin places a greater burden on the ER (Shrestha et al., 2021). The $Lep^{ob/ob}$ and $Lep^{db/db}$ mouse models of obesity both exhibit insulin resistance and accordingly both show elevated beta cell ER stress (Chan et al., 2013). More research is needed into whether dietary excess can (and in what circumstances) tip the balance from an adaptive UPR to a maladaptive UPR, however.

In this chapter, we investigate whether high fat high sucrose feeding in female KINGS mice can exacerbate beta cell ER stress and tip the balance in ER stress signalling to induce overt diabetes seen under normal conditions in male KINGS mice. We chose a high fat high sucrose diet (HFHS: 45kcal% sucrose, 30kcal% fat) as opposed to the more classically used high fat diet which has a higher fat content (60kcal% fat). This was because the lower fat content and addition of sucrose is thought to be more representative of a western diet in humans and therefore this model system is more relevant to beta cell ER stress in the context of human type 2 diabetes.

Understanding whether exacerbation of beta cell ER stress in the female KINGS mice can induce diabetes may give insight into how they are able to adapt to beta cell ER stress and are protected from a more severe phenotype. It may also give insight into what circumstances dietary excess can contribute to loss of glycaemic control which may be relevant to understanding how obesity can lead to T2DM in humans. Indeed, female KINGS mice are glucose intolerant, but blood glucose levels are still below the threshold of overt diabetes, therefore they may represent the pre-diabetic state in T2DM in humans.

6.2 Aims:

- To determine whether high fat high sucrose feeding exacerbates beta cell ER stress in the female KINGS mice.
- To investigate the impact of high fat high sucrose feeding on glycaemic control in the KINGS females.

6.3 Methods:

6.3.1 Experimental outline:

Female WT and KINGS mice were fed a high-fat-high-sucrose diet or a normal chow diet for 17 weeks from weaning (at 3 weeks) in an attempt to exacerbate beta cell ER stress (Figure 62). To assess beta cell ER stress, western blot was used to assess the protein levels of ER stress markers in islet protein lysates. To assess the effect of increased beta cell ER stress on glycaemic control, non-fasted and fasted blood glucose concentration measurements were taken, and glucose tolerance tests were performed. Insulin tolerance tests were also carried out to ascertain whether insulin resistance was in-part responsible for any changes in glucose homeostasis.

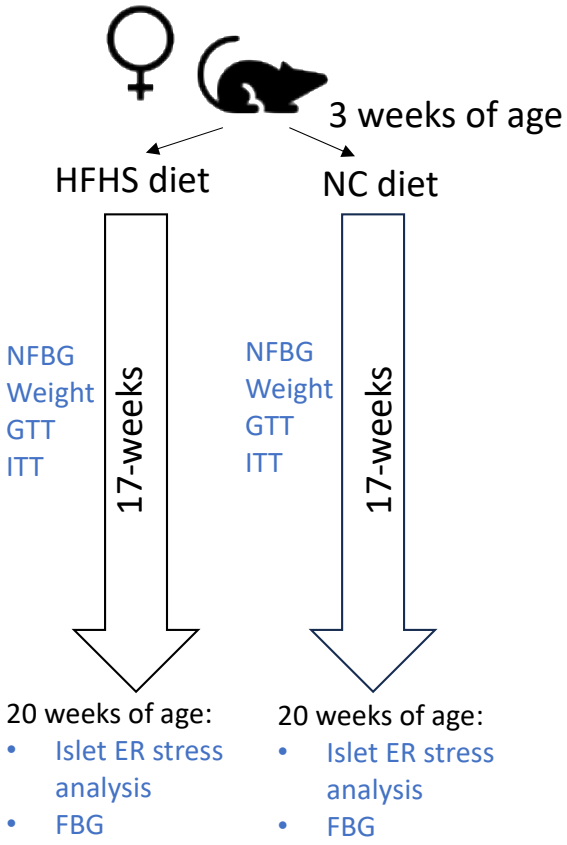


FIGURE 62: *Schematic showing the main experimental outlines to assess whether exacerbating beta cell ER stress in the female KINGS mice can induce the development of overt diabetes:*
Experimental readouts are in blue: non-fasted blood glucose measurements (NFBG), fasting blood glucose (FBG), glucose tolerance test (GTT) and insulin tolerance test (ITT). High-fat-high-sucrose (HFHS), normal chow (NC).

A pilot study was also conducted to investigate whether further stress on the beta cells would exacerbate diabetes male KINGS mice (Figure 63). WT and KINGS male mice were fed a high-fat-high-sucrose diet or normal chow diet for 7 weeks from weaning (3 weeks). This was a shorter time-period compared to the female study due to the ethical concerns of further exacerbating poor glycaemic control over an extended period in these mice. Non-fasted blood glucose concentrations were monitored, and glucose tolerance tests were carried out to assess blood glucose homeostasis. Insulin tolerance tests were also carried out to determine whether insulin resistance contributed to any differences seen in glucose handling.

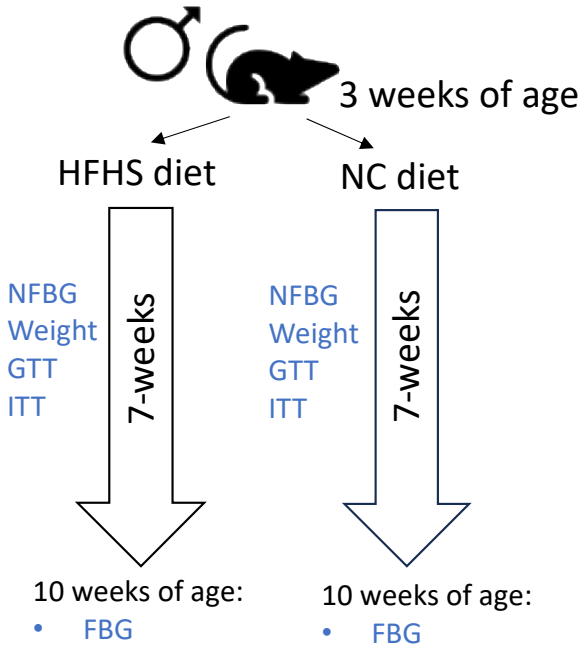


FIGURE 63: *Schematic showing the main experimental outlines to assess whether glucose homeostasis can be further impaired by exacerbating beta cell stress in the male KINGS mice:*

Experimental readouts are in blue: non-fasted blood glucose measurements (NFBG), glucose tolerance test (GTT) and insulin tolerance test (ITT). High-fat-high-sucrose (HFHS), normal chow (NC).

6.3.2 Dietary intervention:

Mice were either fed normal chow (NC: 13.2kcal% fat, 3.18kcal% sucrose, Rodent diet 20, PicoLab, UK), or a high-fat-high-sucrose (HFHS: 45kcal% fat, 30kcal% sucrose, ID: D09112601. Research Diets Inc, USA) diet from weaning at 3 weeks of age. Both diets were irradiated for sterilisation and were provided *ad libitum*. Mice placed on the HFHS diet tended to grind their feed, and as a result rather than placing the food in the cage feeders, food was provided daily at the base of the cages and any food that was soiled was removed.

6.3.3 Non-fasted blood glucose concentration and weight monitoring:

Non-fasted blood glucose measurements were measured in the morning (9-10am) by pricking the end of the tail with a 30G needle and applying the blood droplet generated to an AccuCheck test strip and AccuChek meter. Animal weight was subsequently recorded. Non-fasted blood concentrations were measured after a 16-hour overnight fast at 20 weeks of age (female mice) and 10 weeks of age (male mice).

6.3.4 Glucose tolerance tests:

Glucose tolerance tests were conducted at different intervals throughout the study so that any progressive deterioration in glucose tolerance could be observed. Mice were fasted for 6 hours prior to the glucose tolerance tests and remained fasted throughout the experiment. After a baseline blood glucose measurement was taken, 30% glucose solution (2g/Kg) was injected intraperitoneally. Blood glucose concentrations were subsequently taken at 15, 30, 60, 90 and 120 minutes post injection. Any mouse with a basal blood glucose concentration of >20mM was not subjected to a glucose tolerance test.

6.3.5 Insulin tolerance tests:

Insulin tolerance tests were conducted at different intervals throughout the study so that any progressive deterioration in insulin sensitivity could be observed. Mice were fasted for 2 hours prior to the experiment and remained without food for the duration of the experiment. Baseline blood glucose concentrations were recorded before bovine insulin (0.75IU/kg) was injected intraperitoneally. Blood glucose concentrations were subsequently measured at 15, 30, 45 and 60-mintes post insulin injection. It should be noted that an ITT was conducted at 4 weeks of age with 6-hour fasting, however since high levels of hypoglycaemia occurred (~20% of mice), we subsequently reduced the fasting time to improve animal welfare during the experiment.

6.3.6 Western blotting:

Islets were isolated from 20-week-old WT and KINGS female mice that had been fed HFHS or NC from weaning (3 weeks of age) via collagenase digestion and histopaque separation as previously described (see section 2.5.1). Islets were cultured overnight before being lysed using RIPA buffer and 10µg of islet protein lysate was separated using SDS page and transferred to a PVDF membrane as described previously (see section 2.5.4). Membranes were probed for XBP1s, ATF6, BiP, and p-eIF2-alpha, as well as beta actin which served as the loading control.

6.3.7 Immunofluorescent staining:

BiP expression in islets from pancreas sections was measured as previously described (see section 2.6.2.4) through immunofluorescent staining for BiP and insulin and staining with DAPI. In brief, pancreases were fixed in 3.5% phosphate-buffered formalin and subsequently embedded in paraffin wax and cut into 5mM sections using a microtome. Sections were dewaxed using xylene and rehydrated with incubation in decreasing concentrations of ethanol. Heat mediated citric acid antigen retrieval was then performed and sections were first incubated with primary antibodies towards BiP (rabbit anti-BiP, 1:100 dilution, Cell signalling Technology) and insulin (guinea pig anti-insulin, 1:200 dilution, Abcam) overnight at 4°C, and then incubated with secondary antibodies against rabbit (Alexa Fluor 594, 1:200 dilution, Jackson ImmunoResearch) and guinea pig (Alexa Fluor 488, 1:200 dilution; Jackson

ImmunoResearch), and with DAPI (1:500 dilution). Sections were imaged using a Nikon SE microscope at 20x magnification.

6.3.8 Immunofluorescent image analysis:

FijiImageJ was used to manually draw around islets based on insulin staining and mean fluorescent intensity in the BiP channel was measured within selections (Schindelin et al., 2012).

6.3.9 Statistical analysis:

Graphs were constructed using GraphPad Prism 8.0 or 9.0 software (GraphPad Software, San Diego, CA) and data in the graphs represents mean±SEM unless otherwise stated. SigmaPlot 14.0 (Systat software Inc, San Jose, California) was used for all statistical analysis. A two-way repeated measures ANOVA with Bonferroni post hoc was used to assess statistical significance for repeated measures over time where an additional independent variable existed. For comparisons between multiple groups where there were two independent variables, a two-way ANOVA with Holm-Sidak post hoc test was used. For comparisons between two unpaired groups, a two-way unpaired T test was used. A $p<0.05$ was considered statistically significant.

6.4 Results:

6.4.1 Effects of HFHS feeding on beta cell ER stress and glycaemic control in KINGS female mice:

6.4.1.1 HFHS feeding is associated with increased weight gain in female mice:

HFHS feeding cause an increased body weight in both KINGS and WT mice which was significant in WT mice from 13 weeks of age and in the KINGS mice from 14 weeks of age (Figure 64A-B). In line with this, weight gain was significantly increased with HFHS feeding compared to NC feeding by 14 weeks in the WT mice and 18 weeks in the KINGS mice (Figure 64C-D). By the end of the study, at 20 weeks of age, HFHS fed WT and KINGS mice were on average 7.5g and 5.7g heavier than normal chow fed genotype-matched mice, respectively. Unfortunately, since mice on the HFHS diet tended to

shred the feed, we were unable to record food intake in these mice which may have revealed whether differences in food intake existed and contributed to weight changes.

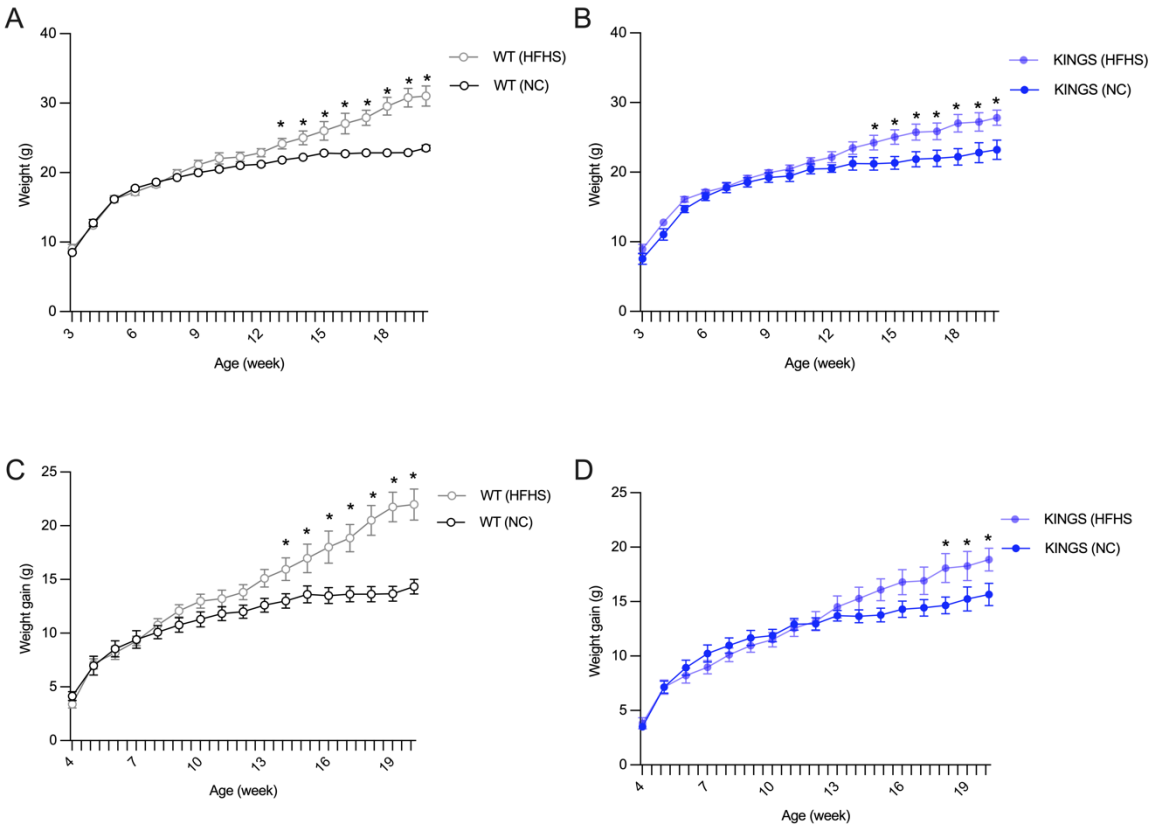


FIGURE 64: *Body weight and weight gain in female mice fed a HFHS or NC diet:*

*Body weight and weight gain over time in (A,C) WT female mice fed a HFHS diet or fed NC and (B,D) KINGS female mice fed a HFHS diet or NC from weaning for 17 weeks. Data represents mean±SEM, two-way repeated measure ANOVA with Bonferroni post hoc test, *p<0.05, n=7-9.*

6.4.1.2 HFHS FEEDING EXACERBATES BETA CELL ER STRESS IN FEMALE MICE:

To establish whether HFHS feeding caused an increase in beta cell ER stress in the KINGS mice, islets were isolated from these mice, cultured overnight to recover from isolation and protein lysates subjected to western blotting to probe for the expression of ER stress markers.

Markers of beta cell ER stress were increased with HFHS feeding in both wildtype and KINGS females (Figure 65). In wildtype mice, a HFHS diet was associated with an increase in islet XBP1s, although we detected no change in phosphorylated eIF2-alpha, BiP or ATF6 protein expression. In contrast BiP expression was elevated with HFHS feeding in the KINGS mice by nearly 2-fold that of normal chow mice. ATF6 protein expression was also increased in KINGS HFHS mouse islets but unlike in the wildtype mice, XBP1s was decreased with HFHS feeding. No changes in p-eIF2-alpha levels were detected between the KINGS groups.

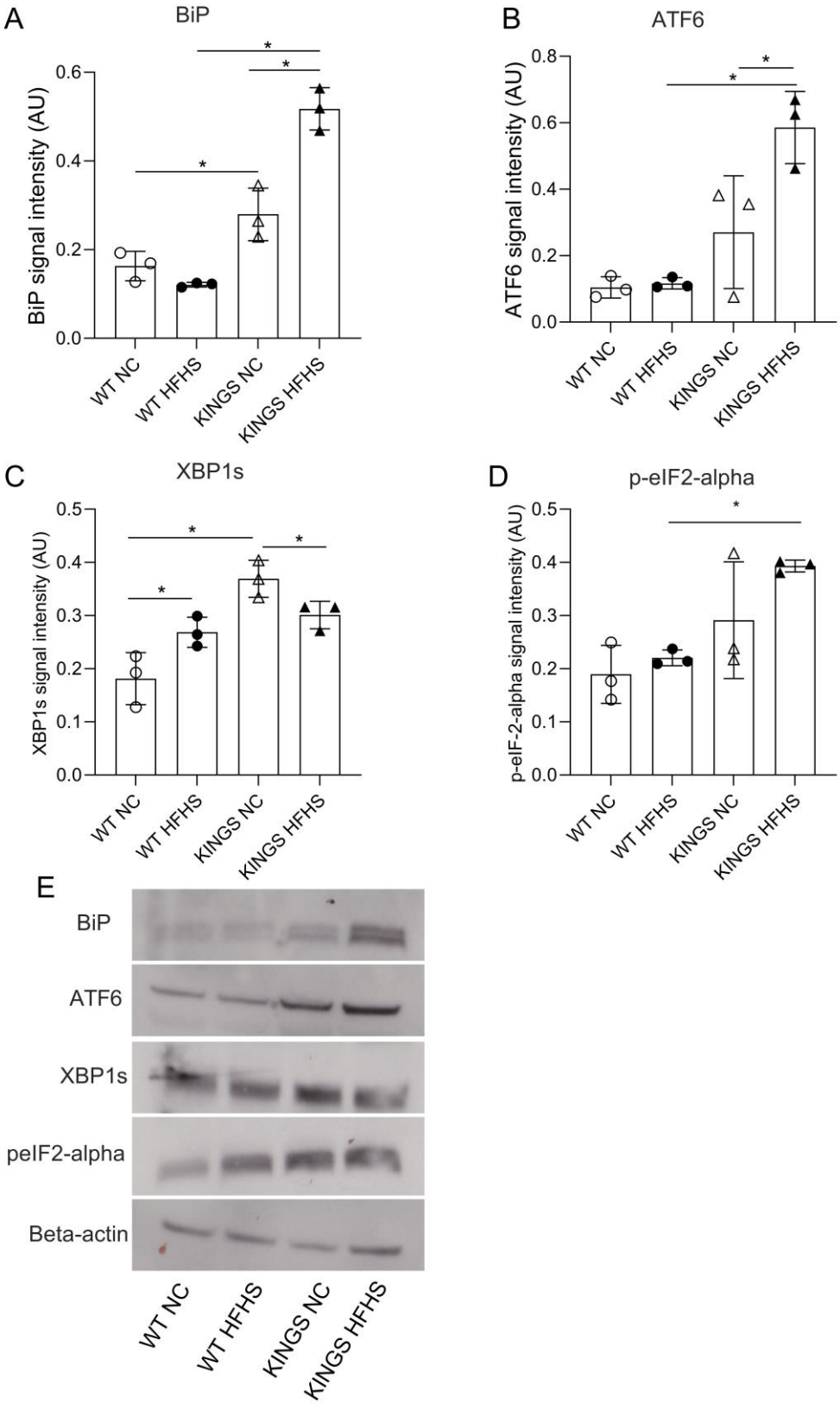


FIGURE 65: *Islet expression of ER stress and UPR markers in female mice fed a NC or a HFHS diet:*

*Protein expression of ER stress markers (A) BiP, (B) ATF6, (C) XBP1s, and (D) phosphorylated eIF2-alpha in islets isolated from WT and KINGS mice fed a HFHS or NC diet. Data represents mean±SEM, two-way ANOVA with Holm Sidak post hoc test, *p<0.05, n=3 experimental replicates, islets from 2-3 mice were pooled per n. (E) Representative western blot images; WT NC (lane 1), WT HFHS (lane 2), KINGS NC (lane 3), and KINGS HFHS (lane 4).*

Expression of the general ER stress marker, BiP, in islets from HFHS and NC fed mice was also investigated through immunofluorescent staining (Figure 66). However, in contrast to the western blot data, no differences were observed in BiP expression between NC and HFHS-fed wildtype and KINGS mice.

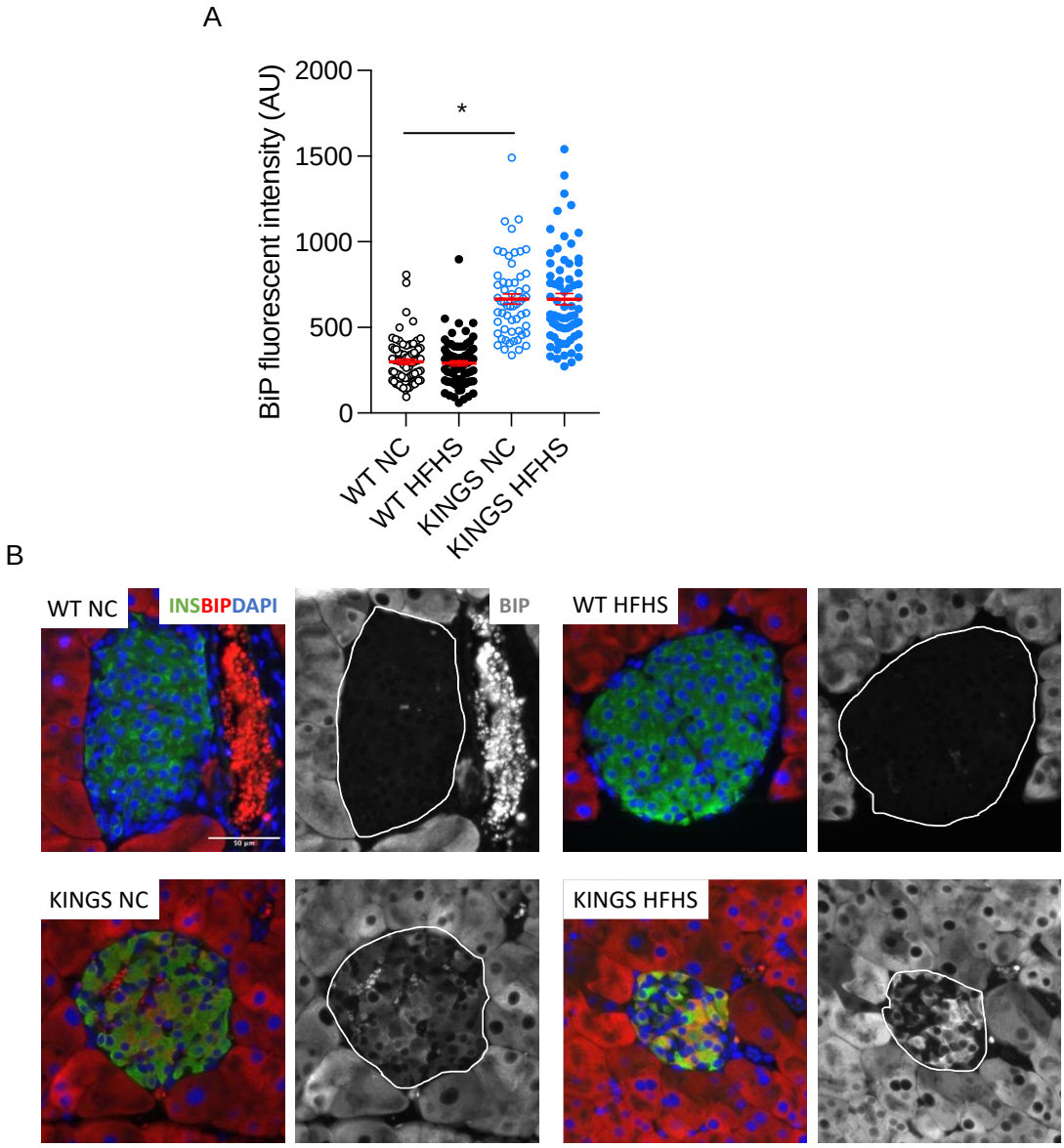


FIGURE 66: *Islet BiP expression in female mice fed a NC or a HFHS diet: (A) Immunofluorescent mean BiP intensity of islets from NC and HFHS fed KINGS and WT female mice. Data represents n=57-79 individual islets from 3 animal per group, mean±SEM is indicated in red. Two-way ANOVA with Holm-Sidak post hoc, *=p<0.05. (B) Representative immunofluorescent images of islets from WT and KINGS, HFHS and NC fed mice stained for insulin (green), BiP (blue and grey) and with DAPI (red). The second panel shows BiP immunofluorescence alone (grey) and the islet has been outlined in white. The scale bar is indicated in the top left image and represents 50mm.*

6.4.1.3 HFHS FEEDING INCREASES NON-FASTED BLOOD GLUCOSE CONCENTRATIONS BUT DOES NOT RESULT IN OVERT DIABETES IN FEMALE KINGS MICE:

To investigate whether the diet-induced increased beta cell ER stress in female KINGS mice resulted in a male-like phenotype (overt hyperglycaemia), non-fasted blood glucose concentrations were measured weekly until 20 weeks of age.

