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The impact of metabolic stress and aging on functionality and integrity of pancreatic islets and beta-cells

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3-NT	3-nitrotyrosine
ADP	Adenosine diphosphate
AGER1	AGE receptor 1
AGEs	Advanced glycation end products
AMPK	AMP-activated protein kinase
ARX	Aristaless related homeobox
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CDK2, 4, 6	Cyclin-dependent kinase 2, 4, 6
CEL	N-ε-carboxyethyllysine
CFD	Carbohydrate-free, high-fat diet
ChoRE	Carbohydrate-response element
ChREBP	Carbohydrate-responsive element-binding protein
CML	N-ε-carboxymethyllysine
COX-2	Cyclooxygenase-2
CRD	Carbohydrate-rich diet
E	Embryonic day
ER	Endoplasmatic reticulum
ETC	Electron transport chain
FA	Fatty acids
FOXO1/M1	Forkhead box protein O/M1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1

GLUT2, 4	Glucose transporter 2, 4
GSH	Reduced glutathione
GSIS	Glucose-stimulated insulin secretion
HNF6	Hepatocyte nuclear factors 6
IL-6, IL-1β	Interleukin-6, Interleukin-1β
iNOS	Inducible nitric oxide synthase
IRS-1, 2	Insulin receptor substrate 1, 2
JNK	C-jun N-terminal kinases
K^+	Potassium
Ki67	Antigen identified by monoclonal antibody Ki67
LKB1	Liver kinase B1
MAFA	MAF BZIP transcription factor A
MAPK	P38 mitogen-activated protein kinase
MG	Methylglyoxal
MG-H1	Methylglyoxal-derived hydroimidazolone
MODY4	Maturity onset diabetes of the young 4
MLX	Max-like factor 1
MLXIPL	MLX interacting protein-like
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NEUROD1	Neural differentiation 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKX2.2, NKX6.1	NK2 homeobox 2, NK6 homeobox 1
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NNT	Nicotinamide nucleotide transhydrogenase
NOD	Non-obese diabetic
NRG3	Neurogenin 3
NZO	New Zealand Obese
PARP	Poly(ADP-ribose)-polymerase
PAX4, 6	Paired box 4, 6
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor

PDX1	Pancreatic and duodenal homeobox 1
PI3K	Phosphoinositide 3-kinase
PK	Protein kinase
PP	Pancreatic polypeptide
PTF1A	Pancreas associated transcription factor 1a
RAGE	Receptor for AGEs
Rb	Retinoblastoma protein
ROS, RNS	Reactive oxygen species, Reactive nitrogen species
SASP	Senescence-associated secretory phenotype
TCA	Tricarboxylic acid cycle
TCF19	Transcription factor 19
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TXN	Thioredoxin
TXNIP	Thioredoxin-interacting protein
UPR	Unfolded protein response
VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cells

Introduction

Pancreas and Langerhans islet development

The pancreas is an endoderm-derived glandular organ and of critical relevance for energy metabolism and nutrient utilization. Usually regarded as two separate organ systems, endocrine and exocrine pancreas are linked closely, both anatomically and physiologically. While the exocrine unit is composed of acinar cells and the ductal epithelium, releasing mainly digestive enzymes, the peptide hormone-producing endocrine compartment forms a network of different cell types, including alpha-, beta-, delta-, epsilon- and PP (Pancreatic polypeptide)-cells, the so-called Langerhans islet.[1] Understanding the development of human pancreas is of high importance for the advancement of therapies or new approaches to treat diabetes[2]. Since studying human pancreatic development is restricted primarily by ethical constraints, but also due to minor availability of donor organs, inability to safely and effectively biopsy as well as tissue autodigestion, rodent pancreas is used to unravel the underlying mechanisms[3].

Pancreatic development in rodents occurs between E9.0 (Embryonic day) and E16.0 (Figure 1)[4-7]. By massive proliferation and differentiation of multipotent progenitor cells in the first phase (E9.0 to E12.5), induced mainly via NOTCH signaling, ventral and dorsal pancreatic buds are formed from the foregut endoderm permeated by microlumina structures[8]. Within the pancreatic buds, differentiated multipotent progenitor cells express two intrinsic hormones, PTF1A (Pancreas associated transcription factor 1a) and PDX1 (Pancreatic and duodenal homeobox 1), both necessarily needed for the generation of all other pancreatic cell types in the second stage of pancreas development[9,10]. At E14.5, PTF1A was identified to be the major transcription factor for the activation of acinar cell generation, whereas increased expression of HNF6 (Hepatocyte nuclear factors 6) induces the differentiation of progenitors into duct cells, forming the exocrine pancreas[10-12]. Simultaneously, PDX1 transcription factor regulates the generation of the endocrine network. By downregulation of NOTCH signaling pathway, transcription factor NRG3 (Neurogenin3) is activated and expressed in progenitor cells, specifying the endocrine

lineage[8,13,14]. The differentiation of endocrine cells involves the coordinated activation and suppression of a large number of transcription factors. Targeted by NRG3 and co-expressed in NRG3-positive cells, ARX (Aristaless related homeobox) or PAX6 (Paired box 6) stimulate the differentiation into alpha-cells[15-17]. In contrast, formation of beta-cells is driven by NEUROD1 (Neural Differentiation 1), NKX6.1 (NK6 homeobox 1) or MAFA (MAF BZIP transcription factor A)[2,18-20]. Transcription factors that determine the phenotype of all other cell types are not fully identified. PAX4 and 6 are supposed to stimulate delta-cell generation, whereas PP-cells are formed presumably via NKX2.2 signals[21]. Remodeling of the pancreatic structure at E17.5 leads to the formation of the main duct connected to the exocrine area, common bile duct and duodenum[22].

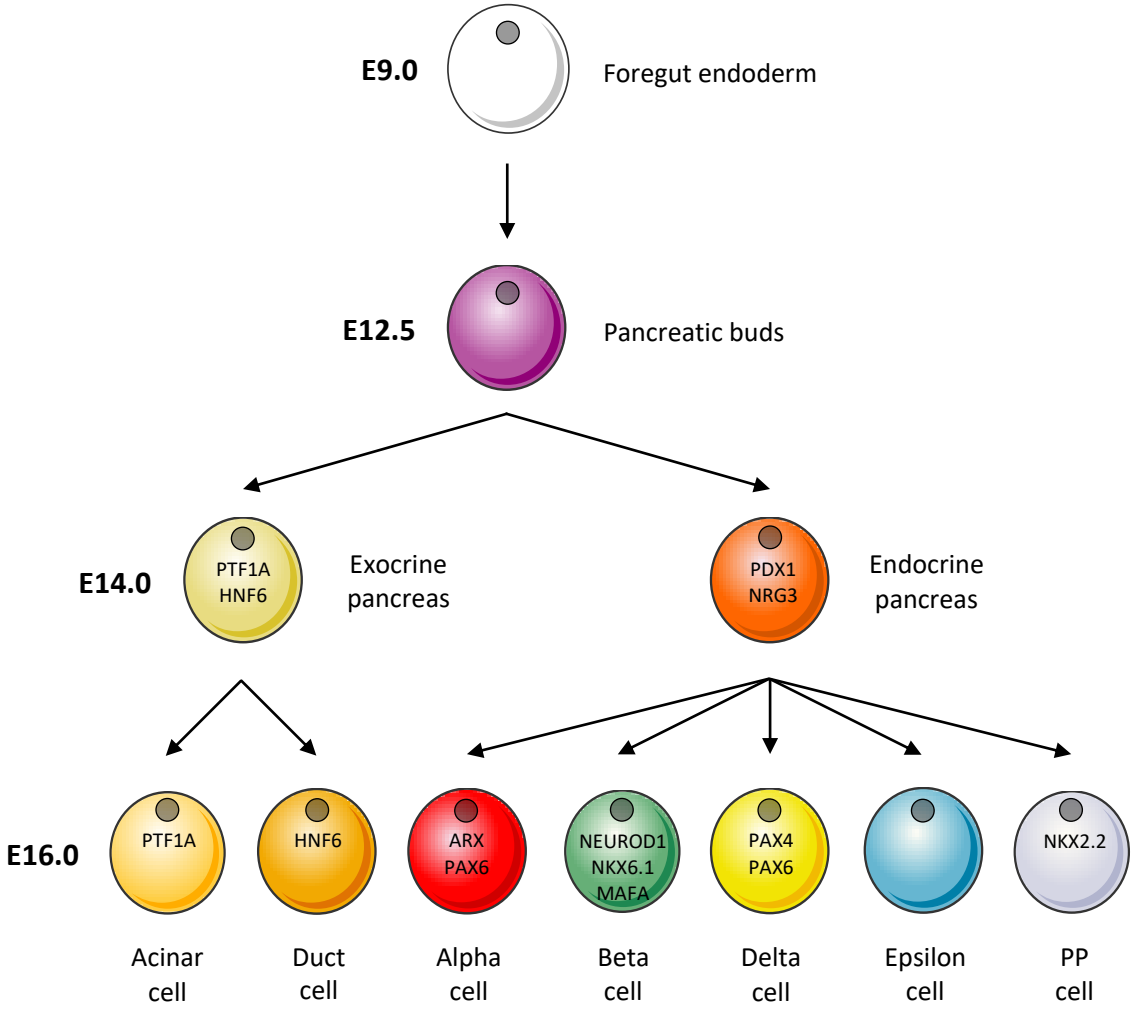

Figure 1 - Pancreas development.
Simplified scheme of pancreas development and subsequent lineage commitment between E9.0 and E16.0, including important transcriptional regulators that define different exocrine and endocrine cell types and stages. ARX (Aristaless related homeobox), E (Embryonic day), HNF6 (Hepatocyte nuclear factors 6), MAFA (MAF BZIP transcription factor A), NEUROD1 (Neuronal differentiation 1), NKX2.2, NKX6.1 (NK2 homeobox 2, NK6 homeobox 1), NRG3 (Neurogenin3), PAX4, 6 (Paired box 4, 6), PDX1 (Pancreatic and duodenal homeobox 1), PP (Pancreatic polypeptide), PTF1A (Pancreas associated transcription factor 1a).

The Langerhans islet - an endocrine machinery

The islets of Langerhans, discovered by the anatomist Paul Langerhans in 1869, represent an endocrine network of multiple cell types[23]. Islets are distributed heterogeneously within the pancreas and account for only 2% of the pancreatic tissue[4]. Nevertheless, they represent one of the most relevant hormone-producing systems in higher organisms[24,25]. As mentioned above, pancreatic islets are organized in clusters of alpha-, beta-, delta-, epsilon- and PP-cells, penetrated and surrounded by a high number of small blood vessels[6,7]. The islet cellular structure and composition is diverse between and within different species. Rodents have a well-designed islet architecture composed of a beta-cell core surrounded mainly by alpha-cells[26-28]. Although it is still a matter of debate, human islets are described to have a more heterogeneous cell distribution (Figure 2)[29,30].

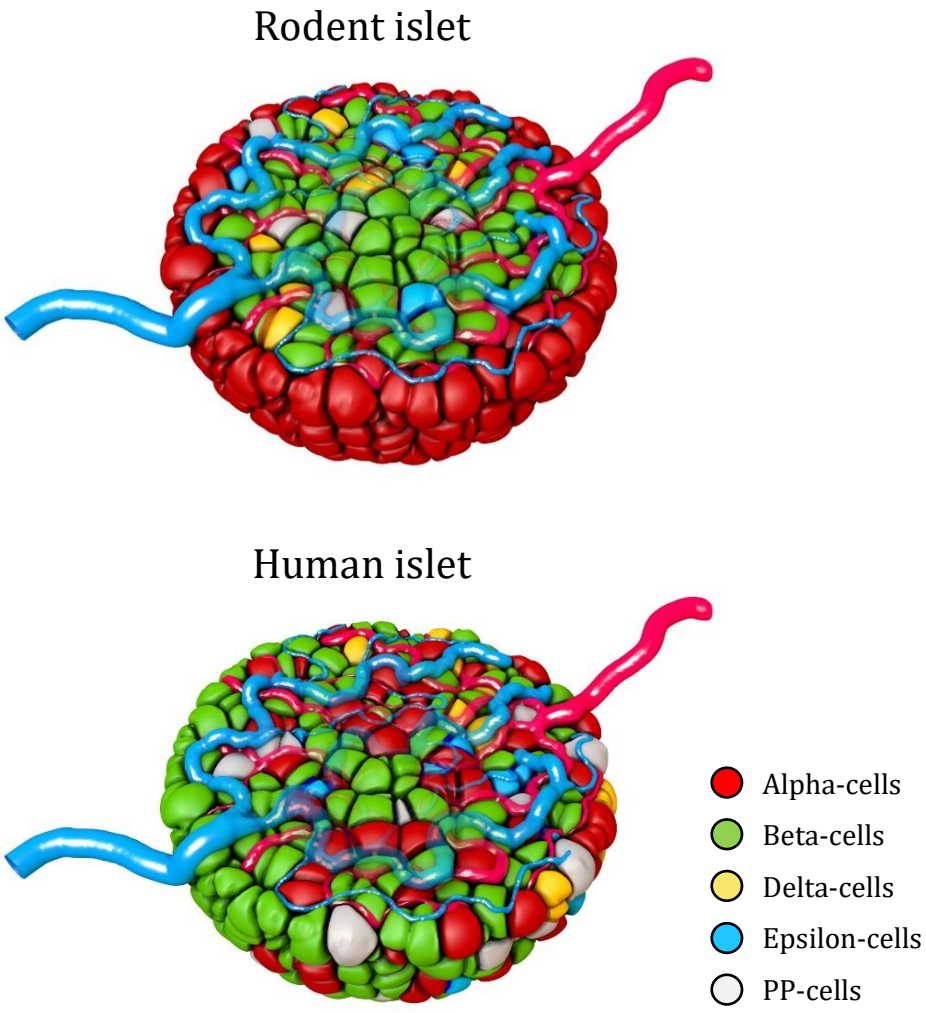

Figure 2 - Architecture of rodent and human islets.
3D-model of cellular organization and composition with sophisticated vasculature of rodent and human pancreatic islets. Red - alpha cells, green - beta-cells, yellow - delta-cells, blue - epsilon cells and grey - PP-cells. PP (Pancreatic polypeptide).

Murine islets contain 60-80% beta-cells, 15-20% alpha-cells and around 10-15% delta-, epsilon- and PP-cells, whereas the population in human islets is approximately 60:30:10[29-33]. Pancreatic islets exhibit a sophisticated vascular system with a high intra-islet capillary density, 5- to 10-fold higher compared to the exocrine tissue, leading to intense islet perfusion[34]. The capillary membrane contains a highly fenestrated endothelium, enhancing their permeability[35,36]. This characteristic guarantees sufficient oxygen and nutrient exchange and rapid sensing of the surrounding metabolic state with subsequent hormone release and transfer into the blood stream[37]. Pancreatic beta-cells synthesize and secrete insulin, the primary peptide hormone within islets, facilitating cellular glucose uptake into the peripheral tissues[38-40]. For the regulation of circulating blood glucose, insulin acts in concert with its antagonist glucagon. This hormone is generated and secreted by alpha-cells when glucose levels are decreased and induces the release of glucose from peripheral stores to prevent hypoglycemia[41-43]. Delta-cells are responsible for the production of somatostatin, inhibiting the release of insulin, glucagon and PP due to nutrient stimuli. However, the exact functional role of somatostatin is not fully understood[42,44]. The PP forms the axis between gastrointestinal tract, brain and pancreas and is secreted postprandially from PP-cells. Furthermore, PP regulates intra-islet secretion by the inhibition of glucagon release[45-47]. Produced in epsilon-cells during fasting, ghrelin reduces insulin secretion to prevent hypoglycemia and acts as paracrine inhibitor of beta-cells[48-50]. Furthermore, two additional cell types were identified in pancreatic islets in the 1970s; serotonin-producing enterochromaffin-cells and gastrin-producing cells[51,52].

The two phases of insulin secretion

Secreting appropriate amounts of insulin when needed is the vital function of pancreatic beta-cells, acting as regulators of circulating nutrient levels[53]. Due to their distinct vascular network as shown in Figure 2, islet beta-cells are able to rapidly respond to nutritional changes[33,54]. The primary stimulus for insulin release is an increase in blood glucose levels. Other nutrients, mainly FA (Fatty acids) and amino acids as well as incretins, such as GLP-1 (Glucagon-like peptide 1) or GIP (Glucose-dependent insulinotropic peptide), might amplify GSIS (Glucose-stimulated insulin secretion)[55,56]. In response to elevated circulating glucose, cytosol-located GLUT2 (Glucose transporter 2) are transferred to the beta-cell membrane,

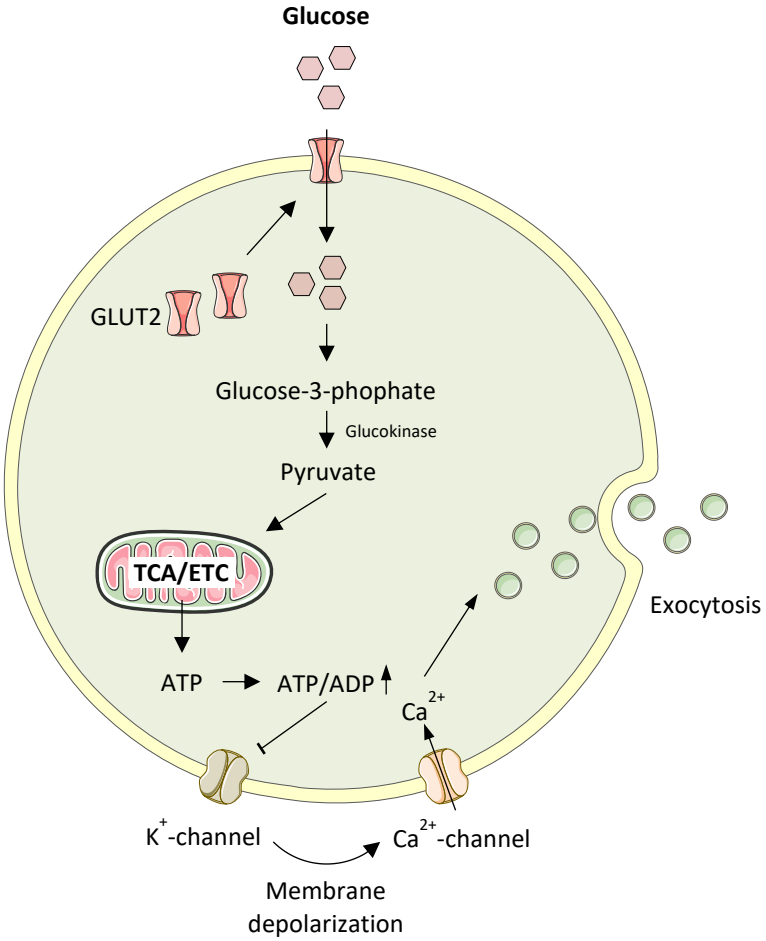

Figure 3 - Glucose-stimulated insulin secretion in pancreatic beta-cells.
Elevated circulating glucose levels lead to increased glucose uptake via GLUT2 and glycolysis induction. Glucose is metabolized to pyruvate, resulting in elevated activity of TCA/ETC cycles and generation of ATP. Subsequent increase of intracellular ATP/ADP ratio results in closure of ATP-sensitive K^+-channels, membrane depolarization, opening of voltage-dependent Ca^{2+}-channels and insulin exocytosis. ADP (Adenosine diphosphate), ATP (Adenosine triphosphate), Ca^{2+} (Calcium), ETC (Electron transport chain), GLUT2 (Glucose transporter 2), K^+ (Potassium), TCA (Tricarboxylic acid cycle).

mediating glucose influx[57,58]. Within the beta-cell, glucose is phosphorylated to glucose-6-phosphate by the rate-limiting enzyme glucokinase, initiating glycolysis[59,60]. Pyruvate is generated at the end of the cascade, serves as substrate for the TCA (Tricarboxylic acid) cycle and is metabolized within the ETC (Electron transport chain) in the mitochondria[61,62]. These reactions induce the generation of ATP (Adenosine triphosphate), a signaling molecule for beta-cell insulin secretion. Elevation of cytosolic ATP leads to an increased ratio of ATP to ADP (Adenosine diphosphate), initiating the closure of ATP-sensitive K^+ (Potassium)-channels, finally causing membrane depolarization[63]. Subsequent opening of voltage-dependent Ca^{2+} (Calcium)-channels mediates an increase in Ca^{2+}-influx and concentration within the beta-cell, facilitating exocytosis of insulin-containing granules from a pool of already synthesized insulin (Figure 3)[53,64]. This signaling cascade is described

as the triggering pathway initiating first phase insulin secretion that occurs within 10-15 min after glucose stimulation and accounts for around 15% of total insulin secretion[65]. The second phase, known as metabolic amplifying pathway, is responsible for successive insulin secretion and dependent on sustained elevated glucose levels and different nutrient-derived metabolites, termed metabolic coupling factors. These factors are generated by anaplerotic conversion of intermediates from pyruvate and TCA cycling[66-68]. By activation of G protein-coupled receptors and cAMP (Cyclic adenosine monophosphate)-dependent PKs (Protein kinases), metabolic coupling factors mediate exocytosis of insulin granules[69,70]. The impairment of insulin secretory function in beta-cells has detrimental effects on overall body energy homeostasis[25,71].

Nutritional impact on beta-cells

The link between obesity and insulin resistance

Basically, chronic intake of high caloric diets combined with physical inactivity are described as fundamental causes for the development of obesity and insulin resistance, in turn, enhancing the risk for health problems, including type 2 diabetes[72,73]. Elevated blood glucose is increasingly metabolized to glycogen and stored in liver and muscle cells, mediated via insulin release from beta-cells[74]. Moreover, insulin regulates the incorporation of large amounts of circulating FA into triglyceride deposits of adipocytes, leading to hyperplastic and hypertrophic expansion of the adipose tissue. Persistent adipocyte overload impairs cellular function and initiates a stress response by infiltration of immune cells into the adipose tissue and the release of pro-inflammatory cytokines, such as IL-6 (Interleukin-6), TNF-α (Tumor necrosis factor-alpha) or iNOS (Inducible nitric oxide synthase). This leads to adipocyte dysfunction and disturbs the insulin receptor signaling cascade, inducing insulin resistance in adipocytes. Dysfunctional adipocytes utilize lower amounts of FA, increasing the pool of circulating FA[75-78]. Additionally, adiponectin release from adipocytes decreases, a hormone acting together with insulin to enhance glucose metabolism in skeletal muscle and liver. Thus, reduced adiponectin amplifies peripheral insulin resistance. Under these conditions, peripheral tissues utilize elevated amounts of circulating FA[79,80].

In skeletal muscle, incorporated FA are converted into triglycerides (stored as ectopic deposits) and disturb mitochondrial function. Increased triglyceride stores are metabolized to diacylglycerol, stimulating PKCθ-dependent phosphorylation and inactivation of the IRS-1 (Insulin receptor substrate-1). Thus, PI3K (Phosphoinositide 3-kinase) and PKB are inactivated, preventing GLUT4 translocation and consequently insulin-mediated glucose uptake[81,82]. In liver cells, elevated triglyceride storage, reinforced by *de novo* lipogenesis from excess glucose supply, leads to the development of steatosis with subsequent functional impairments[83]. ChREBP (Carbohydrate-responsive element-binding protein) has been identified as a possible mediator in this cascade, controlling glycolysis and lipogenesis at transcriptional levels[84,85]. Elevated diacylglycerols, derived from triglyceride deposits, mediate PKCε activation inhibiting insulin-stimulated phosphorylation of IRS-2, followed by impaired generation of glycogen and increased stimulation of gluconeogenesis via FOXO1 (Forkhead box protein O1)[86,87].

Consequently, liver and skeletal muscle become insulin-resistant, resulting in reduced glucose and FA utilization with sustained glucose release from the liver[88]. Additionally, elevated FA levels stimulate the generation of inflammatory pathways by the interaction with TLRs (Toll-like receptors), reinforcing the insulin-resistant state[89]. To compensate insulin resistance, beta-cells increase their secretory function to maintain glucose homeostasis. However, this might initiate a vicious cycle of hyperinsulinemia and elevated insulin resistance, leading to elevated circulating glucose levels and finally beta-cell dysfunction[25,71,90].

Gluco- and lipotoxicity concerts into glucolipotoxicity

Chronically elevated levels of circulating glucose, described as hyperglycemia, have harmful effects on endocrine beta-cell functionality and insulin sensitivity in peripheral tissues[91-93]. As a compensatory mechanism, beta-cells not only exhibit increased insulin synthesis and secretion, rather they expand their mass to appropriately adjust on insulin requirements and maintain normoglycemia[94]. Such structural alterations have been shown in the human situation under certain physiological states, such as obesity and insulin resistance, but also during pregnancy by compensatory beta-cell proliferation[95,96]. However, persistent and repeated hyperglycemia results in depletion of insulin deposits

and beta-cell exhaustion. This mediates beta-cell dysfunction, leading to glucotoxicity and the induction of apoptotic signaling pathways[97]. Besides this, prolonged high levels of FA might have harmful effects on beta-cell functionality (independent of their impact on insulin sensitivity), defined as lipotoxicity[98]. In this context, the degree of saturation seems to determine the outcome. While saturated FA, such as palmitate, are linked to an impairment of insulin secretory capacity, monounsaturated FA might protect beta-cell function due to their anti-oxidative potential[99,100]. Moreover, other lipid-related species, such as diacylglycerol, ceramides, sphingolipids or acyl-coenzyme A, have been associated with beta-cell damage[101,102]. The combined deleterious effects of elevated glucose and FA levels on beta-cell function, but also survival, have been termed glucolipotoxicity, defined as metabolic stress in the present thesis[97,103]. As reviewed by van Raalte and Diamont, continuous glucolipotoxic conditions disturb the balance between beta-cell damage and defense mechanisms, leading to the onset and progression of related diseases, such as type 2 diabetes[104].

The molecular and physiological consequences of glucolipotoxicity on pancreatic beta-cells have been linked primarily to impaired mitochondrial function and redox stress. Persistent elevated workload on mitochondrial TCA and ETC cycles leads to increased production of ROS (Reactive oxygen species)[105,106]. Additionally, this is accompanied by an activation of the NADPH (Nicotinamide adenine dinucleotide phosphate)-oxidase and iNOS, generating RNS (Reactive nitrogen species)[107,108]. This results in oxidative and nitrosative (redox) stress, mediating different mechanisms, such as nitration, carbonylation, peroxidation and nitrosylation from simple sugars, lipids and proteins. Limited by their low anti-oxidative capacity, beta-cells are highly susceptible towards oxidative and nitrosative damage, indicating that changes in redox balance impair beta-cell function and induce apoptosis[109]. Oxidative stress might also perturb protein folding, one of the main functions of the ER (Endoplasmatic reticulum), reflecting the direct connection between oxidative and ER stress[110]. To maintain ER homeostasis, the UPR (Unfolded protein response) is induced at moderate glucolipotoxicity. However, under persistent glucolipotoxic stress, UPR fails to maintain adequate ER function, leading to decreased proinsulin biosynthesis[111,112]. Furthermore, elevated ROS levels have adverse effects on the expression of several beta-cell specific genes, necessary for insulin transcription and survival, including PDX1, NEUROD1 and MAFA[113,114]. Recent research also supports the

hypothesis that chronic lipid exposure inhibits beta cell proliferation and prevents beta cell mass expansion[101]. Moreover, glucose and FA-induced ROS and RNS formation mediates the activation of the inflammasome and NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) pathways followed by pro-inflammatory signals via chemokine and cytokine release[115]. This is also linked to an impairment of the TXN (Thioredoxin)-pathway by increased expression of TXNIP (Thioredoxin-interacting protein)[116,117].

The ChREBP/TXNIP axis - a possible mediator of hyperglycemic damage

In recent years, TXNIP, which belongs to the alpha-arrestin family, has been emerged as potent inducer of beta-cell dysfunction and death under hyperglycemic conditions[118,119]. The effects of TXNIP on beta-cells are mediated via distinct downstream mechanisms dependent on its cellular localization[120]. Nuclear TXNIP reduces insulin synthesis via downregulation of important transcription factors, such as MAFA[121]. Located in the cytosol, TXNIP binds to cysteine residues of the reduced form of TXN1, decreasing its reductase activity and inhibiting the TXN pathway[117]. Moreover, when translocated into the mitochondria, TXNIP competes with ASK1 (Apoptosis signal-regulating kinase 1) for TXN2 binding. This results in ASK1 phosphorylation, triggering the mitochondrial death pathway via cytochrome c release and caspase-3 cleavage (Figure 4)[122,123]. Consequently, TXNIP impairs cellular redox homeostasis leading to oxidative stress and inflammation inducing beta-cell dysfunction and apoptosis. In contrast to glucose, elevated FA does not appear to increase TXNIP expression in beta cells[124,125]. Recent findings also indicate that simultaneous exposure to glucose and palmitate rather decrease the expression of TXNIP, in human as well as mouse islets[126].