In WT mice, a HFHS diet caused a mild elevation in non-fasted blood glucose concentrations that was significantly different from normal chow fed wildtype mice at 10, 11, 14, 17 and 20 weeks of age (Figure 67A). However, there was no progressive difference over time in blood glucose concentrations in the HFHS WT group, with no significant difference between blood glucose concentrations at different ages. Supporting a mild change in phenotype, area under the curve values for blood glucose concentrations over the 17 weeks was not significantly elevated in HFHS WT mice (Figure 67D).

HFHS feeding in the KINGS mice resulted in a more substantial increase in blood glucose concentrations compared to normal chow fed mice, and this reached significance at multiple time points throughout the study including as early as 4 weeks on the diet (Figure 67B). In line with this, area under the curve for blood glucose concentrations over time were increased with HFHS feeding (Figure 67D). However, average blood glucose concentrations in the HFHS KINGS group rarely exceeded the threshold for overt diabetes (16.7mM) with this only occurring at 4, 7, 16 and 17 weeks of age. The maximum average non-fasted blood glucose concentration occurred at 7 weeks of age (4 weeks after dietary intervention) when average blood glucose concentration was 18.2mM. No progressive increase in blood glucose concentrations were seen in the KINGS HFHS group with no significant differences seen in blood glucose levels between different ages. This may have been expected given the increased weight gain over time in this group and the progressive increase in hyperglycaemia seen in KINGS males from 5 weeks of age (see section 3.4.1). Of note, variation in blood glucose concentrations was large for the KINGS females fed a HFHS diet; whilst some

mice only exceeded the threshold for overt diabetes once or twice, one mouse (of seven) consistently showed blood glucose concentrations exceeding 20mM, even exceeding 30mM at 14 weeks and 19 weeks, which is in line with blood concentration concentrations seen in the male KINGS mice in previous studies (Figure 67C).

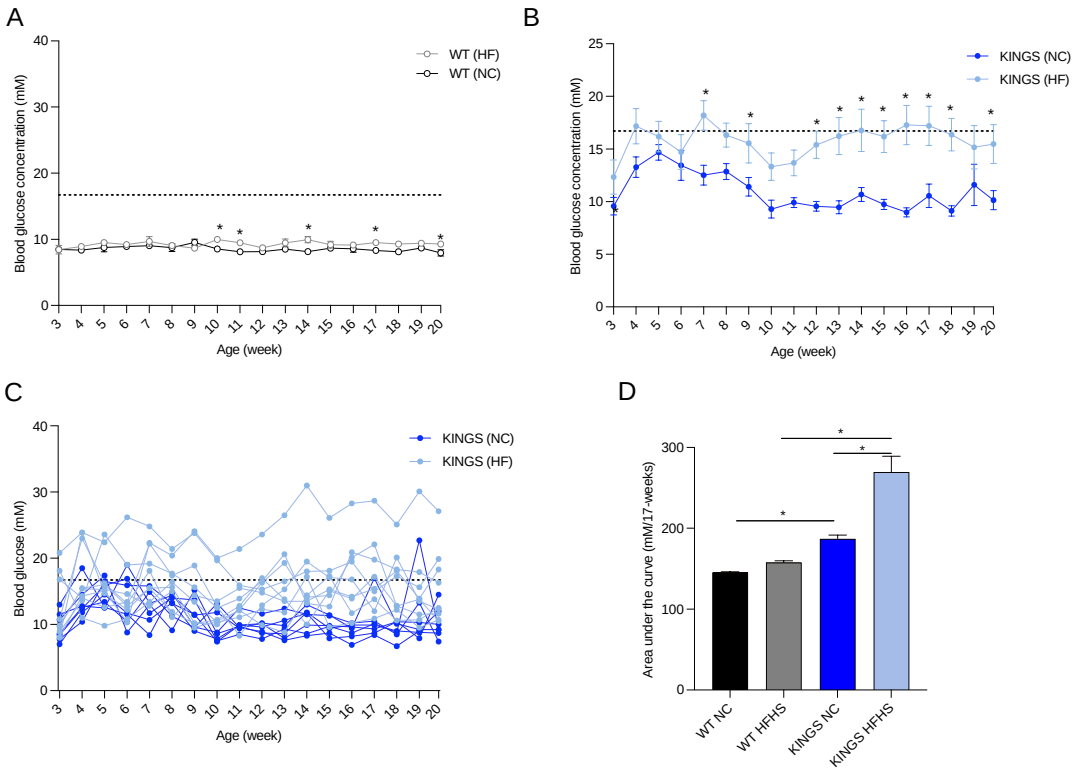


FIGURE 67: *Blood glucose concentrations in female mice fed NC or a HFHS diet:*

*Non-fasted blood glucose concentrations in NC and HFHS fed (A) WT female mice and (B) KINGS female mice. The hashed line represents the threshold for overt diabetes, 16.7mM. Data represents the mean±SEM, two-way repeated measure ANOVA with Bonferroni post hoc, *p<0.05, n=7-9. (C) Non-fasted blood glucose concentrations for individual KINGS mice fed NC and a HFHS diet. One HFHS KINGS female showed a progressive increase in blood glucose concentrations and overt diabetes similar to male KINGS mice. (D) Area under the curve values for non-fasted blood glucose concentrations over time in NC and HFHS fed WT female mice and KINGS female mice. Data*

*represents mean±SEM, two-way ANOVA with Holm-Sidak post hoc, *=p<0.05, n=7-9.*

As our hypothesis was that exacerbating beta cell ER stress in female KINGS mice would cause a more male-like glycaemic phenotype, average non-fasted blood glucose concentrations in the female KINGS HFHS group were compared to historic data from male KINGS mice. Male KINGS mice show elevated blood glucose concentrations compared to all female KINGS mice fed a HFHS at most ages and consistent with this area under the curve values for blood glucose over time are lower (Figure 68). Whilst blood glucose concentrations progressively worsened in the male KINGS mice with increasing age, this was not the case for the majority of HFHS-fed female mice. For the one female mouse that did show a progressive worsening of blood glucose concentrations, blood glucose concentrations were still lower than the average male KINGS blood glucose concentration at most ages.

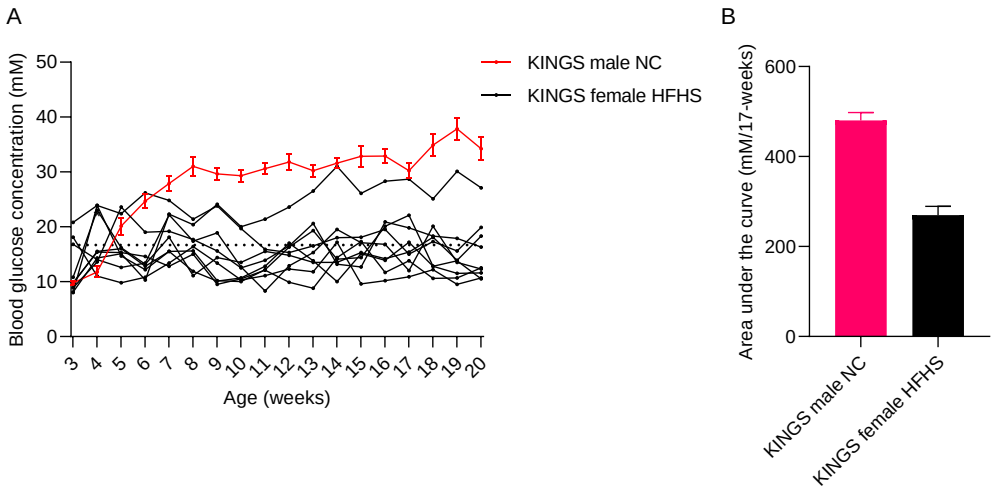


FIGURE 68: *Comparison of non-fasted blood glucose concentrations between KINGS-HFHS female mice and KINGS-NC male mice:*

(A) Average non-fasted blood glucose concentrations in NC male KINGS mice (red line, historic data (Austin et al., 2020) *and individual HFHS fed female KINGS mice (black lines). (B) Area under the curve values for non-fasted blood glucose concentrations for NC fed male KINGS mice and HFHS fed female KINGS mice.*

6.4.1.4 HFHS FEEDING RESULTED IN A MILD INCREASE IN FASTED BLOOD GLUCOSE CONCENTRATIONS IN FEMALE KINGS MICE:

The HFHS diet in this study contains 30kcal% sucrose and since non-fasted blood glucose concentrations are likely taken in the post-prandial state, elevated blood glucose concentrations may be more reflective of the influence of transient blood glucose increases directly after feeding rather than changes at the beta cell level. For this reason, we investigated fasted blood glucose concentrations in these mice at 20 weeks of age.

After a 16-hour fast, blood glucose concentrations were not significantly increased in wildtype mice fed a HFHS diet compared to mice fed normal chow (Figure 69). In contrast, HFHS feeding in the KINGS females increased average fasting blood glucose concentrations by 2.4mM, however the majority of KINGS HFHS female mice were still within a normoglycemic range. It should be noted that one KINGS female HFHS mouse did exhibit hyperglycaemia after a 16-hour fast with a blood glucose concentration of 17.6mM. This was the same mouse that displayed a progressive increase in blood glucose levels and development of hyperglycaemia discussed previously (Figure 68C). Variation in fasting blood glucose concentrations was also higher for the KINGS mice on a HFHS diet compared to normal chow KINGS mice which is also reflective of an impairment in glycaemic control. Of note, the average fasted blood glucose concentration of 7.9mM in the HFHS KINGS female group contrasts previous fasted blood glucose concentrations measured in the male KINGS mice which reached an average of ~17mM (Austin, 2018).

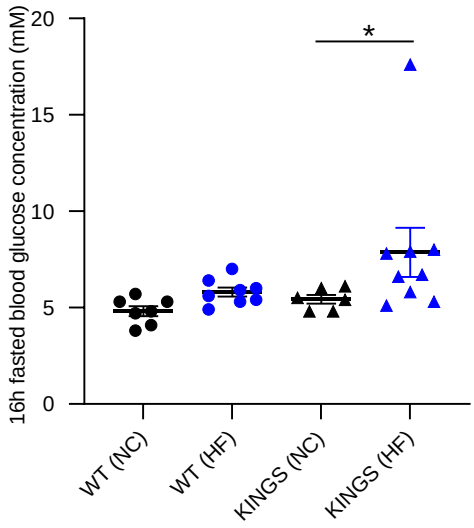


FIGURE 69: *Fasted blood glucose concentrations in female mice fed a HFHS or NC diet:*

*16-hour fasted blood glucose concentrations in WT (circles) and KINGS (triangles) mice fed a HFHS diet (black) or NC (blue). Data represents mean±SEM, two-way ANOVA with Holm-Sidak post hoc, *p<0.05, n=6-9.*

6.4.1.5 HFHS FEEDING IS ASSOCIATED WITH A PROGRESSIVE IMPAIRMENT IN GLUCOSE TOLERANCE IN FEMALE MICE:

To investigate whether glycaemic control under stimulatory conditions was altered with HFHS feeding and whether this progressively worsened over the course of the experiment (in line with a progressive increase in weight gain compared to normal chow controls), glucose tolerance tests were conducted at 4, 8, 10, 12, 16 and 20 weeks of age.

HFHS feeding resulted in an impaired glucose tolerance at 4 and from 10 weeks in the WT mice, indicated by increased area under the curve values compared to chow fed WT mice at these ages (Figure 70A,C,E). There was also a progressive worsening of glucose tolerance with time on diet in the WT HFHS group, with area under the curve values significantly increasing from 4 weeks of age compared to later ages (10, 12, 16 and 20 weeks of age) and values increasing significantly from 8 and 10 weeks to 16 and 20 weeks, and from 12 to 20 weeks. This progressive worsening did not occur in WT mice fed

a normal chow diet, with no significant differences in area under the curve values across all ages.

Similarly, HFHS feeding in female KINGS mice resulted in impaired glucose tolerance, however area under the curve values for the glucose tolerance tests were only significantly increased at 4, 16 and 20 weeks of age (Figure 70B,D,F). A progressive worsening in glucose tolerance in this group was also noted, with glucose tolerance at 20 weeks of age significantly worse than at 4, 8 and 10 weeks of age, whereas this was not the case for KINGS females fed normal chow. Of note, this progressive increase in glucose intolerance is similar to what was observed in the male KINGS mice previously (see Chapter 3 section 3.4.3 and (Austin et al., 2020)).

In line with this, blood glucose levels consistently peaked at a later time point post glucose injection in the HFHS fed mice compared to the NC controls; for example, at 16 weeks of age glucose concentrations peaked at 30 minutes in the KINGS-NC group compared to at 60 minutes in the KINGS-HFHS group, whilst they peaked at 15 minutes in the WT-NC group compared to at 30 minutes in the WT-HFHS group. Interestingly, the fold change in glucose intolerance was higher in the WT NC vs HFHS compared to the KINGS HFHS vs NC. For example, at 20 weeks of age, HFHS feeding increased area under the curve by ~30% in the KINGS mice, compared to ~50% in the WT mice.

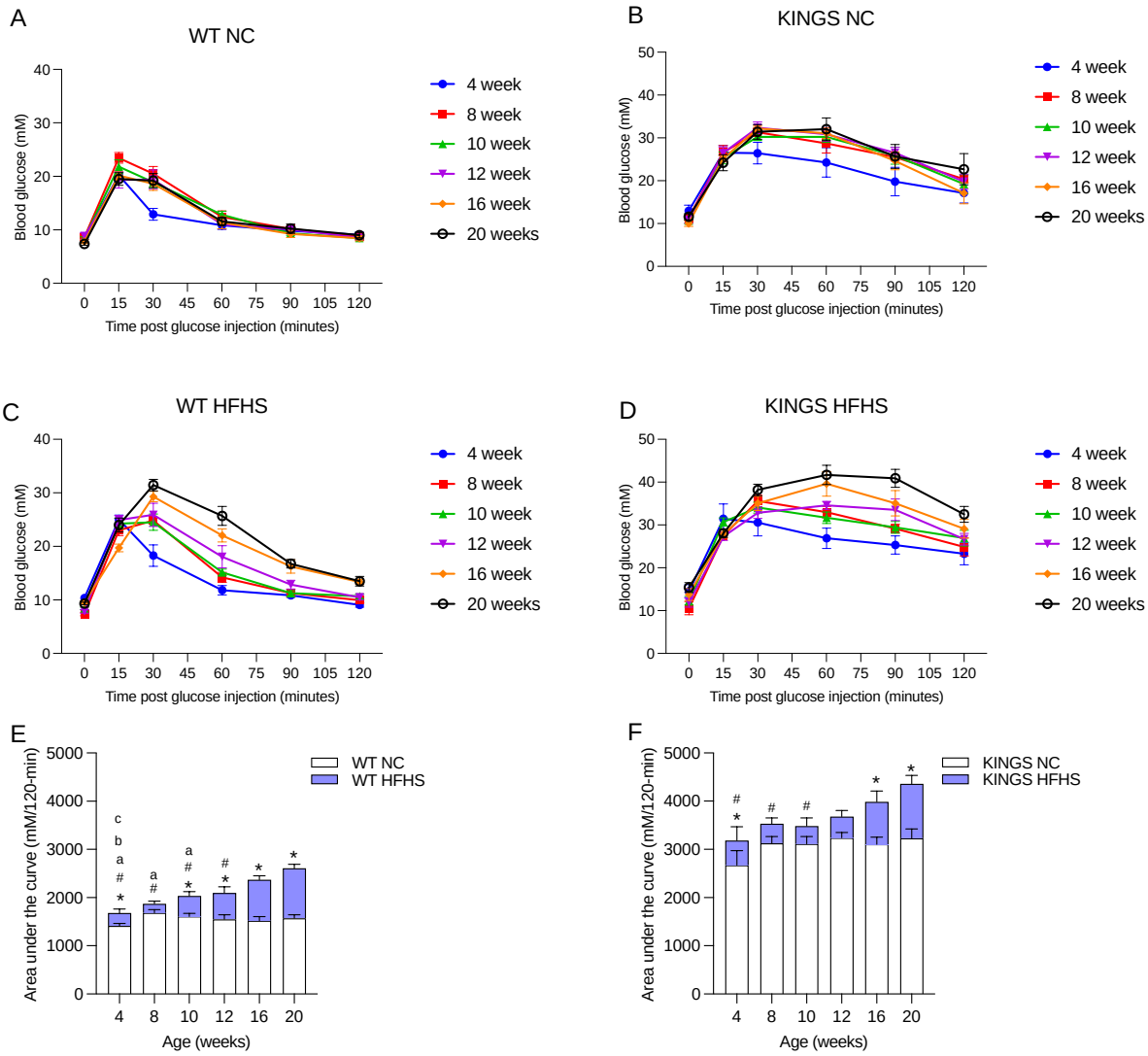


FIGURE 70: *Glucose tolerance over time in female mice fed a HFHS or NC diet:*

*Glucose tolerance tests carried out at 4, 8, 10, 12, 16 and 20 weeks of age in (A) WT female mice fed NC, (B) KINGS female mice fed NC, (C) WT female mice fed a HFHS diet, and (D) KINGS female mice fed a HFHS diet. (E-F) Area under the curve values for the glucose tolerance tests carried out in (E) WT female mice fed NC or a HFHS diet and (F) KINGS female mice fed NC or a HFHS diet. Data represents mean±SEM, two-way repeated measures ANOVA with Bonferroni post hoc test, *=p<0.05 NC vs HFHS, #=p<0.05 HFHS vs 20-week HFHS, a= p<0.05 HFHS vs 16-week HFHS, b=p<0.05 HFHS vs 12-week HFHS, c= p<0.05 HFHS vs 10-week HFHS, n=5-9.*

6.4.1.6 The impact of HFHS feeding on insulin resistance in female mice:

HFHS feeding can influence glycaemic control through mechanisms unrelated to the beta cell including by increasing peripheral insulin resistance. To establish the contribution of the latter to the phenotype seen in HFHS fed KINGS mice, insulin tolerance tests were conducted at 4, 8, 10, 12, 16 and 20 weeks of age. Multiple time points were used to investigate whether the HFHS diet resulted in a progressive increase in insulin resistance. Initially (when mice were 4 weeks of age), mice were fasted for 6 hours prior to the insulin tolerance test, however we found that this caused hypoglycaemia (blood glucose concentrations <2.5mM) in a large proportion of mice (~20%). Therefore, due to ethical concerns fast length was reduced to 2 hours for the insulin tolerance tests conducted after 4 weeks. Basal glucose concentrations, especially in the HFHS male KINGS mice varied at the different ages making comparisons between age groups difficult, for this reason blood glucose measurements as a percentage of basal concentration were used to plot the data and to calculate area above the curve.

Area above the curve values for the insulin tolerance tests were lower with HFHS feeding in the WT mice, although this only reached significance at 8 and 16 weeks of age (Figure 71). Area above the curve values were not significantly different between different ages in the WT HFHS group, but area above the curve values were significantly lower at 12 weeks compared to at 8 weeks in the WT NC group.

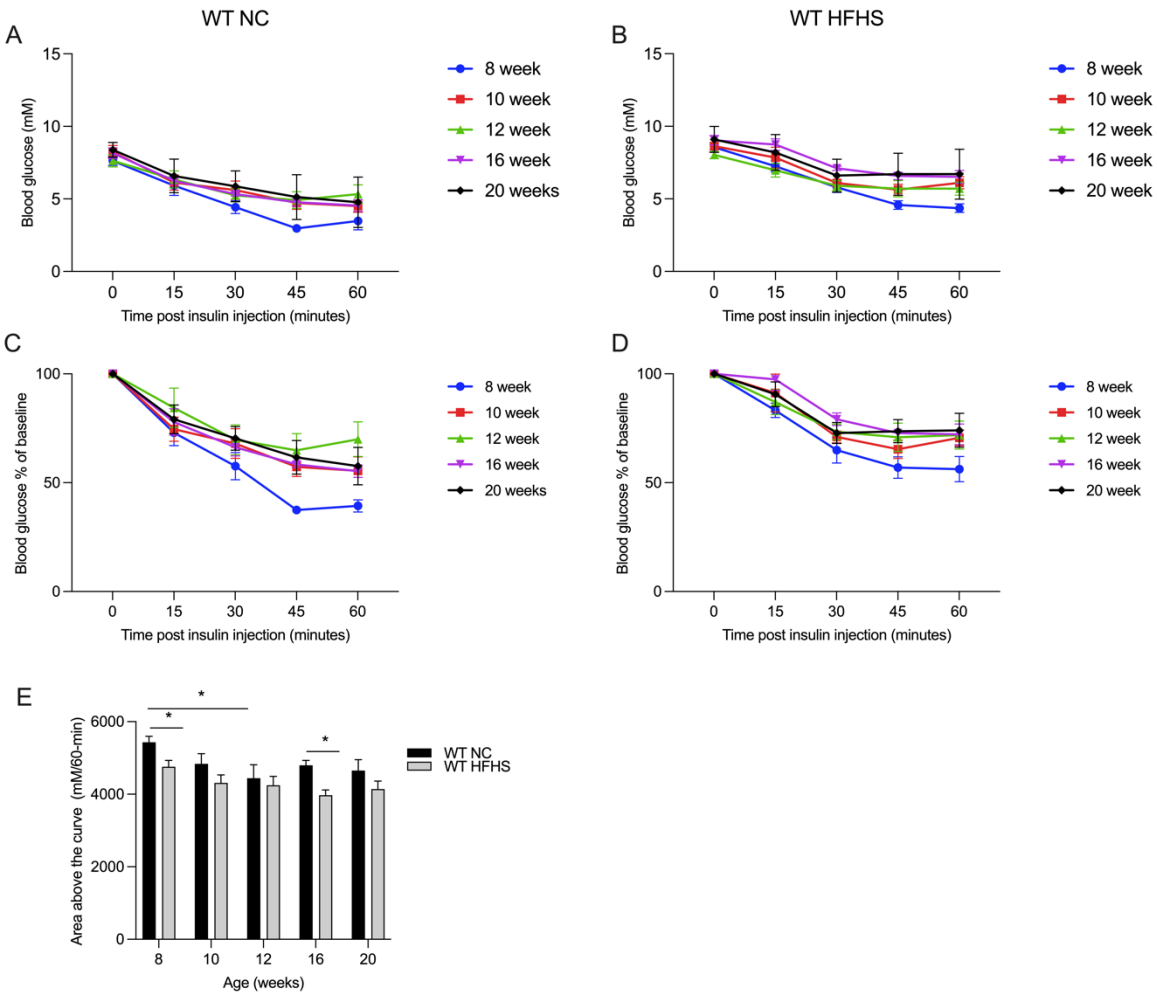


FIGURE 71: *Insulin tolerance tests over time in female WT mice fed a HFHS or NC diet:*

*Insulin tolerance tests carried out at 8, 10, 12, 16 and 20 weeks of age in (A) WT female mice fed NC, (B) WT female mice fed a HFHS diet. Insulin tolerance tests where blood glucose concentrations are expressed as % of basal concentrations in (C) WT female mice fed NC and (C) WT female mice fed a HFHS diet. (E) Area above the curve values for insulin tolerance tests carried out in WT female mice fed either NC or a HFHS diet. Data represents mean±SEM, *P<0.05 Two-way repeated measures ANOVA with Bonferroni post hoc, n=6-8.*

HFHS feeding in the KINGS mice on the other hand had no significant effect on insulin sensitivity, with area above the curve values for the insulin tolerance tests not significantly different compared to NC fed mice at any age and no progression after 8 weeks of age in either the NC or HFHS group (Figure 72).