TXNIP expression is mediated by the activation of the transcription factor ChREBP, discovered in 2001 by Yamashita et al. and emphasized in previous years as one of the major metabolic glucose-dependent regulator in several tissues[127,128]. ChREBP is expressed in the endocrine lineage during pancreatic development and its activity is essential for beta-cell differentiation[129]. Recently, two isoforms of ChREBP (α and β) have been identified. ChREBPα is activated initially by glucose metabolism, stimulating ChREBPβ gene expression that in turn, might inhibit ChREBPα by a negative feedback loop[130,131]. In response to elevated circulating glucose, ChREBP is dephosphorylated and forms a heterodimer with

MLX (Max-like factor 1), initiating cytosolic-nuclear-shuttling[132]. Once translocated, ChREBP binds to the ChoRE (Carbohydrate-response element)-region, inducing the transcription of target genes, including TXNIP[133,134]. Since ChREBP binds to the TXNIP promoter dependent on glucose supply, hyperglycemic conditions are known to reinforce the harmful effects of TXNIP on beta cell function and survival[131]. Moreover, hyperglycemia-induced beta-cell dysfunction are also associated with the formation of AGEs (Advanced glycation end products)[135].

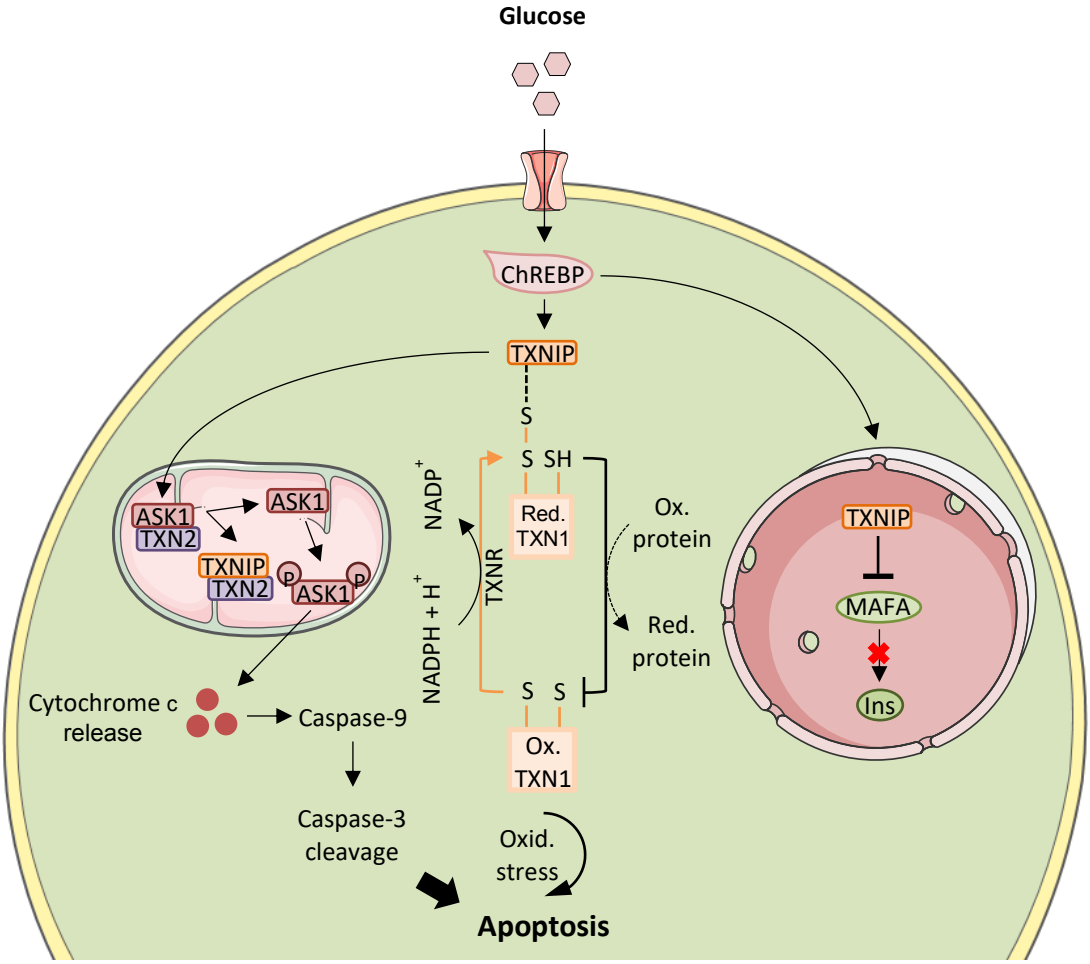

Figure 4 - Downstream mechanisms of TXNIP in beta-cells.
Elevated glucose levels activate ChREBP leading to increased TXNIP expression. Cytosolic TXNIP binds to and inhibits TXN1 resulting in oxidative stress and enhanced apoptosis by declined ability to reduce oxidized proteins. Translocated into the mitochondria, TXNIP interacts with TXN2, leading to phosphorylation and activation of ASK1, cytochrome c release, caspase-9 activation, caspase-3 cleavage and apoptosis. Nuclear TXNIP inhibits the expression of MAFA and decreased insulin transcription. ASK1 (Apoptosis signal-regulating kinase 1), ChREBP (Carbohydrate-responsive element binding protein), Ins (Insulin), MAFA (MAF BZIP transcription factor A), NADPH (Nicotinamide adenine dinucleotide phosphate), TXN (Thioredoxin), TXNIP (Thioredoxin-interacting protein), TXNR (Thioredoxin reductase).

AGEs - critical products of hyperglycemia

AGEs, discovered in the beginning of the 20th century, are a heterogeneous class of non-enzymatically glycated proteins, lipids and nucleic acids, formed during the Maillard reactions [136,137]. Initially, reversible Schiff base intermediates are generated, rearranging to Amadori products and undergo further reactions to form dicarbonyl compounds, such as glyoxal or MG (Methylglyoxal)[138,139]. Besides the Maillard reaction, these highly reactive compounds arise also from glucose autoxidation, lipid peroxidation or the polyol pathway[140,141]. Moreover, MG is generated as byproduct of glycolysis via spontaneous fragmentation of triose phosphate isomers, especially in response to hyperglycemic conditions, and serves as key precursor for various endproducts, such as arg-pyrimidine and MG-H1 (Methylglyoxal-derived hydroimidazolone) [142-145]. Later stages of the Maillard reaction induce the formation of irreversible advanced products, such as ribose-derived cross-linker pentosidine or lysine-derived CML (N-ε-carboxymethyllysine) and CEL (N-ε-carboxyethyllysine)[137,146]. Among these, CML and pentosidine belong to the most frequently used and best-characterized AGEs[137,147]. AGE formation occurs exogenously (*ex vivo*) during thermal processing of foods as well as endogenously (*in vivo*) dependent on the levels of circulating glucose[140,148,149]. About 10% of dietary AGEs are absorbed during digestion of which only 30% are excreted via the urine[150,151]. Endogenous AGE formation increases massively under certain physiological conditions, such as hyperglycemia or oxidative stress, leading to increased amounts of AGEs in the vasculature and in various tissues[143,152]. Both, endogenous and exogenous AGEs are associated with the development of several pathologies and diseases, such as type 2 diabetes and related complications[148,151].

The mechanisms of action of AGEs and their precursors occur by the interaction with their receptors or due to receptor-independent substrate modifications primarily via glycation[137,153]. These modifications affect a wide range of intracellular and extracellular proteins[154]. Glycated intracellular proteins are not eliminated and accumulate, leading to cellular functional impairments and damage[155]. AGE cross-linking and structural deformation of proteins with slow turnover rates, including the extracellular matrix proteins collagen and elastin, contribute to arterial stiffness[152,156]. Moreover, insulin might be a direct target for glycation, resulting in disturbed insulin action[157]. AGE formation also

contributes to insulin resistance by impairing the insulin-signaling pathway of peripheral tissues due to PKCα-mediated increased phosphorylation of IRS-1 and 2[158]. The effects of AGEs via their receptors are dependent on AGE-concentrations[159]. While low AGE levels stimulate the expression of AGER1 (AGE receptor 1), initiating AGE degradation and removal via endocytosis, high AGE concentrations lead to the upregulation of RAGE (Receptor for AGE) expression, inducing different deleterious signaling pathways in variety of cell types and tissues[137,160,161]. AGE-RAGE interaction triggers NADPH-oxidase activation, leading to mitochondrial-derived ROS production and oxidative stress, finally resulting in cellular dysfunction and apoptosis[162,163]. RAGE activation also stimulates PI3K or MAPK (P38 mitogen-activated protein kinase) pathways mediating cytosol-nuclear-shuttling of NF-κB, inducing inflammation and oxidative stress by the expression of pro-inflammatory mediators, such as IL-6, TNFα or VCAM-1 (Vascular cell adhesion molecule-1)[143,164,165]. Moreover, NF-κB binding domain within the RAGE gene promoter enhances RAGE expression by a positive feedback loop between NF-κB and RAGE[166].

In endocrine beta-cells, AGEs have been shown to cause insulin secretory defects due to superoxide generation and mitochondrial dysfunction, reduced Ca^{2+}-flux or increased glucose uptake[167]. By increasing the expression of iNOS, AGEs mediate the inhibition of cytochrome c oxidase, leading to reduced ATP synthesis and insulin exocytosis, thus decreasing beta-cell secretory potential[168,169]. Due to subsequent formation of nitric oxide and peroxynitrite, elevated iNOS expression initiates the generation of nitrated proteins residues, such as 3-NT (3-nitrotyrosine)[170,171]. Combined with elevated generation of PDGF (Platelet-derived growth factor) and proliferation of VSMC (Vascular smooth muscle cells), iNOS activation has been attributed to vascular damage, associated with diabetic complications[153]. AGEs also impair insulin synthesis by reduced phosphorylation of FOXO1, leading to decreased PDX1 expression and downregulation of insulin gene transcription[172]. Moreover, AGEs trigger apoptosis in beta-cells by activation of NADPH oxidase as well as JNK (C-jun N-terminal kinases) and MAPK pathways[173,174]. Beta-cell apoptosis is also induced due to RAGE-mediated mitochondrial stimulation, followed by cytochrome c release and caspase-3 activation as well as reduced expression of anti-apoptotic BCL2 (B-cell lymphoma 2)[175]. Thus, AGEs are involved in the development of insulin resistance and have a high impact on beta-cell functionality and survival, indicating their important role in type 2 diabetes onset and progression as well as related complications[165,176]. The incidence

and prevalence of type 2 diabetes increase massively with age, highlighting the relevance of AGEs in the aging process[177]. To date, a growing body of evidence reveals that the formation and accumulation of AGEs occurs during aging[143,178,179]. For instance, age-dependent CML formations have been observed in various tissues from human non-diabetic and diabetic donors, also correlating with higher risk of all-cause and cardiovascular mortality in older patients[180,181]. The age-related AGE accumulation is dependent on the intake of dietary AGEs and the amount of circulating glucose or the state of hyperglycemia and diabetes, respectively, and increases due to the decline in kidney clearance and reduced activity of repair and defense mechanisms in aging[155,182]. Overall, the formation and accumulation of AGEs is an inevitable component of the aging process promoting the multisystem functional decline observed in advanced age[155,179,183].

Beta-cells in aging

Besides nutritional-related aspects and their physiological consequences, the aging process has been emerged as a particular regulator of insulin action, beta-cell function and survival[184,185]. Aging and related alterations are associated with metabolic disorders, including impaired glucose tolerance and increased peripheral insulin resistance, mediated primarily by physical inactivity, obesity and metabolic stress, indicating that nutrition and aging are indissociable factors affecting beta-cell health[92,184,186,187]. However, whether aging *per se* is responsible for decreased insulin action and beta-cell dysfunction is still under debate[184,188-190]. Basically, aging is defined as an irreversible process characterized by a progressive decline of physiological function and integrity as well as increased susceptibility to stress and apoptosis[191]. In humans and animal models, multiple factors have been associated with beta-cell secretory dysfunction in aging, including downregulated GLUT2 and glucokinase expression, mitochondrial dysfunction, inadequate ion fluxes and reduced expression of beta-cell specific transcription factors. In turn, this leads to deregulated insulin release, diminished proinsulin biosynthesis and lower insulin content[93,192-194]. Moreover, the accumulation and formation of by-products, co-factors and modified proteins during aging, mainly derived from inflammation, oxidative and nitrosative stress, as described before, contribute to an age-related loss of functional beta-cells[105,195]. This might be amplified by their low defense potential due to minor expression

of anti-oxidative enzymes, in combination leading to a higher vulnerability towards apoptosis[196].

In addition, aging plays a crucial role in the regulation of the overall beta-cells mass. Beta-cell mass grows well into adulthood, peaks until middle age and might expand in response to increased metabolic demand during hyperglycemic conditions or pregnancy. Several mechanisms and factors have been emerged to control beta-cell mass expansion during aging, including neogenesis, proliferation and apoptosis (as mentioned above) as well as cell cycle activators and inhibitors (Figure 5)[197-200]. Neogenesis represents the differentiation of pancreatic progenitor cells or trans-differentiation of non-beta-cell fractions into functional beta-cells, occurring during normal embryonic development, but also in adulthood due to increased insulin requirements. However, the role of neogenesis has been discussed controversially and does not appear to contribute to beta cell mass expansion in higher age[201]. In contrast, self-duplication of pre-existing beta-cells is thought to be the major process for cellular mass expansion in aging[200,202]. Mechanistically, proliferation describes the ability to maintain cell cycle entry and progression in response to mitogenic signals, dependent on the expression of several cell cycle activators and transcription factors, including D-type cyclins, CDKs (Cyclin-dependent kinase), forkhead box protein M1 (FOXM1) or PDX1[199,203-205]. Both, cell cycle entry and progression of beta-cells are limited with age, shown by decreased expression of FOXM1, PDX1, Ki67 (Antigen

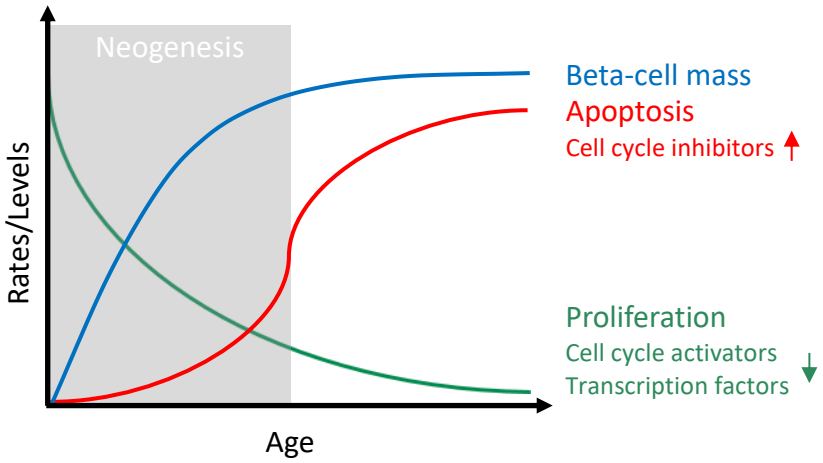

Figure 5 - Mechanisms and factors regulating beta-cell mass during aging.
Proposed model of major mechanisms and factors regulating beta-cell mass in aging, modified from Kushner et al.[202] as well as Gunasekaran and Gannon[185]. Beta-cell mass (blue) increases rapidly during aging and peaks until middle age. Beta-cell proliferation (green) decreases continuously during aging, primarily by reduced expression of cell cycle activators and transcription factors, sustained in higher age. Apoptosis and the expression of cell cycle inhibitors (red) increase more rapidly starting at middle age. Beta-cell neogenesis (grey box) seems to play a minor role in beta-cell mass expansion in advanced age.

identified by monoclonal antibody Ki67) and PCNA (Proliferating cell nuclear antigen)[204,206,207]. Nevertheless, low proliferation was observed in some studies that might increase in response to metabolic stimuli[201,208-210]. The age-related decline in beta-cell proliferation restricts the capacity to regenerate, correlating with elevated expression of cell cycle inhibitors[185,206,211]. The most prominent one in beta-cell aging is the tumor suppressor protein $p16^{INK4a}$, expressed from the *cdkn2a* locus[211]. $P16^{INK4a}$ is known to suppress the interaction between cyclin D2 and CDK4 and 6, preventing the inactivation of Rb. This leads to an irreversible cell cycle arrest by inhibiting the transition from G_1 to S phase (Figure 6), initiating cellular senescence, described as an major hallmark of aging[185,206]. Senescence occurs in response to various cellular stressors, such as DNA damage, mitochondrial deterioration, oxidative stress or oncogenic activation[212]. Cells undergoing senescence exhibit several widespread changes, including a flattened morphology, increased size and protein content or enlarged lysosomes[213]. Moreover, senescent cells express and secrete a variety of pro-inflammatory cytokines and chemokines, growth factors, components of extracellular matrix or ROS and RNS, summarized as the SASP (Senescence-associated secretory phenotype)[214,215]. Consequently, cellular senescence has been linked to obesity and inflammation, promoting insulin resistance as well as tissue and cellular dysfunctions, including beta-cells[216]. However, cellular senescence might also have beneficial effects on beta-cells due to growth induction, increased glucose uptake or enhanced GSIS[217,218].

Collectively, several aspects of aging might affect pancreatic beta-cell function and integrity, contributing to decreased insulin action, impaired glucose tolerance and type 2 diabetes, but most of them are related to certain physiological conditions.

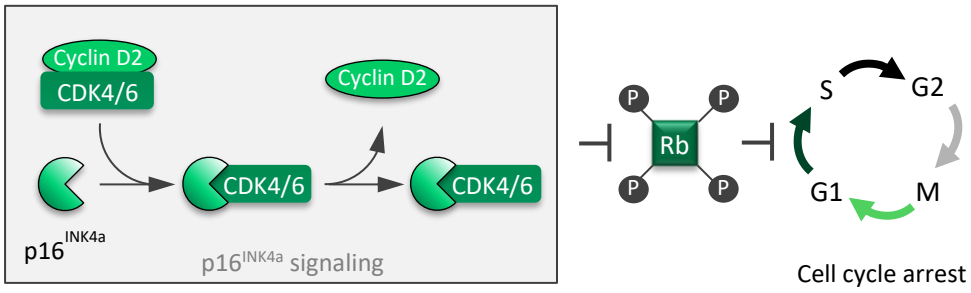

Figure 6 - Induction of cell cycle arrest by $p16^{INK4a}$.
$P16^{INK4a}$ binds to CDK4 and 6, suppressing CDK4/6-cyclin D2 interaction, leading to Rb-mediated inhibition of G1 to 2 phase transition and cell cycle arrest. CDK4, 6 (Cyclin-dependent kinase 4, 6), Rb (Retinoblastoma protein).

Mouse models

The use of mouse models is essential for studying human diseases and the fundamental mechanisms underlying their onset and progression to develop novel treatments and therapies[219]. Due to environmental, ethical and financial benefits, mice are more suitable to research approaches than higher mammals[220]. Although cell lines and cell culture systems are indispensable and a useful tool in biologic research, they poorly mimic conditions in the living organism due to the lack of metabolic interactions. In contrast, anatomical and physiological similarities between mice and humans are of critical relevance to unravel complex metabolic processes and mechanisms[221]. Additionally, by high genetic homogeneity towards humans and relatively short lifespan of about three years, mice are of particular interest for the study of aging and related complications. By using inbred mouse strains and standardized conditions, genetic variations, environmental and lifestyle factors might be excluded, allowing the investigation of diets as well as individual nutrients and their effects on the metabolism[220,222,223].

The C57BL/6J mouse

The C57BL/6 mouse is one of the most widely used inbred strains for neurologic, immunologic and metabolic research, derived from the C57BL strain developed by Clarence Cook Little in the Jackson Laboratory in 1921[224,225]. Substrains, such as C57BL/6J and C57BL/6N established in the 1970s, have been shown to exhibit various phenotypic differences dependent on the deletion of specific genes. Elimination of the nicotinamide nucleotide transhydrogenase (NNT) gene on exons 7-11, observed in the C57BL/6J substrain, leads to an impairment of insulin secretion and glucose homeostasis[226]. Since C57BL/6J mice are susceptible to diet-induced obesity, insulin resistance and dyslipidemia, they are widely used for the investigation of the metabolic syndrome[227-229]. Moreover, previous studies reported that high-fat-diet feeding might initiate type 2 diabetes progression in this substrain[230,231]. However, kept on a chow diet, C57BL/6J mice remain lean and normoglycemic. Besides this, aging in C57BL/6J mice reflects characteristics corresponding to the human situation, such as body weight gain, decreased lean mass, elevated fat mass and stable fasting glucose levels[232]. Thus, C57BL/6J mice were used as

model to investigate the impact of aging on pancreatic islets under normoglycemic and non-pathologic conditions (**publication I**).

The New Zealand Obese mouse

In 1948, Franz Bielschowsky started inbreeding of various mouse colonies at the New Zealand Otago Medical School and established the original New Zealand Obese (NZO) mouse strain[233,234]. NZO mice are a polygenic model exhibiting characteristics comparable to the human metabolic syndrome and type 2 diabetes[235,236]. Hyperphagia and reduced energy expenditure cause early obesity followed by insulin resistance, dyslipidemia and hypertension in male NZO mice. When fed with a carbohydrate-rich diet (CRD), NZO mice develop massive hyperglycemia and hyperinsulinemia, accompanied by insulin secretory dysfunction, depletion of insulin stores and beta-cell death, finally leading to type 2 diabetes-like phenotype[237-240]. Decreased GLUT2 and PDX1 expression as well as increased FOXO1 phosphorylation are defined as main molecular mechanisms. Moreover, previous investigations revealed that NZO mice are protected against loss of functional beta-cell mass by feeding a carbohydrate-free, high-fat diet (CFD), inducing only lipotoxicity and insulin resistance. In turn, re-exposure to a CRD after carbohydrate restriction causes fast and synchronized hyperglycemia and glucolipotoxicity accompanied by beta cell dysfunction and apoptosis[241-243]. By extending the period of carbohydrate restriction, NZO mice were used to investigate the impact of aging on pancreatic islets and insulin-releasing beta-cells, under glucolipotoxic stress (metabolic stress) conditions, focusing on beta-cell functionality (**publication II and manuscript**).

Aims

Dietary imbalance and aging have been described as the ultimate causes for the onset and progression of type 2 diabetes and its complications in humans and rodent models due to their harmful effects on functionality and structural integrity of pancreatic islets and beta-cells accompanied by alterations in redox potential[185,244]. Beta-cell aging is associated with a progressive decline in the proliferative as well as regenerative capacity and affects insulin secretory function[184,202]. However, these age-dependent effects on beta-cells are still discussed controversially and are mainly associated with certain metabolic conditions. Thus, in the first part of the present thesis, age-related changes of pancreatic islets under normoglycemic and non-pathologic (physiological) conditions in C57BL/6J wild-type mice were characterized in order to investigate the following scientific questions:

- Does aging under physiological conditions lead to impaired glucose homeostasis and morphological alterations in pancreatic islets of wild-type mice? (**publication I**)
- Which processes affect the islet morphology during aging? (**publication I**)
- Is aging accompanied by the formation of protein modifications and inflammation in pancreatic islets of wild-type mice and does this have detrimental effects? (**publication I**)

Chronic intake of high-energy-dense diets leads to obesity, peripheral insulin resistance and hyperglycemia, followed by glucolipotoxic (metabolic) stress, contributing to type 2 diabetes by a loss of functional beta-cell mass[92,93]. Whether these damaging effects are amplified in advanced age is barely studied. By investigating diabetes-prone NZO mice, the following scientific questions were examined in the second part of the thesis:

- How does diet-induced metabolic stress affect pancreatic islet and beta cell functionality and structural integrity in obese, diabetes-prone mice? (**manuscript**)

- What mechanisms are involved in metabolic stress-induced beta-cell failure? (**manuscript**)
- Is the formation of AGEs involved in metabolic stress-mediated beta-cell damage? (**publication II**)
- Are the effects of diet-induced metabolic stress on pancreatic beta-cells exacerbated in advanced age of obese, diabetes-prone mice? (**manuscript**)

Publications

Publication I - "Age-related oxidative changes in pancreatic islets are predominantly located in the vascular system."

Richard Kehm, Jeannette König, Kerstin Nowotny, Tobias Jung, Stephanie Deubel, Sabrina Gohlke, Tim Julius Schulz and Annika Höhn

Redox Biol. 15:387-393

Impact factor: 7.793

Own contribution:

- Sample collection
- Majority of experimental work
- Performance of statistical analyses
- Preparation of tables and figures
- Interpretation of results
- Preparation of manuscript

Publication II "Endogenous advanced glycation end products in pancreatic islets after short-term carbohydrate intervention in obese, diabetes-prone mice."

Richard Kehm, Jana Rückriemen, Daniela Weber, Stefanie Deubel, Tilman Grune and Annika Höhn

Nutr Diabetes. 11; 9(1):9

Impact factor: 3.528 (5-year)

Own contribution:

- Preparation of proposal for animal experiments
- Designing and planning of animal study

- Sample collection
- Proportional of experimental work
- Performance of statistical analyses
- Preparation of tables and figures
- Interpretation of results
- Preparation of manuscript

Manuscript "Maintenance of pancreatic beta-cells in middle-aged, diabetes-prone mice under glucolipotoxic stress is mediated by redox balance and cell cycle progression."

Richard Kehm, Markus Jähnert, Jeannette König, Stefanie Deubel, Mandy Stadion, Wenke Jonas, Annette Schürmann, Tilman Grune and Annika Höhn

Prepared for submission

Own contribution:

- Preparation of proposal for animal experiments
- Designing and planning of animal study
- Sample collection
- Majority of experimental work
- Performance of statistical analyses
- Preparation of figures
- Interpretation of results
- Preparation of manuscript

Publication I

"Age-related changes in pancreatic islets are predominantly located in the vascular system"

Richard Kehm, Jeannette König, Kerstin Nowotny, Tobias Jung, Stephanie Deubel, Sabrina Gohlke, Tim Julius Schulz and Annika Höhn

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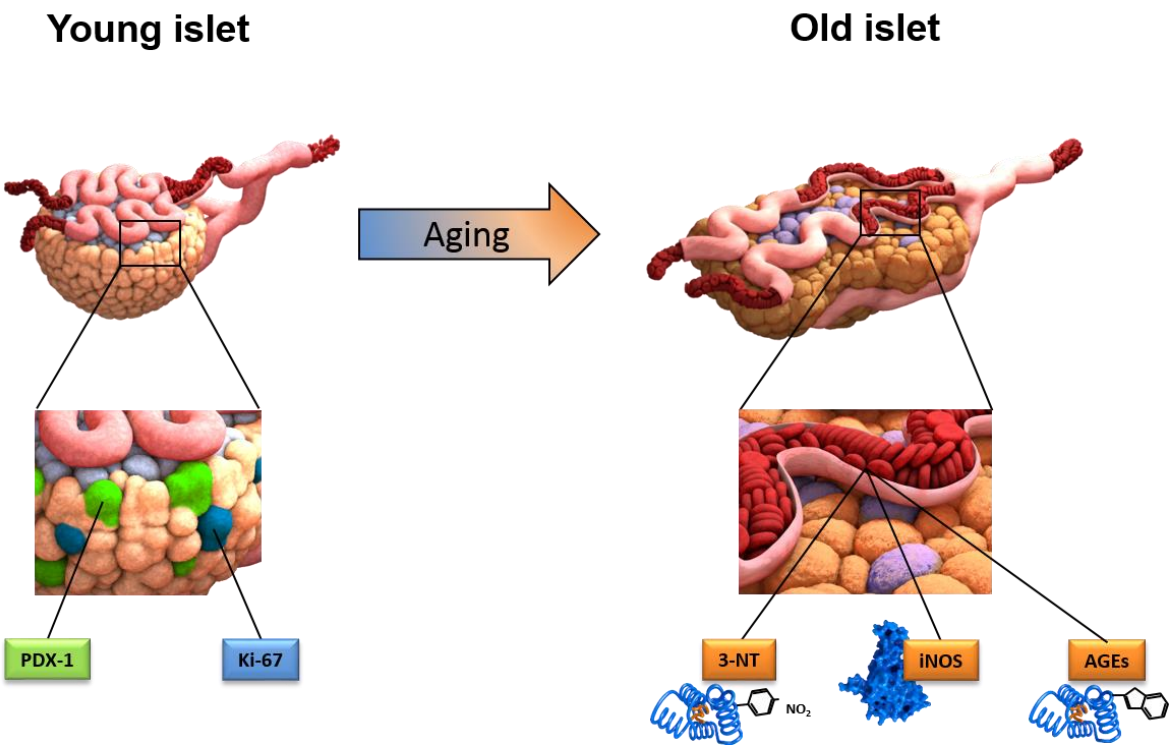
DOI: 10.1016/j.redox.2017.12.015

Due to conversion issues between Microsoft Word and PDF-XChange Editor, Publication I and II have been rebuilt.

Graphical abstract (**Publication I**)



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Redox Biology 15 (2018) 387–395

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Research Paper

Age-related oxidative changes in pancreatic islets are predominantly located in the vascular system

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ABSTRACT

Aged tissues usually show a decreased regenerative capacity accompanied by a decline in functionality. During aging pancreatic islets also undergo several morphological and metabolic changes. Besides proliferative and regenerative limitations, endocrine cells lose their secretory capacity, contributing to a decline in functional islet mass and a deregulated glucose homeostasis. This is linked to several features of aging, such as induction of cellular senescence or the formation of modified proteins, such as advanced glycation end products (AGEs) - the latter mainly examined in relation to hyperglycemia and in disease models. However, age-related changes of endocrine islets under normoglycemic and non-pathologic conditions are poorly investigated. Therefore, a characterization of pancreatic tissue sections as wells as plasma samples of wild-type mice (C57BL/6J) at various age groups (2.5, 5, 10, 15, 21 months) was performed. Our findings reveal that mice at older age are able to secret sufficient amounts of insulin to maintain normoglycemia. During aging the pancreatic islet area increased and the islet size doubled in 21 months old mice when compared to 2.5 months old mice, whereas the islet number was unchanged. This was accompanied by an age-dependent decrease in Ki-67 levels and pancreatic duodenal homeobox-1 (PDX-1), indicating a decline in proliferative and regenerative capacity of pancreatic islets with advancing age. In contrast, the number of $p16^{Ink4a}$-positive nuclei within the islets was elevated starting from 10 months of age. Interestingly, AGEs accumulated exclusively in the islet blood vessels of old mice associated with increased amounts of inflammatory markers, such as the inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine (3-NT). In summary, the age-related increase in islet size and area was associated with the induction of senescence, accompanied by an accumulation of non-enzymatically modified proteins in the islet vascular system.