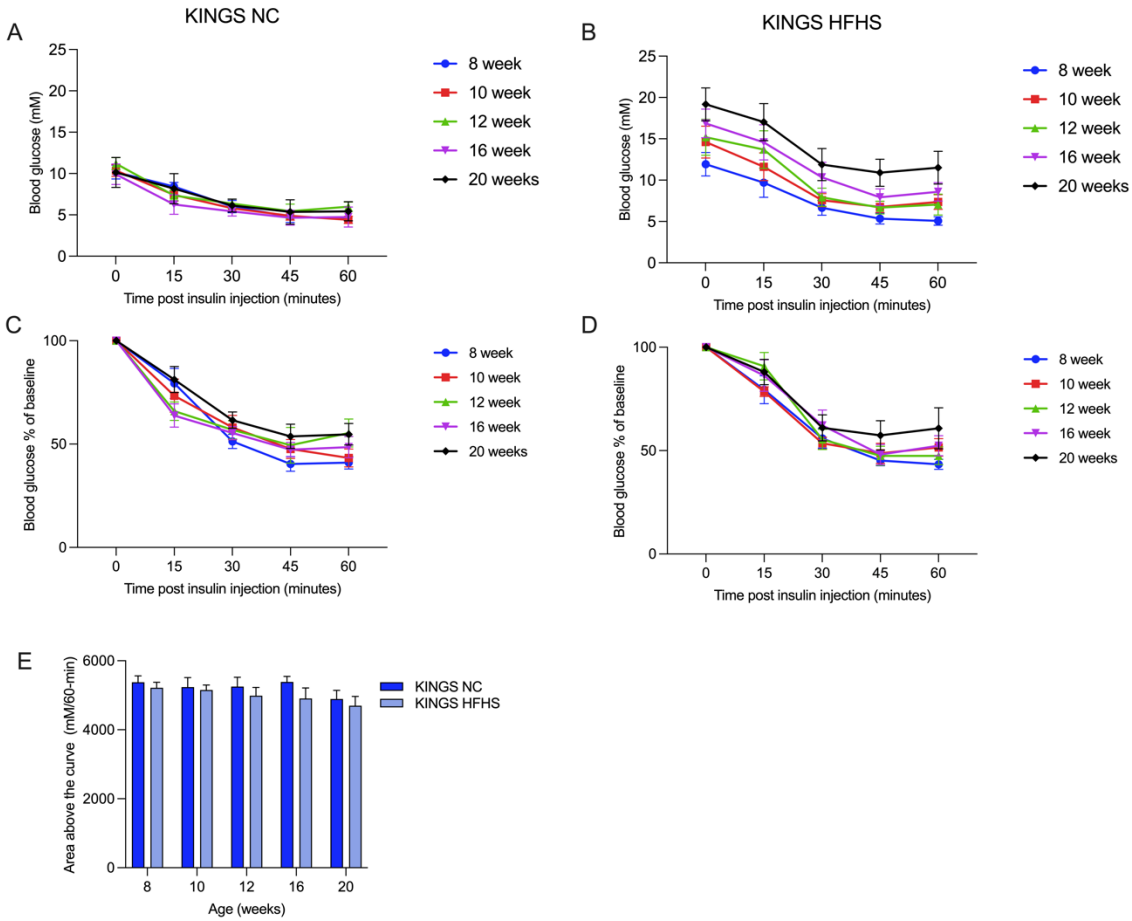


FIGURE 72: *Insulin tolerance tests over time in KINGS female mice fed HFHS or NC diets:*

*Insulin tolerance tests carried out at 8, 10, 12, 16 and 20 weeks of age in (A) KINGS female mice fed NC, (B) KINGS female mice fed a HFHS diet. Insulin tolerance tests where blood glucose concentrations are expressed as % of basal concentrations in (C) KINGS female mice fed NC and (C) KINGS female mice fed a HFHS diet. (E) Area above the curve values for insulin tolerance tests carried out in KINGS female mice fed either NC or a HFHS diet. Data represents mean±SEM, *P<0.05 Two-way repeated measures ANOVA with Bonferroni post hoc, n=5-9.*

6.4.1.7 HFHS IS NOT ASSOCIATED WITH CHANGES IN ISLET AREA IN FEMALE MICE:

KINGS female mice fed a HFHS diet did not develop overt diabetes and the phenotype was not as severe as that seen in male KINGS mice fed a normal

chow diet. This suggests that female KINGS mice are able to adapt to exacerbated beta cell ER stress. One way in which beta cells have been shown to adapt to cellular stress is through proliferation and hypertrophy. The latter both can lead to an increase in islet area, and therefore islet area was measured in these mice using immunofluorescent insulin staining and DAPI staining. No differences were observed in islet area between HFHS and normal chow fed mice of either genotype (Figure 73).

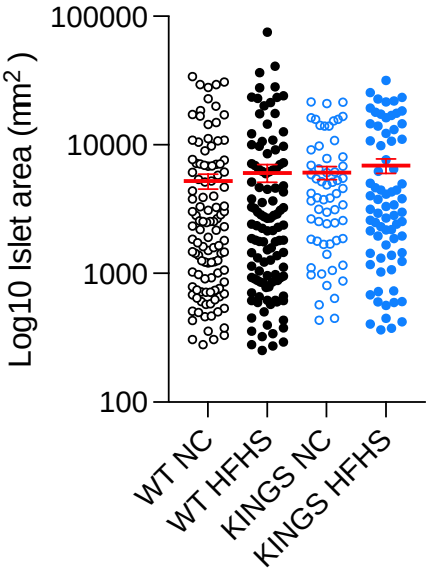


FIGURE 73: *Islet area in female mice fed a HFHS or NC diet:*
Islet area of WT (black) and KINGS (blue) female mice fed either NC (open circles) or a HFHS diet (filled circles). Data represents n=57-79 individual islets from 3 animal per group, mean±SEM is indicated in red. Two-way ANOVA with Holm-Sidak post hoc, p>0.05.

6.4.2 Effects of HFHS feeding on glycaemic control in KINGS male mice:

6.4.2.1 HFHS feeding further exacerbates hyperglycaemia in KINGS males:

Non-fasted blood glucose and fasted blood glucose concentrations in the KINGS females were only moderately increased by HFHS and the majority of study mice remained below the threshold for overt diabetes. This suggests that female KINGS mice can adapt to increased beta cell ER stress. We next

wanted to determine whether this ability to adapt, even under exacerbated beta ER stress, was also present in the male KINGS mice and thus a pilot study was performed where male KINGS mice were fed a HFHS diet for 7 weeks from weaning (until the age of 10 weeks).

A HFHS diet caused a significant increase in body weight in male WT mice from 8 weeks of age (5 weeks on diet) and by the end of the study HFHS mice were on average ~5g heavier compared to normal chow controls (Figure 74A). In line with this, weight gain was significantly elevated with HFHS feeding in the WT mice from 7 weeks of age as well as at 28-30 days of age (Figure 74C). In contrast, male KINGS mice fed a HFHS diet were not significantly heavier at any point through the study and weight gain did not differ compared to NC controls (Figure 74B,D).

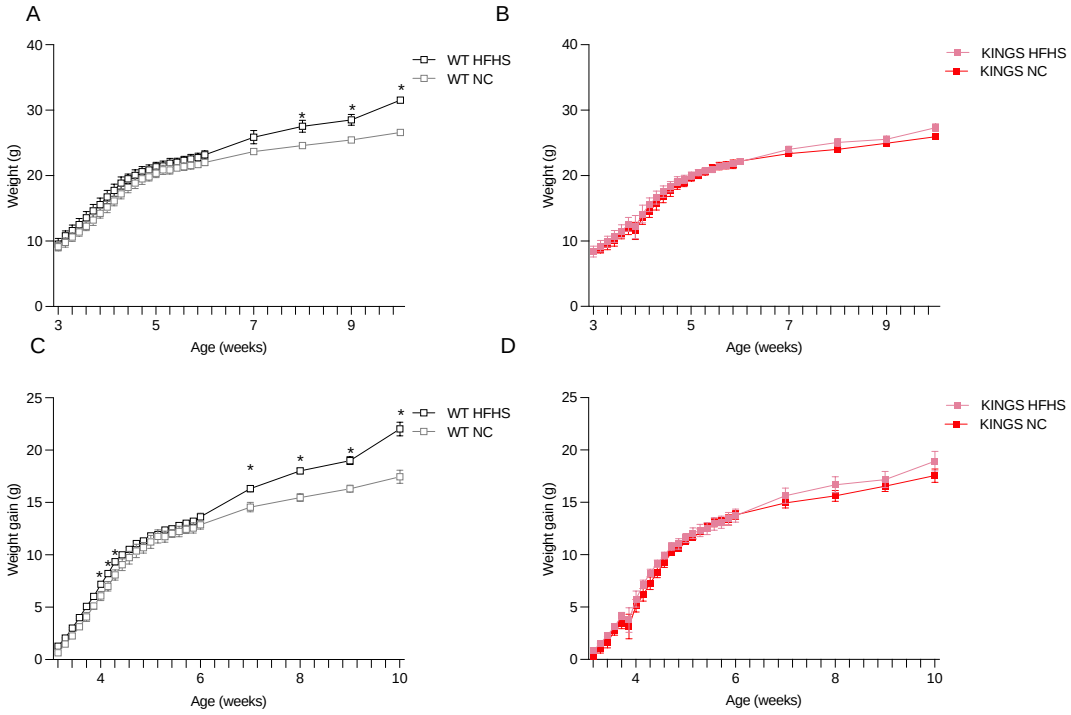


FIGURE 74: *Weight and weight gain in male mice fed a HFHS DIET or NC: Body weight and weight gain over time in (A,C) WT mice fed a HFHS diet or fed NC and (B,D) KINGS mice fed a HFHS diet or NC from weaning for 7 weeks. Data represents mean±SEM, two-way repeated measure ANOVA with Bonferroni post hoc, *p<0.05, n=4-5.*

Despite a lack of weight gain with HFHS feeding in male KINGS mice, the HFHS diet was associated with an elevation in non-fasted blood glucose concentrations which reached significance at 8 weeks of age, although age of diabetes onset was unchanged (Figure 75B). Area under the curve values for blood glucose concentrations over time were also higher with HFHS feeding (Figure 75C). On the other hand, a HFHS diet was not associated with a significant change in blood glucose concentrations in the WT males and area under the curve values were not different (Figure 75A,C).

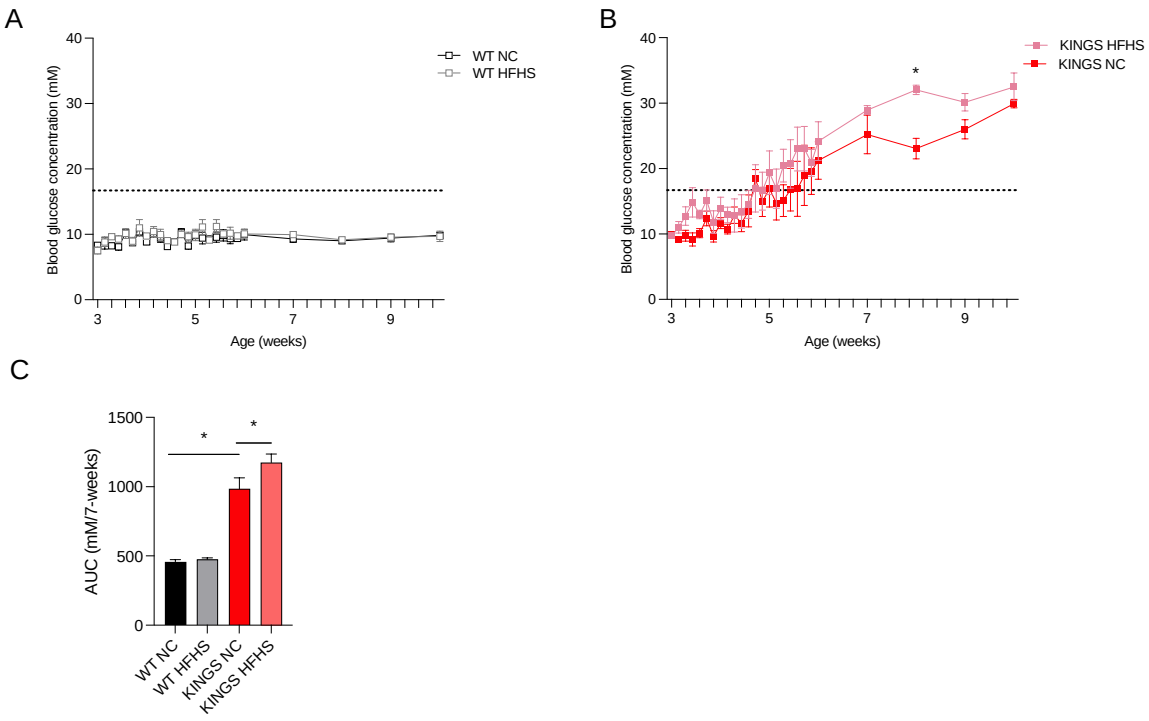


FIGURE 75: *Non-fasted blood glucose concentrations in male mice fed a HFHS or NC diet:*

*Non-fasted blood glucose concentrations in NC and HFHS fed (A) WT mice and (B) KINGS male mice. Data represents mean±SEM, two-way repeated measure ANOVA with Bonferroni post hoc, *p<0.05, n=4-5. (C) Area under the curve (AUC) values for blood glucose concentrations over time in WT and KINGS male mice fed either a NC or HFHS diet. Two-way ANOVA with Holm-Sidak post hoc, *p<0.05, n=4-5.*

6.4.2.2 HFHS FEEDING ELEVATES FASTING BLOOD GLUCOSE CONCENTRATIONS IN THE KINGS MALES:

As mentioned previously, non-fasted blood glucose concentrations are often recorded in the post-prandial state and thus may be influenced directly by the HFHS feed rather than effects that the dietary modification has on the beta cell. To overcome this, 16-hour fasting blood glucose concentrations were measured in male mice fed a HFHS diet.

Fasted blood glucose concentrations were significantly elevated by an average of 5.5mM in male KINGS mice fed a HFHS diet compared to male KINGS mice fed normal chow (Figure 76). However, in the WT mice, HFHS feeding did not cause a significant elevation in fasting blood glucose concentrations.

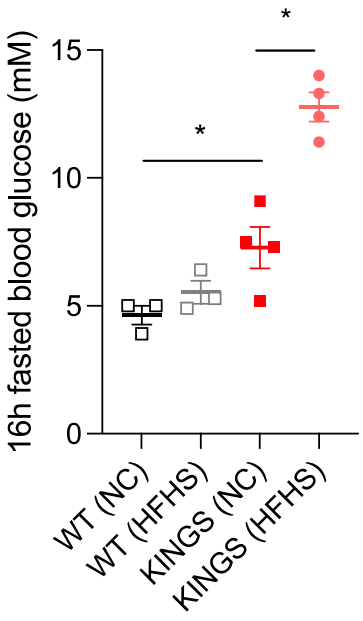


FIGURE 76: *Fasted blood glucose concentrations in male mice fed a HFHS or NC diet:*
*16-hour fasted blood glucose concentrations in WT and KINGS male mice fed a HFHS diet or NC. Data represents mean±SEM, two-way ANOVA with Holm-Sidak post hoc, *=p<0.05, n=3-4.*

6.4.2.3 HFHS FEEDING DOES NOT EXACERBATES GLUCOSE INTOLERANCE IN MALE MICE:

To determine the effect that HFHS feeding had on beta cell function under stimulatory conditions in male mice, glucose tolerance tests were performed.

Due to high basal glucose concentrations (>20mM) in the KINGS mice from 8 weeks of age, we did not perform glucose tolerance tests in KINGS mice from this age and therefore are unable to comment of progressive glucose tolerance caused by the diet in the context of the KINGS mutation.

A HFHS diet did not cause a change in glucose tolerance at 4 weeks of age (1-week of diet) in either wildtype or KINGS males, with no significant differences in area under the curve values for the glucose tolerance tests (Figure 77C,E). Area under the curve values did not differ between HFHS and NC WT groups at 8 or 10 weeks of age. There was a significant increase in area under the curve between 4 and 8 weeks of age in the WT HFHS group possibly suggesting some progressive increase in glucose intolerance, although there was no difference detected between 4 and 10 weeks in this group.

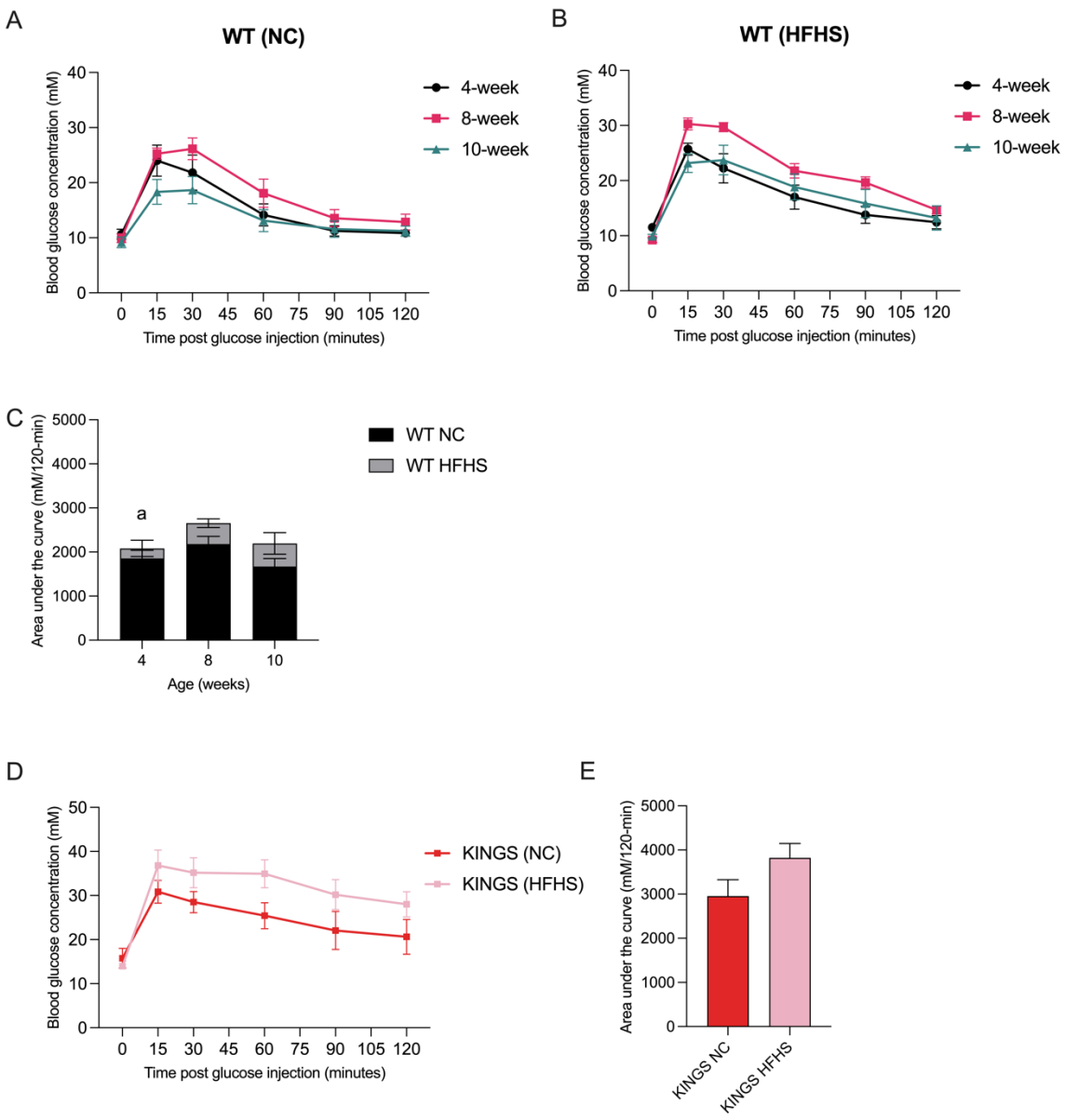


FIGURE 77: *Glucose tolerance in male mice fed a NC or HFHS diet: (A-B) Glucose tolerance tests carried out at 4, 8, 10 weeks of age in (A) WT male mice fed NC and (B) WT male mice fed a HFHS diet. (C) Area under the curve values for the glucose tolerance tests carried out in WT male mice fed NC or a HFHS diet. Data represents mean±SEM, two-way ANOVA with Bonferroni post hoc test, a=p<0.05 HFHS vs 8-week HFHS, n=4-5. (D) Glucose tolerance tests carried out at 4 weeks of age in male KINGS mice fed a HFHS diet or NC. (E) Area under the curve values for the glucose tolerance test carried out in HFHS and NC fed KINGS mice. Data represents mean±SEM, unpaired T-test, p>0.05, n=3-4.*

6.4.2.4 HFHS FEEDING DOES NOT CAUSE OVERT INSULIN RESISTANCE IN MALE MICE:

HFHS feeding increased non-fasted and fasted blood glucose concentrations in male KINGS mice. To determine whether insulin resistance contributed to this impairment in glycaemic control, insulin tolerance tests were conducted at 4 weeks of age (1-week on diet) and at 10 weeks of age (7 weeks on diet) to see whether there was any progressive impairment in peripheral insulin sensitivity.

A reduction in area above the curve for the insulin tolerance tests was observed in all groups between 4 and 10 weeks of age, although this only reached significance in the KINGS mice (Figure 78, Figure 79). Despite this, no difference in insulin sensitivity between HFHS and NC groups was detected in WT or KINGS mice at either of these ages.

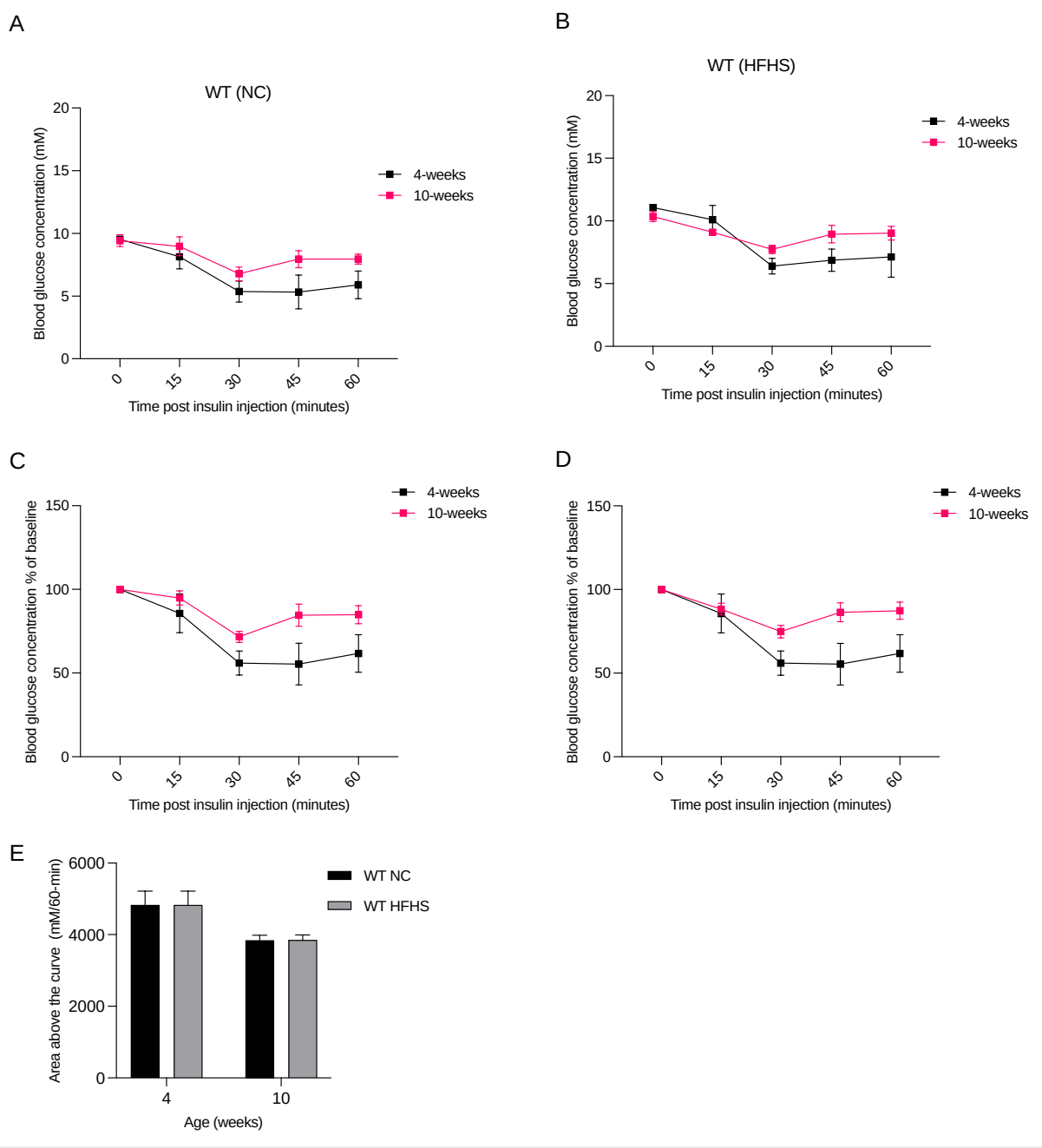


FIGURE 78: *Insulin tolerance tests in male WT mice fed NC or a HFHS diet: Insulin tolerance tests carried out at 4 and 10 weeks of age in (A) WT male mice fed NC, (B) WT male mice fed a HFHS diet. Insulin tolerance tests where blood glucose concentrations are expressed as % of basal concentrations in (C) WT male mice fed NC and (C) WT male mice fed a HFHS diet. (E) Area above the curve values for insulin tolerance tests carried out in WT male mice fed either NC or a HFHS diet. Data represents mean±SEM, two-way repeated measures ANOVA with Bonferroni post hoc, p>0.05, n=3-4.*

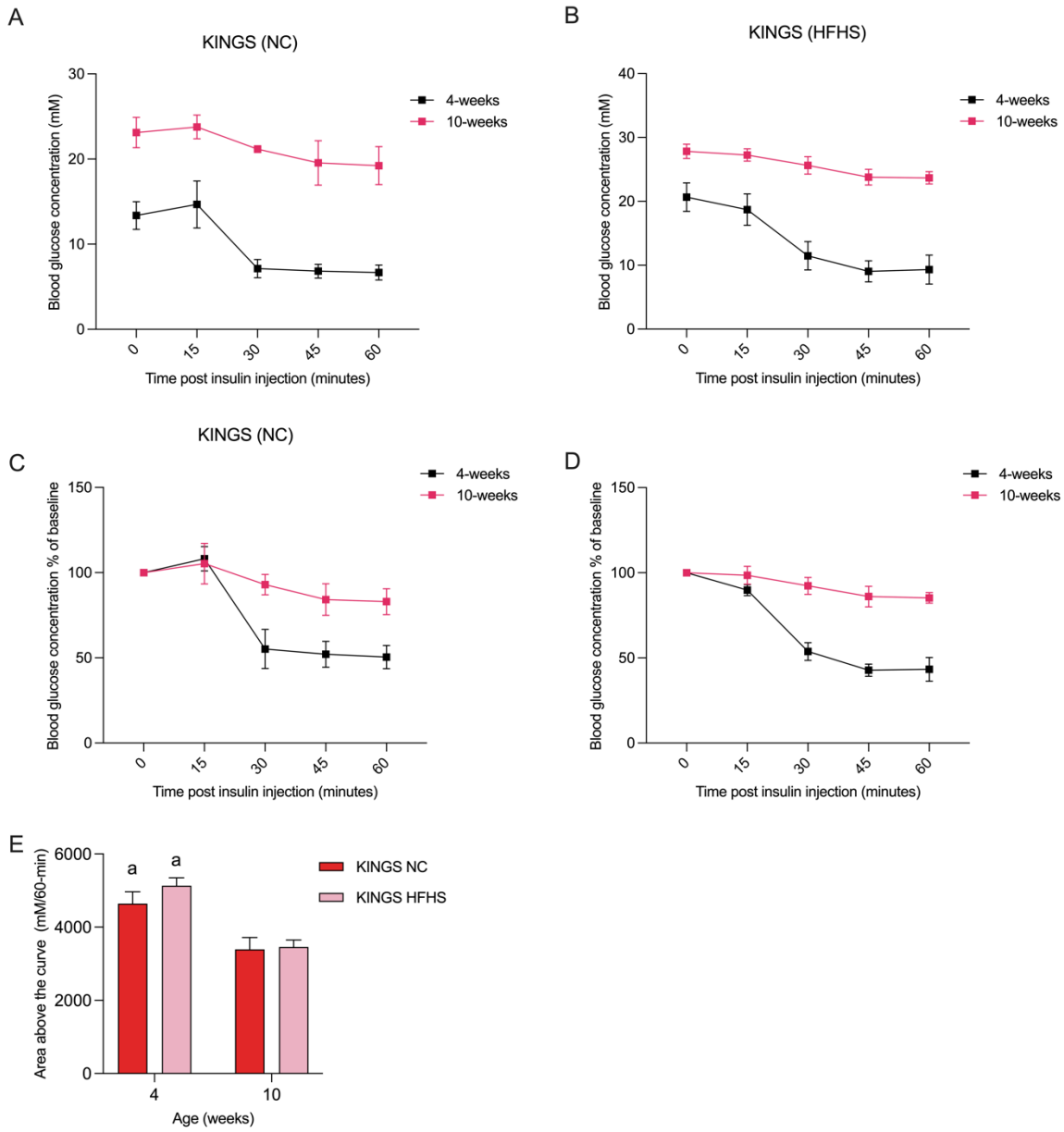


FIGURE 79: *Insulin tolerance tests in male KINGS mice fed a NC or a HFHS diet:*

Insulin tolerance tests carried out at 4 and 10 weeks of age in (A) KINGS male mice fed NC, (B) KINGS male mice fed a HFHS diet. Insulin tolerance tests where blood glucose concentrations are expressed as % of basal concentrations in (C) KINGS male mice fed NC and (C) KINGS male mice fed a HFHS diet. (E) Area above the curve values for insulin tolerance tests carried out in KINGS male mice fed either NC or a HFHS diet. Data represents mean±SEM, a= p<0.05 vs 10 weeks, two-way repeated measures ANOVA with Bonferroni post hoc, n=3-5.

6.5 Discussion:

Beta cell ER stress has been heavily implicated in beta cell dysfunction and death in many types of diabetes. However, mild ER stress is also important for the maintenance of beta cell function and survival. It has been proposed that there exists a fine balance between beta cell adaptive and maladaptive response to ER stress and this model suggests that when ER stress levels reach a critical threshold (where it can no longer be resolved) beta cell maladaptive signalling, which drives dysfunction and death, ensues (Figure 61). Understanding whether this switch can be manipulated, by what factors, and whether some intrinsic factors protect against this shift, may provide insight into novel therapeutics to relieve ER stress and thus a loss of functional beta cell mass in individuals with diabetes. The KINGS mouse represents a model that can be used to give insight into the latter, since female KINGS mice show an adaptive response to beta cell ER stress- they are able to maintain normoglycemia, whereas males show a maladaptive response to ER stress and display stark hyperglycaemia from 5-6 weeks of age. To investigate whether female KINGS mice fail to develop diabetes because their beta cell ER stress levels do not reach a critical threshold (where after a maladaptive response ensues) we attempted to exacerbate ER stress through HFHS feeding and investigating the effects of this on glycaemic control. We also wanted to investigate whether exacerbation of ER stress in the KINGS males, who already have a maladaptive beta cell stress response, would lead to a more severe impairment in glycaemic control. To do this, a pilot study whereby male KINGS mice were fed a HFHS diet was carried out.

Initially it was confirmed that a HFHS diet was capable of exacerbating beta cell ER stress in female mice. XBP1s protein levels were elevated with HFHS feeding in wildtype islets. This is similar to previous findings by Gupta *et al.* who found that high fat feeding in mice increased islet XBP1s mRNA expression from 12 weeks of diet, although by 16 weeks this increase was much less stark (Gupta et al., 2017). Contrasting our findings, the study also found that islet BiP and ATF6 mRNA was increased with high fat feeding, something we did not observe at the protein level in the wildtype mice.

Differences between the findings may be a result of different diets used. The diet used by Gupta *et al* contained 60% fat compared to the 45% fat used in this study and thus likely induces a more severe metabolic syndrome. Indeed, the high 60% fat fed mice gained more weight and had higher blood glucose concentrations compared to the HFHS fed mice in the current study. In addition, whilst we assessed the expression of UPR markers at the protein level, Gupta *et al* used mRNA expression which also may go some way to explaining differences in findings. In another study, Yi *et al* found that 40% high fat feeding C57BL6/J mice increased islet expression of phosphorylated eIF2-alpha and ATF6, which we did not observe (Yi et al., 2020). It is worth noting that both studies used male mice, whereas in this study we were focussing on female mice, and this may also account for the differences.

KINGS females fed normal chow, like the wildtype females fed a HFHS diet, showed increased XBP1s levels compared to wildtype controls. It should be noted that these increases are beyond that of the HFHS wildtype mice suggesting the KINGS mutation induces more severe beta cell ER stress compared to HFHS feeding. KINGS female mice fed normal chow also showed increased BiP compared to wildtype NC fed mice. HFHS feeding in the context of the KINGS mutation further increased BiP and ATF6 levels in the KINGS female islets, suggesting a further exacerbation of beta cell ER stress, but no additional increase was detected in XBP1s or p-eIF2-alpha, suggesting that in this context, signalling through ATF6 may be favoured.

It is unclear why HFHS feeding causes a different UPR response in the context of pre-existing beta cell ER stress (driven by the KINGS mutation) versus minimal ER stress (WT mice), however it is possible that different levels of beta cell ER stress drive the preferential activation of different UPR pathways. This notion is supported by Gupta *et al* who showed that mice fed a high fat diet for different lengths of time showed differential activation of UPR pathways; for example, beta cell ATF6 mRNA was found to be increased at 12 weeks on diet but not at 4 or 16 weeks on diet, and XBP1s mRNA was found to be increased from 12 weeks on diet but not at 4 weeks. *In vitro* experiments have also suggested that different levels of ER stress lead to differential

pathway activation (Kalwat et al., 2021). For example, 6-hour treatment of mouse islets with the ER stress inducer, thapsigargin, was associated with a greater increase in XBP1s mRNA compared to 24-hour treatment (Sharma et al., 2020). The type of ER stressor may also play some role in determining which UPR pathways are activated; ATF6 for example is activated earlier in mouse islets treated with thapsigargin compared to treatment with tunicamycin (Sharma et al., 2020). The latter may also go some way to explaining differences in UPR pathway activation in wildtype *vs* KINGS mice fed a HFHS diet compared to genotype-matched controls. Altogether, our findings and those from others support the notion that an obesogenic diet enhances beta cell ER stress and can exacerbate beta cell ER stress in the context of the KINGS mutation. Given that the female KINGS mice fed a HFHS showed increased expression of ER stress markers but did not show as severe hyperglycaemia as male KINGS mice fed NC, it would be of interest in future to perform a head-to-head comparison of ER stress marker expression between the two groups. This would provide insight into whether female mice are more resistant to increases in ER stress or whether they respond differently to equivalent levels of ER stress.

HFHS feeding resulted in increased weight gain in both KINGS and WT female mice, however this occurred earlier and was more profound in the WT mice. A similar result was found in the male mice, where a HFHS diet was only associated with increased weight gain in wildtype but not KINGS male mice. Uncontrolled diabetes in humans and many animal models of diabetes show weight loss, and as mentioned in section 3.3, male Akita mice show a reduced weight gain compared to wildtype mice. This occurs due to either insufficient total levels of circulating insulin or dysfunctional insulin which limits glucose use as a fuel source and drives the catabolism of muscle and adipose for energy. Insufficient functional insulin in the KINGS mice may explain differences in weight gain between wildtype and KINGS mice placed on a HFHS diet. Indeed, we have previously reported reduced circulating insulin in both male and female KINGS mice (Austin et al., 2020). Another possibility that may explain weight gain differences between WT and KINGS mice placed

on a HFHS diet is differences in dietary intake. Unfortunately, due to the mice grinding the feed, we were unable measure this.

Despite increased body weight and enhanced beta cell ER stress in wildtype female mice fed a HFHS diet, we found that non-fasted blood glucose and fasted blood glucose concentrations were largely unchanged in this group compared to NC fed controls. This suggests that under physiologically normal conditions, enhanced beta cell ER stress does not have a substantial effect of glycaemic control. This is likely because the increases in ER stress markers expression were subtle and didn't reach the levels seen in KINGS females fed normal chow, indicating a mild level of stress which can be compensated for. Interestingly, under conditions of increased beta cell stimulation (a glucose tolerance test), we found that HFHS feeding impaired glycaemic control and this impairment progressively worsened with time on diet. This indicates that whilst enhanced beta cell stress can be compensated for under physiological fluctuations in blood glucose levels, this is not the case under increased beta cell stimulation. It is important to note that glucose tolerance can also be influenced by peripheral insulin resistance, however only a subtle increase was detected in insulin resistance in the HFHS group suggesting that it is mainly a loss of beta cell function that accounts for the glucose tolerance impairment seen. Gupta *et al* also found that 60% high fat feeding causes a progressive worsening of glucose tolerance over time on diet in mice, but additionally found that a progressive worsening in insulin sensitivity contributed to this (Gupta et al., 2017). The starker insulin resistance in Gupta's study may be a result of a higher fat content in the diet as well as the fact that male mice were used.

HFHS feeding in female KINGS mice did result in an increase in non-fasted blood glucose concentration suggesting that the increase in beta cell ER stress in this group does impair glycaemic control under physiological conditions. Surprisingly however, non-fasted blood glucose concentrations in the majority on HFHS KINGS females (with the exception of one) only occasionally exceeded the 16.7mM threshold for overt diabetes and were lower than that seen in male KINGS mice fed normal chow. This suggests that

despite exacerbated beta cell ER stress in these mice, females are still able to adapt and maintain normoglycemia to a certain extent. Supporting this idea, fasted blood glucose concentrations (which removes the direct influence of the HFHS on blood glucose concentrations) were only moderately increased with HFHS feeding in the majority of female KINGS mice and were within the normoglycemic range (apart from one mouse). In line with the findings in wildtype mice, glucose tolerance was impaired from 1-week on diet and progressively worsened with time on diet, which may suggest that beta cell ER stress compromises beta cell function in the context of increased stimulation. Insulin resistance as established through insulin tolerance tests was unchanged with HFHS feeding in the female KINGS mice suggesting that impaired glucose tolerance is not the result of a reduction in peripheral insulin sensitivity. This contrasts the subtle insulin resistance caused by HFHS feeding wildtype mice and this difference may be a result of a more significant increase in weight gain with HFHS in the wildtype mice compared to the KINGS mice; by 20 weeks of age (17 weeks on diet) HFHS mice were on average 7.5g heavier than NC mice in the wildtype groups and 5.7g heavier in the KINGS groups. Reduced weight gain in the KINGS mice on a HFHS diet compared to WT mice may be the result of reduced insulin availability for excess nutrient storage. Reduced weight gain in older male KINGS mice (but not in KINGS females) compared to wildtype mice has also been reported and this may suggest that KINGS-HFHS mice adopt a phenotype more similar to male KINGS mice (Austin et al., 2020).

Despite a lack of weight gain in the KINGS males fed a HFHS diet, area under the curve for non-fasted blood glucose concentrations over time were elevated compared to NC fed KINGS males, indicating a further impairment in glycaemic control caused by the diet. Although not significant, there was also a trend for increased area under the curve for the glucose tolerance tests in the male HFHS KINGS group compared to the NC group. This likely didn't reach significance because 2 out of the 5 mice in the HFHS group were excluded on ethical grounds due to having basal glucose concentrations exceeding 20mM. Indeed, the fact that no mice in the NC KINGS group had basal blood glucose concentrations exceeding 20mM goes some way to

supporting a further impairment of glucose tolerance with HFHS feeding in male KINGS mice. These results suggest that under normal physiological and possibly also under stimulated conditions, beta cell function and thus glycaemic control is impaired with HFHS feeding in male KINGS mice. To investigate the influence of the HFHS diet on glycemia without the direct influence of the diet, blood glucose concentrations were measured following a 16-hour fast. Fasted blood glucose concentrations were 2-fold higher in HFHS fed KINGS males compared to NC mice, which contrasts the more subtle increase (1.4-fold vs NC mice) seen with HFHS feeding in the KINGS females. This may further support the notion that female KINGS mice have an improved capacity to adapt to beta cell ER stress, even when ER stress is exacerbated, whilst male KINGS mice do not. The latter is in line with findings from a previous study from our laboratory where pregnancy was used as an environmental stressor to exacerbate beta cell stress in the KINGS females. Intriguingly, glycaemic control was not impaired with pregnancy (which is associated with enhanced insulin resistance) further emphasising the ability of KINGS females to respond adaptively to further stress, however it is important to note that beta cell ER stress was not directly measured in this study (Austin, 2018).

Islet area was analysed in female KINGS mice fed a HFHS diet to ascertain whether adaptation to exacerbated ER stress may be driven by beta cell hypertrophy or proliferation, however no differences were detected compared to the NC mice. This was despite elevated ATF6 protein levels in islets from HFHS KINGS females, which has previously been associated with increased beta cell proliferation (Sharma et al., 2015). Female KINGS mice may be adapting through increased beta cell insulin secretion, or it is possible that subtle changes in beta cell proliferation or hypertrophy were not detected. In future, it would be interesting to assess glucose stimulated insulin secretion in islets isolated from these mice and to look at beta cell proliferation in a more accurate way for example through Ki67 staining or investigating BrdU incorporation.

Wildtype male mice fed a HFHS diet showed a significant increase in weight gain. Interestingly, the rate of body weight increase seen in wildtype mice with HFHS feeding was greater in the male mice than in the female mice, and this is in line with many previous findings suggesting that females are more resistant to the effects of an obesogenic diet. A recent study has suggested that sex differences in weight gain after high fat feeding may be explained by females maintaining activity levels and having an improved ability to use fat as a fuel source (Casimiro et al., 2021). Despite this, wildtype male mice had no change in non-fasted blood glucose concentrations with HFHS feeding and although they showed a trend for increase area under the curve for glucose tolerance tests conducted at 4, 8 and 10 weeks of age ($p=0.067$), this did not reach significance. This may be due to the fact that this study was a pilot and therefore is likely under powered. Indeed, previous studies have shown that HFHS feeding in male mice results in glucose intolerance from even as early as 1-day on HFHS diet (Demaria et al., 2023). Although it should be noted that other studies have not found a difference in glucose tolerance with HFHS feeding (Omar et al., 2012).

Overall, we have shown that beta cell ER stress can be manipulated through dietary modification which is in line with previous studies and adds support to the notion that beta cell ER stress is an important driver in TD2M which is often associated with dietary excess and obesity. We have also shown that beta cell ER stress can be exacerbated in the context of the KINGS mutation. In the females this drives a further impairment in glycaemic control, with non-fasted blood glucose concentration elevated and glucose tolerance worsened, but did not result in overt diabetes development. This indicates that female KINGS mice have a better capacity to adapt to beta cell ER stress since sex differences remain in diabetes phenotype despite increasing beta cell ER stress levels. In male KINGS mice HFHS feeding further exacerbated impaired glycemia with non-fasted and fasted blood glucose concentrations increased. Since glycemia can be worsened in male KINGS mice, this may suggest that there is still a certain level of beta cell compensation in KINGS males fed NC and thus a window in which beta cell ER stress could be alleviated and glycaemic control improved.

CHAPTER 7: CAN THE DEVELOPMENT OF OVERT DIABETES BE PREVENTED IN MALE KINGS MICE?

Chapter snapshot:

Beta cell ER stress has been implicated in the pathogenesis of diabetes. It is important to understand the extent to which preventing or alleviating beta cell ER stress can prevent the development of diabetes as this may reveal novel diabetes therapeutics.

In this chapter, we used KINGS male mice as a model of beta cell ER stress and determined whether the clinically available drug, liraglutide, could prevent or alleviate diabetes. We also investigated whether TUDCA, a drug not currently used to treat diabetes, could prevent overt diabetes. Finally, the influence of endogenous testosterone removal on preventing diabetes was studied.

7.1 INTRODUCTION:

It is thought that whilst mild ER stress is advantageous for the beta cell, facilitating compensation and adaptation in the face of increased demand, more robust ER stress that cannot be resolved results in beta cell dysfunction and death. The latter has been associated with the development and progression of many types of diabetes, and numerous factors have been implicated in tipping the balance towards maladaptive ER stress signalling. These factors can be subdivided into those that are inherent (including hormones such as oestrogen which was explored in chapter 5) and those that are extrinsic and can be introduced (including an obesogenic diet which was explored in chapter 6). A more robust understanding of what factors can manipulate ER stress levels in the context of diabetes may lead to novel therapeutics or treatment options for diabetes.

In this chapter we used the KINGS male mouse as a model of beta cell ER stress-induced diabetes and determined whether liraglutide, a GLP-1 receptor agonist used to treat diabetes in the clinic, could prevent the development of diabetes. We also investigated whether the FDA approved drug, TUDCA, which is not currently used in diabetes treatment clinically, could prevent diabetes in male KINGS mice. Finally, we assessed whether removal of the major male sex hormone, testosterone, could protect male KINGS mice from becoming overtly diabetic.

Liraglutide is a GLP-1 receptor agonist whose development (along with other GLP-1 receptor agonists such as exenatide and semaglutide) was triggered by the discovery of glucagon-like peptide 1 (GLP-1). GLP-1 is a gut-derived incretin hormone released post-prandially which reduces circulating blood glucose concentrations in numerous ways including through increasing insulin release, delaying gastric emptying, and inhibiting glucagon secretion (Lafferty et al., 2023). Since GLP-1 is rapidly degraded by dipeptidyl peptidase-4 (DPP-4) activity, compounds were developed that mimicked GLP-1's physiological activity whilst resisting this inactivation. The first of these compounds used for the treatment of diabetes was exenatide. Liraglutide was subsequently developed which harbours a longer half-life and is required to be injected once daily compared to twice daily in patients with T2DM (12-hour *vs.* 2.4-hour half-life) (Gorgogietas et al., 2023; Yu et al., 2018).

Several studies have suggested that one of the ways in which GLP-1 receptor agonism may mediate it's anti-diabetogenic effects is through reducing beta cell ER stress mediated beta cell death and dysfunction. *In vitro* studies have found that exenatide treatment improves beta cell survival and insulin translation in primary rat beta cells and INS-1 cells and protects INS-1 cells from ER stress mediated death (Cunha et al., 2009; Yusta et al., 2006). Liraglutide treatment has also been found to reduce ER stress marker expression in INS-1 cells exposed to the ER stress inducer, thapsigargin, improving viability and glucose stimulated insulin secretion (Li et al., 2018). *In vivo* studies have corroborated these findings; treatment of *Lep*$^{db/db}$ mice with exenatide reduced blood glucose concentrations and this was associated with

reduced beta cell ER stress measured through CHOP protein expression and XBP1s/XBP1 mRNA levels in islets (Cunha et al., 2009). Exenatide treatment of mouse models that develop diabetes primarily due to beta cell ER stress (the calmodulin-overexpressing transgenic mouse and the Akita mouse) also reduced blood glucose concentrations and was associated with decreased beta cell CHOP expression (Tsunekawa et al., 2007; Yamane et al., 2011). However, in contrast, more recently Zhao *et al* found that liraglutide treatment in Akita mice failed to reduce non-fasted blood glucose concentrations although it was associated with reduced protein expression of several beta cell ER stress markers (Zhao et al., 2013).

In the present study we wanted to investigate whether liraglutide treatment in a novel model of beta cell ER stress, the KINGS mouse, could prevent diabetes when administered prior to the development of diabetes (before 5-6 weeks of age). We also wanted to establish whether liraglutide treatment could normalise blood glucose concentrations after overt diabetes had been established. Previous studies looking into the effects of GLP-1 receptor agonism on ER stress in animal models have either focussed on preventing diabetes or alleviating established diabetes. This is the first study to our knowledge where the two are directly compared, thus providing an opportunity to gain insight into the extent to which diabetes can be reversed using this therapy.

In this chapter we also performed a pilot study investigating whether Tauroursodeoxycholic acid (TUDCA) treatment could prevent diabetes development in male KINGS mice. TUDCA is a hydrophilic bile acid which is used in the clinic to treat cholestatic liver disease and gall stones (Ajmal et al., 2023). However, exogenous TUDCA administration has also been associated with improved glycemia in preclinical studies (Ajmal et al., 2023). For example, in C57BL6/J mice treated with streptozotocin to induce diabetes, TUDCA administration was associated with a ~40% reduction in blood glucose concentrations and improved glucose tolerance (Bronczek et al., 2019). TUDCA also reduced blood glucose levels in the *Lep*$^{ob/ob}$ model and reduced diabetes incidence in two models of type 1 diabetes; the NOD mouse and the

RIP-LCMV-GP mouse (rat insulin promoter-lymphocytic choriomeningitis virus-glycoprotein) (Engin et al., 2013; Özcan et al., 2006). These anti-diabetogenic effects can be attributed to direct effects on the beta cell since *in vitro* treatment of INS-1 cells with TUDCA prevented palmitate induced death and dysfunction, and TUDCA treatment of pig and mouse islets *ex vivo* improved insulin secretion (Lee et al., 2010; Rosa et al., 2021; Vettorazzi et al., 2016; Zhu et al., 2012).

The mechanisms by which TUDCA reduces beta cell apoptosis and dysfunction are as yet incompletely understood although TUDCA is known to interact with numerous receptors present on the beta cell (Rosa et al., 2021). Studies have suggested that one of these mechanisms involves TUDCA's activity as a pharmaceutical chaperone, improving beta cell protein folding capacity and thus reducing ER stress. Indeed, *in vitro* treatment of beta cell lines and primary islets with TUDCA is associated with a reduced expression of ER stress markers (Lee et al., 2010; Sharma et al., 2015; Zhu et al., 2012). This has also been reported *in vivo*, with TUDCA treatment in Akita and *Lep*$^{db/db}$ mice reducing islet expression of the ER stress marker, BiP (Sharma et al., 2015). However, it is important to note that the latter study did not quantitatively analyse BiP protein levels. Engin *et al.* proposed that TUDCA reduced diabetes incidence in type 1 models (NOD and RIP-LCMV-GP) by restoring UPR signalling in islets; whilst vehicle treated mice had reduced ATF6 and XBP1s protein expression, TUDCA treatment restored this (Engin et al., 2013).

TUDCA has also been shown to reduce ER stress in other cell types *in vivo* including hepatocytes, neurones and adipose tissue (Chen et al., 2022; Özcan et al., 2006). The latter may also go some way to explaining TUDCA's beneficial effects on glycemia outside of direct beta cell effects since enhanced adipocyte and hepatic ER stress is associated with the development of peripheral insulin resistance in obesity and contributes to the development of T2DM (Fernandes-da-Silva et al., 2021). Indeed, TUDCA treatment in *Lep*$^{ob/ob}$ mice improved insulin sensitivity and this was associated with a reduction in hepatic and adipose ER stress (Özcan et al., 2006). Furthermore,

the only clinical study published to date which investigated TUDCA treatment in the context of metabolic syndrome and diabetes, found that patients treated with TUDCA showed a 30% improvement in muscle and hepatic insulin sensitivity, although no change was observed in tissue expression of ER stress markers in this study (Kars et al., 2010). Considering the evidence supporting TUDCA's ability to reduce beta cell ER stress, here we tested whether TUDCA treatment in the KINGS model could prevent the development of diabetes. TUDCA is thought to have beneficial activity on glycemia that extends past improving beta cell ER stress. By investigating the effects of TUDCA on glycemia in a model of diabetes that is primarily driven by ER stress, we may be able to gain insight into whether TUDCA's ability to reduce beta cell ER stress is sufficient to prevent diabetes. The only study to date looking into the effects of TUDCA on glycaemic control in a model of diabetes driven by beta cell ER stress only used 2 days of treatment and did not find any impact of blood glucose levels (Sharma et al., 2015).

Beta cell ER stress has also been shown to be influenced by sex hormones and in particular 17-beta-oestradiol (which we explored in chapter 5) has received the most study. This is perhaps unsurprising given the female protection from diabetes development in many animal models, including the KINGS mouse, and in humans. However, other sex hormones are present at different circulating levels between males and females and may also be responsible for driving these sex differences.

Testosterone is the major male sex hormone. Whilst circulating levels of testosterone are similar between pre-pubescent men and women, levels increase rapidly after the onset of puberty in men and become 15-fold higher compared to levels in women (Handelsman et al., 2018). Although research into the direct influence of testosterone on the beta cell is limited, studies have been conducted investigating the influence of testosterone on diabetes incidences in humans, however these are somewhat conflicting. Some studies have found that low testosterone levels are associated with an increased risk of T2DM in men whilst testosterone excess in women drives an increased risk (Elabbady et al., 2016; Navarro et al., 2015; Yao et al., 2018). Indeed, women

diagnosed with polycystic ovary syndrome, a risk factor for the development of T2DM, exhibit higher circulating levels of testosterone (although other factors also likely play a role such as obesity) (Rubin et al., 2017). However, other studies have found no association between risk of diabetes development and circulating testosterone levels (Holmboe et al., 2016; Lakshman et al., 2010). Interestingly, more recently, testosterone treated overweight or obese men showed a reduced T2DM risk compared to placebo controls (Wittert et al., 2021).