1. Introduction

Pancreatic islets represent a network of endocrine cells, basically divided into two major subgroups (β-cells and non-β-cells). In rodents, β-cells are the most common cell type of the endocrine pancreas (up to 85%) and form the center of the islet. They are surrounded by the non-β-cell fraction (α-, δ-, ε-, and pancreatic polypeptide cells) and penetrated by a large number of blood vessels. In contrast, the human islet architecture exhibits a heterogeneous distribution of endocrine cells, but this remains a matter of discussion. The main function of pancreatic islets is the secretion of hormones (insulin, glucagon, somatostatin, ghrelin and pancreatic polypeptide), essential for the maintenance of homeostatic processes [1–4]. During aging, the endocrine pancreas undergoes morphological and metabolic changes, contributing to an inappropriate regulation of glucose levels. These changes mostly affect the insulin-producing β-cells, whereas in other cell types, only a few modifications were observed [5]. The pancreatic β-cell mass, basically representing the islet mass in rodents, declines with age, induced by an imbalance in β-cell turnover (decreased proliferation and replication, elevated apoptosis). This is accompanied by an increase in β-cell dysfunction, together leading to an overall reduction in functional β-cell mass [6–11].

Abbreviations: 3-NT, 3-Nitrotyrosine; AGE(s), advanced glycation end product(s); IF, immunofluorescence; IHC, immunohistochemistry; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; RT, room temperature

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It has been shown that the limitation of the proliferative and re-plicative capacity of β-cells during aging correlates with the induction of senescence, activated by the transcriptional upregulation of cell cycle inhibitors, such as $p16^{Ink4a}$, preventing the cell cycle entry [12–15]. In addition, Helman and colleagues were recently able to show that an increased expression of $p16^{Ink4a}$ enhances the insulin secretory capacity of β-cells in advanced age, which is in contrast to the previous literature [16–18]. Further well-known age-related changes include accumulation of non-enzymatic modified proteins, such as glycation (formation of advanced glycation endproducts, AGEs), oxidation or nitration of proteins [19–22].

AGEs are formed as products of the Maillard reaction, or precursors are generated as intermediates of glycolysis and lipid peroxidation. Additionally, it is suggested that the development of AGE deposits is accelerated mainly under hyperglycemic conditions and contributes to diabetic complications. However, AGE formation also occurs in normal aging [23–25]. By binding the receptor for advanced glycation endproducts (RAGE), AGEs induce the production of reactive oxygen species (ROS) by activating enzymatic processes. This causes a prionflammatory response mediated by the transcription factor Nuclearfactor Kappa B (NFκB) [26–28]. In addition, peroxynitrite as a product of the proinflammatory response is formed, facilitating protein nitration [29,30]. Since age-related changes in pancreatic islets and their major cell type (β-cells) are associated with the amount of circulating glucose, AGE formation and related processes were mainly investigated under hyperglycemic and disease conditions.

Here, we characterized pancreatic islets of wild-type mice (C57BL/ 6) at various age groups to describe age-related alterations of endocrine islets. C57BL/6J is a widely used inbred strain susceptible to polygenic obesity, type 2 diabetes and atherosclerosis. The observed expansion of islets was associated with the induction of senescence and the maintenance of insulin secretory capacity sufficient for metabolic demand. Additionally, our data show that glycated as well as nitrated proteins are also formed in normal aging, independent of hyperglycemic conditions. We also found that this modified proteins accumulate exclusively in the vascular system of the endocrine pancreas.

2. Material and methods

2.1. Experimental model

Male C57BL/6J mice (2.5, 5, 10, 15 and 21 months) from The Jackson Laboratory were housed in a controlled environment at a temperature of 20 ± 2 °C, with a 12/12 h light/dark cycle and obtained a standard diet (Ssniff, Soest, Germany) as well as water ad libitum. Blood samples were collected before sacrificing the mice, cooled on ice and centrifuged. Subsequently, pancreatic tissues were isolated and fixed in 4% paraformaldehyde solution for 24 h, followed by paraffin embedding according to standard procedures. Mice were kept in agreement with the National Institutes of Health guidelines for care and use of laboratory animals. All procedures are verified and approved by the ethics committee for animal welfare of the State Office Environment, Health, and Consumer Protection (State of Brandenburg, Germany).

2.2. Determination of blood glucose

Blood glucose levels were determined by using an automated ana-lyzer (Cobas Mira S, Hoffmann-La Roche, Basel, Switzerland) and a commercially available reagent kit (Glucose HK CP, Horiba ABX Pentra, Montpellier, France). The method is based on a 2-step enzymatic reaction with Hexokinase followed by Glucose-6-phosphate-dehydrogenase leading to the quantifiable end product D-gluconate-6phosphate.

2.3. Plasma insulin and proinsulin ELISA

The concentration of insulin and proinsulin in murine plasma was determined by using the Mouse High Range Insulin ELISA (ALPCO, Salem, USA) and carried out according to the manufacturer's instructions.

2.4. Immunohistochemistry and immunofluorescence

Longitudinal serial sections (2 μm) were processed for immunohistochemistry (IHC) and immunofluorescent (IF) analysis. The sections were de-paraffinized and re-hydrated in Roti-Histol (Carl Roth, Karlsruhe, Germany) and decreasing serial solutions of ethanol. Heat-mediated antigen retrieval was performed by placing the slides in citrate-buffer (10 mM citrate acid, 0.05% Tween 20 in distilled water) for 20 min at 95–99°C in a water bath, followed by a cooling step of 15 min at room temperature (RT). Pancreatic tissue samples were incubated with blocking solution (Antibody Diluent, Agilent, Waldbronn, Germany) containing 10% goat serum for 1 h. For IHC, sections were blocked with 0.03% hydrogen peroxide (Peroxidase block; Agilent, Waldbronn, Germany) for 10 min at RT. Sections were incubated with primary antibodies, diluted in blocking solution, for 1 h in a lightproof humidified chamber at RT. Rabbit anti-insulin antibody (ab181547, Abcam, Cambridge, United Kingdom), rabbit anti-Ki67 antibody (ab16667, Abcam Cambridge, United Kingdom), mouse MethylglyoxalAGE (Arg-Pyrimidine) (AGE06B, BiLogo, Kiel, Germany), mouse antimethylglyoxal antibody (MG-H1) (STA-011-CB, BioCat, Heidelberg, Germany), and anti-pentosidine antibody (PEN012, BiLogo, Kiel, Germany) were used for IHC staining of Insulin, Ki-67, Methylglyoxalderived AGEs and pentosidine, followed by a 30-min incubation with HRP-labeled polymer. Before mounting with Entellan (Merck Millipore, Darmstadt, Germany), tissue sections were incubated with substratechromogen solution, 3,3′-Diaminobenzidin (EnVision+ system-HRP, Agilent Waldbronn, Germany) and counterstained with hematoxylin (Sigma-Aldrich, Taufkirchen, Germany). Rabbit anti-PDX-1 antibody (07-696, Merck Millipore, Darmstadt, Germany), mouse anti-CDKN2A/ $p16^{INK4a}$ antibody (ab54210, Abcam, Cambridge, United Kingdom), rabbit anti-iNOS antibody (ab178945, Abcam, Cambridge, United Kingdom) and mouse anti-3-Nitrotyrosine antibody (ab110282, Abcam, Cambridge, United Kingdom) were used as primary antibodies for IF staining of PDX-1, $p16^{Ink4a}$, iNOS and 3-NT. All pancreatic slices were co-stained with mouse or rabbit anti-insulin antibodies (L6B10, Cell Signaling, Cambridge, United Kingdom; ab181547, Abcam, Cambridge, United Kingdom) to visualize the β-cell area. Visualization was performed by incubation with secondary antibodies conjugated to AlexaFluor 488 and 594 (Invitrogen, Darmstadt, Germany) and FluorCare including DAPI (Carl Roth, Karlsruhe, Germany) was used as mounting media.

2.5. Quantitative analysis of pancreatic islets

Microscopic analysis was performed by digital imaging of pan-creatic sections using an Olympus IX53 microscope (Olympus, Hamburg, Germany) for IHC or Zeiss LSM 780 confocal microscope (Zeiss, Jena, Germany) for IF. To count the number of positive stained nuclei (Ki-67, PDX-1, $p16^{Ink4a}$) and measure the positive stained area (iNOS, 3-NT, pentosidine, Arg-Pyrimidine, MG-H1) within the pan-creatic islets for the morphometric analysis, Zeiss ZEN 2.3 imaging software (Zeiss, Jena, Germany) was used. Pancreata of 6–8 mice were used for quantification of each staining. At the time of tissue sectioning, weight of the pancreas was not measured routinely. Thus, the islet mass (pancreas weight x islet area) could not be determined. As equivalent marker the islet area was used (%-islet area/slide). Islet size is an absolute parameter in mm^2 (in the meta-data of the original microscopic image files, both the number of pixels is included and the area of a

single pixel in μm^2). To measure islet area, number and size of islets, pancreatic sections were stained with insulin, visualized with 3,3′Diaminobenzidin and counterstained with hematoxylin as described above. Digital images of the entire pancreas were taken with a MIRAXMIDI Scanner (Zeiss, Jena, Germany). Total pancreatic and insulin positive area of each section was quantified with Zeiss ZEN 2.3 imaging software. The software evaluates size of single islets, overall islet size and islet number of a given image

2.6. Statistical analysis

Statistical analysis was performed by using GraphPad Prism version 7.03 (La Jolla, CA, USA). All data presented in the figures are mean values ± SD. Differences between two groups were assessed by Student's t-test and one-way ANOVA was used for multiple comparisons of more than two groups. Differences were considered as statistically significant, if $p < 0.05$ was reached.

3. Results

3.1. Morphological changes of murine pancreatic islets during aging

Aged C57BL/6J mice show increased body weights that remain stable after 10 months of age. In addition blood glucose and plasma insulin levels are unchanged in all age groups, indicating good health conditions also in old C57BL/6J mice. In contrast, the plasma proinsulin level decreases with age. Starting from 10 months of age the levels are significantly lower compared to the youngest mice (2.5 months). Consequently, the proinsulin-to- insulin ratio shifts from 1:5 in young (2.5 months) to 1:14 in old mice (21 months), suggesting a higher conversion rate of proinsulin to insulin in aging to maintain glucose homeostasis (Table 1).

In order to determine age-related morphological changes in whole pancreatic tissue, sections were IHC stained for insulin to visualize the insulin-positive area representing the islets. Islet area was defined as percentage of islet area within the whole pancreatic slice, whereas the islet size represents the area of single islets (in mm^2). IHC analysis revealed an age-dependent increase in pancreatic islet size, starting from the age of 10 months. Additionally, islet size doubled when comparing 2.5 and 21 months old mice (Fig. 1A, B). Islet area was only increased in 21 months old C57BL/6J mice (Fig. 1C). In contrast, almost equivalent islet numbers were observed between age groups (Fig. 1D). Summarized, C57BL/6J mice show an expansion in pancreatic islet size and area during aging, without changes in islet number and exhibit stable blood glucose levels.

3.2. Expansion of islet mass is associated with increased $p16^{INK4a}$-levels

In a next step, replication and proliferation rate of islets was determined. Initially, pancreatic tissue sections were IF stained for the pancreatic duodenal homeobox protein-1 (PDX-1), a specific marker for β-cell replication, differentiation and survival and co-stained for insulin and DAPI. The number of positive-labeled nuclei within the insulin-positive area was quantified and indicated as percentage of the entire nuclei. As shown in Fig. 2A, the relative number of PDX-1-positive βcells decreases with advancing age. At 10, 15 and 21 months of age, a decline of 25% compared to 2.5 months and 15% compared to 5 months old mice was found. In addition to PDX-1, tissue sections were stained with Ki-67 confirming an age-dependent decrease in proliferation capacity. After 5 months of age the number of Ki-67-positive nuclei was reduced by 50% compared to 2.5 months old mice. Moreover, Ki-67levels of 15 months old mice were lowered by further 30% (Fig. 2B). At 21 months of age, the average of Ki-67-positive nuclei was approximately 0.3%.

The age-dependent decline in proliferative capacity of pancreatic islets has been previously correlated with the transcriptional activation of the cell cycle inhibitor protein $p16^{Ink4a}$, reflecting the induction of cellular senescence [12]. Thus, to investigate the potential connection between proliferation and senescence, pancreatic tissue sections were IF labeled for $p16^{Ink4a}$ and co-stained for insulin and DAPI. As expected, a higher number of $p16^{Ink4a}$-positive nuclei within the insulin-positive area of islets were observed with advancing age. After 10 months of age, mice showed elevated levels of $p16^{Ink4a}$ compared to animals aged 2.5 and 5 months (Fig. 2C). In summary, the decrease in Ki-67-positive nuclei is accompanied by reduced levels of the transcription factor PDX1, and an age-related increase in $p16^{Ink4a}$-levels, indicating an association between the induction of cellular senescence and the expansion of pancreatic islet size with age.

3.3. AGE formation and nitric oxide production increase with age in the vascular system

Besides cellular senescence and proliferative modifications, aging is also associated with the accumulation of AGEs [31,32]. Therefore, different AGEs were analyzed by IHC staining of pancreatic tissue sections. Comparing 2.5 and 21 months old mice, a 9.5-fold increase for methylglyoxal-derived Arg-Pyrimidine was observed, MG-H1 increased 8-fold (Fig. 3A, B). Equally, the levels of the 3-deoxyglucosone-formed pentosidine were higher in the old mice (Fig. 3C). Interestingly, the agerelated formation of all analyzed AGEs was not prominent in endocrine cells but rather located in the blood vessels of the pancreatic islets (Fig. 3D). In addition to glycation, nitration of proteins is another important non-enzymatic modification in aging [19]. In order to investigate this, iNOS and 3-NT-levels were determined by IF labeling. iNOS and 3-NT-levels of old C57BL/6J mice were also increased and located in the blood vessels of the endocrine islets comparable to AGE accumulation (Fig. 4A, B). Altogether, these results suggest that the agedependent formation of AGEs is associated with higher amounts of nitrated proteins in the vascular system of the pancreatic islets.

4. Discussion

In general, aging affects the endocrine pancreas by a decline in islet turnover [10,33]. This contributes to a decreased islet mass and functionality, associated with deregulated glucose utilization and

Table 1
Body weight and plasma parameters of C57BL/6J mice at different age groups.

	Age (months)				
	2.5	5	10	15	21
Body weight (g)	23 ± 3.8	34.3±1.4a	40.4±3.74a,b	37.5±5a,b	41.5±5a,b
Glc (mmol/l)	8.45 ± 1.06	8.24 ± 1.25	8.43 ± 0.77	7.48 ± 1.66	6.85 ± 0.81
Ins (nM)	0.62 ± 0.21	0.71 ± 0.15	0.74 ± 0.16	0.8 ± 0.32	0.7 ± 0.2
ProIns (nM)	0.12 ± 0.03	0.08 ± 0.02	0.06±0.04[a]	0.07±0.07[a]	0.05±0.03[a]
ProIns/Ins	0.19 ± 0.11	0.12 ± 0.04	0.09 ± 0.08	0.12 ± 0.09	0.07±0.05[a]

Data are presented as mean values ± SD of n = 6–8 mice. Glc = blood glucose, Ins = insulin, ProIns = Proinsulin. Statistical significance was assessed by one-way ANOVA, [a,b]$p < 0.05$ (a = significance compared to 2.5 months, b = significance compared to 5 months).

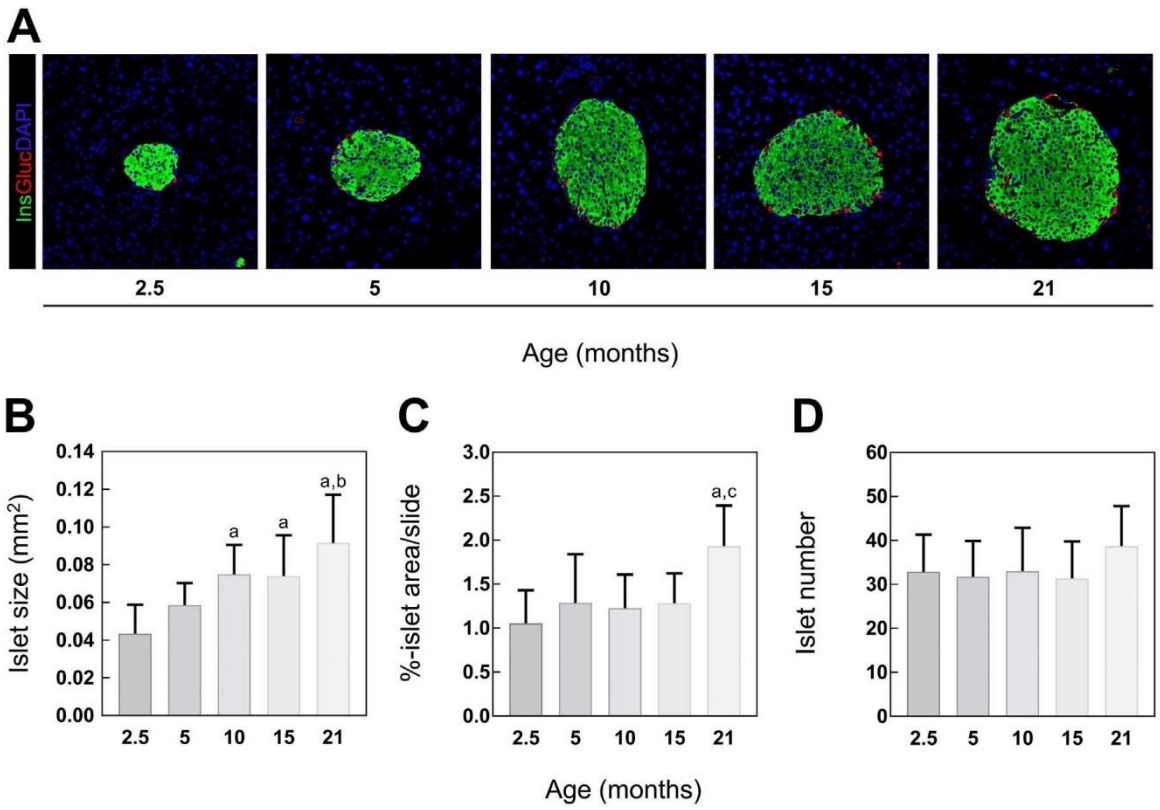

Fig. 1. Islet size, area and number in 2.5, 5, 10, 15 and 21 months old C57BL/6 mice. (A) Representative images of IF labeled insulin (Ins), glucagon (Gluc) and DAPI at indicated age. Quantification of IHC stained insulin of whole pancreatic sections represented as **(B)** islet area, **(C)** islet size and **(D)** islet number (n = 7–8 mice). Green: insulin, red: Glucagon, blue: DAPI. Data are presented as mean values ± SD. Statistical significance was assessed by one-way ANOVA, $^{a,b,c}p < 0.05$ (a = significance to 2.5 months, b = significance to 5 months, c =significance to 10 months).

hyperglycemia [34–36]. In contrast, our data show that pancreatic islets of normal aged wild-type C57BL/6J mice were able to secrete adequate amounts of insulin to compensate the metabolic demand, thus preventing hyperglycemic conditions. This was accompanied by an agedependent increase in islet area and size, whereas the islet number was unchanged, reflecting an expansion of the entire islet mass. Similar results were found by Sone and Kagawa and Tschen et al., both showing an adaptive increase in beta cell and islet mass in aging rodents under standard conditions [37,38]. Several reasons, mainly self-renewal and growth of β-cells as well as replication and differentiation of pancreatic progenitor cells, were used to explain the age-induced expansion of the pancreatic islets [39–41]. Here, the differentiation capacity of murine islets was determined by using the major pancreatic transcription factor, PDX-1, showing a decline with advancing age. This was directly associated with an age-dependent decrease in islet proliferation rate under normoglycemic conditions indicated by Ki-67. Our findings are in agreement with previous investigations, revealing that β-cells of normal aged mice show low PDX-1 expression and a decreased proliferative capacity [7,38]. Additionally, the age-related proliferative limitation of pancreatic islets correlates with the increased expression of the cell cycle regulator $p16^{Ink4a}$, shown by Krishnamurthy and colleagues [12]. By blocking the cell cycle due to the inhibition of the cyclin-dependent kinases 4 and 6, $p16^{Ink4a}$ induces senescence and restricts the proliferative capacity of cells [13,42]. According to that, the decreased proliferation shown in the present study was also accompanied by increased $p16^{Ink4a}$-levels with advancing age. Consequently, the $p16^{Ink4a}$induced reduction of cell cycle progression contributes to limited regenerative potential of pancreatic islets in old mice. However, given that islet expansion continues until advanced age, these results suggest that endocrine cells have a relatively long lifespan and the reduced growth rate seems to be sufficient to maintain an increase in islet size [43,44]. Another factor, possibly contributing to an increase in islet size, is the senescence-induced structural and functional reprogramming of cells. By increasing protein and RNA content including an overproduction of cytoskeleton and membrane proteins, such as vimentin and caveolin-1, senescent cells are known to enlarge [45–47]. Furthermore, a recent investigation [16] revealed a beneficial role of the senescence inducer $p16^{Ink4a}$ towards an increase in β-cell functionality possibly responsible for the maintenance of glucose homeostasis by generating adequate amounts of insulin as seen in this study. In contrast, it has been reported that functional impairments of pancreatic β-cells with age are associated with the formation of AGEs [48]. Zhao et al. and Coughlan et al. showed that circulating AGEs are associated with a decline in insulin secretory capacity of β-cells, mainly mediated by impaired mitochondrial functionality [49,50]. In addition, it has been shown by Puddu et al. that AGEs downregulate the protein expression of PDX-1 [51]. Thus, the observed reduction in PDX-1 levels together with the accumulation of AGEs in advanced age indicates that AGEs may contribute to the decline in regenerative potential of pancreatic islets. AGEs are formed under hyperglycemic conditions, but occur also as part of the normal aging process and contribute to complications in age-related diseases [50,52]. Here, we observed an accumulation of AGEs, such as pentosidine, Arg-Pyrimidine and MG-H1, in old mice confirming their increased formation at an advanced age. Interestingly, AGEs were found only in the blood vessels of the pancreatic islet, contrary to a recent finding [53]. Our observations demonstrate a local limitation of AGE deposits within the vascular system of the endocrine pancreas. In accordance, other investigations demonstrate an AGE accumulation in the vessel wall [54–57]. Extracellular matrix proteins, especially their most common form collagen, constitute the scaffold of the vascular system. Due to the slow turnover of collagen, this structural protein increases constantly with age [58–60]. Moreover, the formation of cross-linking products such as pentosidine diminishes the protein turnover

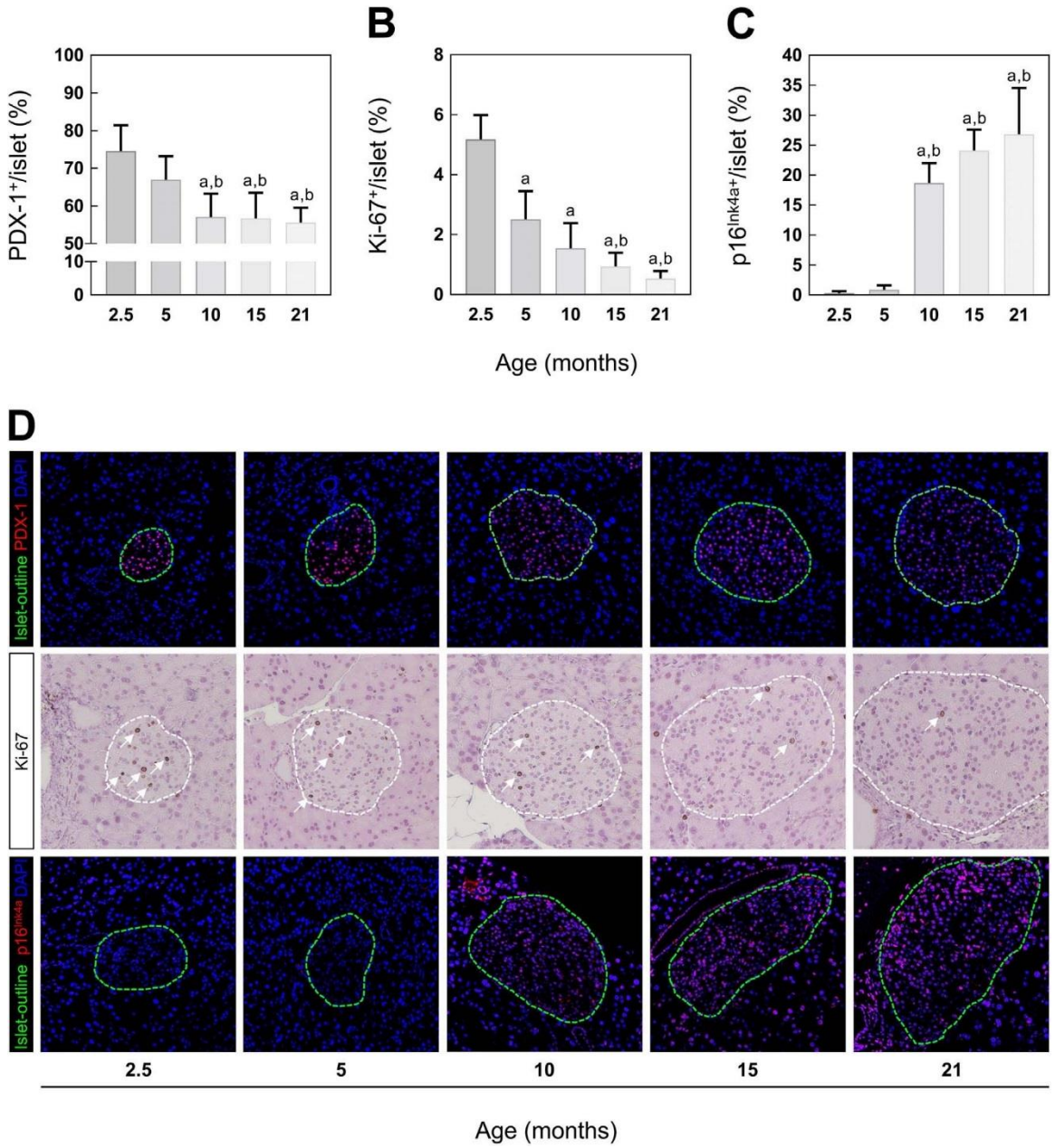

Fig. 2. β-cell regeneration and proliferation in 2.5, 5, 10, 15 and 21 months old C57BL/6 mice. Quantitative analysis of IF labeled **(A)** PDX-1, IHC stained **(B)** Ki-67 and IF labeled **(C)** $p16^{Ink4a}$ (n = 7–8 mice). **(D)** Representative images of each staining at indicated age. For better visibility of PDX-1 (first row) and $p16^{Ink4a}$ (third row), islets are outlined in green (according to insulin staining, not shown). For Ki-67, white lines mark the islet area and white arrows show positive stained nuclei. Green: islet-outline, red: PDX-1/$p16^{Ink4a}$, blue: DAPI. Data are presented as mean values ± SD. Statistical significance was assessed by one-way ANOVA, $^{a,b}p < 0.05$ (a = significance to 2.5 months, b = significance to 5 months).

contributing to AGE accumulation as well as vascular stiffening [61–63]. In addition to protein modifications, AGEs directly mediate their detrimental effects by binding to their major receptor, RAGE that is expressed, among others on the surface of endothelial cells [64,65]. It has been shown that this interaction activates the NADPH oxidase and causes intracellular ROS production [27,66]. As a feedback mechanism, the formation of AGEs is increased followed by an activation of NF-κB and its downstream pathways [67]. It was shown in different cell types that both processes contribute to the induction of iNOS expression [68–70]. This promotes the formation of nitric oxide able to react with superoxide anions to form peroxynitrite, leading to the production of nitrated proteins such as 3-NT [30,71,72]. This is in accordance with our findings showing that age-related generation of AGEs is accompanied by higher levels of iNOS in the blood vessels. Furthermore, we observed an accumulation of nitrated proteins, quantified via the typical product 3-NT. This modification was again only found in the blood vessels of pancreatic islets, indicating an association between the formation of AGEs and the generation of nitrosative stress in the vascular system of the endocrine pancreas during aging.

In summary our data show an age-related expansion of endocrine islets associated with increased $p16^{Ink4a}$-levels and the induction of cellular senescence. This is accompanied by an accumulation of AGEs and nitrated proteins occurring exclusively in the islet vascular system of normal aged wild-type C57BL/6J mice. Further investigations with isolated islets are necessary, to unravel the mechanism behind these age-related changes.

Declaration of interest

The authors declare that there is no conflict of interest associated with this manuscript.