Testosterone mediates its activity through the androgen receptor (AR) which acts as a transcription factor when activated. Both male and female beta cells show AR expression, suggesting that testosterone signalling can directly influence beta cell function (Xu et al., 2019). Indeed, male mice with beta cell specific knockdown of AR have reduced glucose stimulated insulin secretion which results in mild glucose intolerance, exacerbated further with high fat feeding (Navarro et al., 2016). Xu *et al.* recently showed that AR signalling increases insulin secretion by enhancing the activity of GLP-1 (Xu et al., 2023). Testosterone has also been found to be protective against INS-1 cell apoptosis in the content of hydrogen peroxide-induced oxidative stress (Kang et al., 2021). On the other hand, some studies have suggested that testosterone contributes to beta cell dysfunction. For example, pre-treatment of male mice with anti-androgen or performing orchidectomy reduced the hyperglycaemic effect of streptozotocin, which is classically more potent in male mice. Additionally, pre-treatment with androgens in female mice increased the hyperglycaemic effect of streptozotocin (Maclaren et al., 1980; Paik et al., 1982). More study is required into the direct effects of testosterone on the beta cell and its contribution to sex differences in diabetes. One area in particular that has received very little study is the role of testosterone in beta cell ER stress. A recent study by Rossetti *et al.* found that the pubertal testosterone increase in male rats is associated with an increase in UPR markers in hepatocytes, suggesting that testosterone could play a role in enhancing ER stress in other cell types including the beta cell (Rossetti et al., 2019). Therefore, in this study we used the KINGS mouse as a model of beta

cell ER stress to ascertain whether endogenous testosterone removal though orchidectomy could protect male mice from the development of diabetes.

7.2 AIMS:

- Determine whether liraglutide treatment can prevent diabetes development in the male KINGS mice and investigate whether any effects on blood glucose homeostasis are transient or permanent.
- Perform a pilot study to determine whether TUDCA treatment can prevent diabetes onset in male KINGS mice.
- Investigate whether endogenous testosterone removal through performing orchidectomy can prevent diabetes development in male KINGS mice.

7.3 METHODS:

7.3.1 EXPERIMENTAL OUTLINE:

Liraglutide study (Figure 80): KINGS male mice were administered liraglutide (200µg/kg) via subcutaneous injection daily from weaning (3 weeks) for 3 weeks (until the age of 6 weeks) and blood glucose concentration measurements were taken daily to determine whether liraglutide administration could prevent diabetes development. Treatment was subsequently stopped for 2 weeks and during this time blood glucose measurements were taken to determine whether the effects of liraglutide treatment on blood glucose homeostasis were transient or permanent. At 8 weeks of age, liraglutide treatment was resumed to determine whether liraglutide treatment could alleviate established diabetes. As a positive control for normoglycemia, WT male mice were also injected with PBS from 3 weeks until 10 weeks of age.

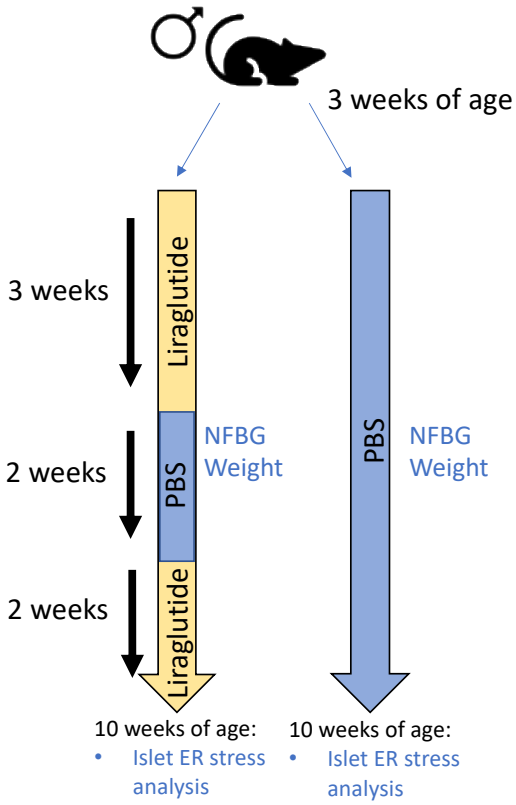


FIGURE 80: *Schematic showing the experimental outline to assess whether liraglutide can prevent the development of diabetes in the KINGS male mice and whether it can alleviate diabetes once this is established: Experimental readouts are depicted by the blue text: non-fasted blood glucose concentration measurements (NFBG).*

TUDCA pilot study (Figure 81): 3-week-old male KINGS were administered TUDCA through twice-daily intraperitoneal injections (500mg/kg total daily dose) for 3 weeks and blood glucose concentrations were monitored once daily to investigate the effects of this on blood glucose homeostasis. Glucose tolerance tests were performed at 6 weeks of age, after the 3-week treatment course.

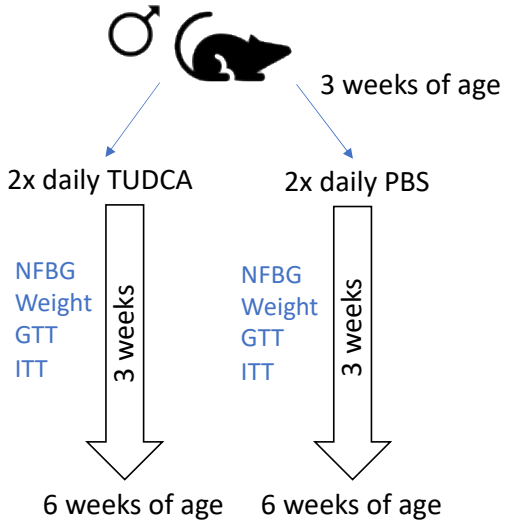


Figure 81: *Schematic showing the experimental outline to assess whether TUDCA treatment can prevent the development of diabetes in the KINGS male mice:*
Experimental readouts are depicted by the blue text: non-fasted blood glucose concentration measurements (NFBG), glucose tolerance test (GTT), insulin tolerance test (ITT).

Orchidectomy study (Figure 82): KINGS male mice were orchiectomised at 3 weeks of age (prior to puberty and the onset of diabetes). Blood glucose concentrations were monitored, and glucose tolerance tests performed to determine the effect of endogenous testosterone removal on diabetes development. Insulin tolerance tests were also carried out to assess whether changes in insulin resistance as a result of orchidectomy contributed to changes in glycaemic control.

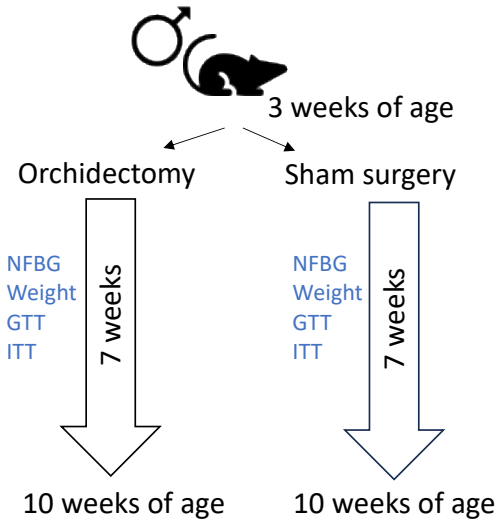


FIGURE 82: *Schematic showing the experimental outline to assess whether pre-pubertal removal of endogenous testosterone can prevent the development of diabetes in the KINGS male mice:*
Experimental readouts are depicted by the blue text: non-fasted blood glucose concentration measurements (NFBG), glucose tolerance test (GTT), insulin tolerance test (ITT).

7.3.2 Drug administration:

Liraglutide was administered mice starting from 3 weeks of age at a daily dose of 200µg/kg through subcutaneous injections for 3 weeks until mice were 6 weeks of age. At 6 weeks of age all mice were injected with PBS daily for 2 weeks and at 8 weeks of age liraglutide was re-administered for a further 2 weeks. Control groups received a PBS injection daily throughout.

TUDCA was administered through intraperitoneal injection twice a day (250mg/kg per injection, a total of 500mg/kg daily) in mice from 3 weeks of age for 3 weeks until mice were 6 weeks of age. The injection site was alternated between left and right daily to reduce any pain experienced by the mice and mice were monitored throughout the experiment for any signs of ill health. Control groups received twice-daily PBS injections throughout.

7.3.3 Orchidectomy:

Orchidectomy was carried out as previous described (see section 2.3.3). Briefly, 3-week-old mice were anaesthetised using 5% isoflurane, 95% oxygen in an induction chamber. The lower abdomen was shaved and washed using Hibiscrub and ethanol. Mice were maintained under anaesthesia using ~2-3% isoflurane supplied through a nose cone on a heated mat and viscotears was applied to the eyes. A 1cm incision was made using a scalpel to the lower abdomen and the skin was bluntly dissected away from the underlying fascia. A further 0.5cm incision was made in the fascia directly below the initial incision site and the perigonadal fat pads were located. Sequentially, each fat pad was gently pulled from the abdomen using forceps to expose the testes and the blood vessel supplying the testes was cauterised using a cautery pen. A scalpel was used to remove each testis and suture was used to close both incisions. Lidocaine (2mg/kg) was administered subcutaneously at the site of incision and Carprofen (4mg/kg) was administered intraperitoneally. For mice receiving sham surgery, the testes were removed and replaced but all other surgical procedures were the same. Mice were placed in a heated chamber and were monitored for appropriate recovery.

7.3.4 Assessment of preputial separation:

To determine whether orchidectomy surgery was successful in male KINGS mice, onset of puberty was assessed by checking for preputial separation (an external indicator of puberty onset in male mice). After the mice were culled at the end of the experiment, the abdomen either side of the penis was gently pushed inwards to determine whether the penis was separate from the prepuce (Figure 83). Separation indicated puberty onset, whereas no separation indicated no onset of puberty and hence successful orchidectomy.

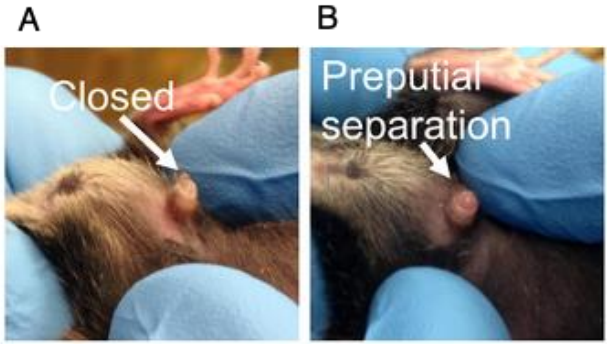


FIGURE 83: *Assessment of preputial separation used to investigate puberty onset and thus success of orchidectomy in male mice:*
(A) No preputial separation suggesting that puberty has not occurred (B) Preputial separation which is an external puberty marker. Images taken and adapted from (Hoffmann, 2018).

7.3.5 Food intake monitoring:

Food intake was measured per cage over a 24-hour period. The food hopper was removed and weighed at ~9am, any food pellets at the base of the cage were removed and the food hopper was replaced. At 9am the following day, the food hopper was re-weighed, and the base of the cage was checked for any food pellets whose weight was also added. The difference between the food hopper weight over the 24-hour period was divided by the number of mice in the cage to get an estimate of food intake per mouse. Since there were only 2 cages of WT-PBS mice, the n value for this group was 2.

7.3.6 Blood glucose concentration and weight monitoring:

Non-fasted blood glucose concentrations and weight was monitored in mice administered liraglutide as previously described once a day for 7 weeks (see section 2.2.1). For mice administered TUDCA, blood glucose concentrations were monitored once a day for 3 weeks, and for orchiectomised mice, blood glucose concentrations were measured once a week for 7 weeks.

7.3.7 Glucose tolerance tests:

Glucose tolerance tests were carried out in orchiectomised mice at 10 weeks of age and in TUDCA treated mice at 6 weeks of age as previously described (see section 2.2.3). Briefly, blood glucose concentrations were measured after a 6-hour fast and before intraperitoneal administration of a glucose solution (2g/kg) and subsequent measurement of blood glucose concentrations at 15, 30, 60, 90 and 120 minutes post injection.

Initially glucose tolerance tests were carried out at 5 weeks of age for a subset of mice administered liraglutide, however we found that this caused non-fasted blood glucose concentrations on the subsequent day to be elevated compared

to previous days. We believed that delivery of such a high bolus of glucose may alter the effects of liraglutide on glycaemic control therefore glucose tolerance tests were not carried out for the remaining mice.

7.3.8 Insulin tolerance tests:

Insulin tolerance tests were carried out for orchiectomised mice at 10 weeks of age and TUDCA-treated mice at 6 weeks of age, at least 2 days after the glucose tolerance tests were performed, as previously described (see section 2.2.4). Briefly, blood glucose concentrations were measured after a 2-hour fast and before a 0.75IU/kg intraperitoneal administration of insulin solution and subsequent measurement of blood glucose concentrations at 15, 30, 45 and 60 minutes post injection.

7.3.9 Immunofluorescent staining and analysis:

To investigate the effect of liraglutide treatment on beta cell ER stress, BiP expression was measured in islets from fixed pancreas sections derived from mice treated with liraglutide or vehicle through immunofluorescent staining (see section 2.6.2.4). In brief, pancreases were fixed using phosphate-buffered formalin (3.5%), embedded in paraffin wax and cut into 5μM sections which were then mounted on superfrost glass slides. Sections were dewaxed with histoclear and rehydrated through incubation with decreasing concentrations of ethanol. Heat mediated citric acid antigen retrieval was subsequently carried out and sections were incubated with primary antibodies towards BiP (1:100 rabbit anti-BiP, Cell signalling technology) and insulin (1:200, guinea pig anti-insulin, Abcam) overnight at 4ºC. The next day, sections were washed and incubated with relevant secondary antibodies against rabbit (1:200, anti-rabbit Alexa Fluor 594, Jackson ImmunoResearch) and mouse (1:200, anti-guinea pig Alexa Fluor 488, Jackson ImmunoResearch) as well as with DAPI (1:500). Sections were imaged using a Hamamatsu fluorescent slide scanner. FijiImageJ was used to semi-automatically draw regions of interest around islets based on insulin staining and mean fluorescent intensity in the BiP channel was measured as previously outlined (see section 2.6.4.4) (Schindelin et al., 2012).

7.3.10 Statistical analysis:

All graphs were constructed using GraphPad Prism 8.0 or 9.0 software (GraphPad Software, San Diego, CA) and data represents mean±SEM. All statistical analysis was carried out in SigmaPlot 14.0 (Systat software Inc, San Jose, California). For comparisons between two groups, a two-tailed unpaired T test was used. For comparisons between multiple groups where there were two independent variables, a two-way ANOVA with Holm-Sidak poc hoc test was used to assess statistical significance. A two-way repeated measures ANOVA with Bonferroni post hoc was used to assess statistical significance for repeated measures over time where time represented one independent variable and there was an additional independent variable. A $p<0.05$ was considered statistically significant.

7.4 Results:

7.4.1 Liraglutide treatment alleviates diabetes in the KINGS male mice but this is not sustained post-treatment:

Daily liraglutide treatment in male KINGS mice starting prior to the normal onset of overt diabetes (3 weeks of age) prevented diabetes development (Figure 84). Whilst PBS treated KINGS male mice crossed the threshold for overt diabetes (16.7mM) by 41 days of age, KINGS males treated with liraglutide remained below this threshold for entire liraglutide administration period and only reached a maximum average blood glucose concentration of 11.8mM. In line with this, blood glucose concentrations were significantly lower in liraglutide treated KINGS males compared to PBS-treated KINGS males from 37 days of age. In addition, blood glucose concentrations in liraglutide treated KINGS males were not found to significantly differ from WT-PBS mice for the initial liraglutide treatment period.

Following cessation of liraglutide treatment at 42 days of age, blood glucose concentrations progressively increased in the KINGS-liraglutide group, crossing the threshold for overt diabetes by 47 days of age and becoming significantly elevated compared to 42 days of age by 48 days. By 47 days of age, blood glucose concentrations did not differ between PBS-treated and liraglutide pre-treated KINGS mice and in line with this, from 44 days of age

blood glucose concentration significantly differed between wildtype-PBS and KINGS-liraglutide groups.

When liraglutide treatment was resumed in the KINGS male mice, blood glucose concentrations were lowered, becoming consistently reduced compared to PBS-treated KINGS mice by 60 days of age. However, blood glucose concentrations remained close to the threshold for overt diabetes and were higher compared to blood glucose concentrations during the first treatment with liraglutide at 21-42 days of age and compared to wildtype-PBS mice.

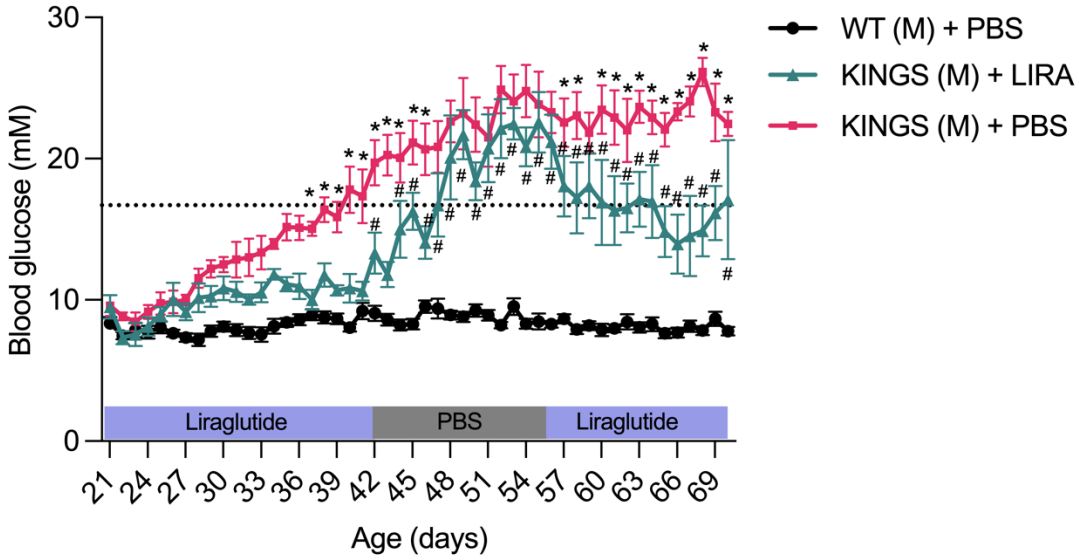


FIGURE 84: *Non-fasted blood glucose concentrations in male KINGS mice treated with liraglutide:*

*(A) Non-fasted blood glucose concentrations over time in WT and KINGS male mice administered PBS for the entirety of the study and KINGS mice administered liraglutide (LIRA) for 3 weeks at the beginning of the study followed by administration of PBS for 2 weeks and finally 2 subsequent weeks of liraglutide treatment. Data represents the mean±SEM. Two-way repeated measures ANOVA with Bonferroni post hoc test, *= p<0.05 KINGS-PBS vs. KINGS-LIRA, #= p<0.05 WT-PBS vs. KINGS-LIRA, n=6-8.*

7.4.2 No significant change in weight or food intake with liraglutide treatment in male mice:

GLP-1 receptor agonism has been associated with weight loss in animal models as well as in humans and this has been in-part associated with changes in appetite. Since changes in glycaemic control seen in the KINGS mice treated with liraglutide could be in-part the result of changes in body weight, weight was monitored in these mice throughout the study. Weekly food intake was also measured during the initial period of liraglutide treatment (3-5 weeks).

Liraglutide did not impact weight in the KINGS mice, with no differences detected in weight or weight gain at any age between the different groups (Figure 85A-B). In line with this, no differences in food intake were detected between the groups (Figure 85C). Of note there is a trend for reduced food intake in the liraglutide treated KINGS mice compared to the PBS-treated KINGS mice after 1-week of treatment ($p=0.081$). As expected with increased weight gain with age, we also found that both PBS and liraglutide treated mice showed increased food intake between 1 and 3 weeks of treatment. Since there were only 2 cages housing the WT mice treated with PBS, the n number (2) was too low for statistical analysis.

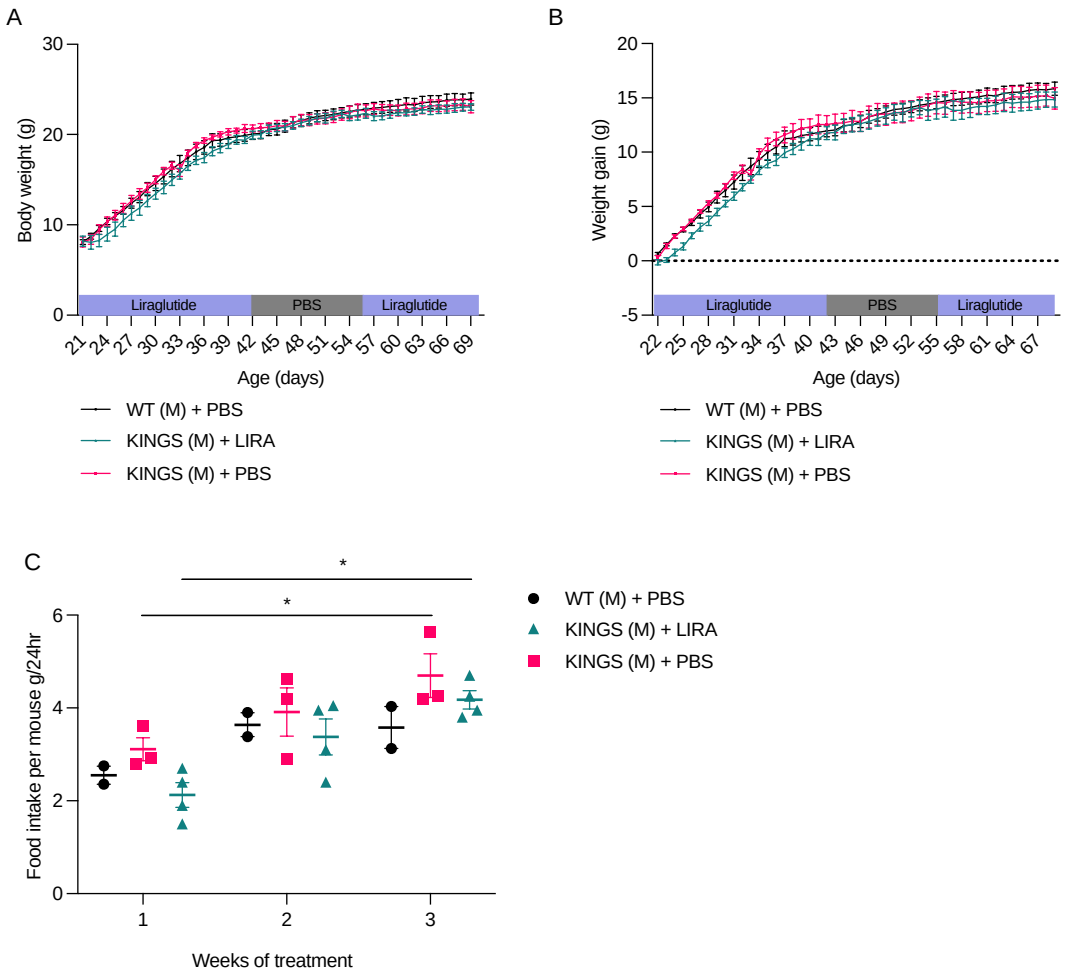


FIGURE 85: *Body weight, weight gain and food intake in male mice treated with liraglutide:*

(A-B) Body weight and weight gain over time in WT and KINGS male mice administered PBS for the entirety of the study and KINGS mice administered liraglutide (LIRA) for 3 weeks at the beginning of the study followed by administration of PBS for 2 weeks and finally 2 subsequent weeks of liraglutide treatment. Data represents the mean±SEM. Two-way repeated measures ANOVA with Bonferroni post hoc test, n=6-8. (C) Weekly food intake during the initial treatment of KINGS or WT mice with LIRA or PBS. Food intake was measure per cage over a 24-hour period and this was divided by the number of mice in the cage to give an estimate of food intake per mouse. Two-way repeated measures ANOVA with Bonferroni post hoc test, n=3-4 (statistics was not carried out on the WT-PBS group as n=2).

7.4.3 Liraglutide treatment is not associated with reduced beta cell BiP expression or changes in islet size:

Liraglutide's beneficial effects on glycaemic control have been associated with a reduction in beta cell ER stress as well as increased beta cell proliferation (Fusco et al., 2017; Moffett et al., 2015). To ascertain whether improvements in glycaemic control were the result of improved beta cell ER stress in the KINGS mice, protein expression of the general ER stress marker, BiP, was assessed through immunofluorescence staining of islets from pancreatic sections of liraglutide or PBS treated KINGS mice. In addition, to determine whether liraglutide enhanced beta cell proliferation, islet size was assessed through immunofluorescent staining for insulin.

In line with our previous findings, BiP expression was significantly increased in PBS treated KINGS mice compared to PBS treated WT mice (Figure 86A). Liraglutide treatment in KINGS mice did not result in a reduction in islet BiP expression, with BiP fluorescent intensity not found to be different between PBS and liraglutide treated KINGS mice and significantly increased compared to WT-PBS mice.

Islet area was increased in KINGS-PBS mice compared to WT-PBS mice, in contrast to our previous findings in chapter 3 suggesting no difference in islet size (Figure 86B). Liraglutide treatment in male KINGS mice did not result in an increase in islet area, with area no different from KINGS-PBS or WT-PBS mice.