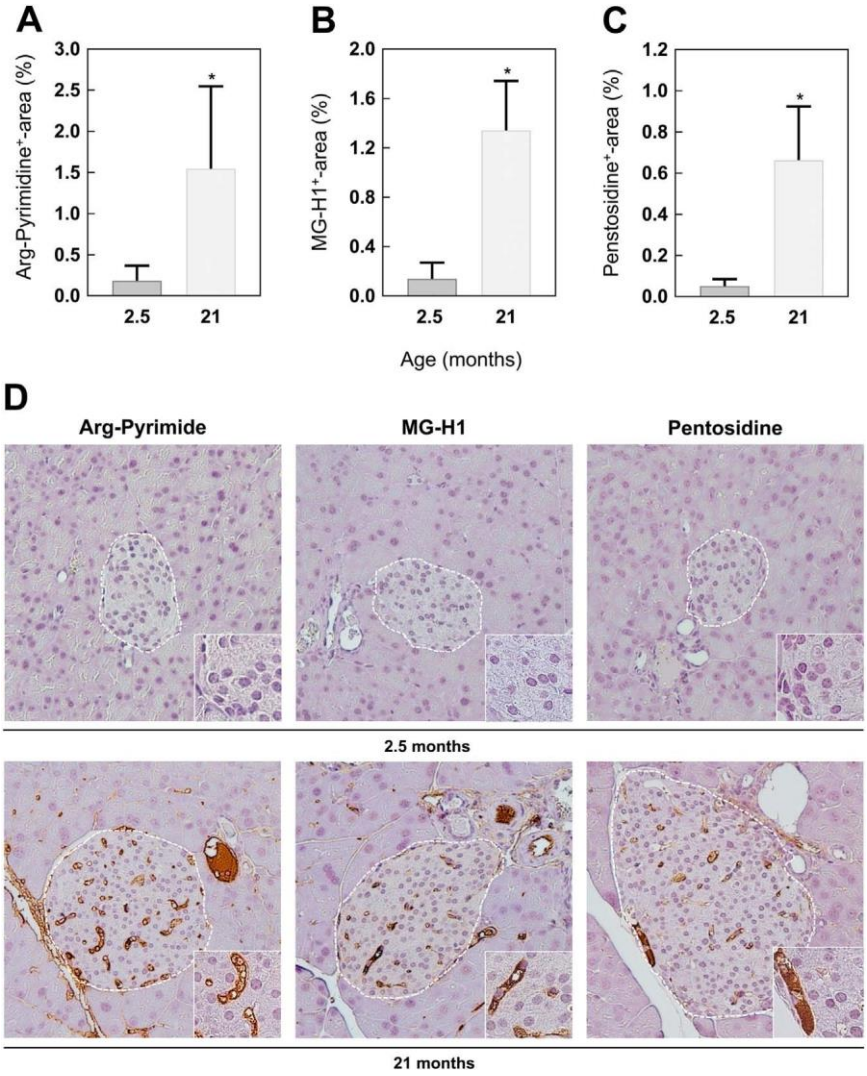

Fig. 3. Formation of AGEs in pancreatic islets of 2.5 and 21 months old C57BL/6 mice. Quantitative analysis of IHC stained **(A)** Arg-Pyrimidine, **(B)** MG-H1 and **(C)** pentosidine. **(D)** Representative images of each staining in a magnification of 20× and 60× (inset). For better visibility, white lines mark the islet area. Data are presented as mean values ± SD. Statistical significance was assessed by student's t-test (unpaired), *p < 0.05.

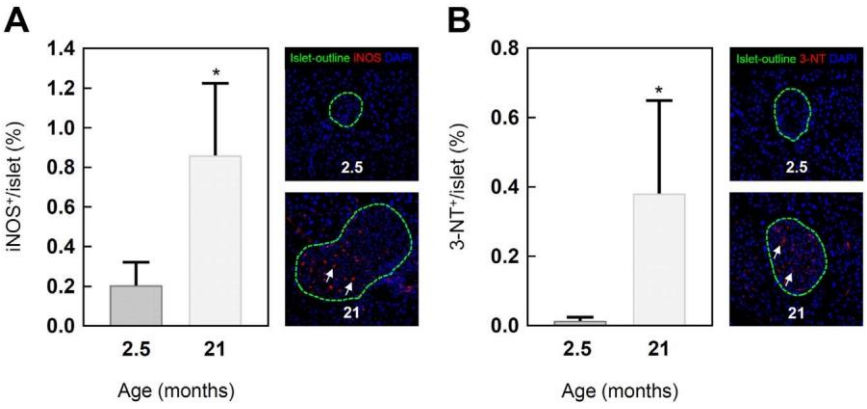

Fig. 4. Induction of nitrosative stress and nitration products in pancreatic islets of 2.5 and 21 months old C57BL/6J mice. Quantitative analysis and representative images of IF labeled **(A)** iNOS and **(B)** 3-NT sections. For better visibility of iNOS and 3NT, the islets are outlined in green (according to insulin staining, not shown). White arrows show iNOS and 3-NT positive stainings. Green: islet-outline, red: iNOS/3-NT, blue: DAPI. Data are presented as mean values ± SD. Statistical significance was assessed by student´s t-test (unpaired), *p < 0.05.

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Publication II

"Endogenous advanced glycation end products in pancreatic islets after short-term carbohydrate intervention in obese, diabetes-prone mice"

Richard Kehm, Jana Rückriemen, Daniela Weber, Stefanie Deubel, Tilman Grune and Annika Höhn

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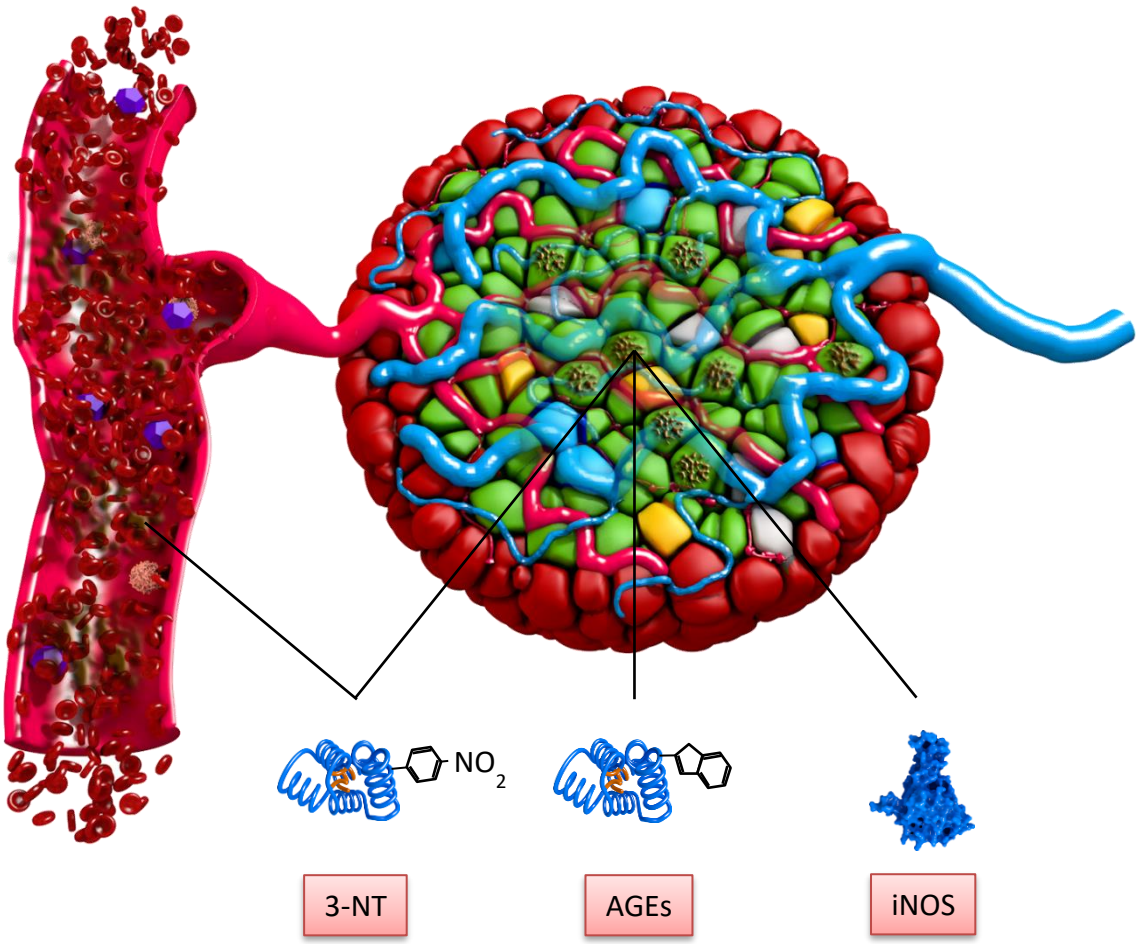
Due to conversion issues between Microsoft Word and PDF-XChange Editor, Publication I and II have been rebuilt.

Graphical abstract (**Publication II**)



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BRIEF COMMUNICATION Open Access

Endogenous advanced glycation end products in pancreatic islets after short-term carbohydrate intervention in obese, diabetes-prone mice

Richard Kehm[1,2], Jana Rückriemen[1], Daniela Weber[1,3], Stefanie Deubel[1], Tilman Grune[1,2,3,4,5] and Annika Höhn [1,2]

Abstract

Diet-induced hyperglycemia is described as one major contributor to the formation of advanced glycation end products (AGEs) under inflammatory conditions, crucial in type 2 diabetes progression. Previous studies have indicated high postprandial plasma AGE-levels in diabetic patients and after long-term carbohydrate feeding in animal models. Pancreatic islets play a key role in glucose metabolism; thus, their susceptibility to glycation reactions due to high amounts of dietary carbohydrates is of special interest. Therefore, diabetes-prone New Zealand Obese (NZO) mice received either a carbohydrate-free, high-fat diet (CFD) for 11 weeks or were additionally fed with a carbohydrate-rich diet (CRD) for 7 days. In the CRD group, hyperglycemia and hyperinsulinemia were induced accompanied by increasing plasma 3-nitrotyrosine (3-NT) levels, higher amounts of 3-NT and inducible nitric oxide synthase (iNOS) within pancreatic islets. Furthermore, N-ε-carboxymethyllysine (CML) was increased in the plasma of CRD-fed NZO mice and substantially higher amounts of arg-pyrimidine, pentosidine and the receptor for advanced glycation end products (RAGE) were observed in pancreatic islets. These findings indicate that a short-term intervention with carbohydrates is sufficient to form endogenous AGEs in plasma and pancreatic islets of NZO mice under hyperglycemic and inflammatory conditions.

Introduction

Obesity and hyperglycemia, induced by nutritional overload and characterized by chronic inflammation, are among the major risk factors for the development of type 2 diabetes. Increasing evidence indicates that the formation of advanced glycation end products (AGEs), initiated and accelerated under hyperglycemic and inflammatory conditions, plays a crucial role in this metabolic axis[1,2]. AGEs and their precursors are compounds formed via nonenzymatic glycoxidation reactions, induced by the nucleophilic addition of free amino groups from proteins, lipids or nucleic acids to carbonyl groups of monosaccharides[1,3]. The so-called Maillard reaction leads to the generation of a reversible Schiff base adduct that spontaneously rearranges to Amadori products or reactive dicarbonyls[4,5]. During the advanced stage of the Maillard reaction, stable modifications such as N-ε-carboxymethyllysine (CML), N-ε-carboxyethyllysine (CEL) and amino acid crosslinking products such as pentosidine are formed[6]. AGEs-formation occurs exogenously during food processing or endogenously due to the presence of high amounts of reducing carbohydrates[7]. Exogenously generated AGEs are ingested with diets of which about 10% are absorbed by the body leading to an increase of circulating AGEs[8–10]. On the other hand, sustained

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elevated glucose levels trigger inflammatory processes, resulting in the endogenous generation of AGEs accelerated by high sugar consumption. High levels of AGEs were shown in plasma after glucose load in diabetic patients or after long-term intake of high carbohydrate diets in rat tissues, such as tail tendon and skin[11,12]. However, the short-term effects of carbohydrates on AGE-formation in pancreatic islets have not been yet investigated. Therefore, we determined the endogenous formation of AGEs in pancreatic tissue as well as plasma of diabetes-prone mice fed a carbohydrate diet for 7 days after carbohydrate restriction.

Materials and methods

Animal procedures and study design

Experiments were performed in male, obese and diabetes-prone New Zealand Obese (NZO) mice (NZO/HIBomDife mice, German Institute of Human Nutrition, Potsdam-Rehbruecke, Germany), housed in groups of four animals under standardized conditions (20 ± 2 °C, 12/12 h light/dark cycle) and had free access to food and water. Seven-week-old animals were randomly assigned into two groups (n= 8 per group). Group one received a carbohydrate-free, high-fat diet (CFD, 32% (wt/wt) protein and 31% (wt/wt) fat, C 105789, Altromin, Lage, Germany) for 11 weeks, the second group was additionally fed with a carbohydrate-rich diet for 7 days (CRD, 20% (wt/wt) protein, 28% (wt/wt) fat and 40% (wt/wt) carbohydrates), containing sucrose and starch, after carbohydrate restriction (for detailed diet compositions, see ref. [13]). At the end of the trial, animals were sacrificed by acute exposure to isoflurane and blood and tissue samples were collected. Mice were kept in agreement with the National Institutes of Health guidelines for the care and use of laboratory animals. All procedures were verified and approved by the ethics committee for animal welfare of the State Office Environment, Health, and Consumer Protection (Germany, Brandenburg, 2347–21–2015). No sample size estimation was carried out.

Body composition, blood glucose, and plasma analysis

Fat and lean mass of NZO mice were determined by nuclear magnetic resonance (NMR, EchoMRI™-100H, EchoMRI LCC, Houston, USA). Blood glucose levels were measured by using a CONTOUR® XT glucometer (Bayer, Leverkusen, Germany) immediately before sacrificing the mice. The concentration of plasma insulin and proinsulin was determined by performing a Mouse Ultrasensitive Insulin and Proinsulin ELISA (ALPCO, Salem, USA) according to the supplier's instructions. Plasma 3-nitrotyrosine (3-NT) levels were determined with an in-house-designed ELISA (for detailed information, see ref. [14]).

Immunohistochemical analysis

Immunohistochemical labeling and quantitative analysis was performed as previously described[15]. A list of used antibodies and applied concentrations are provided as supplementary information (SI Table 1). Imaging and quantification of all stainings were performed blinded; only the study leader had access to the code list.

UPLC-MS/MS measurement

Sample preparation for protein-bound CML and CEL analysis in murine plasma as well as the detection via UPLC-MS/MS was performed as described in the SI. For determination of AGEs in the experimental diets, 25 mg of the diets were used and AGE content was measured following the procedure of CML and CEL plasma analysis.

Statistical analysis

Statistical analysis was performed by using Graph Pad Prism version 7.04 (San Diego, USA). Group variances were similar and appropriate tests were performed to evaluate statistical differences. Shapiro−Wilk normality test was used to assess normal distribution. Accordingly, group comparisons were performed by two-tailed unpaired Mann−Whitney U test. All data are presented as mean values ± SD. Statistically significant differences were considered if $p < 0.05$.

Table 1 Body composition, plasma parameters, and inflammatory markers

	CFD	CRD
Body composition (NMR)		
Body weight (g)	79.93 ± 7.10	81.00 ± 4.99
Fat mass (g)	36.76 ± 3.06	37.19 ± 1.74
Lean mass (g)	34.36 ± 1.07	34.13 ± 0.92
Plasma parameters		
Glucose (mM)	7.58 ± 1.51	17.19 ±3.38*
Insulin (nM)	0.72 ± 0.49	7.61 ± 3.67*
Proinsulin (nM)	0.07 ± 0.03	0.27 ± 0.09*
3-NT (pmol/mg)	3.36 ± 0.46	9.42 ± 3.32*
Islet parameters		
3-NT (%-area/ins$^+$-area)	0.90 ± 0.59	2.80 ± 1.62*
iNOS (%-area/ins$^+$-area)	0.54 ± 0.17	2.11 ± 0.99*

NZO mice received a CFD for 11 weeks and were fed subsequently with a CRD for 7 days (NMR n= 4, proinsulin n= 6, other experiments n= 8). All results are presented as mean ± SD and statistically significant differences were considered if *p< 0.05. CFD carbohydrate-free, high-fat diet, CRD carbohydrate-rich diet, iNOS inducible nitric oxide synthase, NMR nuclear magnetic resonance, 3-NT 3-nitrotyrosin

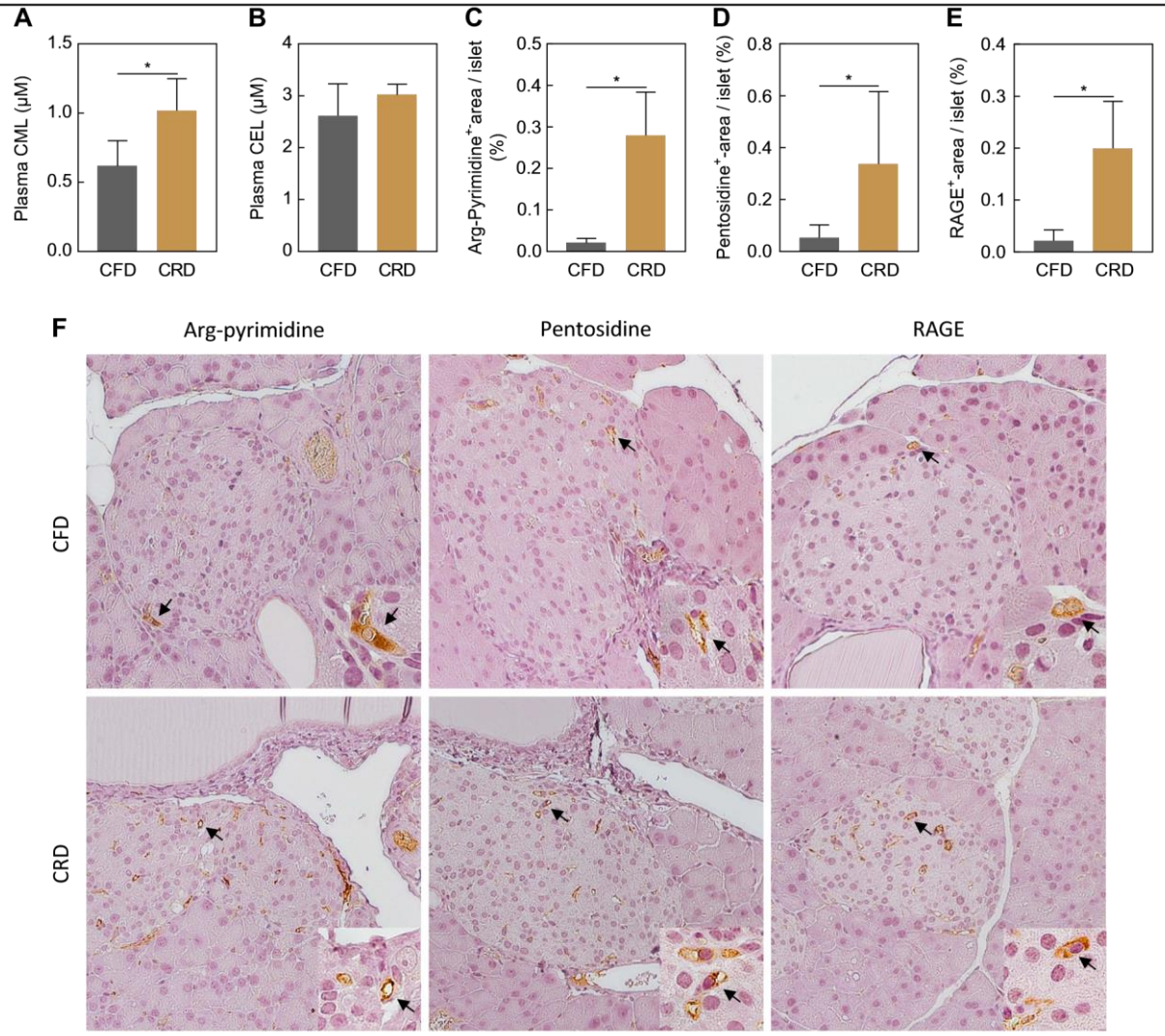

Fig. 1 Short-term intervention with carbohydrates (7 days) induces the formation of AGEs in plasma and pancreatic islets of NZO mice. Plasma levels of CML (**a**) and CEL (**b**) in CFD- or CRD-fed animals measured by UPLC-MS/MS ($n = 6$). Quantitative analyses and representative images of immunohistochemical stained arg-pyrimidine ($n = 8$) (**c**, **f**), pentosidine ($n = 8$) (**d**, **f**) and RAGE ($n = 8$) (**e**, **f**). Images are represented in a magnification of 20x or 63x (inserts). White lines mark the islet area and black arrows indicate the positive-stained (brown) area. All results are presented as mean ± SD and statistical significance was assessed by Mann−Whitney *U* test (unpaired), $*p < 0.05$. AGE advanced glycation end product, CML *N*-ε-carboxymethyllysine, CEL *N*-ε-carboxyethyllysine, CFD carbohydrate-free, high-fat diet, CRD carbohydrate-rich diet, RAGE receptor for advanced glycation end products

Results

Feeding a CFD for 11 weeks induced body weight gain and obesity in both experimental groups (Table 1—Body composition). Subsequent carbohydrate-challenge for 7 days increased blood glucose, insulin and proinsulin levels, indicating conditions of hyperglycemia and hyperinsulinemia. Moreover, plasma levels of 3-NT were almost threefold higher in the CRD group compared to the CFD group (Table 1—Plasma parameters). Quantitative analyses of immunofluorescent stainings revealed an increase of 3-NT and the inducible nitric oxide synthase (iNOS) by threefold and twofold, respectively, within the insulin-positive area of pancreatic islets in CRD-fed NZO mice (Table 1—Islet parameters, images not shown). Plasma analysis of protein-bound CML and CEL showed that CRD-challenged animals had 40% higher levels of plasma CML compared to the CFD group. In contrast, CEL plasma levels were unchanged but more than twofold higher in both groups compared to CML levels (Fig. 1a, b). Determining the AGE content of the experimental diets revealed higher CML-amounts in the CFD (CML: CFD 12.8 ± 1.9 mg/kg, CRD 6.2 ± 1.4 mg/kg), whereas the amount of CEL was higher in the CRD (CEL: CFD 28.4 ± 1.4 mg/kg, CRD 79.4 ± 1.2 mg/kg). As illustrated in

immunohistochemical stainings revealed an eightfold increase of arg-pyrimidine in murine islets of CRD-fed NZO mice compared to the CFD group (Fig. 1c, f) accompanied by 5.2-fold higher amounts of the crosslinker-AGE pentosidine (Fig. 1d, f). Furthermore, the positive-stained islet area of the receptor for advanced glycation end products (RAGE) was ninefold higher in the NZOs challenged with carbohydrates (Fig. 1e, f).

Discussion

In the present study, we demonstrated that 7-day intake of a CRD, containing sucrose and starch, after preceding carbohydrate restriction is sufficient to increase the levels of protein-bound plasma CML as well as the amount of arg-pyrimidine, pentosidine and RAGE in pancreatic islets of hyperglycemic NZO mice. This reflects the high impact of short-term dietary sugars on AGE-formation in pancreatic islets.

It has been described that hyperglycemia in response to excess nutrients causes conditions of inflammation by the induction of multiple pathways linked to the formation of AGEs[3,5,7]. In accordance with this, we observed that NZO mice on a CRD develop hyperglycemia and hyperinsulinemia creating a proinflammatory environment, shown by an increase in plasma 3-NT levels and the amount of 3-NT and iNOS within the pancreatic islets. In addition, plasma levels of protein-bound CML, the reaction product of glyoxal and lysine, were increased after short-term feeding with carbohydrates. The lower amounts of CML in the CRD compared to the CFD indicate that higher plasma CML levels do not originate from the diet directly but were generated endogenously. In contrast, CEL-content of the CRD was higher but no differences were found in CEL plasma levels, indicating that neither carbohydrates nor dietary-derived AGEs had an influence on plasma CEL levels. In line with this, Schindhelm et al. found rising free plasma CML levels and unchanged CEL levels in type 2 diabetic women after a carbohydrate-rich meal[16]. By investigating the pancreatic islets of NZO mice an increase in methylglyoxal-derived arg-pyrimidine was observed after challenging with carbohydrates. Besides single-amino acid modifications, irreversible cross-linking of proteins represents another type of AGE-formation that is assumed to occur in at least weeks to months[17]. In contrast, we demonstrated that feeding a CRD for 7 days is sufficient to form cross-linked pentosidine, the product of the reaction between a pentose sugar with arginine and lysine residues in pancreatic islets of NZO mice. Furthermore, higher levels of AGEs and their precursors induce the expression of RAGE[1,18]. This is in accordance with our findings demonstrating that RAGE was increased within the pancreatic islets. The AGE-RAGE interaction initiates a signaling cascade leading to the activation of NFκB and NADPH-oxidase shown to trigger the generation of reactive oxygen species and oxidative damage[7,17]. Our study shows that although the measurement of plasma AGEs only reveals minor changes between CRD and CFD, murine pancreatic islets appear to be susceptible to glycation reactions. Increased glycation of pancreatic islets might promote the AGERAGE system and thereby lead to a vicious cycle of hyperglycemic damage. In conclusion, short-term intervention with a CRD for 7 days led to hyperglycemic and inflammatory conditions in obese NZO mice associated with the endogenous formation of AGEs in plasma and pancreatic islets.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary information

SI Table: Antibodies

Antibody	Product #	Company	Dilution
Primary			
Rabbit polyclonal anti-nitrotyrosine	06-284	Merck Millipore	0.5 µg/ml
Mouse monoclonal anti-3-nitrotyrosine [7A12AF6]	ab110282	abcam	1:200
Rabbit monoclonal iNOS (D6B6S)	13120S	Cell Signaling	1:200
Rabbit monoclonal anti-Insulin [EPR17359]	ab181547	abcam	1:10.000
Mouse monoclonal insulin (L6B10)	8138S	Cell Signaling	1:200
Mouse monoclonal methylglyoxal-AGE (Arg-Pyrimidine)	AGE06B	BioLogo	1:1000
Mouse monoclonal pentosidine	PEN012	BioLogo	1:800
Mouse monoclonal RAGE	sc-365154	Santa Cruz	1:100
Secondary			
Goat anti-Rabbit IgG (H+L) Alexa Fluor 488	A-11008	Invitrogen	1:200
Goat anti-Mouse IgG (H+L) Alexa Fluor 633	A-21052	Invitrogen	1:200

UPLC-MS/MS measurements

Sample preparation for CML and CEL analysis in plasma

Plasma protein-bound N-ε-carboxymethyllysine (CML) and N-ε-carboxyethyllysine (CEL) were analyzed by using UPLC MS/MS. 50 µl plasma were mixed with 100 µl of ultrapure water. Reduction of early glycation products such as fructoselysine was performed to avoid neo-formation of CML during heating in hydrochloric acid. Therefore, 250 µl sodium borate buffer (0.4 N, pH 10.2) and 250 µl sodium borohydride (1 M in 0.1 M NaOH) was added followed by incubation for 2h at room temperature. For protein precipitation, 1 ml of trichloroacetic acid (20%, w/v) was added followed by centrifugation (4 °C, 10000 rpm, 10 min). The supernatant was discarded, the precipitate was washed with trichloroacetic acid (5%, w/v) and the centrifugation step was repeated. After removal of the supernatant, 10 µl of internal standard (containing 20 µM $^{2}H_{4}$-CML and 20 µM $^{2}H_{4}$-CEL dissolved in water) and 1 ml of 6 M hydrochloric acid were added to the protein pellet and the sample was incubated at 110°C for 23 h. The hydrolyzed samples were evaporated to

dryness with a vacuum concentrator (SpeedVac, Thermo Fisher Scientific, Schwerte, Germany). The residue was dissolved in 100 µl eluent B (10 mM ammonium formiate) and after centrifugation (4°C, 10000 rpm, 10 min) an aliquot of 90 µl was subjected to UPLC-MS/MS analysis. For a description of the UPLC MS/MS detection, see ESM methods.

Ultra-High Performance Liquid Chromatography with MS/MS detection

UPLC analysis was performed with an Acquity Ultra Performance LC system coupled to a Waters Quattro Premier XE mass spectrometer (both Waters Corporation, Milford, USA). For chromatographic separation, an Intrada Amino Acid column at a column temperature of 40 °C was used. Solvent A was of 0.3% formic acid in ACN and solvent B was 10 mM ammonium formiate. The solvents were pumped at a flow rate of 0.6 ml/min in gradient mode (0 min, 0% B; 2 min, 0% B; 6.5 min, 40% B; 8 min, 90% B; 10 min, 100% B; 12 min, 100% B; 12.01 min, 0% B; 15 min, 0% B). The injection volume was 10 µl. Data were acquired and evaluated with the MassLynx Software (Waters, version 4.1). The ESI source was operated in positive mode and nitrogen was utilized as the nebulizing gas with a gas flow of 650 l/h and gas temperature of 350 °C. The capillary voltage was set to 2.5 kV, the source temperature was 150 °C and the cone voltage was 24 V. Analytes were measured in MRM mode with the following transitions and optimized collision energies (CE). Transitions used for quantification are labelled with q and transitions used for the confirmation of the presence of the analyte are labelled with Q. CML: 204.9→84.2 (q,CE: 18 V), 204.9→130.2 (Q,CE: 12 V); $^{2}H_{4}$-CML: 209.2→88.1 (q, CE: 20 V), 209.2→134.1 (Q, CE: 12 V); CEL: 219.1→84.1 (q,CE: 18 V), 219.1→130.1 (Q,CE: 12 V); $^{2}H_{4}$-CEL: 223.2→88.1 (q, CE: 20 V), 223.2→134.1 (Q, CE: 12 V). For quantification, external calibration with standard solutions in water containing CML, CEL and $^{2}H_{4}$-CML, $^{2}H_{4}$-CEL as internal standards was used. Calibration curves for CML and CEL, obtained by linear regression of a plot of the analyte/internal standard peak area ratio versus analyte concentration, were used to calculate concentrations in plasma samples. All plasma samples were analysed in duplicate.

Manuscript

"Maintenance of pancreatic beta-cells in middle-aged, diabetes-prone mice under glucolipotoxic stress is mediated by redox balance and cell cycle progression"

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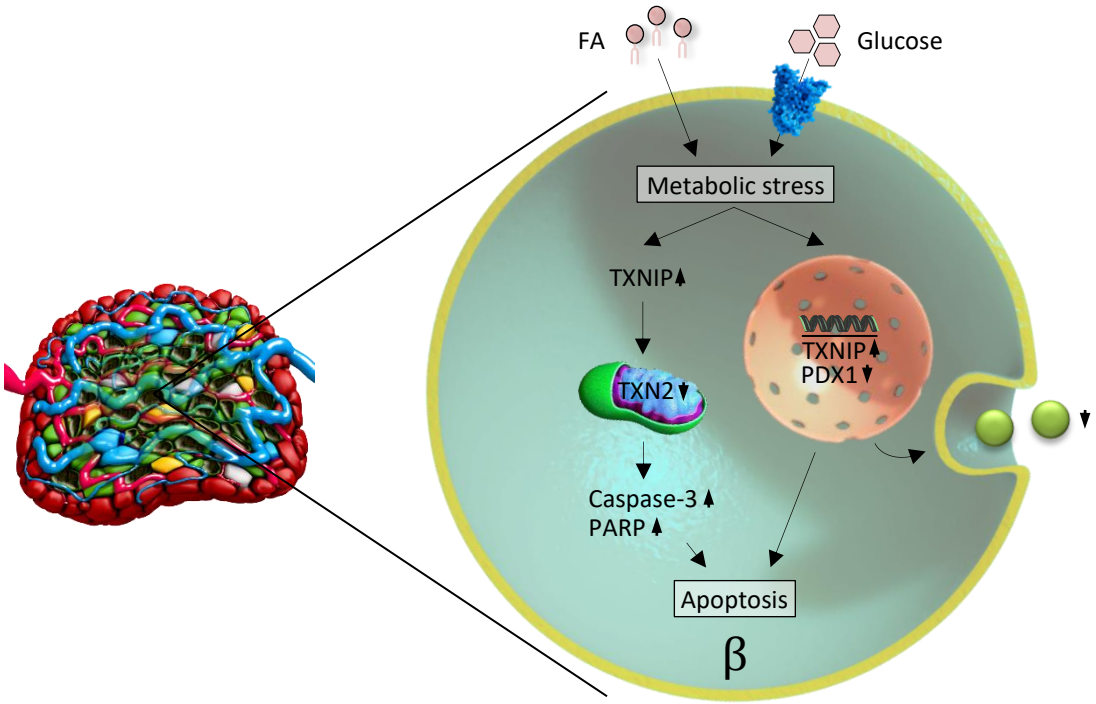
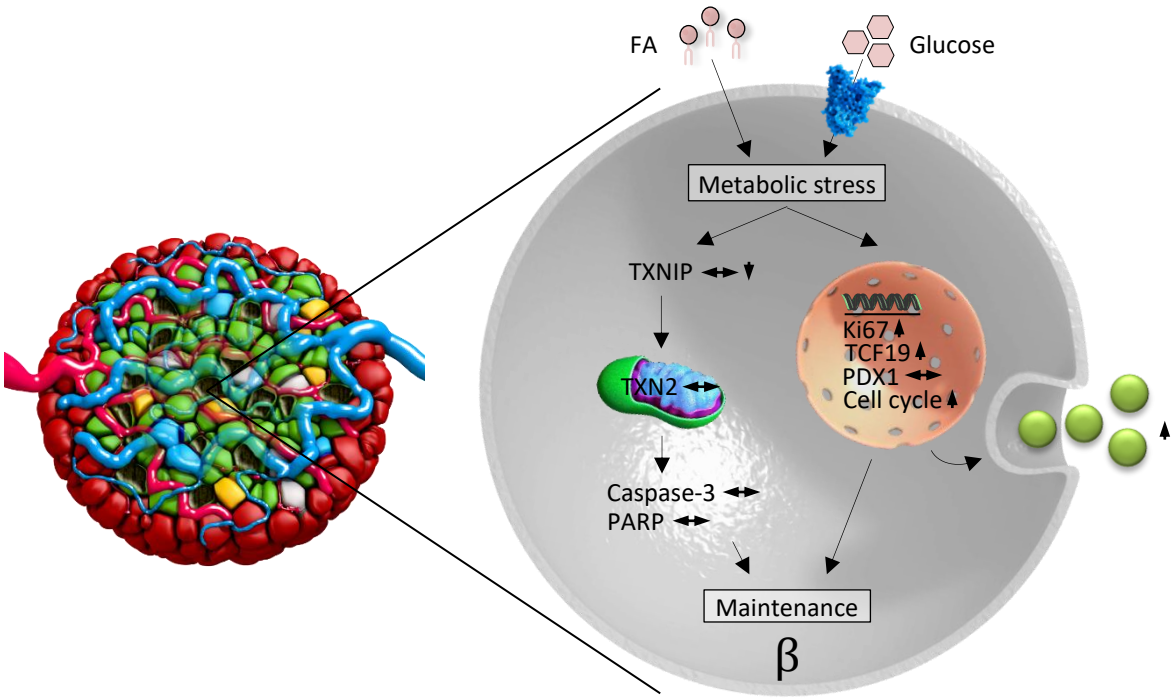
Prepared for submission

Graphical abstract (**Manuscript**)

Young NZO mice



Middle-aged NZO mice



Maintenance of pancreatic beta-cells in middle-aged, diabetes-prone mice under glucolipotoxic stress is mediated by redox balance and cell cycle progression

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Keywords

Aging, Hyperglycemia, New Zealand Obese mice, Pancreatic islets, Thioredoxin-interacting protein

Abbreviations

ASK1, Apoptosis signal-regulating kinase 1; ChREBP, Carbohydrate-responsive element-binding protein; ChoRE, Carbohydrate-response element; CFD, Carbohydrate-free, high-fat diet; CRD, Carbohydrate-rich diet; GAPDH, Glycerinaldehyde-3-phosphate dehydrogenase; GO, Gene ontology; GSIS, Glucose-stimulated insulin secretion; HBSS, Hank´s buffered salt solution; HRPT, Hypoxanthine guanine phosphoribosyl transferase; IPA, Ingenuity pathway analysis; MLX, Max-like factor 1; MLXIPL, MLX interacting protein-like; NZO, New Zealand Obese; oGTT, Oral glucose tolerance test; PARP, Poly(ADP-ribose)polymerase; PCNA, Proliferating cell nuclear antigen; PDX1, Pancreatic and duodenal homeobox 1; RPL13a, Ribosomal protein L13a; TCF19, Transcription factor 19; TXN1, Thioredoxin 1; TXN2, Thioredoxin 2; TXNIP; Thioredoxin-interacting protein

Abstract

Excess intake of high caloric diets associated with persistent elevated levels of circulating glucose and fatty acids are known to initiate a cascade of obesity, insulin resistance and hyperglycemia-induced glucolipotoxicity, finally leading to functional impairment and apoptosis of pancreatic beta-cells. However, the impact of aging on beta-cell functionality and integrity under prolonged glucolipotoxic (metabolic stress) conditions remains widely unknown. To investigate this, diabetes-prone New Zealand Obese (NZO) mice were kept on a carbohydrate-free, high-fat diet (CFD) for 11 (young) or 32 (middle-aged) weeks with subsequent carbohydrate-rich diet (CRD) for 21 days. Young NZO mice developed early and massive hyperglycemia accompanied by a depletion of insulin stores, abnormal insulin release and beta-cell death in response to the carbohydrate challenge. This was associated with an increased expression of thioredoxin-interacting protein (TXNIP), initiating an apoptotic signaling cascade via caspase-3. Interestingly, CRD intervention had a less harmful effect on middle-aged NZO mice. These animals exhibited moderate hyperglycemia and regular beta-cell function, presumably by sustained redox balance as well as proliferative potential, leading to beta-cell maintenance. These findings indicate that higher age had no adverse effects on beta-cells of metabolic-stressed NZO mice, even more it seems that maturation processes are promoted and defense mechanisms are maintained.

Introduction

Pancreatic islets, especially their insulin-releasing beta-cells are crucial for the regulation of glucose homeostasis in response to nutrient supply. Thus, an impairment of beta-cell functionality has harmful effects on the entire body metabolism[1,2]. In this context, chronic intake of high caloric diets, composed of carbohydrates and fats, initiates a cascade of obesity, systemic inflammation and peripheral insulin resistance followed by hyperglycemia and metabolic stress. Elevated circulating glucose and fatty acids have been linked to a gradual decline in the proliferative and secretory potential of beta-cells and promote apoptotic cell death[3-6]. Besides dietary factors, aging and related alterations have been shown to affect beta-cell characteristics. Recently, we demonstrated that pancreatic islets of normoglycemic wild-type mice lose their proliferative and regenerative potential starting at the age of 10 months associated with the induction of cellular senescence[7]. Furthermore, different well-known hallmarks of aging, such as mitochondrial dysfunction,

elevated oxidative stress and inflammation, all linked to a dysregulated redox balance, affect beta-cells by impairing secretory function. In combination, this leads to a decline of insulin action in peripheral tissues and beta-cell dysfunction[8-10]. As it was demonstrated in a study by Fontes et al. comparing 2 and 6 months old Wistar rats, an age-related functional impairment might be reinforced by metabolic stress factors, such as glucolipotoxicity[11]. To date, several investigations demonstrate that the thioredoxin-interacting protein (TXNIP) serves as a critical mediator of glucotoxicity-induced beta cell failure and death via different mechanisms. TXNIP inhibits the thioredoxin-pathway by binding to the reduced form of Thioredoxin 1 (TXN1), downregulates essential transcription factors responsible for insulin synthesis and triggers the mitochondrial death pathway by binding to mitochondrial Thioredoxin 2 (TXN2) and caspase-3 activation[12-16]. Thus, TXNIP regulates beta-cell redox homeostasis by inhibiting their anti-oxidative potential, leading to higher susceptibility towards oxidative damage and apoptosis[13,17]. It has been shown that Txnip expression is mediated by the carbohydrate-responsive element-binding protein (ChREBP). Due to glucose stimuli, ChREBP is dephosphorylated and translocated from the cytosol into the nucleus where it binds to the carbohydrate-response element (ChoRE)-region, inducing the transcription of target genes, such as Txnip[18-21].

Previous reports demonstrate that New Zealand Obese (NZO) mice, a polygenic model for type-2-diabetes and obesity, develop glucolipotoxicity-induced beta cell death under a specific dietary regimen composed of a carbohydrate-rich diet (CRD) subsequent to a carbohydrate-free, high-fat diet (CFD)[22-26]. In this study, by extending the period of carbohydrate restriction, middle-aged NZO mice (hereinafter indicated to as aged mice) were used to investigate the effects of aging on beta-cell functionality and damage under glucolipotoxic conditions. Interestingly, our study provide data demonstrating that higher age has no adverse effects on metabolically stressed beta-cells, but rather lead to a preservation of their functional, proliferative and redox balance.

Results

Different effects of the carbohydrate challenge on glucose homeostasis in young and aged NZO mice.

Young and aged diabetes-prone NZO mice were kept on a specific dietary regimen composed of initial carbohydrate restriction with a subsequent carbohydrate intervention (Fig. 1A). Even before the diet switch, young and aged NZO mice exhibited massive body weight gain that was reinforced by prolonged CFD-feeding in aged animals (Fig. 1B). In response to the CRD challenge, circulating glucose concentrations of both young and aged NZOs increased continuously. However, higher blood glucose levels were found in young mice after 2 and 21 days of the CRD intervention compared to aged animals (Fig. 1C). After oral glucose administration, blood glucose curves indicate impaired glucose tolerance in all groups. Calculation of the area under the curve (AUC) revealed impaired glucose tolerance in young CRD-fed mice compared to the untreated animals. In addition, glucose intolerance was more pronounced in the aged NZOs compared to their young counterparts, even without the carbohydrate challenge, whereas the CRD for 14 days did not induce changes in glucose tolerance in mice at advanced age (Fig. 1D). After the 2-day CRD intervention elevated plasma insulin levels were observed in young mice, returning to the initial levels after 21 days. In contrast, plasma levels of insulin in aged NZO mice increased continuously during CRD feeding and were higher after 21 days compared to the young animals (Fig. 1E). Determination of total pancreatic insulin revealed lower insulin content in young NZOs in response to the diet change after 21 days. In contrast, aged CRD-fed animals exhibited unchanged values and had a 3-fold higher pancreatic insulin content in comparison to their young counterparts (Fig. 1F). Total values of energy expenditure normalized to body weight were comparable between young and aged NZOs at the end of the CRD intervention (Fig. 1G).

To evaluate beta-cell functionality *in vitro*, a glucose-stimulated insulin secretion (GSIS) was performed by perifusion experiments in freshly isolated islets before and 2-days after CRD challenging. Insulin secretion was similar in islets of young and aged CFD-fed mice. Two days after the diet change, islets of young mice reflected elevated first and second insulin release after administration to high glucose levels and were unable to adjust to low glucose conditions. In comparison, islets of aged animals after CRD feeding exhibited only tendentially increased insulin secretion under high glucose conditions and respond with

gradual decreasing release of insulin when kept on low glucose. However, no difference was found comparing islet insulin secretion of young and aged NZO mice after the CRD intervention (Fig. 1H).

Beta-cell maintenance in aged NZO mice in response to carbohydrate intervention.

To assess islet morphology in young and aged NZO mice, a multi-color staining of insulin, glucagon, somatostatin and DAPI was performed. In young NZO mice, the insulin^{+}-area was smaller after the CRD intervention compared to their aged counterparts (Fig. 2A). This was confirmed by morphometric analysis, demonstrating reduced insulin^{+}-area (mirrors beta-cell area) and number of nuclei per islet in young mice after 21 days of the CRD intervention in comparison with young CFD-fed animals. Comparing young and aged NZOs, total islet-area (ghrelin- and pancreatic polypeptide-area was not considered) and insulin^{+}-area were larger in the aged animals after challenging with a CRD (Fig. 2C-E). By using TUNEL assay technique - apoptotic cells were detected and counted automatically with an in-house programmed macro - a marked increase in the percentage of TUNEL^{+}-cells was observed in young NZO mice due to a 21-day CRD feeding but not in aged mice. Moreover, a 2-fold higher amount of TUNEL^{+}-nuclei was found when young and aged NZOs were compared 21 days after the diet switch (Fig. 2B and F).

Differences in transcriptomic profile and differentiation between young and aged NZO mice after carbohydrate intervention.

To unravel the molecular mechanisms leading to the differences in carbohydrate response in young and aged NZO mice, a microarray-XS-based transcriptomic analysis of isolated pancreatic islets before and after 2 days of the diet change was performed. As shown in figure 3A, 1.461 transcripts in young mice and 1.440 transcripts in aged animals were expressed differentially after CRD feeding. Among these, a total number of 519 transcripts were regulated equally in young and aged NZO islets. To identify in which biological processes the differentially expressed transcripts are involved, Gene Ontology (GO) analysis was performed. This analysis showed enrichments of pathways related to proliferation and cell cycle progression, especially in islets of aged NZO mice in response to the CRD intervention (Fig. 3B). By using QIAGEN ingenuity pathway analysis (IPA), ranking of identified transcripts was conducted, indicating differences in transcripts related to proliferation in young and aged NZO mice after the carbohydrate challenge. Antigen Ki67

(MKi67) was among the top 5 upregulated molecules in islets of both, young and aged NZO mice after CRD feeding. Furthermore, an upregulation of proliferating cell nuclear antigen (PCNA) was found exclusively in aged CRD-fed animals. Although transcription factor 19 (TCF19) was upregulated in both young and aged NZO mice after CRD intervention, direct comparison revealed higher expression levels in aged mice (Fig. 4A, IPA ranking not shown).

To confirm the data obtained from IPA, RT-PCR analysis was performed in isolated islets. *Mki67* mRNA expression was higher after CRD feeding in young and aged mice compared to their CFD-fed groups. Moreover, islets of aged NZOs on a CRD had a 2-fold higher *Mki67* expression in comparison to their young counterparts. Determining the mRNA levels of pancreatic and duodenal homeobox 1 (PDX1) revealed no differences between the groups, similar to microarray data. *Pcna* expression was 5-6 folds higher in both age groups after the CRD challenge in comparison to their CFD-fed groups. Furthermore, *Tcf19* mRNA levels were increased exclusively in islets obtained from aged NZO mice 2 days after the CRD intervention (Fig. 4B).

As shown in Figure 4C and E, Ki67 staining was used to determine the percentage of proliferating cells in pancreatic tissue sections. Consistent with microarray and RT-PCR data, the amount of $Ki67^{+}$-cells increased markedly in response to CRD feeding in both young and aged NZO mice. However, no difference was found between these groups. This was followed by a simultaneous decrease in the number of proliferating cells after 21 days of CRD challenging in young and aged animals. Immunofluorescent nuclear labeling of PDX1, co-stained with insulin to highlight the beta-cell area, revealed a decrease by 20% of $PDX1^{+}$-nuclei in young NZO mice after a 21-day CRD intervention, whereas PDX1-levels in aged animals were unchanged. Thus, $PDX1^{+}$-nuclei were more abundant in aged NZOs when compared with their young opponents at this time point (Fig. 4D, F).

Beta-cell loss in young NZO mice is mediated by increasing TXNIP expression.

Besides islet proliferation, IPA identified differences in transcripts related to the TXN pathway. *Txnip* and *Txn1* expression were exclusively upregulated in islets of young NZO mice after CRD intervention. Furthermore, comparison of young and aged NZOs after 2-days of CRD feeding showed markedly lower expression levels of *Txnip* in aged animals. In contrast, *Txn2* levels were comparable in all groups (Fig. 5A). Again, RT-PCR analysis of islets was used to validate microarray data. As displayed in Figure 5B, *Txnip* mRNA levels were

increased in young and aged NZO mice in response to the CRD after 2 days. The comparison of these two groups revealed that mRNA expression of *Txnip* was lower in aged mice, confirming microarray data. Furthermore, *Txn1* mRNA levels were increased in both, islets of young and aged NZOs after CRD feeding. However, no difference was observed between CRD-fed animals. Young NZO mice on a CRD showed lower expression of *Txn2* compared to the untreated ones, whereas in aged animals no difference was observed (Fig. 5B). The impact of carbohydrates on TXNIP protein levels was assessed via immunoblotting of islet lysates. Confirming array and RT-PCR data, TXNIP protein expression increased in young NZO mice after a 2-day CRD intervention and returned to the initial level after 21 days. Additionally, protein levels of TXNIP were lower in aged NZO mice compared to young animals after 2 and 21 days of CRD feeding, respectively (Fig. 5C).

To investigate further downstream mechanisms of TXNIP, caspase-3 immunostaining and western blot analysis of poly(ADP-ribose)-polymerase (PARP) were performed. As shown in figure 5D and E, caspase-3^{+}-nuclei were more abundant in young CRD-challenged NZO mice after 21 days that was also observed when compared to their aged counterparts. Consistently, PARP protein levels were elevated exclusively in young mice at the end of the CRD intervention (Fig. 5F).

Induction of TXNIP expression is not mediated by ChREBP in NZO mice.

To understand the upregulation of TXNIP expression, the upstream mechanism via ChREBP was investigated. Gene expression of Max-like factor 1 interacting protein-like (MLXIPL) and its complex partner Max-like factor 1 (MLX) as well as ChREBP protein levels were determined in islets of young and aged NZO mice before and after 2 days of the CRD intervention. RT-PCR analysis of *Mlxipl* revealed lower mRNA levels in islets of young and aged NZO mice after the CRD intervention without a difference between CRD-fed groups. In contrast, gene expression of *Mlx* doubled in young and aged animals in response to CRD feeding. Again, no difference was found between young and aged NZOs (Fig. 6A). Immunoblotting of ChREBP revealed lower protein levels in young NZO mice after 21 days of the CRD intervention, whereas the levels of aged animals were unchanged. Moreover, higher ChREBP protein concentrations were found in aged NZOs compared to their young counterparts at the end of the trial (Fig. 6B). In contrast, morphometric analysis of ChREBP^{+}-cells after immunostaining showed a threefold increase in ChREBP^{+}-cells, when young NZO

mice before and after 21 days of the CRD challenge were compared that was not observed in aged animals. Comparing young and aged CRD-fed NZO mice a lower percentage of ChREBP^{+}-stained nuclei was found in aged ones (Fig. 6C, D).

Discussion

In the present study, we investigated the impact of aging on beta-cell functionality and integrity under diet-induced glucolipotoxic (metabolic) stress conditions in diabetes-prone NZO mice. Young NZO mice exhibited fast and massive hyperglycemia followed by beta-cell dysfunction and apoptosis in response to the carbohydrate challenge. This was associated with an increased expression of the scaffold protein TXNIP and activation of related downstream pathways, initiating capase-3-induced apoptosis. Interestingly, middle-aged mice (indicated as aged mice) develop only moderate hyperglycemia accompanied by high insulin levels after carbohydrate exposure presumably mediated by maintenance of proliferation and redox potential, preserving functional beta-cells.

It has been shown in previous investigations that NZO mice develop obesity and insulin resistance when kept on a CFD, followed by depletion of insulin stores and a loss of pancreatic beta-cells in response to a CRD intervention[22,23,26,27]. In accordance with this, we observed comparable results in our young NZO cohort. Prolonged CFD feeding lead to increasing body weight and impaired glucose tolerance in aged mice. Interestingly, CRD-fed animals were protected against pronounced hyperglycemia by persistently high insulin levels. Perifusion experiments indicated that islets of aged animals were able to adjust to changing glucose levels, whereas young islets lose their adaptability and exhibit abnormal insulin secretion. This indicates dysfunction of beta-cells in response to metabolic stress followed by an induction of apoptosis in young NZO mice. This was confirmed by a visible loss of beta-cell- and islet-area as already described in previous studies[22,23,26,27]. In contrast, aged animals seem to maintain their beta-cells and showed no evidence of beta-cell apoptosis.

Data obtained from GO term enrichment analysis and mRNA profiling indicate that islets of aged mice have a higher proliferative potential and maintain cell cycle progression under metabolic stress conditions. Interestingly, this outcome is comparable with findings in islets of obese, diabetes-resistant B6-*ob/ob* mice, demonstrating a compensation of glucolipotoxicity by induction of proliferation, resulting in increased beta-cell mass[23,26]. In

252 contrast, cell cycle in young NZO islets seems to be negatively regulated, even though islets
253 of CRD-fed young as well as aged mice start to proliferate in order to compensate the
254 elevated carbohydrate supply. Furthermore, beta-cells of aged animals showed stable
255 expression of PDX1 transcription factor, shown to be crucial for beta-cell differentiation,
256 replication and insulin synthesis[28-30]. Consistent with a previous study, PDX1 was decreased
257 in young NZOs paralleled by reduced insulin levels, confirming the essential role of this
258 transcription factor in the regulation of insulin gene expression[22]. TCF19, another
259 transcription factor that has been shown to induce growth of beta-cell mass and survival in
260 INS-1 cells, was exclusively upregulated in aged CRD-fed mice, suggesting a potential role
261 in beta-cell maintenance of CRD-fed aged mice[31]. As mentioned above, we observed an
262 increase in Ki67 proliferation factor in young animals that is in contrast with data from Kluth
263 et al. demonstrating unchanged beta-cell proliferative capacity[23,26]. This discrepancy might
264 be explained by higher levels of apoptotic cells in our study compared to their recent
265 report[26]. Furthermore, we found that beta-cell apoptosis occurred after 21 days of CRD
266 feeding, whereas proliferation started already after 2 days. Thus, it might be presumed that
267 increased proliferation observed in our young NZO model has been covered by parallel
268 induction of massive apoptosis. Taken together, these data suggest that sustained
269 replicative and proliferative capacity as well as lower levels of apoptosis, found in aged
270 CRD-fed mice, might be responsible for the maintenance of functional beta cells.
271 In addition, our data suggest that the pro-oxidative and pro-apoptotic protein TXNIP is
272 of high relevance for the decline of functional beta-cells in young NZO mice. In previous
273 years, TXNIP has been identified as a major player in hyperglycemia-induced beta-cell
274 apoptosis[12-14,16]. In a microarray analysis of human islets by Shalev et al. in 2002, TXNIP was
275 discovered as the highest glucose-induced gene in diabetic patients[32]. In line with this, *Txnip*
276 was among the top 3 upregulated transcripts in our IPA of young NZO islets in response to
277 the carbohydrate challenge. This was confirmed by a marked increase in *Txnip* mRNA and
278 TXNIP protein expression. Furthermore, our data demonstrate consistently, that pancreatic
279 islets of young mice express higher amounts of TXNIP after CRD feeding compared to their
280 aged counterparts (although aged NZO islets showed increased mRNA levels after 2 days).
281 Importantly, despite moderate hyperglycemia observed in aged animals, TXNIP expression
282 was decreased at the end of the study. TXNIP mediates its effects by several downstream
283 mechanisms dependent on its localization[33]. Cytosolic TXNIP inhibits the TXN pathway by

binding to cysteine-residues of the reduced form of TXN1[34]. Thus, TXNIP regulates the cellular redox state by decreasing the anti-oxidative capacity of the TXN system, initiating beta-cell inflammation and apoptosis[34-36]. Here, we found elevated TXN1 gene expression in both, islets of young and aged NZOs that might be explained by a compensatory mechanism to maintain the anti-oxidative defense of the TXN pathway. In contrast, previous studies demonstrate that *Txnip* upregulation induces a decrease in *Txn1* expression and activity[36]. Thus, we cannot assume that beta-cell apoptosis in young mice was mediated by TXNIP-induced inhibition of TXN1. Besides this, TXNIP has been shown to interfere with TXN2 after shuttling into the mitochondria, where it triggers an apoptotic cascade via caspase-3 activation[12,37,38]. Interestingly, only islets of young NZO mice exhibited decreasing *Txn2* mRNA expression in response to the carbohydrate challenge. As previously described, this might lead to increased phosphorylation and activation of apoptosis signal-regulating kinase 1 (ASK1) followed by cytochrome c release and caspase-3 cleavage, finally inducing beta-cell apoptosis[38,39]. Similarly, we observed elevated amounts of caspase-3^{+}-nuclei exclusively within pancreatic islets of young CRD-fed NZO mice, consistent with a previous investigation[22]. This was accompanied by increased PARP protein concentrations in islets of young mice at the end of the CRD intervention. Thus, reduced *Txn2* gene expression with elevated capase-3 and PARP protein levels suggest a TXNIP-related induction of mitochondrial apoptotic signaling cascade in response to metabolic stress that might initiate beta cell destruction in young NZO mice.

It has been demonstrated that ChREBP acts upstream of TXNIP, dependent on glucose supply[40,41]. Due to glucose stimulation, ChREBP forms a heterodimer with MLX, inducing cytosolic-nuclear shuttling[42]. Once translocated, this complex binds to the ChoRE-region of the DNA and activates the transcription of target genes, such as TXNIP[43,44]. In contrast to the general knowledge, our findings indicate reduced ChREBP expression in islets of young and aged NZO mice, in conflict with increasing gene expression of its complexing agent MLX. Although, we detected elevated ChREBP-signals in beta-cells of young mice, located perinuclear, these observations were made after 21 days of the carbohydrate exposure, whereas increasing TXNIP protein content was already found after 2 days of CRD feeding in islets of young NZO mice. Furthermore, cytosolic localization of ChREBP indicates that its translocation into the nucleus has not been occurred. Thus, we found no association

between TXNIP and its glucose-dependent mediator ChREBP in our model, indicating that TXNIP might be activated by other transcription factors in young NZOs.

In conclusion, young NZO mice developed hyperglycemia in response to a carbohydrate challenge accompanied by beta-cell dysfunction and loss, mediated by TXNIP-related beta-cell apoptosis. Although aged NZO mice were not fully protected against hyperglycemia after carbohydrate exposure, islets maintain their proliferative, replicative as well as anti-oxidative potential that ensure beta-cell survival and functionality. However, it has to be considered that aged mice exhibited increasing glucose levels combined with insulin hypersecretion after 21-days of the CRD intervention. Thus, beta-cell dysfunction and death in aged NZOs might occur theoretically after extending the CRD feeding period. Thus, we provide data that aging had no adverse effects on maintenance of pancreatic beta-cells and their redox balance in NZO mice under metabolic stress.

Material and methods

Animal procedures, diets and study design.