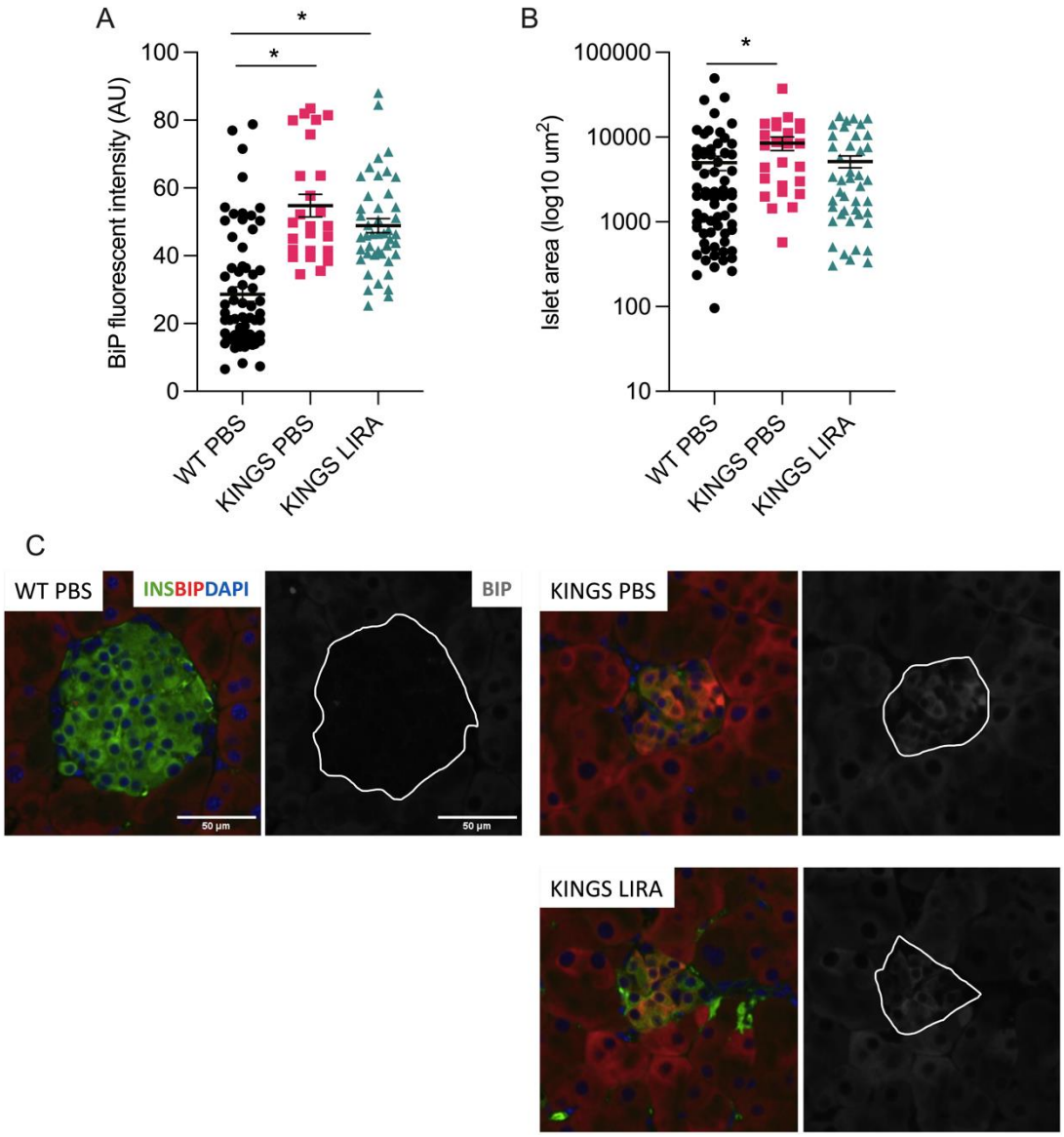


FIGURE 86: *Islet BiP expression and islet area in male mice treated with liraglutide:*

(A) BiP fluorescent intensity of islets from WT and KINGS mice treated with PBS for 7 weeks from weaning (3 weeks) and KINGS mice treated initially with liraglutide for 3 weeks, then PBS for 2 weeks and finally liraglutide was re-administered for a further 2 weeks. (B) Islet area in WT and KINGS mice treated with PBS for 7 weeks from weaning (3 weeks) and KINGS mice treated initially with liraglutide for 3 weeks, then PBS for 2 weeks and finally liraglutide was re-administered for a further 2 weeks. Data represents n=25-68 individual islets from 4-8 mice per group. Mean±SEM is shown. One-way ANOVA on ranks with Dunn's post hoc test, p<0.05. (C) Representative immunofluorescent images of islets from WT-PBS, KINGS-PBS and KINGS-

LIRA male mice stained for insulin (green), BiP (red and grey) and with DAPI (blue). The BiP channel is also shown alone (grey), and the outline of the islet is indicated with a white line. A scale bar representing 50µm is shown in the first image.

7.4.4 TUDCA TREATMENT DOES NOT PREVENT DIABETES DEVELOPMENT IN KINGS MALES OR IMPROVE GLUCOSE TOLERANCE:

Twice daily administration of TUDCA to KINGS mice starting from weaning (3 weeks of age) until 6 weeks of age did not prevent the development of overt diabetes in male KINGS mice (Figure 87B). KINGS mice administered TUDCA crossed the threshold for overt diabetes at 42 days of age which was only one day after the PBS control KINGS mice crossed this threshold. Moreover, blood glucose concentrations were not found to be significantly different between PBS and TUDCA treated KINGS mice at any age and area under the curve values for blood glucose concentrations over time were similar (Figure 87C).

WT mice administered TUDCA showed similar blood glucose concentrations compared to the PBS treated WT mouse (Figure 87A,C), however since n numbers were low (n=1-2) we were not able to perform statistical analysis.

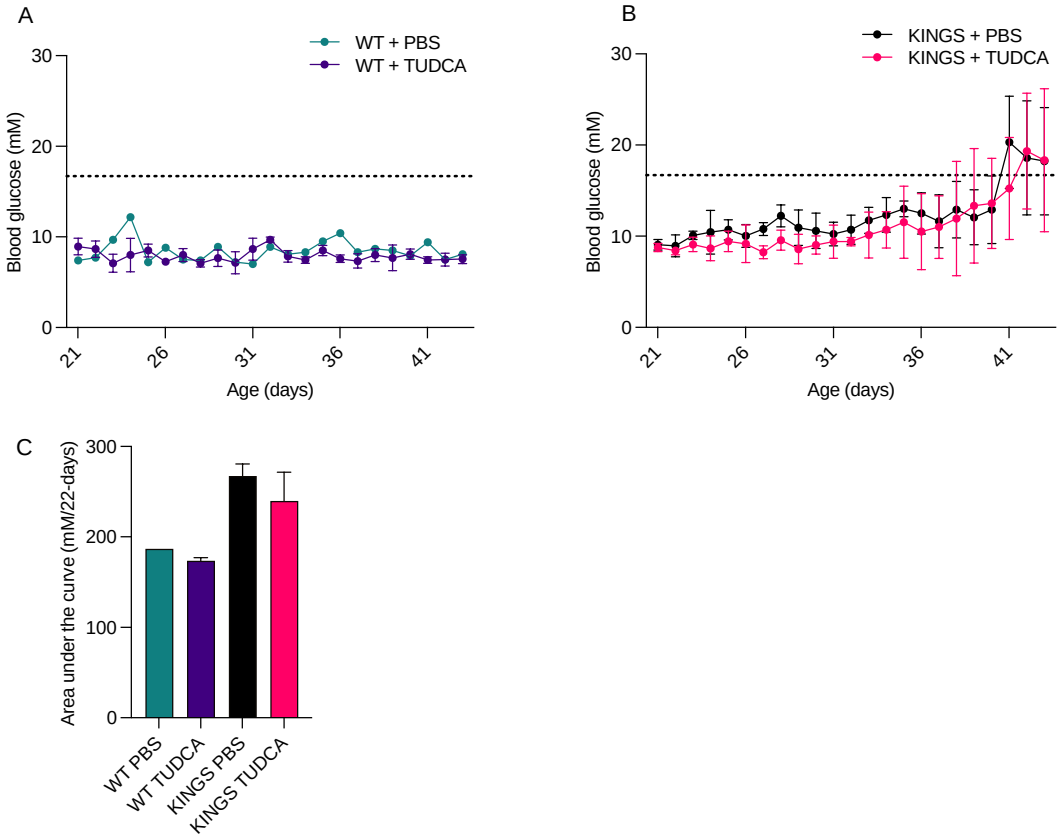


FIGURE 87: *Non-fasted blood glucose concentrations in male mice treated with TUDCA:*

(A) Non-fasted blood glucose concentrations over time in WT male mice treated with TUDCA or PBS vehicle control. Where n numbers are more than 1, data represents the mean±SEM, n=1-2 (statistical analysis was not carried out) (B) Non-fasted blood glucose concentrations in KINGS male mice treated with TUDCA or PBS control. Data represents the mean±SEM, two-way repeated measures ANOVA with Bonferroni post hoc, n=3. (C) Area under the curve values for non-fasted blood glucose concentrations over time in WT and KINGS males treated with TUDCA or PBS. Data represents the mean±SEM. Unpaired T-test, n=3 (statistics was not carried out on the WT groups as n=1-2).

Body weight and weight gain were not significantly affected in KINGS mice administered with TUDCA compared to PBS control KINGS mice (Figure 88B,D), and although n numbers are too low to determine whether this was

the case for wildtype mice, weight over time and weight gain in wildtype mice treated with TUDCA and PBS was similar (Figure 87A,C).

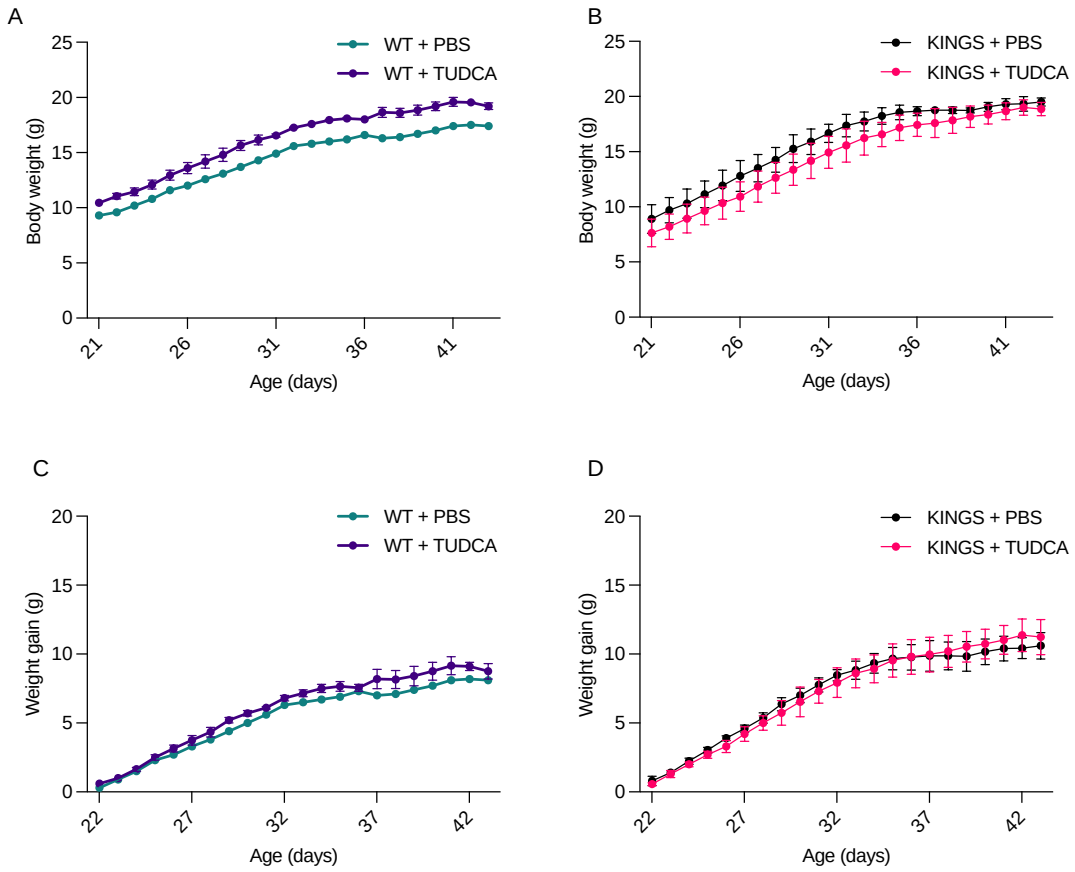


FIGURE 88: *Body weight and weight gain in male mice treated with TUDCA: (A, C) Body weight and weight gain over time in WT male mice treated with TUDCA or PBS vehicle control. Where n numbers are more than 1, data represents the mean±SEM, n=1-2 (statistical analysis was not carried out) (B, D) Body weight and weight gain over time in KINGS male mice treated with TUDCA or PBS control. Data represents the mean±SEM, two-way repeated measures ANOVA with Bonferroni post hoc, n=3 (statistics was not carried out on the WT groups as n=1-2).*

To investigate whether TUDCA treatment could improve glycaemic control under stimulatory conditions, mice treated with TUDCA or PBS were subjected to a glucose tolerance test at 6 weeks of age (after 3 weeks of TUDCA/PBS treatment). TUDCA treatment had no effect on glucose tolerance in the KINGS mice, with area under the curve values for the glucose tolerance tests no

different between KINGS-PBS and KINGS-TUDCA mice (Figure 89). Area under the curve values were also similar between WT mice treated with TUDCA and PBS, however n numbers were too low to determine whether this was statistically significant.

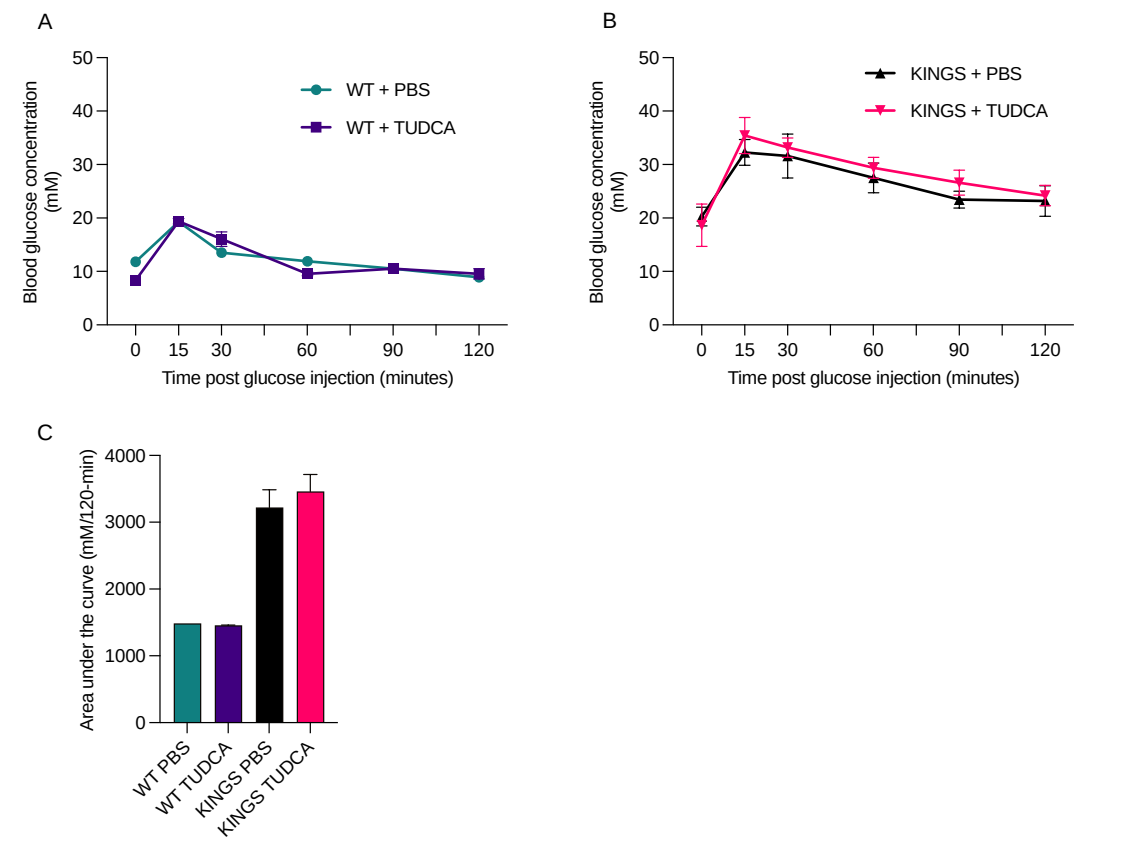


FIGURE 89: *Glucose tolerance in male mice treated with TUDCA: (A) Glucose tolerance tests performed in WT male mice treated with TUDCA or PBS vehicle control. Where n numbers are more than 1, data represents the mean±SEM, n=1-2. (B) Glucose tolerance tests performed in KINGS male mice treated with TUDCA or PBS control. Data represents the mean±SEM. (C) Area under curve values for the glucose tolerance test carried out in WT and KINGS mice treated with TUDCA or PBS. Data represents the mean±SEM. Unpaired T-test, n=3 (statistics was not carried out on the WT groups as n=1-2).*

We attempted to investigate whether TUDCA had any effect on peripheral insulin sensitivity by performing insulin tolerance tests in mice at 6 weeks of

age, however none of the groups responded to insulin (data not shown). We hypothesised that this was the result of stock insulin degradation.

7.4.5 Orchidectomy prevents the development of diabetes in KINGS males:

Endogenous testosterone was removed by carrying out orchidectomies in KINGS mice at 3 weeks of age, before the onset of overt diabetes and before the onset of puberty. Preputial separation was not observed in any of the mice receiving orchidectomy, confirming success of the surgery.

Orchidectomy was associated with a protection from the development of overt diabetes (Figure 90B). KINGS orchiectomised mice had reduced non-fasted blood glucose concentrations compared to the sham operated KINGS mice by 5 weeks of age, and unlike the KINGS-sham mice which developed overt diabetes by 5 weeks, KINGS-orchiectomised mice never crossed the threshold for overt diabetes with a maximum average blood glucose concentration of 12.8mM at 4 weeks of age. Blood glucose concentrations progressively worsened in the KINGS-sham group, with blood glucose concentrations significantly higher from 7 weeks compared to at 3 weeks of age, however there were no significant differences between the different ages in the orchiectomised KINGS mice suggesting that blood glucose concentrations were stable. Despite this, orchiectomised KINGS mice still had higher non-fasted blood glucose concentrations compared to wildtype orchiectomised mice with higher area under the curve values for blood glucose concentrations over time (Figure 90C).

Orchidectomy was only associated with an increase in non-fasted blood glucose concentrations in WT mice at 5 weeks of age but not at any other ages (Figure 90A). Accordingly, area under the curve values for non-fasted blood glucose over time was not significantly different between the two groups.

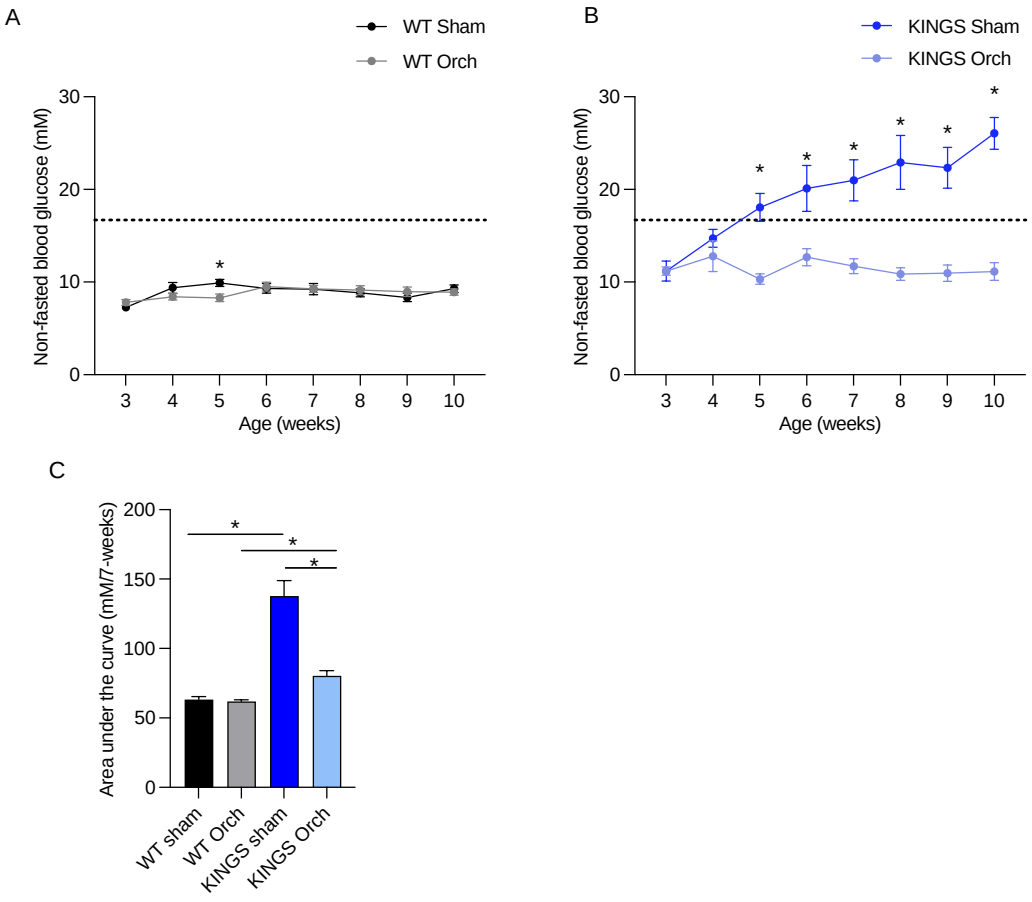


FIGURE 90: *Non-fasted blood glucose concentrations in sham operated or pre-puberty orchiectomised male mice:*
*Non-fasted blood glucose concentrations over time in (A) WT and (B) KINGS mice that were either sham-operated on or orchiectomised (orch) at 3 weeks of age. Data represents mean±SEM. Two-way repeated measures ANOVA with Bonferroni post hoc, *p<0.05, n=5-9. (C) Area under the curve values for non-fasted blood glucose concentrations over time. Data represents mean±SEM. Two-way ANOVA with Holm Sidak post hoc test, *p<0.05, n=5-9.*

7.4.6 The effect of orchidectomy on weight in male KINGS mice:

The improvements in blood glucose levels seen with orchidectomy in the KINGS mice may be in-part due to changes in weight. To investigate this, weight over time was monitored.

Orchidectomy resulted in a subtle decrease in body weight in the WT males and this reached significance at 5, 6, 7, 9 and 10 weeks of age (Figure 91A). The largest difference in weight between the groups was seen at 6 weeks of

age when sham WT mice were 2.7g heavier than orchiectomised mice. However, no significant differences were observed in weight gain between the groups (Figure 91C).

In the KINGS mice, orchidectomy had no significant impact on weight at any age, however there was a slight trend for reduced weight in the orchiectomised group from 5 weeks of age and this reached a maximum difference of 1.9g (Figure 91B). In line with this, weight gain was significantly higher in the sham-operated KINGS mice at 6 and 10 weeks of age (Figure 91D).

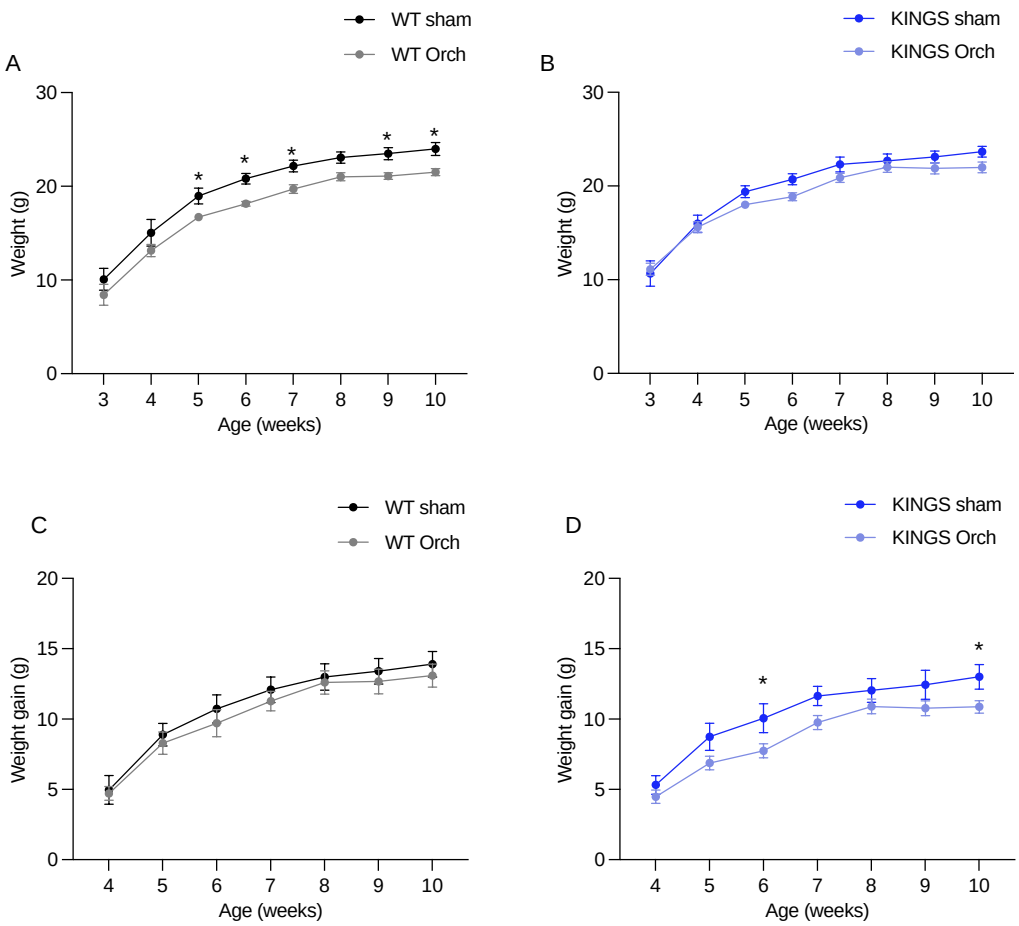


FIGURE 91: *Body weight of sham or pre-puberty orchidectomised male mice: (A-B) Body weight measurements in (A) WT and (B) KINGS mice that were either sham-operated or orchiectomised (orch) at 3 weeks of age. (C-D) Weight gain in (C) WT and (D) KINGS mice that were either sham-operated or orchiectomised (orch) at 3 weeks. Data represents mean±SEM. Two-way repeated measures ANOVA with Bonferroni post hoc, *=p<0.05, n=5-9.*

7.4.7 Orchidectomy has no effect on glucose tolerance:

To investigate whether removal of endogenous testosterone improved glycaemic control under stimulatory conditions, glucose tolerance tests were conducted at 10 weeks of age. Orchidectomy in wildtype male mice resulted in no change in glucose tolerance, with comparable area under the curve values for the glucose tolerance test between sham operated and orchiectomised mice (Figure 92A,C).