Male, diabetes-prone New Zealand Obese (NZO) mice (NZO/HIBomDife mice, German Institute of Human Nutrition, Potsdam-Rehbruecke, Germany), were housed in open cages of 4-5 animals at a controlled environment (20±2°C, 12/12hr light/dark cycle) with *ad libitum* access to diets and water. As shown in Figure 1A, after weaning and feeding a control diet (V1534-300, Ssniff, Soest, Germany), seven-weeks old mice were randomly assigned into young and middle-aged (indicated as aged) groups. Both groups received a carbohydrate-free, high-fat diet (CFD, 20% (wt/wt) protein and 68% (wt/wt) fat, C 1057-89, Altromin, Lage, Germany) for 11 weeks (young) or 32 weeks (aged). Afterwards, subgroups were fed with a carbohydrate-rich diet (CRD, 20% (wt/wt) protein, 28% (wt/wt) fat and 40% (wt/wt) carbohydrates) for a maximum of 21 days (for detailed diet compositions, see reference[25]). After sacrificing by acute isoflurane exposure, blood samples were taken and pancreatic tissue samples were either fixed in 4% paraformaldehyde for 24h with subsequent paraffin embedding or shock-frozen in liquid nitrogen. Furthermore, endocrine islets were isolated by collagenase digestion as described below. Mice were kept in agreement with the NIH guidelines for the care and use of laboratory animals. All experiments were verified and approved by the animal welfare committee of the DIfE and

the ethics committee of the State Office Environment, Health, and Consumer Protection (Germany, Brandenburg, approval number: V3-2347-21-2015).

Assessment of glucose utilization.

Body weight and blood glucose measurements were monitored in a 2-weeks interval and immediately before sacrificing the mice at indicated time points by using a CONTOUR® XT glucometer (Bayer, Leverkusen, Germany). For oral glucose tolerance test (oGTT), blood samples of fasted mice (16h overnight) were taken from the tail vein and blood glucose was measured at 0, 15, 30, 60 and 120min after oral administration with 20% glucose solution (Braun, Melsungen, Germany). Energy expenditure in young and aged animals before and after 21 days of the carbohydrate intervention was determined by using indirect calorimetry (PhenoMaster, TSE Systems, Bad Homburg, Germany). Animals were adapted to respiratory cages for 24h followed by a 72h measurement in a controlled environment at 22°C with free access to food and water. Mean values over 48h starting after 1 day of the measurement were included in the calculation. Energy expenditure was normalized to body weight and expressed in kcal/g/h.

Determination of plasma insulin and pancreatic insulin content.

The concentration of plasma insulin was measured by performing a Mouse Ultrasensitive Insulin ELISA (ALPCO, Salem, USA), according to supplier's instructions. The entire pancreatic insulin content was determined in freshly isolated pancreas. Placed in ice-cold acidic ethanol, samples were homogenized with a tissue lyser for 5min at maximum speed and incubated overnight at 4°C. Pancreatic lysates were centrifuged for 10min (16.000 rpm, 4°C) and insulin was detected in the supernatant fraction, again by using Mouse Ultrasensitive Insulin ELISA (ALPCO, Salem, USA).

Isolation of pancreatic islet and microarray-XS-based transcriptomics.

The isolation of islets from NZO mice before and after 2 and 21 days of CRD feeding was performed as previously described with slight modifications[23]. Due to the small number of isolated islets, some samples had to be pooled. After perfusion by an injection of 3 ml Collagenase Type IV from *Clostridium histolyticum* (Sigma-Aldrich, Taufkirchen, Germany) diluted in Hank´s buffered salt solution (HBSS, plus 25 mM HEPES, 0,5% BSA) into the common bile duct, the pancreas was digested in 2 ml of collagenase for 12min at 37°C.

After mechanical tissue disruption by using a 18G needle, samples were washed and sedimented three times in HBSS and islets were separated from exocrine tissue by manual selection in RPMI 1640 (plus 10% FCS, 2% L-Glutamine, 1% penicillin/streptomycin).

Total RNA isolation and microarray-XS-based transcriptomic analysis of NZO islets on day 0 and after 2 days of the diet change was performed by Oak-Labs GmbH (Berlin, Germany). Briefly, RNA extracts underwent a quality control with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and samples with a minimal RNA integrity number (RIN) of 8.0 were included in microarray analysis. For the generation of fluorescent cRNA, the low Input QuickAmp Labeling Kit (Agilent Technologies, Waldbronn, Germany) was used. Fluorescent signals were detected by a SurePrint G3 Mouse GE 8 × 60 k chip (Agilent Technologies, Waldbronn, Germany). Microarray data were analyzed with Software R (version 3.4.1)[45]. Web-based GOrilla tool was used to identify biological processes influenced by differentially expressed transcripts (http://cbl-gorilla.cs.technion.ac.il/, 05/16/2019). The list of differentially expressed transcripts was ranked by log2 fold change (log2FC) to detect enriched GO terms (enrichment p value < 10^{-3}) of young and aged NZO mice before and after 2 days of the diet switch[46]. Top ten lists of differentially regulated transcripts were generated by using Qiagen IPA (content version: 44691306, QIAGEN Bioinformatics, Hilden, Germany). After quantile normalization, fold change and p-values were calculated. For p-values, two-tailed Students t-test with a threshold of $p \leq 0.05$ with the "stats" R-package version 3.1.1 was used. Different expressions between groups were significant when a threshold of log2 fold change of |0.7| was reached. The accession ID (GEO) for raw data of murine islet microarray analysis has not been generated, yet.

Glucose-stimulated insulin secretion (GSIS) in primary mouse islets.

Immediately after isolation and 1h recovery in RPMI1640 (containing 10% FCS, 2% L-Glutamine, 1% penicillin/streptomycin) at 37°C, 45 hand-picked primary islets per sample were used to determine insulin secretion. Islets were recovered in Krebs-Ringer solution (plus 0.2% BSA, pH 7.4) under low glucose conditions (2.8 mmol/l) for 1h. Subsequent to the islet transfer into perifusion chambers, GSIS was performed following a sequence of low glucose conditions (2.8 mmol/l) for 18min, high glucose conditions (20.0 mmol/l) for 35min, low glucose conditions for 30min and finally potassium chloride (40 mmol/l) for

406 20min with a continuous flow of 0.5 ml/min. Insulin secretion was measured in intervals of
407 2-4min. Levels of insulin were evaluated by performing a Mouse Ultrasensitive Insulin ELISA
408 (ALPCO, Salem, USA) normalized to DNA content determined with the Quanti-iT PicoGreen
409 dsDNA Assay Kit (Invitrogen, Carlsbad, USA).

410 **Immunostaining and TUNEL assay in pancreatic tissue sections**.
411 For immunohistochemical and immunofluorescent analysis, longitudinal sections (2 µm) of
412 young and aged NZO mice before and after 2 and 21 days of the CRD intervention were
413 processed as previously described[7]. Pancreatic tissue slices were incubated with primary
414 antibodies against rabbit Ki67 (1:1000, Abcam, Cambridge, UK), rabbit PDX1 (1:200, Merck
415 Millipore, Darmstadt, Germany), mouse caspase-3 (1:100, Life Technologies, Darmstadt,
416 Germany) and rabbit ChREBP (1:100, Invitrogen, Carlsbad, USA) for 1h at room
417 temperature. For multi-color staining, sections were incubated overnight at 4°C with rabbit
418 insulin antibody (1:1000, Abcam, Cambridge, United Kingdom). After washing and a second
419 blocking step, this was followed by 1h incubation with primary antibodies against mouse
420 glucagon (1:200, ab10988, Abcam, Cambridge, United Kingdom) and rat somatostatin
421 (1:75, ab30788, Abcam, Cambridge, United Kingdom). Detection of primary antibodies was
422 achieved with cross-adsorbed secondary antibodies conjugated to AlexaFluor 488, 546 and
423 633 (1:200, Invitrogen, Darmstadt, Germany). Nuclei were visualized by embedding in
424 FluorCare including DAPI (Carl Roth, Karlsruhe, Germany). Apoptotic cells in pancreatic
425 tissue sections were detected via TUNEL assay - TdT *In Situ* Apoptosis Detection Kit
426 Fluorescein (R&D Systems, Minneapolis; USA), according to supplier´s instructions. Imaging
427 was performed by using Olympus IX53 (Olympus, Hamburg, Germany) or Zeiss LSM 780
428 confocal microscopes (Zeiss, Jena, Germany). Immunofluorescent images, except for
429 ChREBP, were evaluated via automated quantification with an in-house programmed
430 macro for ImageJ software package (Version 1,52h, NIH, USA)[47]. Images of Ki67 and
431 caspase-3 stainings were quantified by using Zeiss ZEN2.3 imaging software (Zeiss, Jena,
432 Germany). Number of $ChREBP^+$-nucei was calculated manually in a blinded manner. Nuclei
433 were defined as $ChREBP^+$, when surrounded by $ChREBP^+$-signal.

434 **Real-time PCR analysis.**
435 The mRNA extraction from 70-100 pancreatic islets was performed by using Dynabeads™
436 mRNA Purification Kit (Thermo Fisher Scientific, Darmstadt, Germany), following

manufacturer´s guidelines. Due to small amounts of available material, supernatants of islet lysates obtained during the mRNA isolation step were preserved for western blot analysis. Subsequent to extraction, mRNA samples were reverse transcribed by SensiFAST cDNA Synthesis Kit (Bioline, London, UK) and diluted 1:10 in water, followed by RT-PCR reactions with the Dream-Taq-Hot Start-DNA Polymerase (Thermo Fisher Scientific, Darmstadt, Germany) and SYBR Green (Invitrogen, Carlsbad, USA). Standard curves of diluted PCR products were monitored in parallel and used for quantification. The following murine primers obtained from Sigma-Aldrich (Taufkirchen, Germany), designed with the Primer-Blast tool of NCBI, were used: *Mki67* (fwd 5'-TGGCGTGAAACAAACACAAACGA-3', rev 5'-TTGCTTGTGGGTTTCTTTGGAGTG-3'), *Mlxipl* (fwd 5'-ATCTCCAGCCTCGTCTTCCT-3', rev 5'-GTTGGGATCCAAGGGTCCAG-3'), *Mlx* (fwd 5'-TGGGTCAAGGTCGAGTATGC-3', rev 5'-GCCTCCGGTCTTTGTAGGAC-3'), *Pcna* (fwd 5´-AGAGGAGGCGGTAACCATAGAG-3´, rev 5´-ACTGTAGGAGACAGTGGAGTGG-3´), *Pdx1* (fwd 5´-CTTAACCTAGGCGTCGCACAA-3´), rev 5´-GAAGCTCAGGGCTGTTTTTCC-3´), *Tcf19* (fwd 5´-AAGCTGCTCTGATAGCCAGTG-3´, rev 5´-TCTGTCACTTCATTTGCAAGC-3´), *Txn1* (fwd 5´-AGTGGATGTGGATGACTGCC-3´, rev 5´-CCTTGTTAGCACCGGAGAACT-3´), *Txn2* (fwd 5´-CGGACAGTACACACCACCAG-3´, rev 5´-GCGGTCCTAGGATCTTGCAG-3´) and *Txnip* (fwd 5´-TAGTGATTGGCAGCAGGTCTGG-3´, rev 5´-ATAGCAAGGAGGAGCTTCTGGG-3´). Target gene expression was normalized to a reference factor composed of hypoxanthine guanine phosphoribosyltransferase (*Hrpt*) (fwd 5'-GCAGTCCCAGCGTCGTG-3', rev 5'-GGCCTCCCATCTCCTTCAT-3´), ribosomal protein L13a (*Rpl13a*) (fwd 5'-GTTCGGCTGAAGCCTACCAG-3', rev 5'-TTCCGTAACCTCA AGATCTGCT-3´) and glycerinaldehyde-3phosphate dehydrogenase (*Gapdh*) (fwd 5'-GGGTGTGAACC ACGAGAAAT-3', rev 5'-GTCTTCTGGGTGGCAGTGAT-3´).

Western immunoblot analysis.

As mentioned above, islet supernatants from mRNA isolation were used for immunoblotting. Protein concentrations were determined with Lowry assay (Bio-Rad Laboratories, Munich, Germany) followed by acetone precipitation overnight at -20°C. Afterwards, samples were centrifuged for 10min (14.000 rpm, 4°C), pellets were transferred in loading buffer and incubated for 5min at 95°C. Subsequently, immunoblot analysis was performed by using a 15% polyacrylamide gel (20 slots chamber), loaded with 7 µg of sample. After gelelectrophoresis, proteins were transferred to 4.5 µm nitrocellulose

membrane via semi-dry blotting for 30min. Membranes were then blocked in Odyssey© blocking buffer (LI-COR Biosciences, Lincoln, USA) for 1h at room temperature, cut horizontally to detect a maximum number of proteins and incubated with primary antibodies against rabbit TXNIP (1:1000, Thermo Fisher Scientific, Darmstadt, Germany), rabbit PARP (1:1000, Cell Signaling, Boston, USA), rabbit ChREBP (1:500, Invitrogen, Carlsbad, USA) and mouse GAPDH (1:20.000, Abcam, Cambridge, UK) overnight or for 48h at 4°C. This was followed by washing steps and secondary antibody incubation with mouse and rabbit fluorescent-conjugated secondary antibodies from LI-COR Biosciences (Lincol, USA). Signals were detected with an Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, USA).

Statistical analysis.

Graph Pad Prism version 7.04 (San Diego, USA) was used for the generation of all graphs, heatmaps and statistical analysis (statistical parameters are included in the figure legends). Data are represented as mean values ±SD. For group comparison, Two-way ANOVA with Sidak´s multiple comparison tests were performed. Values of $p < 0.05$ were considered statistically significant, represented as letters.

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Conflict of interest

The authors declare that they have no conflict of interest.

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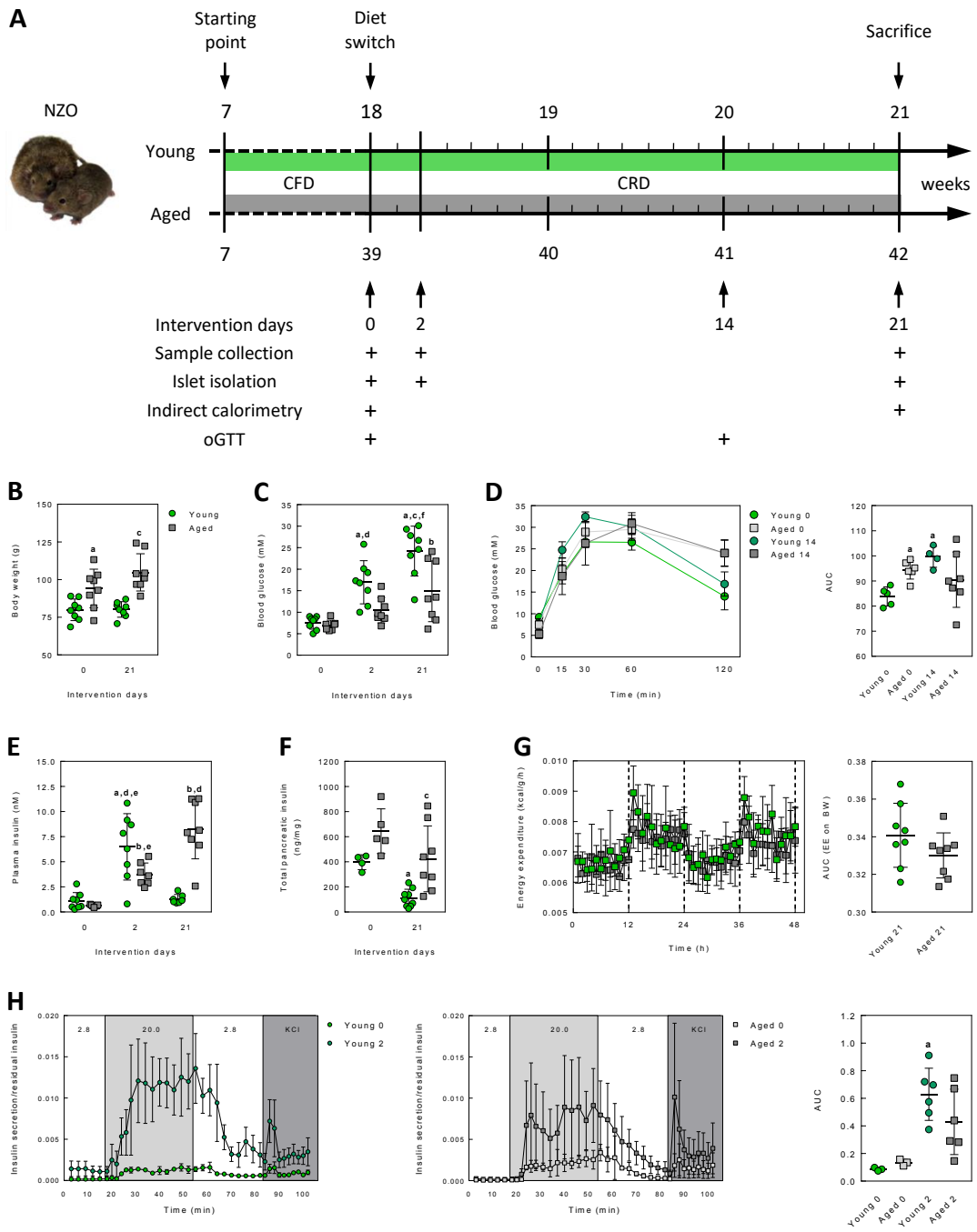

Fig. 1 Glucose utilization and GSIS of young and aged NZO mice in response to the CRD intervention.
(A) Study design and experimental setup. Young (green) and aged (grey) New zealand obese (NZO) mice received a carbohydrate-free, high-fat diet (CFD) for 11 or 32 weeks, followed by a carbohydrate-rich diet (CRD) for a maximum of 21 days. Plus symbols (+) at indicated time points mark different animal experiments and sample collections. **(B)** Body weights (n = 8) and **(C)** blood glucose levels (n = 7-8) of young and aged NZOs at indicated time points. **(D)** Oral glucose tolerance test (oGTT) with corresponding area under the curve (AUC) of young and aged animals at day 0 and 14 days after the diet change (n = 4-7). **(E)** Plasma insulin (n = 7-8) and **(F)** total pancreatic insulin content of isolated pancreas (n = 4-9) in young and aged NZO mice at indicated time points. **(G)** Energy expenditure measured by indirect calorimetry with corresponding AUC (referred to body weight) of young and aged NZO mice 21 days after CRD feeding (n = 8-9). **(H)** Glucose-stimulated insulin secretion (GSIS) of pancreatic islets isolated from young and aged NZOs before and after 2 days of carbohydrate feeding obtained from perifusion experiments (n = 3-6). Data are represented as mean ±SD. Statistical significance was assessed by Two-way ANOVA with Sidak´s multiple comparison test, $^{a,b,c,d,e,f}p < 0.05$ (a = significant to young 0, b = significant to aged 0, c = significant to young 2, d = significant to aged 2, e = significant to young 21, f = significant to aged 21).

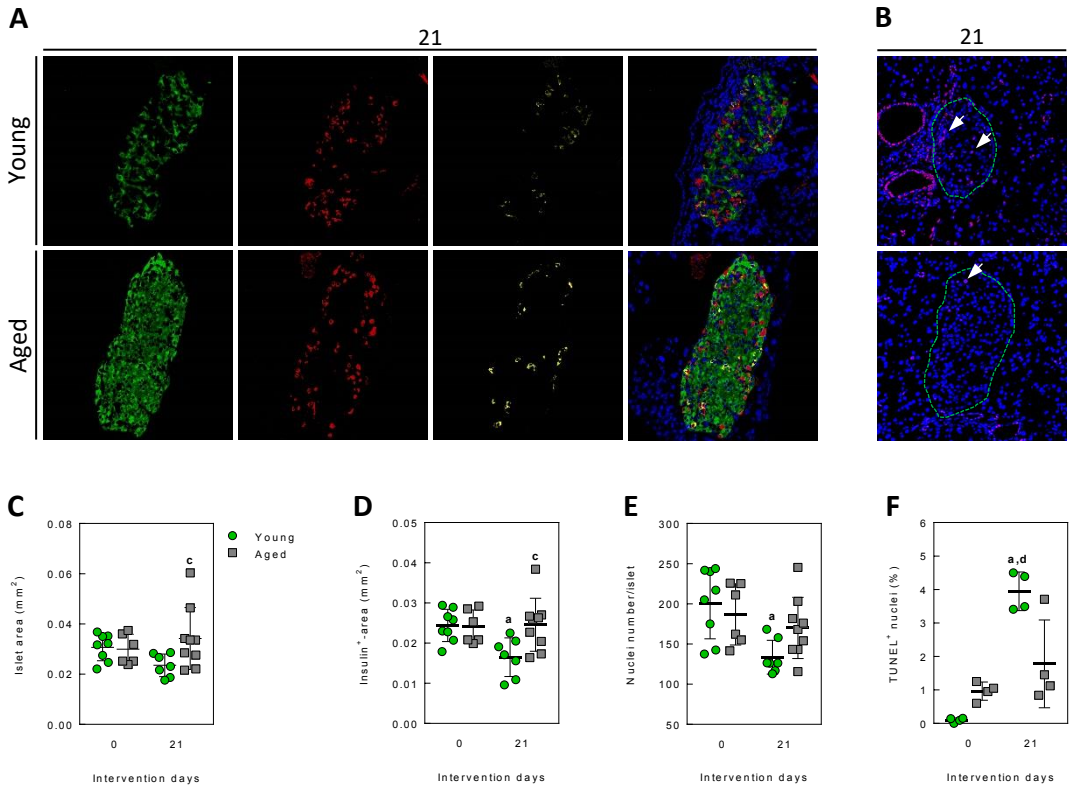

Fig. 2 Morphology and TUNEL-assay of young and aged NZO mice before and after CRD challenging.
(A) Representative images (20x magnification) showing multi-color immunofluorescent stainings of insulin (green), glucagon (red), somatostatin (yellow) and DAPI (blue) as well as **(B)** TUNEL assay in longitudinal, serial sections (0.2 µm thickness) from young and aged NZO mice after 21 days of CRD feeding. Quantitative analysis corresponding to multi-color stainings of **(C)** islet-area in mm^2, **(D)** $insulin^+$-area in mm^2 and **(E)** total nuclei number (n = 6-8) of young (green) and aged (grey) NZO mice at indicated time points. **(F)** Quantification of $TUNEL^+$-nuclei, given as percentage of the total nuclei number within $insulin^+$-area (n = 4). Red = $TUNEL^+$-nuclei, blue = DAPI, green line = $insulin^+$-area. White arrows show $TUNEL^+$-nuclei. Results are represented as mean values ±SD. Statistical significance was assessed by Two-way ANOVA with Sidak´s multiple comparison test, $^{a,c,d}p < 0.05$ (a = significant to young 0, c = significant to young 2, d = significant to aged 2).

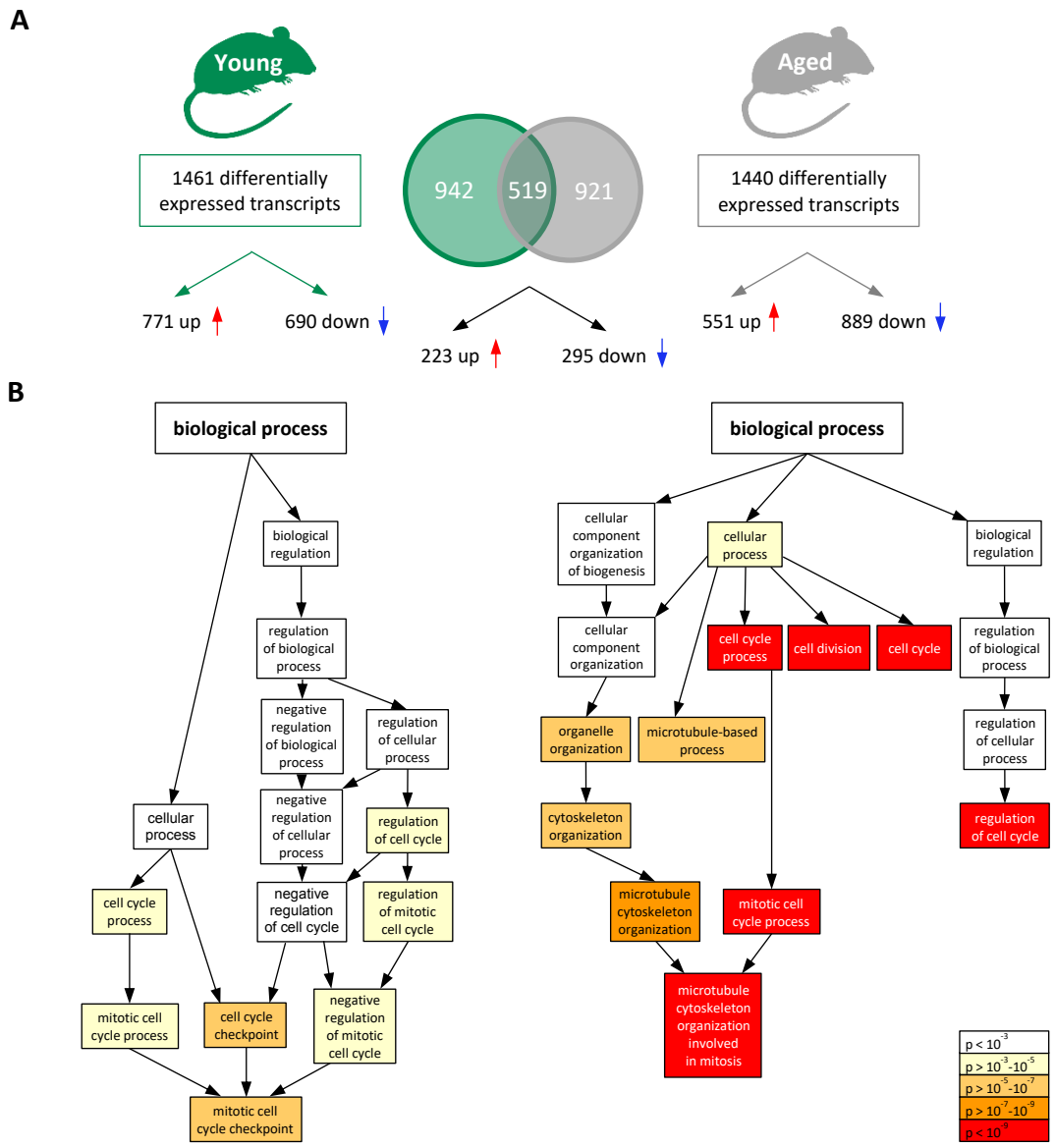

Fig. 3 Microarray-based transcriptomics and GO term enrichment analysis in islets of young and aged NZO mice in response to the CRD intervention.

(A) Total numbers of differentially expressed transcripts obtained from microarray in pancreatic islets isolated from young (green) and aged (grey) NZO mice before and 2-days after the CRD intervention **(B)** Visualization of mainly enriched Gene ontology (GO) terms by upregulated transcripts in islets of young and aged NZO mice before and after 2 days of the CRD feeding. P-values for enrichment are $p < 10^{-5}$ in young mice and $p < 10^{-8}$ in aged mice.

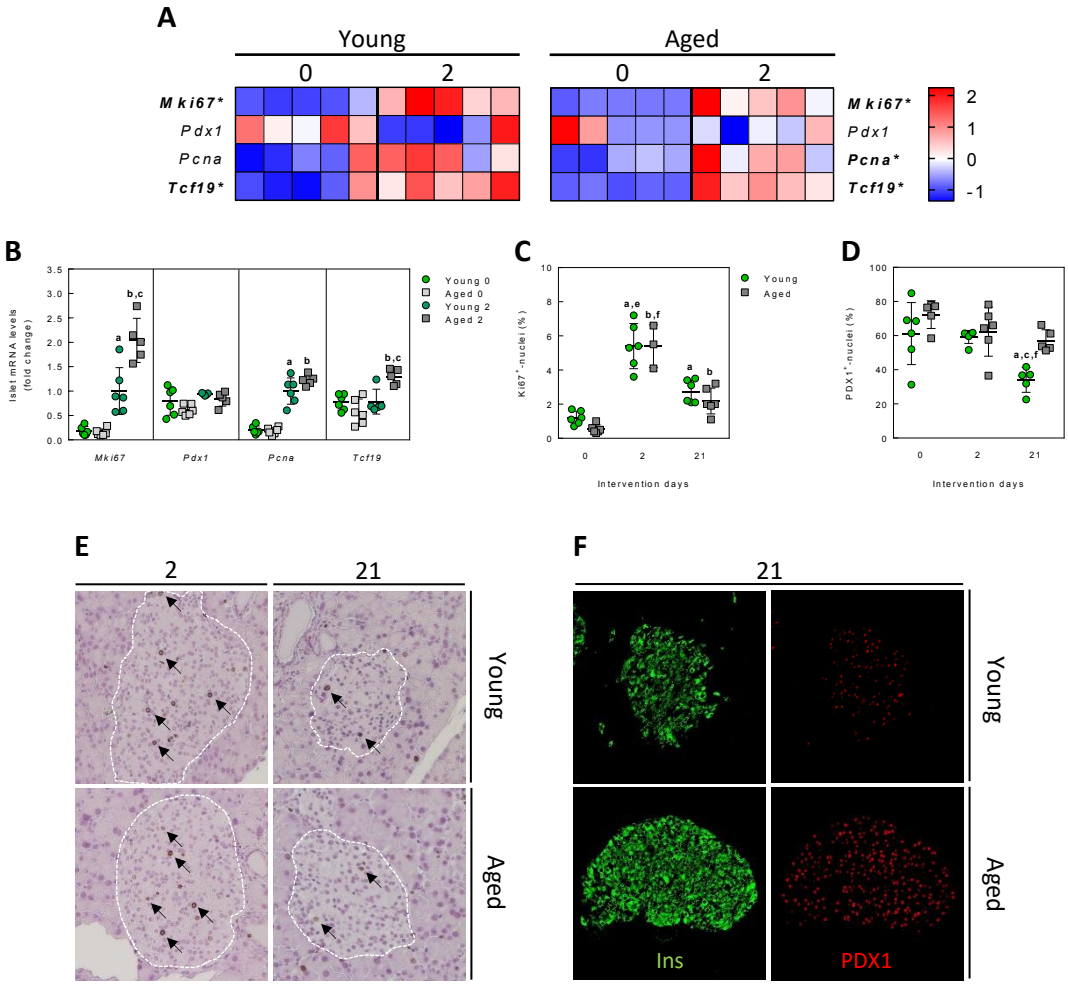

Fig. 4 Proliferation and differentiation in isolated islets and pancreatic tissue sections of young and aged NZO mice before and after CRD feeding.

(A, B) Heatmap and corresponding RT-PCR analysis of selected transcripts before (0) and after (2) the CRd intervention in islets of young and aged NZO mice (red squares = upregulation, blue squares = downregulation, n = 5 for transcriptomics, n = 5-6 for RT-PCR). Bold letters with asterisk highlight differentially expressed transcripts. **(C, E)** Quantification of immunostainings against Ki67 (n = 4-6) and PDX1, co-stained with insulin (n = 5-6), given as percentage of positive stained nuclei per islet and **(D, F)** representative images (20x magnification) of pancreatic tissue sections of young (green) and aged (grey) NZOs at indicated time points. White lines mark the islet area and black arrows show Ki67^{+}-nuclei. Green = insulin, red = PDX1. Data are represented as mean ±SD. Statistical significance was assessed by Two-way ANOVA with Sidak´s multiple comparison test, $^{*,a,b,c,d,e,f}p < 0.05$ (a = significant to young 0, b = significant to aged 0, c = significant to young 2, d = significant to aged 2, e = significant to young 21, f = significant to aged 21).

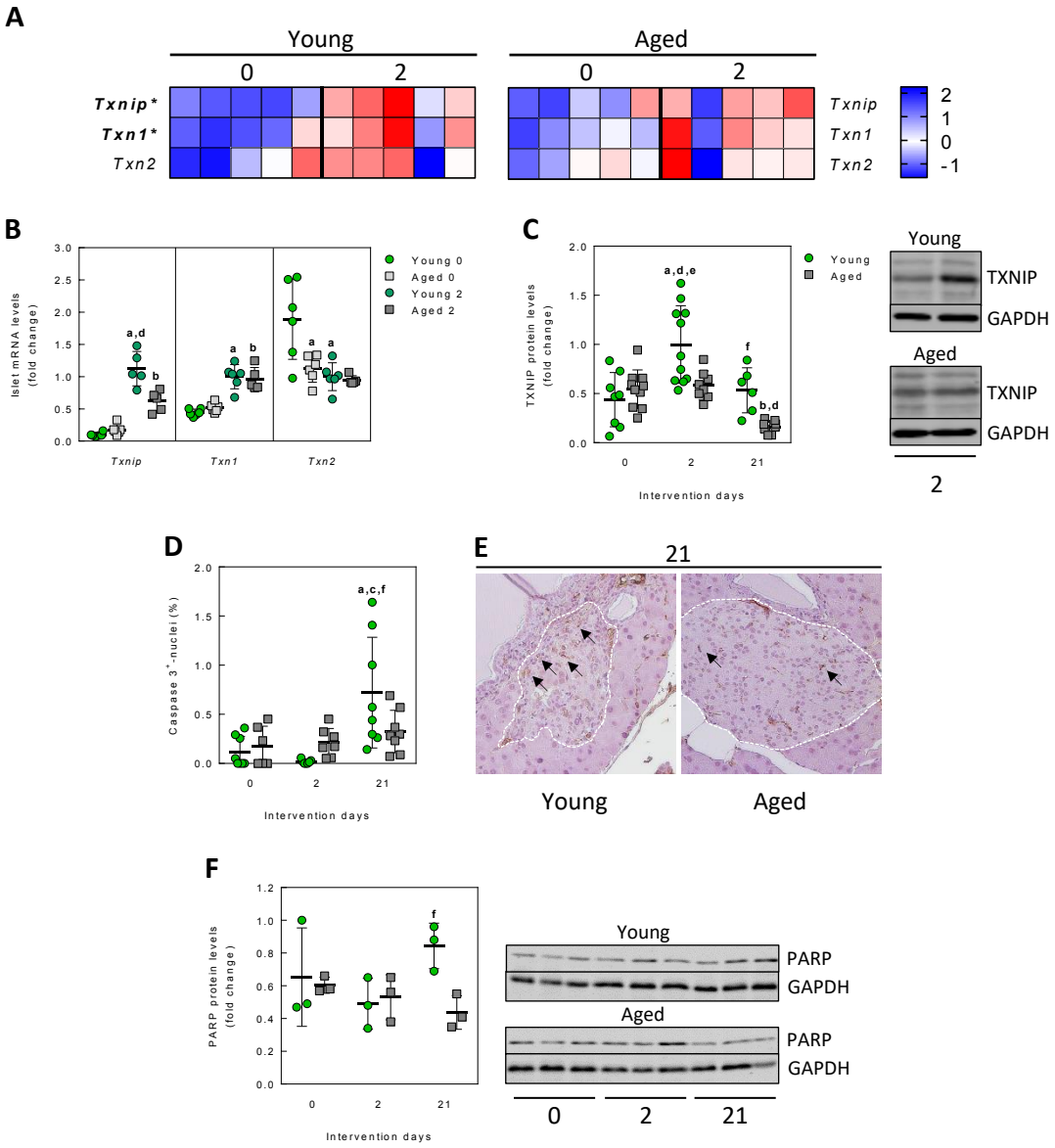

Fig. 5 TXNIP expression and downstream mechanisms in young and aged NZO mice in response to CRD feeding.

(A-B) Heatmap of thioredoxin (TXN)-pathway-related transcripts selected from microarray-based transcriptomics and corresponding RT-PCR analysis of isolated islets from young and aged NZO mice at day 0 and day 2 after the CRD challenge (red squares = upregulation, blue squares = downregulation, n = 5 for transcriptomics, n = 6 for RT-PCR). Bold letters with asterisk mark differentially expressed transcripts. **(C)** Thioredoxin-interacting protein (TXNIP) immunoblot analysis in pancreatic islets of young (green) and aged (grey) NZOs at indicated time points with representative blots after 2 days of CRD feeding (n = 6-11). **(D)** Quantitative analysis of caspase-3 immunostaining, shown as percentage of positive stained nuclei per islet in pancreatic tissue sections of young and aged NZO mice at indicated time points and **(E)** representative images after 21 days of the CRD intervention (n = 6-8). Images are presented in a magnification of 20x. White lines mark the islet area and black arrows indicate positive stained nuclei. **(F)** Western blot analysis of PARP in islets isolated from young and aged NZO animals with representative blots at given time points (n = 3). For immunoblot analysis, protein levels were normalized to GAPDH. Data are presented as mean values ±SD. Statistical significance was assessed by Two-way ANOVA with Sidak´s multiple comparison test, $^{*,a,b,c,d,e,f}p < 0.05$ (a = significant to young 0, b = significant to aged 0, c = significant to young 2, d = significant to aged 2, e = significant to young 21, f = significant to aged 21).

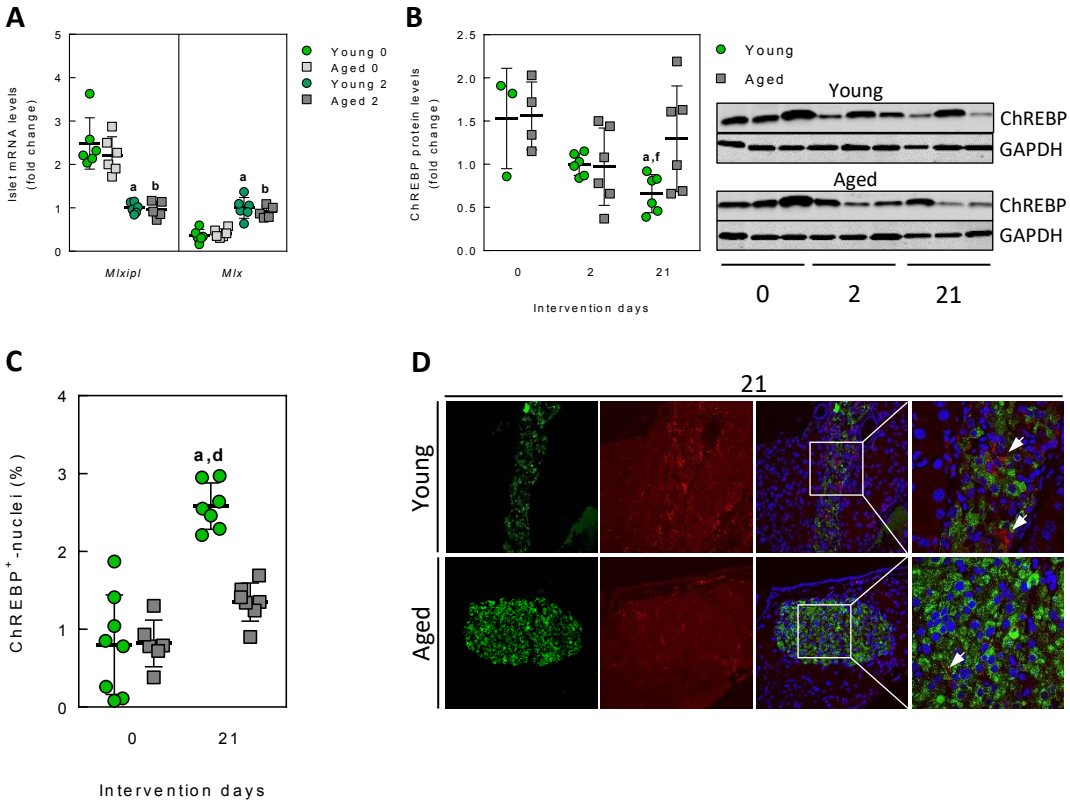

Fig. 6 Upstream mechanism of thioredoxin-interacting protein in islets and tissue sections of young and aged NZO mice before and after CRD challenging.

(A) RT-PCR analysis of Max-like factor 1 interacting protein like (*Mlxipl*) and Max-like factor 1 (*Mlx*) and **(B)** Carbohydrate-responsive element-binding protein (ChREBP) immunoblot analysis in isolated islets from young (green) and aged (grey) NZO mice with representative blots at indicated time points. For immunoblot analysis, protein levels were normalized to GAPDH. **(C)** Quantification of immunostaining against ChREBP, shown as percentage of surrounded (counted as positive stained) nuclei per islet (n = 6-8) in tissue sections of young and aged NZOs at indicated time points (n = 5-6) with **(D)** representative images in pancreatic tissue sections after 21 days of CRD feeding. Images are represented in a magnification of 20x or 63x (inserts). White arrows show ChREBP^{+}-nuclei. Green = insulin, red = ChREBP, blue = DAPI. Data are presented as mean values ±SD. Statistical significance was assessed by Two-way ANOVA with Sidak´s multiple comparison test, $^{a,b,d,f}p < 0.05$ (a = significant to young 0, b = significant to aged 0, d = significant to aged 2, f = significant to aged 21).

Discussion

Worldwide population steadily grows older and human life expectancy has doubled in the last two centuries. Since aging is one of the main risk factors for most diseases and conditions limiting health span, such as type 2 diabetes and related complications, the dramatic increase in population aging has become a major public health care problem[177,245]. Therefore, the impact of aging and associated consequences on metabolic health has gained scientific attention in recent years. In general, the aging process is described as a time-dependent progressive decline of physiological integrity and function, accompanied by enhanced vulnerability towards stress conditions[191]. On a cellular level, aging is characterized among others by a progressive loss of proteostasis (proteasomal and autophagy-lysosomal system), increased oxidative and nitrosative (redox) stress, accumulation of modified proteins and an induction of cellular senescence, reviewed recently by our group (Figure 7)[213]. These age-related alterations are shown to have a wide range of detrimental effects on cellular morphology and functionality as well as proliferative and regenerative potential, also affecting pancreatic islets and their beta cells, regulating glucose homeostasis by secreting several hormones[184,202]. However, beta-cell aging has been discussed controversially, associated with both diminished but also unchanged beta cell function as well as islet morphological alterations[185]. More

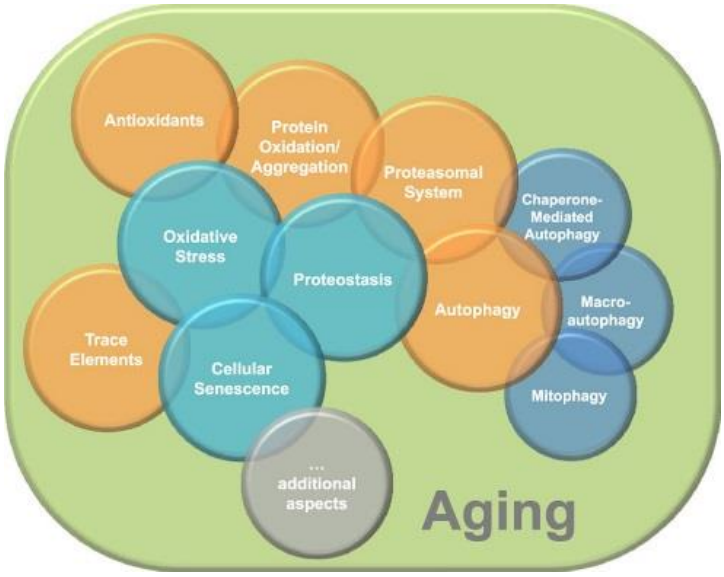

Figure 7 - Effects of aging on cellular mechanisms.
Overview of several hallmarks affecting cell physiology and integrity during aging. Adopted from Höhn et al.[213]

importantly, malnutrition by excess intake of high caloric diets, containing large amounts of carbohydrates and fats, triggers a cascade of obesity, peripheral insulin resistance and systemic inflammation. This results in dyslipidemia and hyperglycemia due to sustained elevated circulating glucose and FA levels followed by glucolipotoxic (metabolic) stress, in turn, leading to a loss of functional beta-cell mass[73,92,93]. Thus, aging and nutrition have been described as major risk factors for the onset and progression of type 2 diabetes and its complications in humans and rodents, but are mainly studied separately in current research[177,244,246]. Therefore, the impact of aging on the functionality and structural integrity of beta cells under diet-induced metabolic stress conditions was investigated in the present study.

In the first part, age-related changes in endocrine islets of C57BL/6J mice were examined under normoglycemic and non-pathological (physiological) conditions at various age groups (2.5, 5, 10, 15 and 21 months) (**publication I**). In the second part, the combination of diet-induced metabolic stress and advanced age on beta-cell functionality and integrity was investigated by comparing young (18-21 weeks) and middle-aged (39-42 weeks) male, diabetes-prone NZO mice (**publication II and manuscript**). Originally, the purpose was to use mice at the age of 12 months; however, this was inconvertible for animal welfare reasons. The duration of the dietary regimen was reduced therefore by about 3 months. Thus, mice age at the end of the study corresponds to the human age of 38-40 years, when the prevalence of type 2 diabetes is already increasing[247,248].

Characterization of pancreatic islets during aging

The age-related islet mass expansion - one factor ensuring normoglycemia

Endocrine islet development occurs during prenatal life after the formation of pancreatic buds (Figure 1)[8]. In neonatal and fetal life, the majority of islet cell population is formed from undifferentiated precursor cells, stimulated by multiple growth and transcription factors, hormones and nutritional compounds, initiating mitosis and neogenesis[249]. Studies in rodents and humans suggest that islet mass grows slightly until adulthood and is under homeostatic control in advanced age[95,202,206,250]. The expansion of islet mass in aging occurs primarily under conditions of increased metabolic demand, such

as obesity, hyperglycemia but also pregnancy[184,202,251,252]. However, our data in C57BL/6J mice show an increase in islet size as well as elevated islet area with advancing age under physiological conditions accompanied by stable circulating insulin levels. Since islet number was unchanged, it might be assumed that hypertrophy of endocrine cells rather than the generation of new islets is responsible for islet size expansion, in agreement with the data from Dolborg et al.[253]. Moreover, islet growth seemed to occur by increasing beta-cell area as observed by immunohistochemical analysis. Such morphological alterations were found even in 1981 by Robert C. McEvoy, describing an increase in islet size of Sprague Dawley rats due to elevated beta-cell volume until the age of 7 months [254]. Moreover, an age-dependent increase of beta-cell area and mass in normoglycemic Lewis rats as well as lean, non-diabetic human subjects has been associated with beta-cell hypertrophy, rather than hyperplasia[95,255]. This suggests that the islet expansion in our wild-type mice arises from beta-cell growth.

Islet mass expansion is dependent on the interaction of several mechanisms, such as neogenesis, proliferation and apoptosis, as illustrated in figure 5. However, it has been shown in previous years that neogenesis from pancreatic precursor cells or non-beta-cell fractions occurs rarely in aged endocrine tissues, whereas self-duplication of existing cells seems to be the crucial mechanism for cellular mass expansion in aging islets[200,201]. In this context, PDX1 has been emerged as a key transcriptional regulator for beta-cell growth but also function and survival[185,256]. As shown by Johnson et al., even a partial PDX1-deficiency of 50% in 1-year old mice increased the vulnerability towards apoptosis compared to their aged controls. This was accompanied by islet morphological abnormalities, islet mass reduction and beta-cell dysfunction[257]. A more dramatic phenotype was observed in mice with a beta-cell specific deletion of PDX1 during embryogenic stages but also in adult mice, initiating decreased insulin secretion and progressive beta-cell loss followed by diabetes progression[256,258,259]. Morover, mutations in the human orthologue of the PDX1 gene represent an important risk factor for pancreatic agenesis or MODY4 (maturity onset diabetes of the young 4)[260]. In addition to artificially-induced and genetically-engineered PDX1 deficiency, the expression of this transcription factor decreases during the normal aging process, as it was shown by Maedler and colleagues, comparing islets of Sprague Dawley rats aged 2 or 8 months[261]. Similarly, we observed a decline in beta-cell specific PDX1 expression until middle age, indicating a decline in beta-cell functionality. However,

since plasma insulin levels remained stable in C57BL6/J mice throughout lifespan, it might be assumed that the islet expansion serves as compensatory mechanism to persistently adjust on insulin requirements and maintain glucose homeostasis during aging. Additionally, sustained PDX1 expression combined with unchanged islet size and circulating insulin levels, observed between middle age and advanced age, suggest that insulin secretory function and beta-cell survival are maintained. Besides this, PDX1 expression is associated with the proliferative potential of beta-cells, as indicated by Maedler et al. They revealed a simultaneous reduction in PDX1 and the proliferation marker Ki67 with age in pancreatic tissue sections of lean, non-diabetic human donors between 17 and 74 years[261]. This is in line with our findings, indicating that reduced PDX1 expression until middle-age was accompanied by an age-related proliferative decline in endocrine cells of normoglycemic wild-type mice. Consistently, several studies performed in the past years described that proliferation of pancreatic islets and their beta-cells decreases with age. Montanya and colleagues observed an early reduction in beta-cell replicative potential of Lewis rats[255]. Moreover, 50% partial pancreatectomy and subsequent administration with Bromdesoxyuridin in mice at various age groups (2, 8, 12, 14 and 19 months) was associated with an age-dependent decline of the regenerative potential of beta-cells, shown by Rankin and Kushner[209]. In line with the data from Tschen and colleagues, they revealed further that young mice were able to maintain their proliferative potential in response to exendin-4 or streptozotocin treatment, whereas aged mice did not[206,209]. Reduced proliferation as function of age was also shown in the human situation, by using pancreatic tissue sections of non-diabetic donors ranging from 2 to 66 years[199]. Consistently, all of these studies found minimal proliferation rates of pancreatic beta-cells in advanced age. Dor et al. proposed that this serves to maintain beta-cell regenerative capacity for changing metabolic demands throughout lifespan[208]. Thus, given that the islet size and area of our C57BL/6J mice continued to increase in advanced age, it might be assumed that reduced proliferative potential under physiological conditions is sufficient to maintain adaptive islet expansion.

The present study is limited by the missing link between aging and apoptosis induction. However, since several studies in rodents and humans revealed that apoptosis of endocrine cells is unchanged during normal aging and we observed an increase in islet size and area, it might be assumed that this process does not play a fundamental role[95,199,253]. Kushner as

well as Finegood and colleagues proposed further that adult beta-cells are very long-lived and maintain their functionality throughout lifespan, coinciding with our data[202,207].

The proliferative potential of beta-cells is dependent on the ability to enter the cell cycle, regulated by cell cycle activators and inhibitors[203,204,211]. As indicated by global knockout models and transgenic mice, several D-type cyclins and CDKs have been emerged to play a central role in beta-cell proliferation, most importantly cyclin D2 as well as CDK4 and 6. In response to mitogenic signals, cyclin D2 interacts with CDK4 and 6, inducing the phosphorylation and inactivation of the Rb. This stimulates E2F transcription factors, initiating cell cycle progression and beta-cell proliferation[205,262,263]. However, it has been shown that the expression of cell cycle activators declines with age accompanied by a simultaneous increase in cell cycle inhibitors, such as $p16^{INK4a}$ tumor suppressor protein[185,202,211]. To date, $p16^{INK4a}$ is accepted to be one of the most useful markers of senescence[264]. $P16^{INK4a}$ prevents the interaction between cyclin D2 and CDK4 and 6, inhibiting cell cycle entry (Figure 6)[265]. Thus, $p16^{INK4a}$ accumulation leads to a loss of cellular and tissue proliferative and regenerative, inducing cellular senescence[202,213,265]. Moreover, recent studies showed that $p16^{INK4a}$ correlates with increased age and decreased proliferation[193,211]. In agreement with this, the gradual proliferative decline with age observed in the present study was accompanied by an increase in $p16^{INK4a}$ expression, indicating beta-cell senescence. Interestingly, by using transgenic or diabetogenic mice, recent work by Helman and colleagues revealed that $p16^{INK4a}$-expressing, senescent beta-cells exhibit enhanced insulin secretion and improved glucose homeostasis mediated by mitochondrial stimulation, elevated glucose uptake and cellular enlargement[217,218]. The cellular growth might occur by an increased formation of cytoskeleton and membrane proteins, such as vimentin and caveolin-1[266,267]. Thus, the age-dependent islet growth, observed in the present study, might be associated with senescence-induced cellular expansion, potentially contributing to maintained insulin secretory capacity in higher age.

Taken together, aging in C57BL/6J mice was associated with islet expansion to maintain normoglycemia and metabolic homeostasis throughout lifespan. Minimal but sustained proliferative potential and induction of cellular senescence in advanced age presumably contribute to cellular enlargement and maintenance of insulin secretion, but further investigations are needed to identify additional causes for the observed morphological alterations.

The formation of AGEs in aging - products associated with islet redox stress

Besides morphological changes as well as proliferative and regenerative limitations, the formation and accumulation of protein aggregates, such as AGEs, have been emerged as a critical feature of aging, associated with cellular and tissue dysfunctions[143]. AGE formation occurs exogenously by the intake of high AGE-containing diets or endogenously due to increased protein glycation, especially under hyperglycemic conditions[148,268]. In general, several studies describe that AGEs accumulate during the normal aging process in almost all tissues throughout the body, caused by an imbalance of AGE generation and renal clearance as well as decreased functionality of AGE detoxifying enzymes[137,178,183,269]. Although AGE formation and accumulation have been strongly associated with aging, diabetes and its complications, a direct connection between these factors and deposition of glycation products within pancreatic islets has been barely investigated, yet.

Our data revealed an increased formation of several AGEs, such as MG-derived arg-pyrimidine and MG-H1 as well as ribose-derived pentosidine within pancreatic islets of old C57BL/6J mice under normoglycemic conditions. Recently, our group found similar results in skin biopsies of young and old C57BL/6J mice, indicating that arg-pyrimidine and pentosidine accumulate with advancing age[270]. This goes along with a comprehensive study by Schleicher et al., revealing an age-dependent increase in CML formation in various tissues, such as skin, lung, heart, kidney and intestine that was further enhanced in some organs and serum samples of long-standing diabetes patients[181]. Interestingly, the results of our study showed that AGE formation in aging was predominantly located within the vascular system of islets, whereas endocrine cells seemed to be less affected. In a recent study, Morioka and colleagues found co-localizations of MG-derived AGEs within pancreatic beta cells and their insulin-secretory granules in healthy rats, partially applicable to our observations. However, they used self-designed antibodies that might react with different immunological epitopes of AGEs and AGE-modified proteins[271].

AGEs and AGE-precursors mediate their detrimental effects in the vasculature primarily by modifying circulating proteins, such as hemoglobin, direct binding and activation of RAGE or cross-linking with extracellular matrix proteins, especially their most common form collagen. This structural protein has a slow turnover rate and is highly vulnerable towards AGE modifications[137,272,273]. Elevated AGE levels in aging, such as of pentosidine

and MG-H1, correlate with vascular stiffness by targeting collagen, independent of diabetic pathologies, as shown by several studies[270,273,274]. Furthermore, elevated AGE content within aortic tissue samples of normoglycemic rats and rabbits is associated with increased vascular permeability and impaired elasticity, inducing vascular dysfunctions[275]. Thus, it might be speculated that AGE formation in the islet vasculature of our old C57BL/6J mice modifies membrane-standing proteins, presumably impairing vascular functions. Besides protein modifications, the interaction of circulating AGEs with their major receptor RAGE, expressed among others on the surface of endothelial cells, has a damaging potential on the vasculature, shown to increase age-dependently. However, it is still a matter of debate, which AGEs and AGE-precursors bind to RAGE[137,153,276]. Mechanistically, AGE-mediated activation of RAGE promotes the activity of NF-κB and NADPH oxidase, leading to the induction of redox stress, inflammation and apoptosis, observed in a pancreatic islet endothelial cell line or endothelial progenitor cells, respectively, isolated from Sprague Dawley rats[277,278]. Additionally, the latter revealed that cellular proliferation was also impaired. Transferred to our data, the formation of AGEs within islet cells but primarily within the vasculature might be associated further with a cellular proliferative decline in advanced age. Similarly, Zhu et al. indicated that the proliferative capacity of cultured human keratinocytes was diminished in response to AGE treatment[279]. Furthermore, shown by cell culture experiments in several cell lines and primary cells, AGE-RAGE interaction mediates NADPH oxidase and NF-κB activation, leading to increased iNOS expression and induction of nitrosative stress[280-282]. In turn, this promotes the generation of nitrated protein residues, such as 3-NT, initiated by enhanced nitric oxide and peroxynitrite formation[170,283]. Accordingly, AGE formation observed in the present study was associated with elevated iNOS and 3-NT expression in advanced age, located again primarily in the islet vasculature but also within endocrine cells of old wild-type mice. This suggests that aging under physiological conditions might lead to oxidative and nitrosative stress. However, this seemed to have no adverse effects on islet functionality, indicated by unchanged circulating blood glucose and insulin levels. In contrast, Zhao and colleagues revealed that AGE-mediated increase in iNOS expression and nitric oxide production inhibits GSIS in isolated islets from C57BL/6J mice[169]. Disturbed glucose uptake, mitochondrial dysfunction, declined ATP generation and reduced calcium influx or inhibited glucokinase activity are further factors, connecting AGE formation and beta-cell

dysfunction[167,284,285]. Moreover, it has been shown consistently in INS-1 as well as HIT-T15 cells that AGE administration decreases the expression of PDX1, leading to reduced insulin transcription and synthesis[172,286]. This is in contrast to our data, indicating that AGE formation occurred in advanced age during sustained PDX1 expression accompanied by unchanged insulin levels, also suggesting a missing link between AGE formation and islet functional decline. A potential explanation was given recently by Elmhiri et al., showing that the effect of acute MG treatment on the functionality of islets isolated from adult rats is dependent on glucose concentrations. While MG-induced an inhibition of insulin secretion under high glucose conditions, basal glucose stimulation enhances insulin release[287]. Thus, it might be speculated that the effects of MG-derived arg-pyrimidine and MG-H1 does not impair islet cellular function under normoglycemic conditions.

In summary, aging was accompanied by the formation and accumulation of AGEs, predominantly but not exclusively located in the islet vasculature. Although this is associated with the induction of redox stress, it seemed to have no adverse effects on islet and beta-cell functionality in aged wild-type C57BL/6J mice.

The impact of metabolic stress on beta-cell functionality and structural integrity in young and middle-aged NZO mice

Diet-induced metabolic stress - initiator of beta-cell dysfunction and loss in young NZO mice

Chronic intake of high caloric diets combined with an akinetic lifestyle leads to energy surplus, stored in the peripheral tissues. This mediates obesity, closely associated with insulin resistance, inducing dyslipidemia and hyperglycemia by increased circulating glucose and FA, finally resulting in metabolic stress conditions[92,93,110]. Consequently, beta-cell mass of most obese individuals expands to adjust on elevated insulin requirements and maintain normoglycemia. However, when individuals fail to compensate the increased insulin demand, the onset and progression of type 2 diabetes is induced by a loss of functional beta-cell mass[288,289]. Obese mouse models susceptible for postprandial metabolic stress mimic the human obesity-associated type 2 diabetes and were used in

previous research to investigate the molecular basis of this disease[243,290]. One of these models is the diabetes-prone NZO mouse, exhibiting a progressive loss of functional beta-cell mass in response to a specific dietary regimen[235]. Work from the lab of Joost and colleagues demonstrated that young NZO mice develop early hyperglycemia and type 2 diabetes in response to chow or high-fat diets, whereas CFD feeding was associated with normoglycemia in the presence of obesity and insulin resistance[241,291]. In turn, exposure to a CRD after carbohydrate restriction initiates hyperglycemia followed by metabolic stress, finally leading to beta-cell dysfunction and apoptosis in NZO mice[242]. Due to the maintenance of normoglycemia by CFD feeding and the possibility to induce hyperglycemia at a specific time point by a CRD intervention, this mouse model represents a useful tool to examine the impact of diet-induced metabolic stress on pancreatic beta-cell functionality and integrity in different ages. Therefore, 7-week old NZO mice were fed a CFD for 11 (young) or 32 (middle-age) weeks with subsequent CRD intervention for 2, 7 or 21 days. The results of this study are shown in **publication II** and **manuscript**.