All male KINGS mice that received sham surgery had a 6-hour fasting baseline glucose concentration exceeding 20mM, and therefore as outlined previously, we did not conduct a glucose tolerance test owing to ethical concerns. Since baseline blood glucose concentrations in the orchiectomised KINGS mice were below 20mM (13.2mM average) glucose tolerance tests were carried out in this group, however we cannot directly compare this to sham controls. Despite the normalisation of non-fasted blood glucose seen with orchidectomy in KINGS mice, glucose tolerance was still significantly impaired compared to WT mice, indicated by the higher area under the curve values (Figure 92B-C).

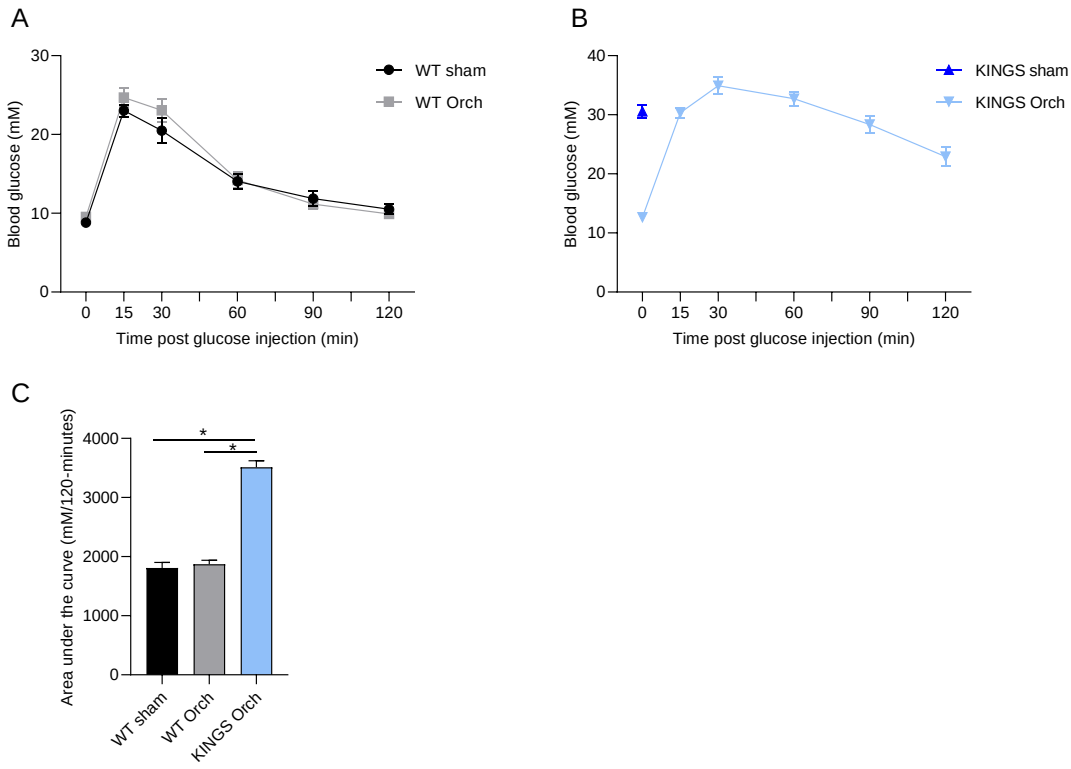


FIGURE 92: *Glucose tolerance in sham or pre-puberty orchidectomised male mice:*

*(A) Glucose tolerance test in orchiectomised (orch) and sham-operated WT mice at 10 weeks of age (7 weeks post-surgery). (B) Glucose tolerance test in orchiectomised KINGS mice at 10 weeks of age (7 weeks post-surgery) and 6-hour basal blood glucose concentration in sham-operated male KINGS mice at 10 weeks. (C) Area under the curve values for the glucose tolerance tests conducted at 10-weeks of age in WT sham operated mice, WT orchiectomised mice and KINGS orchiectomised mice. One way ANOVA with Holm-Sidak post hoc, *p<0.05, n=6-9.*

7.4.8 ORCHIDECTOMY HAS NO EFFECT ON INSULIN SENSITIVITY:

To determine whether changes in peripheral insulin sensitivity may be responsible for the reduction in non-fasted blood glucose concentrations seen with orchidectomy in the KINGS mice, insulin tolerance tests were conducted at 10 weeks of age.

All groups showed a reduction in blood glucose concentrations in response to insulin, however since baseline blood glucose concentrations differed in the KINGS mice (Figure 93A-B), data was also expressed as percentage of basal blood glucose levels (Figure 93C-D). The area above the curve values for this data was not found to be significantly different between any of the groups, suggesting that orchidectomy does not alter peripheral insulin sensitivity (Figure 93E).

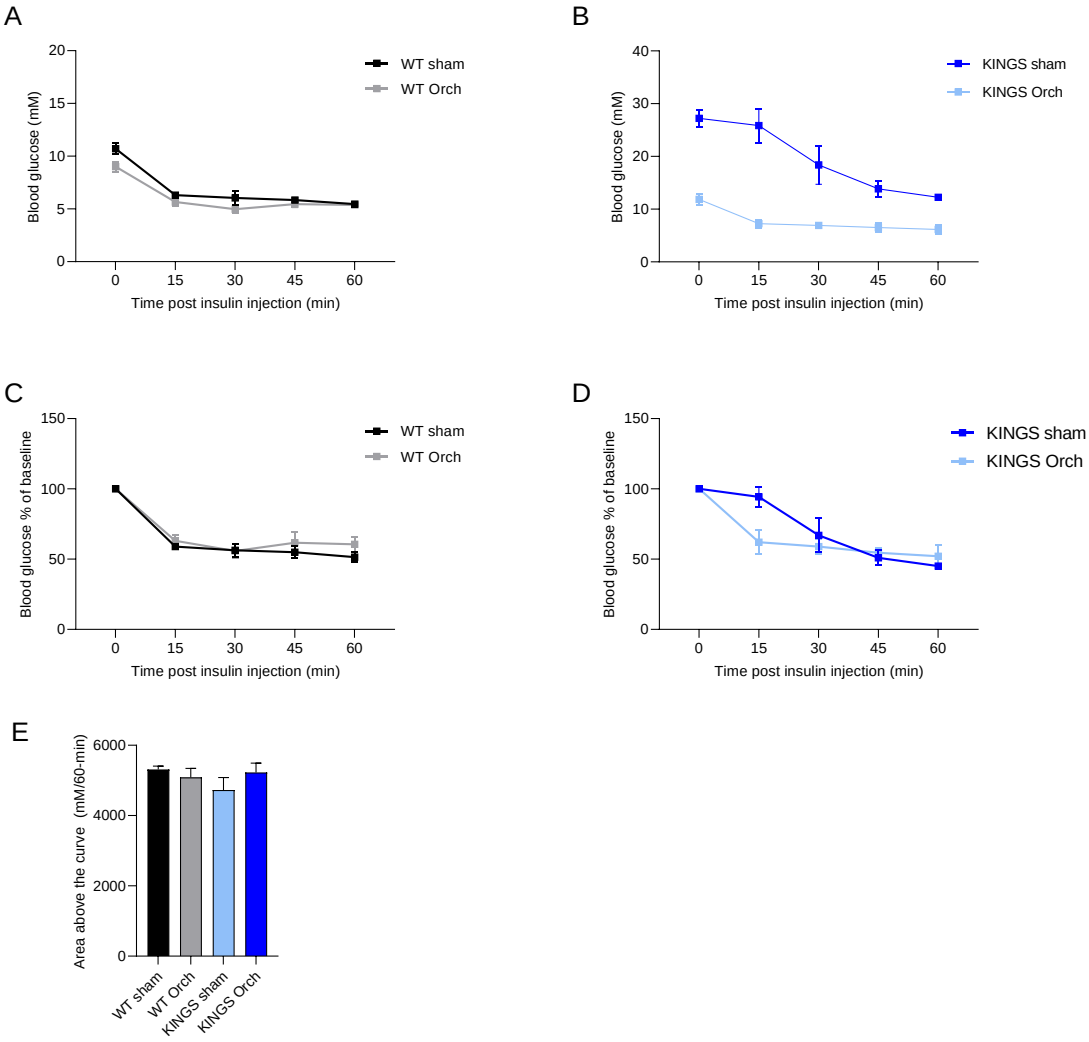


FIGURE 93: *Insulin tolerance tests in sham or pre-puberty orchidectomised male mice:*

(A) Insulin tolerance test in orchiectomised (orch) and sham-operated WT mice at 10 weeks of age (7 weeks post-surgery). (B) Insulin tolerance test in orchiectomised and sham-operated KINGS mice at 10 weeks of age (7 weeks post-surgery). (C) Insulin tolerance test data expressed as percentage of basal glucose concentrations in orchiectomised and sham-operated WT mice. (D) Insulin tolerance test data expressed as percentage of basal glucose concentrations in orchiectomised and sham-operated KINGS mice. (E) Area above the curve values for data represented in figures C-D. Two-way ANOVA, p>0.05, n=3-4.

7.5 Discussion:

Beta cell ER stress has been implicated in the pathogenesis of many types of diabetes and thus understanding how we can alleviate this may provide novel therapeutics for diabetes treatment. To shed light on this, here we assessed whether two FDA approved drugs, liraglutide which is currently used in the treatment of diabetes, and TUDCA which is used in the treatment of liver disease, could prevent diabetes development in a mouse model of beta cell ER stress induced diabetes, the KINGS mouse. We also investigated whether removal of endogenous testosterone could prevent diabetes development in the same model.

Daily liraglutide administration prevented the development of overt diabetes in the male KINGS mice. However, this protective effect was lost when liraglutide administration was stopped, with blood glucose concentrations increasing to levels seen in the vehicle controls, suggesting that this GLP-1 agonist has no permanent beneficial effects on glucose homeostasis. We believe this is the first study to show that the protective effects of GLP-1 receptor agonism in the context of a beta cell ER stress model are reversible.

The complete protection that liraglutide treatment offered from diabetes development in the KINGS mice differs from what has been reported in the literature in the Akita mouse, a similar model of beta cell ER stress induced diabetes. Although one study found that twice daily exendin-4 treatment in Akita males reduced blood glucose concentrations compared to vehicle-treated controls, these mice still developed overt diabetes with blood glucose levels at ~19mM by the end of the study (Yamane et al., 2011). In addition, Barbetti *et al*. found that once daily exendin-4 treatment prior to diabetes development had no effect on blood glucose levels (Barbetti et al., 2016).

In the present study we also found that when liraglutide treatment was re-administered to hyperglycaemic KINGS mice, the reduction in blood glucose concentrations was less stark than what was seen when liraglutide was administered prior to the development of overt diabetes. This suggests that GLP-1 receptor agonists may be more effective prophylactically compared to when used therapeutically, possibly supporting the use of incretin-based

therapy in individuals with pre-diabetes. Indeed, a plethora of clinical studies have suggested that GLP-1 receptor agonist treatment in individuals with prediabetes improves glucose homeostasis and decreases the prevalence of T2DM. However, these studies were conducted in obese individuals, and thus it's hard to determine whether these effects are mediated solely due to weight loss associated with treatment. Further clinical studies are warranted to determine whether GLP-1 receptor agonist treatment in individuals with prediabetes in the absence of obesity reduces diabetes incidence (Papaetis, 2014).

The blood glucose concentration lowering effect of GLP-1 receptor agonist treatment in KINGS males with established diabetes contrasts previous findings that liraglutide treatment in Akita males with established diabetes had no effect on blood glucose concentrations (Zhao et al., 2013). This was unexpected given that the liraglutide dose used for both studies were similar (188µg/kg vs 200ug/kg). A possible reason for the differences in findings may be that the Akita mouse represents a more severe models of beta cell ER stress; as mentioned in previous chapters, blood glucose concentrations were found to be higher in Akita mice compared to age-matched KINGS mice and whereas Akita males suffer mass beta cell loss this is not observed in KINGS males (Austin et al., 2020).

Intriguingly, the same study found that liraglutide treatment was associated with a reduction in Akita mouse beta cell ER stress (with reduced protein expression of markers including BiP and phosphorylation eIF2-alpha) whilst we found no change in beta cell ER stress with treatment, assessed through the protein expression of BiP (Zhao et al., 2013). These findings suggest that the mechanism by which liraglutide reduced blood glucose concentrations in the KINGS males was not through reducing beta cell ER stress. Indeed, as mentioned previously, liraglutide is known to improve glycaemic control through a plethora of mechanisms which are both beta cell and non-beta cell directed. For example, liraglutide has been found to improve peripheral insulin sensitivity via multiple mechanisms including reducing peripheral tissue inflammation and upregulating the expression of glucose transporters at

peripheral sites. Unfortunately, in the present study we did not investigate insulin sensitivity in mice treated with liraglutide, however it would be interesting to perform insulin tolerance tests in future to determine whether improved insulin sensitivity contributes to the improvement we see in blood glucose concentrations with liraglutide treatment. It is notable however that previous chapters have not found an impairment in insulin resistance in the KINGS mice, and thus arguably liraglutide is unlikely to be mediating normalisation of blood glucose concentrations by improving upon insulin resistance alone.

It is possible that no differences were detected in beta cell ER stress owing to the low numbers of islets analysed for this study. For example, for some mice only 3 islets were present on the chosen immunofluorescent stained section. This paired with a high inter-islet BiP variability may have meant that subtle differences in islet BiP expression between liraglutide and PBS treated KINGS mice was missed. In addition to this, islet BiP expression was measured after liraglutide re-administration when blood glucose levels were still significantly higher than the initial liraglutide administration to pre-diabetic mice and treatment only lasted for 2 weeks. In the study which found that liraglutide reduced islet BiP expression in the Akita mice, the treatment period was significantly longer, 7 weeks, which may explain why we did not observe a reduction in beta cell ER stress (Zhao et al., 2013). It would be interesting to investigate islet ER stress in mice treated for a longer period with liraglutide, as well as in pre-diabetic mice treated with liraglutide but not followed by treatment cessation. Interestingly, a study by Cunha *et al.* attributed the protective effects of exendin-4 on lipotoxic ER stressed INS-1 cells in-part to upregulated BiP expression which was hypothesised to enhance ER folding capacity (Cunha et al., 2009). Since both an upregulation and downregulation of BiP with GLP-1 receptor agonism has been reported, it would be beneficial in future to investigate the expression of other ER stress markers in the beta cells of the KINGS mice treated with liraglutide.

In the present study we also investigated whether the bile acid, TUDCA, could prevent male KINGS mice from developing overt diabetes. We found that there

were no differences in blood glucose concentrations between TUDCA and vehicle treated KINGS mice at any point through the experiment, and both groups developed overt diabetes by the end of the study. Although this was a pilot study, and only n=3 mice were used for each KINGS group, these results suggest that TUDCA has no effect on glucose homeostasis. Further supporting this, we found no differences in glucose tolerance when mice were treated with TUDCA. This contrasts findings in other animal models of diabetes, including the NOD, streptozotocin and *Lep*$^{ob/ob}$ mouse, where TUDCA treatment has been associated with improved blood glucose concentrations (Bronczek et al., 2019; Engin et al., 2013; Özcan et al., 2006; Zangerolamo et al., 2021). However, our results are in line with findings that TUDCA treatment did not improve blood glucose homeostasis in Akita mice, although it should be noted that treatment time for this study was only 2 days (Sharma et al., 2015). TUDCA dosing regimen was similar between these studies and the present one (500mg/kg per day in the present study vs 300-500mg/kg per day) and therefore this does not explain differences between findings. Differences in results may be explained by differing levels of basal beta cell ER stress between the mouse models; for example, beta cell ER stress in *Lep*$^{ob/ob}$ mice is milder when compared to ER stress levels in the Akita mice (Sharma et al., 2015). A higher level of beta cell ER stress may reduce the capacity for TUDCA to alleviate this and therefore have a more limited impact on reducing blood glucose levels. In future, it would be interesting to determine the effect of administering a higher TUDCA dose on blood glucose concentrations in the male KINGS mice or to determine whether TUDCA treatment in the female KINGS mice (which show reduced beta cell ER stress levels) can reverse impaired glucose tolerance. It would also be interesting to assess beta cell ER stress in TUDCA treated KINGS mice through assessing the protein expression of ER stress markers. Pancreases from TUDCA-treated KINGS mice were excised and formaldehyde-fixed and so this can be done through immunofluorescent staining in future.

Removal of endogenous testosterone from the KINGS males prior to the onset of puberty and diabetes onset prevented diabetes. However, glycaemic control was not completely normalised in these mice since they were still significantly

glucose intolerant compared to wildtype littermates. This may suggest that removal of testosterone is not protective under conditions of increased beta cell stimulation and only under normal physiological blood glucose fluctuations. This is similar to what is seen in KINGS female mice, which are glucose intolerant despite having the capacity to maintain blood glucose concentrations below the threshold for overt diabetes (see chapter 3). Interestingly, non-fasted blood glucose concentrations in the wildtype mice were largely unchanged with orchidectomy surgery, apart from a mild reduction of 1.6mM at 5 weeks of age, implying that testosterone has no effect of blood glucose concentrations under healthy physiological conditions. In line with this, orchidectomy was not associated with any improvement in glucose tolerance when mice were subjected to a glucose bolus.

Existing literature on the effects of orchidectomy on glucose homeostasis is conflicting. Supporting our findings, a study performed in Sprague-Dawley rats found whilst orchidectomy had no effect on glucose homeostasis under normal conditions, it reduced glucose intolerance and increased blood glucose concentrations when diabetes was induced through alloxan treatment (Morakinyo et al., 2014). Another study found that both bi and uni-orchidectomy was associated with reduced blood glucose concentrations in normoglycemic rats, and subsequent exogenous administration of testosterone reversed this effect (Barazandeh-Asl, 2006). Orchidectomy has also been shown to reduce blood glucose concentrations in C57BL6/J, Swiss nu/nu and CB17 mice (Inoue et al., 2010; Ganouna-Cohen et al., 2022). Other studies contradict these findings and ours, for example, orchidectomy was found to increase blood glucose concentrations in high fat fed C57BL6/J mice and increase diabetes incidence in NOD mice (Fitzpatrick et al., 1991; Harada et al., 2016; Rosmalen et al., 2001). Findings are similarly unclear in human studies, with some studies suggesting that low testosterone is associated with higher diabetes incidences in men. For example, Kumari *et al.* found that men with diabetes showed lower serum testosterone levels compared to non-diabetic men (Kumari et al., 2021). In contrast, other studies have found no effect of testosterone level on diabetes incidence in men and hyperandrogenism in woman has frequently been associated with poorer

glucose homeostasis and increased diabetes risk (Holmboe et al., 2016; Lakshman et al., 2010). Rasmussen *et al.* recently found that in women followed over 6.6-9.4 years, those who were in the higher quartile for plasma testosterone levels had an increased risk of developing T2DM (Rasmussen et al., 2020).

Differences in findings between pre-clinical studies and the present study could be attributed to differences in pathophysiology underlying diabetes. To date, the effect of orchidectomy has only been studied in autoimmune induced diabetes, chemically induced diabetes models and high fat fed models. No other studies have investigated the effect of orchidectomy in a model of beta cell ER stress induced diabetes. Functional studies have suggested that testosterone promotes glucose stimulated insulin secretion via amplification of the insulinotropic action of GLP-1. Since enhanced insulin secretion increases the burden on the beta cell ER to fold and process insulin, it is possible that testosterone action in the context of KINGS mutated insulin leads to more robust ER stress which drives a maladaptive beta cell response. This may explain why removal of endogenous testosterone in the context of beta cell ER stress alleviates hyperglycaemia, whilst removal of testosterone in contexts where beta cell ER stress is not as robust (for example in the NOD mouse) may increase diabetes incidence.

Interestingly, a similar mechanism has been proposed to explain why androgen excess in female animal models and women increases the incidence of diabetes; Xu *et al* suggested that high testosterone levels in females drives insulin hypersecretion and subsequent beta cell exhaustion and failure (Xu et al., 2019). In support of this, it was found that testosterone administration in female mice fed a high fat diet resulted in hyperinsulinemia and resulting hyperglycaemia whereas this effect was lost when mice with beta cell specific AR knockdown were used (Navarro et al., 2018). ER stress is thought to play a major role in beta cell exhaustion, and therefore it is possible that testosterone enhances beta cell ER stress, however neither the study by Xu *et al* or the present study investigated beta cell ER stress levels. It would be interesting to investigate this in future.

As well as the direct effects of testosterone on the beta cell, it has also been implicated in impairing insulin sensitivity. Despite this, we found no change in insulin sensitivity, assessed through insulin tolerance tests, in orchiectomised KINGS or wildtype mice. This suggests that the improvement in glycaemic control seen with orchidectomy in the KINGS mice is not the result of peripheral effects of testosterone removal and supports the idea that testosterone may be acting directly on the beta cell. Although not significant in the male KINGS mice, orchidectomy in this study was associated with a slight reduction in body weight. Moreover, weight gain was significantly lower with orchidectomy in the KINGS mice at two time points, suggesting a subtle impact of orchidectomy on weight. In line with these findings, testosterone is also known to play an important role in increasing muscle and fat mass (Alrabadi et al., 2020). A reduced body weight would likely reduce demand for insulin and therefore the workload of the beta cell, possibly contributing to differences seen in glycaemic control with orchidectomy.

Overall, in this chapter, we have found that liraglutide treatment prevented diabetes development in the KINGS mice and alleviated hyperglycaemia in KINGS mice with established diabetes. However, this protective effect was lost upon cessation of treatment suggesting that liraglutide has no permanent effects on glycaemic control. In addition to this, we found no effect of liraglutide treatment on beta cell ER stress levels, in contrast to the existing literature. Removal of endogenous testosterone was also found to protect male KINGS mice from developing overt diabetes, however further study is required to assess whether this is mediated through direct effects on the beta cell including through reducing ER stress. TUDCA, on the other hand, was found to have no effect on glycemia in KINGS males, although this was a pilot study and repeating this study as well as investigating the effects of higher TUDCA doses on glycemia and TUDCA treatment in female KINGS mice is warranted.

CHAPTER 8: GENERAL DISCUSSION:

8.1 INTRODUCTION:

Diabetes mellitus is thought to affect over 9.3% of the global population and is recognised as the 9th leading cause of global deaths by the world health organisation (Saeedi et al., 2019; World Health Organisation, 2020). It therefore represents a significant health problem, not only to individuals living with this disease whose quality of life is profoundly impacted, but also to the wider society. Indeed, diabetes healthcare costs and costs associated with early retirement, absenteeism and social benefits are thought to be in excess of £23.7 billion a year in the UK (Hex et al., 2012). This situation is only set to worsen as global obesity rates increase, driving a surge in T2DM cases. Moreover, modelling studies have suggested that rates of T1DM may double by 2040 and whilst it is unclear what is causing this increase, factors such as increased childhood obesity, viral infections (for example COVID-19) and pollution have been implicated (Abela & Fava, 2021; Gregory et al., 2022). Gaining further insight into diabetes pathogenesis is therefore imperative as it can guide the development of novel therapies for this disease.

Intriguingly sex differences exist in diabetes, with pre-menopausal women more protected from the development of T1DM and T2DM (Wild et al., 2004). This phenomenon also extends to animal models of diabetes which generally show a male predominance. Although sex hormones, specifically oestrogen, have been implicated in mediating sex differences, the precise mechanisms of this and whether any other factors are involved are unknown. Beta cell ER stress is a prominent pathogenic mechanism that can drive beta cell failure and has been implicated in many types of diabetes. Differences in how males and female beta cells resist and respond to beta cell ER stress may contribute to sex differences in diabetes incidences. Whilst recent studies have shed light onto this, including Brownrigg *et al's* finding that female beta cells maintain functionality better under ER stress, more research is needed in this area (Brownrigg et al., 2023).

The KINGS mouse harbours a heterozygous mutation in *Ins2* which causes the development of early onset diabetes in the male mice whilst female mice are protected. A previous study revealed ultrastructural signs of beta cell ER stress in these mice, however ER stress was not confirmed (Austin et al., 2020). In this thesis, we established the KINGS mouse as a model to investigate sex differences in beta cell ER stress induced diabetes and used it to further understand how beta cell ER stress can be manipulated and the consequence of this on glycaemic control.

8.2 The KINGS mouse as a model of beta cell ER stress:

Animal models are fundamental for preclinical diabetes research, and this extends to gaining insight into beta cell ER stress and sex differences in diabetes (King & Bowe, 2016; King, 2012). To establish whether the KINGS mouse could be used as a model for the latter, we initially needed to re-characterise the phenotype since inbred colonies are subject to genetic drift. We subsequently investigated whether protein markers of ER stress were elevated in the beta cells of these mice.

A subtle reduction in the male KINGS mouse blood glucose levels in the current study compared to the previous study were observed. Changes in phenotype can be mediated by genetic drift over time or changes in the animal environment. Our findings re-emphasise the need to incorporate appropriate control groups rather than relying on historical controls even when using mice derived from the same colony.

Despite differences between the previous and current characterisation studies it is important to note that day of diabetes onset in male KINGS mice was still similar suggesting that overall, the KINGS mouse is a robust model of impaired glycaemic control with a predictable diabetes onset. This is supported by the fact that blood glucose concentrations of the control KINGS male mice in each of the experiments conducted in this thesis (which were carried out over a 4-year period) are largely similar (Figure 94).

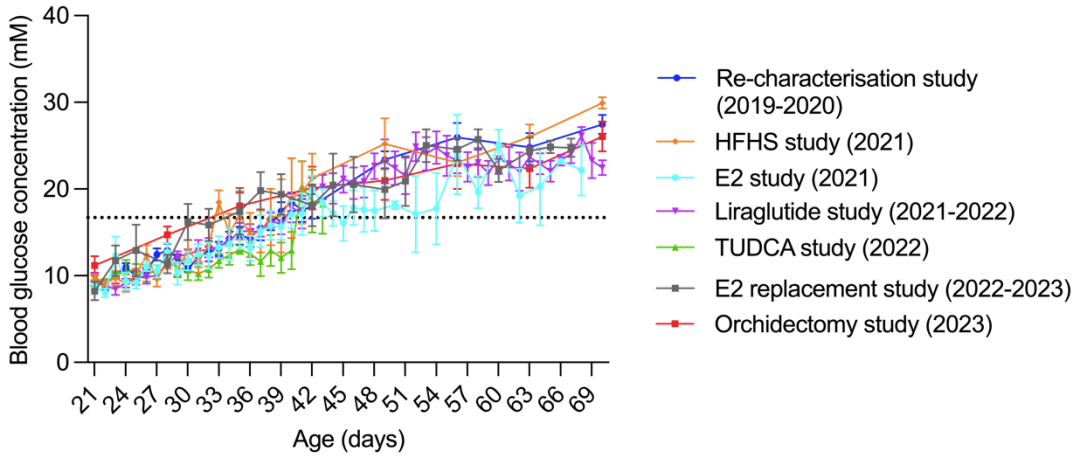


FIGURE 94: *Blood glucose concentrations in control group male KINGS mice used for each of the in vivo experiments conducted in this thesis: Re-characterisation study (blue), orchidectomy study (red), TUDCA study (green), liraglutide study (pink) and HFHS study (orange).*

The Akita mouse model also harbours an *Ins2* mutation and similarly shows a predictable and robust diabetes onset (Yoshioka et al., 1997). However, hyperglycaemia seems to be milder in the KINGS mice. Indeed, onset of diabetes occurs earlier in Akita mice (3-4 weeks) compared to the KINGS mice (5-6 weeks), and previous studies have found that blood glucose concentrations exceed 30mM by 9 weeks in Akita mice whereas average blood glucose concentrations in the KINGS mice remained below 28mM at least up until 10 weeks of age (Vastani et al., 2018). A head-to-head study directly comparing blood glucose concentrations in these two models confirmed this (Austin et al., 2020). *In vitro* studies whereby the Akita proinsulin showed a more severe retention in the ER and drove higher levels of ER stress in MIN6 cells compared to the KINGS proinsulin also add support to this notion (Rajan et al., 2010). Beta cell ER stress in T1DM and T2DM in humans is thought to be mild, and therefore the KINGS mice may offer a more appropriate model for this. Additionally, since different ER stress levels may result in differing outcomes on the beta cell, having a variety of animal models to reflect this is undoubtedly useful for gaining more insight into the mechanisms behind these responses.

Beta cell ER stress was confirmed in the KINGS mice in this study with protein markers of ER stress and the UPR elevated in the islets from these mice. This supports previous findings of ultrastructural signs of beta cell ER stress (Austin et al., 2020). We additionally found that ER stress preceded diabetes onset, indicating its involvement in diabetes initiation rather than being a side effect of hyperglycaemia. It is worth noting that ER stress levels increased in male KINGS mice over time, suggesting that chronic hyperglycaemia may exacerbate ER stress, which is in line with previous findings in the literature (Johnson & Kaufman, 2021). These findings are similar to those in the Akita mouse where ER stress marker expression also precedes diabetes and adds further support to the notion that beta cell ER stress alone is sufficient to drive beta cell failure and diabetes (Riahi et al., 2018).

Overall, we have re-characterised the phenotype of the KINGS mouse, re-emphasising that loss of glycaemic control is robust and diabetes onset is predictable in male mice making it an appealing model of hyperglycaemia. We have also shown that the KINGS mutation drives beta cell ER stress and as such it can be used as a model to study this in the context of diabetes. Since the female KINGS mice show a phenotype more similar to pre-diabetes (impaired glucose tolerance but no overt hyperglycaemia) they offer a model of beta cell ER stress in the context of pre-diabetes.

8.3 Beta cell response to ER stress in the KINGS mouse:

Beta cell response to a given stressor ultimately determines cell fate and thus whether diabetes develops. In response to ER stress the beta cell can activate the adaptive UPR, which attempts to restore protein homeostasis and thus reduces ER stress, or it can activate the maladaptive UPR. The latter has been associated with beta cell failure and is activated upon robust ER stress that cannot be resolved through adaptive mechanisms.

The UPR is activated as early as 4 weeks of age in the beta cells of the KINGS mice, preceding diabetes onset and thus implicating it in this. Although it is difficult to conclusively determine whether UPR signalling is adaptive or maladaptive (since the same pathways are used for each), chronic and robust

signalling through each arm of the UPR is associated with a switch towards more maladaptive outcomes for the cell (Lenghel et al., 2021). Male KINGS islets showed a greater UPR upregulation compared to female KINGS islets indicating that UPR signalling is more maladaptive. In line with this, reduced beta proliferation was seen at 4 weeks of age in male KINGS mice but not in the females. Other studies have also found that beta cell ER stress and the UPR mediates a reduction in beta cell proliferation including Balboa *et al.* who showed that insulin mutations drive ER stress and reduced proliferation in beta-like cells (Balboa et al., 2018; Riahi et al., 2018).

Previous studies have also shown that ER stress drives beta cell death, but despite this we found no increase in beta cell apoptosis at the time of diabetes development and no differences in beta cell mass or islet size between KINGS and WT mice. Our lab has however previously found impaired glucose stimulated insulin secretion and reduced insulin content in KINGS islets and this is more severe in male KINGS mice which likely also contributes to sex differences (Austin et al., 2020). Together, our findings and previous findings suggest that ER stress mediates diabetes development in the KINGS mice through driving beta cell dysfunction rather than death, and greater dysfunction in the KINGS males may explain sex differences in phenotype.

These findings contrast the proposed mechanism by which ER stress mediates diabetes in the Akita mice, beta cell apoptosis. Akita mice show up to a 60% loss of beta cell mass which has been attributed to increased proapoptotic CHOP expression, a consequence of maladaptive UPR signalling (Oyadomari, Koizumi, et al., 2002). Interestingly, we found no increase in CHOP in KINGS islets and supporting this, a higher level of CHOP mRNA has been detected in MIN6 cells expressing Akita proinsulin compared to those expressing KINGS proinsulin (Rajan et al., 2010). The same study found that mutant KINGS proinsulin showed reduced retention in the ER and better recruitment to secretory granules compared to Akita proinsulin which may result in reduced ER stress. Collectively these findings may imply that beta cell ER stress is milder in the KINGS mice, although future studies where

markers of ER stress are directly compared between beta cells from the two models is required to conclude this.

An important limitation of this study is that direct causality between ER stress/a maladaptive UPR and KINGS male diabetes development was not shown. In an attempt to investigate this, a pilot study whereby male KINGS mice were treated with the ER-stress lowering compound, TUDCA, prior to diabetes onset was conducted. However, TUDCA treatment showed no impact on blood glucose concentrations. Despite this, it is important to note that ER stress levels were not investigated in this study and since the KINGS proinsulin is still likely to mis-fold even upon increased chaperone action, it is possible that TUDCA does not reduce ER stress in this model. Another option to investigate direct causality between ER stress/UPR activation and diabetes in this model would be to selectively knockout or inhibit components of the UPR in the KINGS mice and ascertain whether this improves glycaemic control. This has been done in the Akita mice where knockout of CHOP delayed diabetes whilst XBP1s knockout worsened diabetes (Lee et al., 2011; Oyadomari, Koizumi, et al., 2002).

Taken together, our data suggests that beta cell ER stress drives the loss of glycaemic control seen in the KINGS mice, and differing levels of beta cell ER stress and activation of the UPR may account for sex differences in phenotype. Moreover, ER stress likely mediates beta cell failure through beta cell dysfunction rather than through a loss of beta cell mass, setting it apart from the Akita mouse model and possibly implying that beta cell ER stress is milder.

8.4 Exacerbating beta cell ER stress in female KINGS mice:

Our finding that UPR activation is greater in the KINGS male mice led us to hypothesise that increasing beta cell ER stress in the KINGS females may abolish the sex differences seen in diabetes phenotype. In line with previous findings that high fat diets can increase beta cell ER stress, KINGS and WT mice fed a western diet did exhibit increased beta cell ER stress and expression of UPR markers. This adds further support to the notion that dietary

excess associated with obesity in humans may contribute to beta cell failure through enhancing ER stress. Despite exacerbating beta cell ER stress, sex differences remained in glycaemic control in the KINGS mice with female KINGS mice fed a western diet still showing superior glycaemic control compared to male KINGS mice fed normal chow. The latter may suggest that female KINGS mice have an improved capacity to adapt to beta cell ER stress. These findings are supported by a previous unpublished study conducted in our laboratory which found that pregnancy, which increases beta cell demand through a transient increase in peripheral insulin resistance, was not associated with any impairment in glycaemic control in the KINGS female mice (Austin, 2018).

No differences were found in islet size between KINGS mice fed normal chow or a western diet, suggesting that they are not adapting to exacerbated ER stress through increased beta cell proliferation or hypertrophy. This contrasts reports in the literature that high fat feeding increases islet size through enhanced proliferation, reduced beta cell apoptosis and beta cell hypertrophy (Ahrén et al., 2010). It is possible that subtle but biologically relevant differences in islet size were not detected, and therefore in future it would be beneficial to directly investigate beta cell proliferation and apoptosis in this model. Moreover, female KINGS mice may be adapting to increased beta cell ER stress through other means such as increasing beta cell insulin secretion, it would also therefore be worthwhile to measure islet glucose stimulated insulin secretion in these mice.

It would also be interesting to investigate the level of beta cell ER stress in the HFHS female KINGS mice relative to male KINGS mice fed normal chow. Direct comparison of the protein expression of ER stress and UPR markers between the two may provide insight into whether females are able to resist beta cell ER stress or respond more adaptively towards it. Intriguingly, a study whereby male and female mouse islets were subjected to ER stress through thapsigargin treatment found that whilst no differences were seen in protein expression of UPR markers, female islets were more resistant to cell death

and repression of protein synthesis suggesting that they may respond more adaptively to ER stress (Brownrigg et al., 2023).

Overall, our data suggests that female beta cells are more resistant to ER stress induced failure since even under exacerbated ER stress they resist the development of diabetes. Further studies are needed to ascertain how females adapt to ER stress.

8.5 The role of sex hormones in mediating sex differences in the KINGS mouse:

Oestrogen has been heavily implicated in sex differences seen in diabetes. The findings presented in this thesis go some way to supporting this notion, with oestradiol treatment in male KINGS mice associated with reduced beta cell ER stress and protection from diabetes. This is in line with findings in the Akita mice that oestradiol treatment reduces blood glucose concentrations and more general findings in humans that the menopause (and associated reduction in circulating oestradiol) is associated with increased diabetes risk. However, in contrast to the Akita and Munich models, we found that ovariectomy prior and post puberty only subtly further impaired glycaemic control and did not induce overt diabetes despite increasing beta cell ER stress. These results collectively suggest that whilst oestradiol may play some role in protecting female KINGS mice from diabetes, it is not solely responsible for the sex differences seen and other factors must play a role.

Since we also present evidence that endogenous testosterone removal in the KINGS male mice prior to puberty prevents diabetes development, testosterone may represent another factor driving sex differences in diabetes in this model. Whilst our findings are supported by previous studies showing that testosterone exacerbates STZ -induced hyperglycaemia in rodents, it conflicts with a whole body of literature suggesting that testosterone deficiency in men predisposes to diabetes development and its removal in NOD males induces diabetes (Maclaren et al., 1980; Rosmalen et al., 2001; Wittert et al.,

2021). A possible reason for differences in findings may be that testosterone actions on the beta cell can be beneficial under certain conditions but detrimental under others, and this may depend upon the beta cell stressor. To our knowledge, no other studies have investigated the effect of testosterone in the context of beta cell ER stress. Based on our findings, it is possible that testosterone drives a maladaptive response to beta cell ER stress. Indeed, testosterone has been associated with beta cell dysfunction in females and this has attributed to it driving insulin hypersecretion which can lead to beta cell exhaustion and failure (Xu et al., 2019). In the context of ER stress, testosterone driven increases in beta cell insulin secretion may exacerbate the pre-existing ER stress caused by the KINGS mutation, driving beta cell failure. Unfortunately, beta cell ER stress was not investigated in the beta cells of orchiectomised KINGS mice, but pancreases were collected, and future studies will investigate whether changes in BiP fluorescence in islets can be detected. More study in this area is warranted in future and this includes administering testosterone to female KINGS mice to ascertain whether this is sufficient to drive diabetes. Importantly, testosterone is converted into oestradiol *in vivo* and therefore use of dihydrotestosterone (DHT) which cannot be converted to oestradiol would avoid inadvertently elevating oestradiol levels in such a study. It would also be worth investigating the effect of testosterone removal in other models of diabetes where underlying pathogenesis of beta cell failure differs as this may give insight into other contexts in which testosterone is beneficial/harmful. Finally, future investigation into the effects of removing testosterone post-puberty would give insight into the activational vs organisational effects of this sex hormone on diabetes development in the male KINGS mice.

A distinct limitation of both studies is that plasma levels of oestradiol and testosterone were not investigated, although we attempted to measure plasma oestradiol levels the ELISA kit used was found to be unreliable. Though success of the orchidectomy and ovariectomy studies was confirmed by analysis of preputial separation, vaginal opening and oestrous cycling (all of which require normal physiological levels of testosterone and oestrogen to occur), both hormones can be produced at non-gonadal sites albeit at lower

levels. Another important factor to consider is that sex hormones have previously been shown to influence blood glucose levels through non-beta cell effects including through influencing peripheral insulin sensitivity. Emphasising this fact, changes in weight (which is closely associated with insulin sensitivity) were observed upon removal/addition of both hormones. To overcome this in future studies, beta cell specific AR and ER/GPER knockout mice could be crossed with the KINGS mice.

Collectively our results show that oestradiol and testosterone likely contribute to the sex differences seen in diabetes in the KINGS mouse. Whilst oestradiol in part mediates its protective effects through reducing beta cell ER stress and increasing insulin secretion, further study is required to determine whether testosterone influences beta cell ER stress and function.

8.6 Therapeutic intervention to alleviate diabetes in KINGS male mice:

As outlined throughout this thesis, beta cell ER stress has been heavily implicated in the pathogenesis of diabetes initiation and progression. For example, insulin resistance in the pre-T2DM state drives hyperinsulinemia as beta cells try and functionally compensate for increased relative demand, resulting in increased proinsulin misfolding and beta cell ER stress which is associated with beta cell failure. In T1DM, insulitis has been directly associated with mediating beta cell ER stress. Moreover, as beta cell numbers decline and the remaining beta cells attempt to functionally compensate, increased insulin secretion may drive beta cell ER stress that contributes to diabetes progression. Agents that act to alleviate beta cell ER stress may therefore represent promising therapies for diabetes. In this thesis we investigated whether liraglutide, an FDA approved drug for diabetes, and TUDCA, an FDA drug approved for liver disease, could protect against diabetes in the KINGS model of beta cell ER stress.

GLP-1 agonists have been shown to reduce beta cell ER stress *in vitro* and *in vivo* in models including the Akita and Lep$^{db/db}$ mouse (Cunha et al., 2009;

Yamane et al., 2011). Despite not showing that ER stress was reduced through islet BiP expression in the KINGS mice, we did find that liraglutide treatment prevented diabetes development and reduced blood glucose concentrations in overtly diabetic KINGS mice. It is important to note that these drugs have pleiotropic effects and thus the mechanism by which diabetes is rescued in the KINGS mouse may not be dependent on reducing ER stress (and these effects may also indirectly reduce beta cell ER stress) and this represents a limitation of our study. Nevertheless, since diabetes is primarily driven by beta cell ER stress in this model, our findings add support to the notion that GLP-1 receptor agonists can alleviate ER stress induced diabetes.

GLP-1 receptor agonists are frequently prescribed only to individuals with established T2DM and whose blood glucose concentrations are not adequately controlled with exogenous insulin treatment (Hinnen, 2017). Considering the role that beta cell ER stress plays in the initiation of T2DM, further study is warranted into whether earlier treatment with GLP-1 receptor agonists, possibly even at the pre-diabetes stage, delays beta cell failure. In line with this idea, we found that prophylactic liraglutide treatment in the KINGS males was more effective at maintaining normal blood glucose concentrations compared to therapeutic treatment. GLP-1 receptor agonists are not currently used clinically to treat individuals with T1DM; however, animal studies have suggested that their benefits on glucose homeostasis and beta cell function (Gao et al., 2021; Issa & Azar, 2012). Since ER stress is implicated both in the progression and initiation of T1DM, further study into whether GLP-1 receptor agonists can reduce ER stress and preserve beta cell functional mass in the context of T1D would be interesting.

TUDCA represents a chemical chaperone thought to reduce ER stress by improving ER protein folding capacity. In this study we presented pilot data showing that TUDCA treatment in male KINGS mice fails to protect from the development of overt diabetes. Although we did not look at the effects of this drug on beta cell ER stress in these mice, our findings contrast others in the literature who have found that TUDCA is associated with alleviated beta cell ER stress and reduced blood glucose concentrations in several models

including the Lep$^{ob/ob}$ and NOD mouse (Bronczek et al., 2019; Engin et al., 2013). Since the KINGS mouse likely represents a model where beta cell ER stress is more robust than previous models used to test TUDCA, further study looking into the effect of an increased TUDCA dosage may be worthwhile. In addition to this, both the Lep$^{ob/ob}$ and NOD mouse represent models where ER stress is driven by proteins that misfold as a result of environmental changes rather than as a result of misfolding mutations. TUDCA may be able to facilitate proper folding in the case of the former but not for the latter which may explain our findings. Future research should investigate the effect of TUDCA on ER stress in KINGS beta cells to give further insight into this. It would also be valuable to investigate the effect of TUDCA treatment on female KINGS mouse glucose tolerance since these represent a milder beta cell ER stress model.

Overall, we have shown that the clinically available drug, liraglutide, is able to reduce blood glucose concentrations in the context of beta cell ER stress induced diabetes, but its effects are more beneficial when the drug is used prophylactically. This suggests that clinical study into GLP-1 receptor agonist use in pre-diabetes is warranted. Although TUDCA on the other hand had no effect on blood glucose concentrations in the KINGS mice, more study into the effect of higher TUDCA dosage, TUDCA treatment of female KINGS mice and the effects of TUDCA on beta cell ER stress level would be of interest.

8.7 Future research directions:

The findings presented here have further established the KINGS mouse as an appropriate model to study beta cell ER stress induced beta cell failure and diabetes. We have used this model to shed light onto sex differences in beta cell ER stress, to gain insight into how beta cell ER stress can be manipulated, and the effect that ER stress manipulation has on glucose homeostasis.

8.7.1 Establishment of the KINGS mice as a model for diabetes:

Our data has confirmed the KINGS phenotype as a robust one, where hyperglycaemia predictably develops in KINGS males. However, the phenotype induced by the KINGS mutation has only ever been investigated

on a C57BL/6J genetic background. It is well recognised that background strain can have a significant impact on glycaemic phenotype, and indeed C56BL/6J mice are known to be more susceptible to STZ-induced hyperglycaemia compared to ICR mice (Daniels Gatward et al., 2021; Shimizu et al., 2012). In future, investigating whether a similar phenotype is induced by the KINGS mutation on different genetic backgrounds would give insight into the robustness of this model.

Throughout this thesis we have suggested that the KINGS mouse represents a milder model of beta cell ER stress compared to the Akita mouse. This is based on findings by our laboratory and others that blood glucose concentrations are higher in the Akita mice, the Akita mice suffer a larger loss of beta cell mass and *in vitro* studies that suggest that the Akita mutation leads to more severe retention of proinsulin in the ER. In order to establish whether this is the case, future studies could directly compare the expression of ER stress markers and activation of the UPR between beta cells from age-matched Akita and KINGS mice maintained in the same animal facility.

Lastly, there exist numerous health complications associated with chronic hyperglycaemia. Since the KINGS model maintains weight and does not require exogenous insulin despite showing overt hyperglycaemia, it may represent an attractive model to study these complications. Currently cardiac tissue has been provided to collaborators to investigate the long-term effects of hyperglycaemia on cardiomyocyte health in the KINGS mice. In addition, another collaborator is currently investigating whether the vasculature of the retina is augmented in the KINGS model. These studies may give insight into cardiovascular pathology and retinopathy that is associated with diabetes and provide insight into whether the KINGS mouse represents an appropriate model to study this.

8.7.2 Diabetes pathogenesis in the KINGS model:

In the present study 1-2 markers of each individual UPR pathway were investigated. Although this allows us to determine which of the UPR arms are activated, this is relatively crude since the UPR is complex, has many

components and each component is regulated at different levels. More detailed study into these components may reveal further differences between the sexes which may go some way to explaining phenotype differences as well as reveal factors which may be driving beta cell dysfunction. A more sophisticated method to do this would be to use mass spectroscopy which can be used to analyse the expression of thousands of proteins within a given sample. The latter is gaining traction since it is perceived to provide a more accurate view of cell processes and states compared to RNA sequencing (Kolic et al., 2023). The UPR is also thought to be highly dynamic and thus likely changes significantly over the course of disease in the KINGS mouse, it would therefore be beneficial to assess expression of UPR components at a broader range of ages including at 20 weeks of age when male KINGS mice have had chronic hyperglycaemia. The latter may also give insight into the increased beta cell death that was seen at this age.

The impact of beta cell ER stress on beta cell apoptosis, proliferation and glucose stimulated insulin secretion have all been investigated in the KINGS mice. However, ER stress is also known to drive beta cell failure through dedifferentiation. In future, it would be of benefit to investigate the gene expression of beta cell identity genes such as *Pdx1* in KINGS beta cells. It would also be interesting to investigate whether these genes are differentially expressed between male and female KINGS mice at different ages encompassing the disease course.

Finally, as touched on previously, to determine a direct causality between beta cell ER stress/UPR activation and diabetes development in the KINGS mice, future studies should look at knocking down or inhibiting components of the UPR prior to the development of diabetes in male mice. This would also give insight into which UPR pathways are more important in driving beta cell failure and which are more important for protecting against this.

8.7.3 Influence of testosterone on beta cell ER stress and sex differences in diabetes:

We have presented evidence that testosterone may promote a maladaptive beta cell response to ER stress which may go some way to explaining sex differences in the KINGS phenotype. However, we have not shown direct evidence for this in the current project. In future it would be valuable to investigate protein expression of ER stress markers in orchiectomised and sham-operated KINGS mouse beta cells to see if these differ. It would also be interesting to investigate whether dihydrotestosterone administration to female KINGS mice promotes a diabetic phenotype.

It is important to consider that since the testes are also responsible for producing anti-Müllerian hormone and inhibin and activin proteins, orchidectomy also results in a removal of these (Victor et al., 2022). The latter may influence blood glucose homeostasis, although research into this is currently lacking. To confirm it is the removal of testosterone that protects male KINGS mice from diabetes, future studies should investigate whether testosterone administration after orchidectomy results in diabetes onset.

Testosterone may impact blood glucose concentrations through directly acting on the beta cell or indirectly by acting at peripheral sites. It is difficult to distinguish between the latter. To specifically investigate the effect of testosterone on the beta cell, islets could be isolated from WT and KINGS mice and treated *ex vivo* with testosterone to study the effects of this on ER stress levels, glucose stimulated insulin secretion and beta cell turnover. In addition, KINGS male mice could be crossed with mice harbouring beta cell specific knockdown of the androgen receptor to investigate whether this, like orchidectomy, prevents diabetes development. Prevention of diabetes through the latter would indicate that testosterone acts directly on the beta cell to induce diabetes in the KINGS male mice.

Lastly, our findings that testosterone removal through orchidectomy protects against diabetes contrasts with some of the literature suggesting that testosterone is beneficial for blood glucose homeostasis in males. However, it is important to note that research into this area is currently limited. Therefore,

it would be interesting to investigate the effect of testosterone removal in other models of diabetes which have a different underlying pathogenesis.

8.8 Conclusion:

To conclude, the findings presented in this thesis have established the KINGS mouse as a suitable model for investigating beta cell ER stress in the context of impaired glycaemic control and sex differences in diabetes. Furthermore, we have used this model to show that females respond more adaptively to beta cell ER stress even when this is exacerbated and that both oestradiol and testosterone likely play some part in generating sex differences in phenotype. Finally, we have shown that liraglutide can protect from and alleviate diabetes in a model of beta cell ER stress. These findings give further insight into the role that ER stress plays in beta cell failure and sex differences in response to this which may have important implications for the development of novel diabetes therapeutics and sex-tailored therapies.

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