Young NZO mice fed a CFD exhibited continuous and marked body weight gain with approximately 45% body fat mass (**publication II**) by visceral fat storage accompanied by impaired glucose tolerance (**manuscript**), indicating dyslipidemia and insulin resistance, as described by Jürgens et al.[241]. Subsequent CRD intervention leads to hyperglycemia in young animals even after 2 days with a simultaneous increase in plasma insulin and proinsulin levels (Figure 12 - Appendix 1) due to elevated insulin secretion. However, perifusion experiments in young NZO islets revealed an abnormal first and second phase insulin secretion (insulin hypersecretion) with impaired adaptability on changing glucose levels. Although islets of young mice exhibited increased proliferation in order to compensate for increased insulin requirements, continued CRD feeding leads to beta-cell dysfunction and reduced circulating insulin levels associated with decreased PDX1 levels. This was accompanied by enhanced glucose intolerance and hyperglycemia, finally resulting in apoptosis and a loss of functional beta-cells in young NZO mice under metabolic stress conditions (Figure 8). Although Kluth et al. demonstrated nearly equal findings in NZO mice by using the same dietary regimen, they revealed no changes in islet proliferative potential[242,292,293]. However, compared to their recent report, apoptotic levels of beta-cells in our NZO cohort were 4-fold higher[294]. Thus, it might be postulated that increased proliferation has been covered by the induction of massive beta-cell apoptosis in islets of

our young NZO mice, leading to similar outcomes, the loss of functional beta-cells. In line with our data, Donath et al. indicated that early hyperglycemia in response to high caloric diets was accompanied by transient beta-cell proliferation and increased beta-cell apoptosis followed by destructed islet morphology in *Psammomys obesus*[295]. Moreover, beta-cell apoptosis under high glucose concentrations has been shown in a series of studies in islets from non-diabetic but also type 2 diabetic patients by Maedler et al.[296-298].

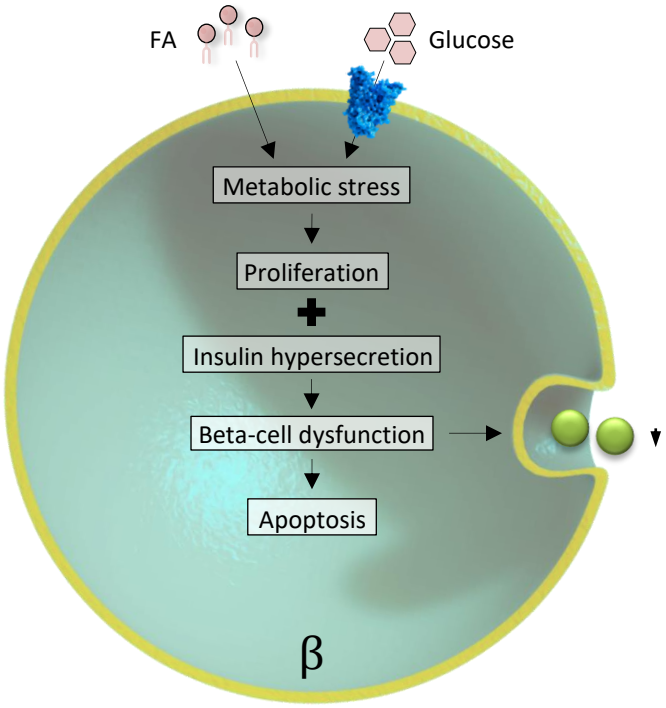

Figure 8 - Effects of metabolic stress in beta-cells of young NZO mice.
Diet-induced metabolic stress leads to increased beta-cell proliferation and insulin hypersecretion. This was insufficient to compensate elevated glucose supply, resulting in beta-cell dysfunction and apoptosis. FA (Fatty acids).

On a molecular level, several mechanisms underlying nutrient-induced beta-cell dysfunction and apoptosis have been proposed in recent years, such as ROS generation, inflammation, UPR and PKC activation or AGE formation[109]. However, most of the mechanisms belong to the induction of ER as well as redox stress, interrelated with each other[110]. Conditions of chronically high insulin demand increase the proinsulin biosynthesis and might overwhelm the ER protein folding potential. Initially, excess ER stimulation is accompanied by elevated circulating insulin but also protein misfolding, leading to the activation of ER stress response via UPR to preserve ER homeostasis. When UPR fails to restore ER homeostasis, pro-inflammatory and pro-apoptotic pathways via different signaling molecules are initiated, inducing beta-cell apoptosis. This indicates the important

role of the ER in maintaining functional beta-cell mass[110,299,300]. However, we did not focus on the impact of diet-induced metabolic stress on ER-mediated beta-cell damage in this study. Nevertheless, it was suggested in a study by Sachdeva et al. that PDX1 transcription factor regulates the susceptibility of beta-cells to ER stress responses. Their findings revealed enhanced vulnerability towards ER stress-related apoptosis by impairing the expression of multiple genes essential for ER homeostasis and the UPR in *Pdx1*-deficient MIN6 cells as well as *Pdx1*-haploinsufficient primary beta-cells[301]. Our data showed decreased beta-cell specific PDX1 levels in young NZO mice paralleled by apoptosis in response to metabolic stress. Although the expression of PDX1 is regulated by various factors, there might be a potential link between declined PDX1 and ER stress in our young NZO mice. Besides this, PDX1 is also targeted by oxidative and nitrosative stress in beta-cells[302]. In general, the induction of beta-cell redox stress occurs mainly via nutrient-mediated mitochondrial overstimulation, leading to the generation of ROS, emerged as one of the major drivers of beta-cell failure. This is underlined by the particular sensitivity of beta-cells towards ROS, because of their reduced anti-oxidative capacity, indicated by low expression levels of detoxifying enzymes, such as superoxide dismutase, catalase or glutathione peroxidase [110,303,304]. Moreover, hydrogen peroxide exposure of rat islets was associated with a decreased DNA binding activity to PDX1, assumed to be mediated by the activation of JNK pathway and enhanced nuclear uptake of FoxO1, thus reducing insulin gene expression[109]. Kluth et al. proposed further that glucolipotoxic stress conditions induce the inhibition of FOXO1-PKB-pathway, resulting in beta-cell apoptosis presumably by increased expression of BAX (BCL2-associated X protein) or reduces insulin syntheses by suppressed expression of PDX1 downstream transcription factors, such as MAFA and NKX6.1 in young NZO mice[242]. On the other hand, PDX1-deficiency has been shown to induce hyperglycemia, impair glucose tolerance and beta-cell secretory potential by the induction of apoptosis and the loss of regular islet morphology[257,305]. Consequently, beta-cell functional decline and apoptosis in young NZO mice after diet-induced metabolic stress seemed to be associated with reduced PDX1 levels. Besides the damaging potential on insulin synthesis, hydrogen peroxide treatment has been also shown to inhibit GSIS in INS-1 or isolated islets of female NMRI mice by induction of impaired mitochondrial membrane potential and decreased intracellular ATP concentration resulting in plasma membrane hyperpolarization[306,307]. An inhibition of ATP generation mediated by ROS has been also

proposed to disturb GSIS by downregulating GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) activity[308,309]. In turn, this induces the accumulation of glycolytic metabolites and the formation of AGEs especially under conditions of hyerpglycemic and metabolic stress, shown to be one of the most important causes for the loss of functional beta-cell mass and the onset and progression of type 2 diabetes[310,311]. Thus, we investigated the impact of AGE formation and its role in diet-induced metabolic stress on beta-cell dysfunction and death (**publication II**). Since the detection of AGEs and their precursors was aggravated after 21 days due to advanced islet destruction, measurements were performed in animals after 7 days of CRD feeding when hyperglycemia was already existent.

Hyperglycemia drives AGE formation and redox stress - a harmful axis contributing to beta-cell dysfunction and apoptosis in young NZO mice

The formation and accumulation of AGEs is an age-related process, as described in **publication I**, but occurs also due to the intake of high AGE-containing foods and in particular in response to chronic hyperglycemia as well as oxidative and nitrosative stress. Under these conditions, AGEs and their precursors are formed at a higher rate via non-enzymatic protein glycation or glucose autoxidation, in turn, promoting redox stress and inflammation via distinct mechanisms[303,310]. In agreement with this, CRD-induced hyperglycemia in young NZO mice was associated with the development of a pro-oxidative and pro-inflammatory environment, indicated by elevated plasma 3-NT levels as well as whole blood GSH (reduced glutathione, Figure 13 - Appendix 2) after 7 or 21 days, respectively (**publication II**). Additionally, young hyperglycemic NZO mice exhibited elevated plasma levels of protein-bound CML, originating from endogenous formation since CML amounts were comparable in both diets. In contrast, CEL-content of the CRD was higher but CEL plasma levels were similar, indicating that neither carbohydrates nor dietary-derived AGEs are affecting plasma CEL levels in young NZO mice. Elevated plasma CML levels accompanied by unchanged CEL levels were shown also by Schindhelm et al. in type 2 diabetic women after a carbohydrate-rich meal. Such differences might be explained by enhanced formation of glyoxal in response to hyperglycemia, shown to be the major precursor for CML generation[171,312].

Besides this, we observed an increased formation of MG-derived arg-pyrimidine as well as pentosidine within pancreatic islets of NZO mice even after 7 days of CRD feeding. Especially the formation of pentosidine within days is of high importance, because it has been shown that this occurs in at least months to years, also supported by our data (**publication I**)[313,314]. Moreover, in contrast to the predominant accumulation of arg-pyrimidine and pentosidine in the vasculature of pancreatic islets during aging (**publication I**), glycation products were found mainly within the endocrine cells in response to hyperglycemia (**publication II**), indicating the high impact of diet-induced metabolic stress conditions on intracellular AGE formation. In line with the current knowledge, increased AGE formation was accompanied by elevated levels of RAGE, suggesting enhanced AGE-RAGE interaction. As discussed earlier, this initiates a pro-inflammatory signaling cascade by NF-κB and NADPH-oxidase activation, among others leading to increased iNOS expression and 3-NT generation. Accordingly, we observed elevated levels of iNOS and 3-NT, indicating that pancreatic islets of young NZO mice are exposed to redox stress conditions. Since these observations were not limited to endocrine cells, it has to be also considered that the islet vasculature is affected by these alterations. AGE-mediated induction of iNOS followed by increased formation of nitric oxide and nitrosative stress has been proposed to suppress the activity of cytochrome c oxidase and the production of ATP, leading to decreased insulin secretion[169]. Recently, it has been also demonstrated that exposure to different concentrations of BSA-AGE (Bovine serum albumin) deteriorates insulin secretion in INS-1-3 cells by activation of p38/MAPK pathway, disturbing microtubule dynamics[315]. Besides this, AGE treatment in different beta-cell lines has been linked to impaired insulin gene transcription, described consistently in two independent studies. Shu et al. demonstrated that AGE treatment initiates dephosphorylation of FOXO1 in rat INS-1 cells, increasing its nuclear accumulation, leading to an inhibition of cellular PDX1 levels that impairs insulin synthesis[286]. HIT-T15 cells, cultured in the presence of AGEs, showed similarly reduced insulin content due to decreased expression of PDX1 and increased dephosphorylation as well as acetylation of FOXO1[172]. Transferred to our data, it might be assumed that the observed AGE formation with simultaneous increase of iNOS and 3-NT (**publication II**) followed by decreased beta-cell specific PDX1 levels (**manuscript**) are associated with reduced insulin secretion and synthesis in pancreatic islets of young NZO mice in response to metabolic stress conditions. However, it has to be considered that

decreased insulin secretion occurred earlier as elevated iNOS and 3-NT were determined, indicating that AGE-mediated redox stress might be contributor rather than initiator of beta-cell functional decline. Moreover, since AGE formation in pancreatic islets (**publication II**) was followed by decreased beta-cell specific PDX1 levels (**manuscript**), AGEs seem to directly affect insulin synthesis in young NZOs.

In addition to the impact on beta-cell function, AGEs have been shown to mediate beta-cell apoptosis by the induction of redox stress conditions in several studies. In INS-1 cells, Lim et al. demonstrated that the incubation with AGEs stimulates the generation of ROS, thus, initiating cellular apoptosis due to the activation of RAGE. In addition, AGE administration in INS-1 cells promotes ROS generation and beta-cell apoptosis driven mainly by mitochondrial ETC and NADPH oxidase but also due to JNK and p38 MAPK signaling pathways[174]. Moreover, it was demonstrated by Zhu et al. that AGE treatment of INS-1 and primary rat islets is characterized by elevated RAGE protein expression, inducing apoptosis by downregulation of *Bcl2* gene and increased cytochrome c release as well as caspase-3 and 9 activation[175]. Similarly, AGE incubation dose-dependently increased RAGE

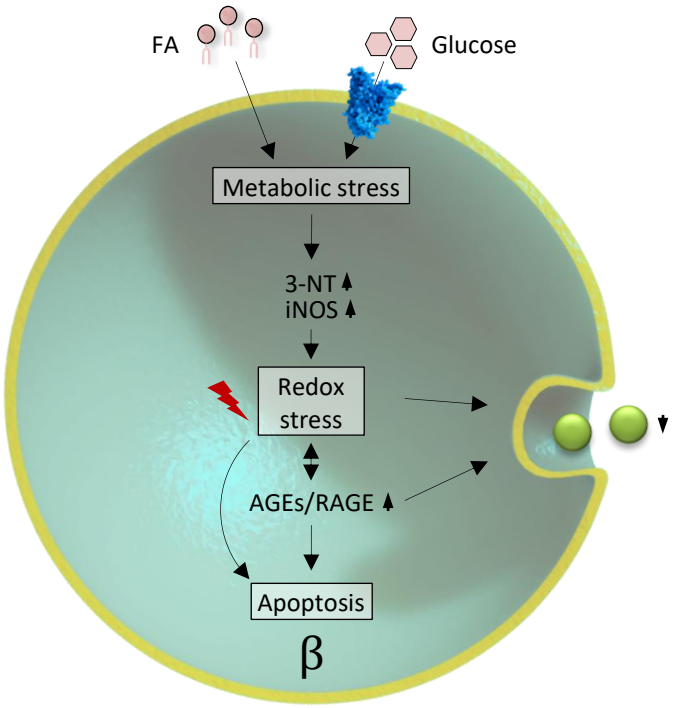

Figure 9 - Redox stress and AGE-mediated beta-cell dysfunction and apoptosis in young NZO mice.
Potential mechanism of metabolic stress-induced beta-cell dysfunction and apoptosis due to oxidative and nitrosative (redox) stress and AGE formation. Increased 3-NT and iNOS levels mediate redox stress associated with the formation of AGEs, in turn, promoting redox stress via elevated RAGE expression and interaction. Both, increased redox stress and AGE formation contribute to decreased insulin synthesis and secretion as well as beta-cell apoptosis in young NZO mice. 3-NT (3-nitrotyrosine), AGEs (Advanced glycation end products), FA (Fatty acids), iNOS (Inducible nitric oxide synthase), New Zealand obese (NZO), RAGE (Receptor for AGEs).

and COX-2 (Cyclooxygenase-2) protein expression as well as NFκB-p65 phosphorylation, as recently described in MS1 islet endothelial cells. This was accompanied by apoptotic cell death, indicated by elevated caspase-3 and PARP (Poly(ADP-ribose)-polymerase) levels[277]. In line with this, we found higher caspase-3 and PARP protein amounts (**manuscript**) that might be associated with the formation of AGEs and simultaneously elevated RAGE levels within NZO islets (**publication II**). Thus, it might be assumed that AGE-RAGE interaction, mediating redox stress, might contribute to increased beta-cell apoptosis in islets of young hyperglycemic NZO mice (Figure 9).

Interestingly, previous investigations have shown that insulin might be a direct target for glycation. Incubation of human insulin with MG *in vitro* lead to the attachment of MG at arginine residues, generating MG-insulin adducts, evidenced by additional peaks observed in mass spectrometric analysis. In turn, this alters the structure and function of insulin, resulting in decreased glucose uptake by reduced insulin receptor-binding in peripheral cells, declined insulin clearance and impaired autocrine control of insulin secretion, finally promoting insulin resistance[157]. Furthermore, a study performed by Abdel-Wahab and colleagues indicated that insulin and proinsulin glycation occurs in pancreatic islets of different animal models[316]. It might be therefore speculated that localization of AGEs directly at the site of insulin origin in endocrine cells and in the islet vasculature (**publication II**), might lead to increased insulin glycation, thus, contributing to glucose intolerance and increased insulin resistance in young NZO mice (**manuscript**).

Taken together, although diet-induced metabolic stress conditions induce minor changes in plasma AGEs, endocrine cells appear to be highly affected by glycation reactions. The generation of redox stress was associated with the formation of AGEs observed in NZO islets accompanied by increased RAGE levels, indicating enhanced AGE-RAGE interaction. In turn, this seems to mediate redox imbalance, in combination contributing to a decline in functional beta-cell mass in young NZO mice.

Hyperglycemia mediates TXNIP-dependent death pathway - a crucial player of beta-cell apoptosis in young NZO mice

Substantial research in recent years emerged TXNIP as one of the major redox-sensitive and pro-apoptotic proteins responsible for beta-cell dysfunction and apoptosis under hyperglycemic conditions[120,317]. Even in 2002, Shalev et al. discovered TXNIP as the highest glucose-induced gene in intact islets of diabetic human donors, by using oligonucleotide microarray analysis[318]. Accordingly, data obtained from our microarray-based transcriptomic analysis identified TXNIP among the top 3 upregulated transcripts in islets of young hyperglycemic NZO mice. Further investigations on mRNA and protein levels confirmed an increase in TXNIP expression even after 2 days of the diet switch. Although circulating glucose levels continued to increase due to prolonged CRD feeding, TXNIP protein expression declined. However, TXNIP expression is known to increase in a glucose-dependent manner, mediated by the transcription factor ChREBP, as described recently[126,128]. ChREBP is one of the major transcription factors in glucose metabolism, triggering the expression of glycolytic, gluconeogenic as well as lipogenic genes[319]. Although ChREBP has been shown to mediate glucose-stimulated beta-cell proliferation, it is associated with detrimental effects on beta-cells under hyperglycemic conditions[130]. Mechanistically, increasing glucose levels induce the dephosphorylation of ChREBP, forming a heterodimer with MLX, in turn, leading to cytosolic-nuclear-shuttling. Within the nucleus, ChREBP-MLX complex interacts with the DNA-ChoRE-region, stimulating the transcription of several target genes, such as TXNIP. In INS-1 cells, ChREBP induces TXNIP expression in response to increasing glucose concentrations by increased binding activity of ChREBP-MLX complex to the TXNIP promoter[131]. Moreover, overexpression of a constitutively active mutant of ChREBP dramatically induces TXNIP expression[320]. In contrast, *Mlxipl* mRNA (Gene symbol of ChREBP: MLXIPL - MLX interacting protein-like) and ChREBP protein expression were reduced in islets of our young NZO mice despite hyperglycemia and elevated TXNIP levels. In accordance with increased circulating glucose levels, but in conflict with decreased ChREBP levels, *Mlx* expression was elevated. In addition, we observed a perinuclear formation of ChREBP in beta-cells of young mice at the end of the CRD intervention, but enhanced TXNIP protein expression was increased already after 2 days. Perinuclear localization of ChREBP indicated further that ChREBP cytosolic-

nuclear-shuttling has not been occurred. Consequently, we found no association between TXNIP and its glucose-dependent mediator ChREBP. Interestingly, a study published recently by Richards et al. revealed that islets of ChREBP-knockout mice upregulated *Txnip* in response to glucose stimulation similarly as their controls did, indicating that ChREBP might be not required for *Txnip* expression. By contrast, they found that the ChREBP homolog, MondoA was the predominant glucose-responsive transcription factor, mediating the expression of *Txnip* in human beta-cells, although this has been assumed to occur only in muscle cells[321,322]. Thus, it might be speculated that MondoA rather than ChREBP is induced in response to elevated circulating glucose levels, which would explain the increase of TXNIP as well as *Mlx* in our young NZO mice under metabolic stress.

The detrimental effects of TXNIP on beta-cell function and survival in response to elevated glucose are mediated via various mechanisms. By using INS-1 cells and beta-cell specific *Txnip* knockout mice, Jing et al. demonstrated that TXNIP promotes the expression of amylin via the downregulation of miRNA-124a, associated with a progressive loss of functional beta-cell mass[323]. Moreover, a recent study by Xu and colleagues identified a novel pathway for TXNIP-induced beta-cell dysfunction in the context of diabetes. By using several *in vivo* and *in vitro* models, they showed that TXNIP induces the expression of miRNA-204, thus, inhibiting insulin secretion and synthesis by the downregulation of *Mafa*[121]. Although this indicates that PDX1, known as upstream regulator of MAFA in beta-cells, might be also targeted by TXNIP, *Pdx1* expression was unaffected, similar to the data by Jing et al.[121,323]. Transferred to our data, it might be speculated that increased *Txnip* mRNA expression is associated with reduced insulin synthesis in young NZO mice.

By binding of redox-active cysteine residues to reduced TXN1, TXNIP suppresses the oxidoreductase activity of TXN1 and contributes to a reduced cellular anti-oxidative capacity, leading to oxidative stress and induction of apoptosis[118,120]. TXN1 serves as negative regulator of pro-apoptotic ASK1 by binding to its N-terminal region, leading to the inhibition of ASK1-mediated apoptosis[324]. Shown in NOD (Non-obese diabetic) as well as *db/db* mice, overexpression of *Txn1* protects beta-cells from hyperglycemia-induced oxidative stress and prevents the progression of type 2 diabetes[325,326]. However, chronic hyperglycemia and oxidative stress increase the expression of TXNIP, leading to reduced TXN1 expression and activity[327,328]. In contrast, our data show increased *Txn1* mRNA levels in young NZO mice despite elevated circulating glucose levels with simultaneous rise in

TXNIP expression, indicating that beta-cell apoptosis is not associated with TXNIP-induced inhibition of *Txn1* in these animals. Increased expression of *Txn1* might rather serve as adaptive response on metabolic stress conditions to maintain cellular anti-oxidative defense of the TXN pathway in our model.

Besides this, TXNIP-induced beta-cell apoptosis occurs by the activation of the mitochondrial death pathway. First described by Saxena et al., TXNIP localized primarily in the cytosol of beta-cells under physiological conditions, is translocated into the mitochondria in response to hyperglycemia and interacts with TXN2. Consequently, TXN2 binding to ASK1 is inhibited, leading to increased ASK1 phosphorylation and activation of apoptotic signaling pathway, shown by enhanced mitochondrial cytochrome c release and caspase-3 cleavage[122]. Similarly, islets of our young NZO mice exhibited decreasing *Txn2* mRNA expression in response to hyperglycemic conditions followed by increased caspase-3 and PARP protein levels. Thus, decreased *Txn2* mRNA expression combined with enhanced capase-3 and PARP protein expression in young NZO mice under metabolic stress, suggest a TXNIP-related induction of the mitochondrial death pathway, inducing beta cell apoptosis (Figure 10).

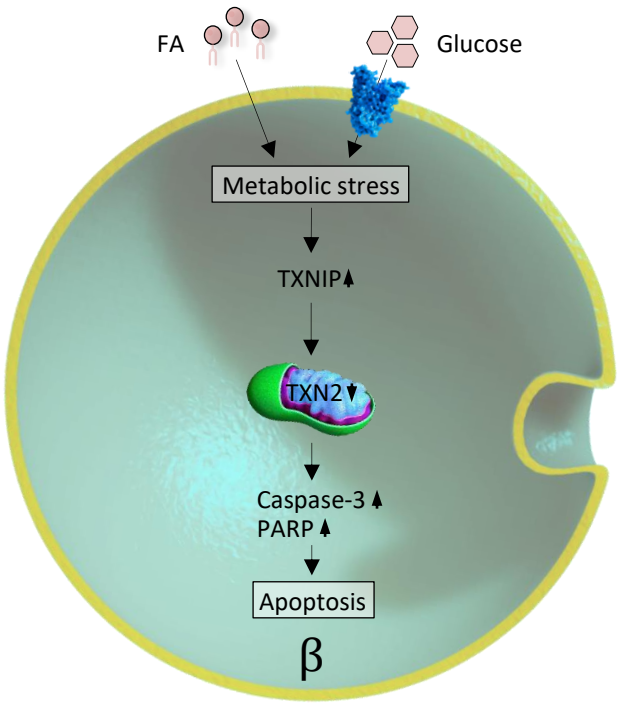

Figure 10 - TXNIP-induced beta-cell apoptosis in young NZO mice.
Possible mechanisms of metabolic stress-induced beta-cell apoptosis by increased expression of TXNIP, leading to reduced mitochondrial *Txn2* expression, elevated caspase-3 and PARP levels. FA (Fatty acids), New Zealand obese (NZO), PARP (Poly(ADP-ribose)-polymerase), TXN (Thioredoxin), TXNIP (Thioredoxin-interacting protein).

Interestingly, TXNIP has been also shown to stimulate the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome, resulting in caspase-1 activation and IL-1β cleavage that might mediate inflammation in beta-cells[120]. Moreover, a recent study by Kong et al. in C57BL/6J mice demonstrated that increased TXNIP-NLRP3 interaction occurs in response to AGE-induced ROS formation, associated with impaired beta-cell function[116]. This implicates a potential link between hyperglycemia-induced formation of AGEs and elevated expression of TXNIP in our study, working in concert at least to drive beta-cell dysfunction. However, to confirm this connection, it has to be determined whether AGE formation occurred even after 2 days of CRD feeding in pancreatic islets of young NZO mice.

In summary, increased TXNIP expression was associated with a decline of functional beta-cells in young NZO mice under metabolic stress conditions, indicated by the activation of the mitochondrial death pathway and presumably due to the interaction with the AGE-ROS pathway.

No adverse effects of aging - middle-aged NZO mice maintain functional beta-cells under metabolic stress conditions

Beta-cell functionality and integrity are not only affected by diet-induced metabolic stress but also due to the aging process. However, the combined effect has received minor scientific attention with controversial outcomes. By using young (14 weeks) and aged (14 months) C57BL/6J mice, He and colleagues revealed that aging exacerbates the consequences of an 8-weeks high-fat diet intervention. While young mice were able to compensate the increased metabolic demand due to elevated beta-cell mass accompanied by mild glucose intolerance and reduced insulin secretion, aged animals exhibited impaired glucose tolerance and decreased beta-cell function and survival[329]. Fontes et al. reported from an age-dependent functional decline in pancreatic islets isolated from Wistar rats exposed to glucolipotoxicity by 72h infusion with glucose and fat emulsion, despite a doubling in beta-cell mass by increased proliferation. However, this study was performed in rats aged 2 and 6 months and the age of the latter group is more comparable to the age our young NZO cohort[330]. Indeed, direct comparison showed various similarities between both groups, such as impaired insulin secretion, reduced insulin content, decreased PDX1 expression as well as increased proliferation. In contrast, Leon et al. found an age-

associated protection of beta-cell function in old (22 months) compared to young (4-6 months) C57BL/6J mice in response to high-fat diet feeding over 4 weeks, although body weight gain and glucose intolerance were enhanced. Beta-cell mass expansion was observed in both age groups but *ex vivo* islet insulin secretion and pancreatic insulin content of young animals were reduced, whereas old mice maintained beta-cell secretory function[331]. Although we used a different dietary regimen, the response towards diet-induced metabolic stress conditions in advanced age of NZO mice was comparable. Prior to CRD exposure, prolonged CFD feeding in NZO mice lead to further body weight gain with increasing obesity and impaired glucose tolerance. This was accompanied by adipocyte dysfunction, shown by reduced plasma adiponectin levels compared to young animals (Figure 14 - Appendix 3), indicating increased peripheral insulin resistance. Interestingly, middle-aged mice exhibited only moderate hyperglycemia in response to CRD feeding although metabolic stress conditions were enhanced. Perifusion experiments revealed that the amounts of secreted insulin were comparable in both age groups, but isolated islets of middle-aged mice were able to adjust appropriately on changing glucose concentrations and prevent insulin hypersecretion. Similar to young mice, islets of mice with advanced age start to proliferate in order to compensate the increase glucose supply. This was followed by continuously increasing plasma insulin and proinsulin levels (Figure 12 - Appendix 1) as well as sustained pancreatic insulin content and PDX1 levels. Furthermore, middle-aged mice revealed unchanged beta-cell- or islet-area without evidence of apoptosis. Thus, NZO mice at middle age were able to prevent massive hyperglycemia by maintaining insulin secretory function, ensuring beta-cell maintenance.

An adaptive response on elevated glucose levels by increased beta-cell proliferation has been shown in a series of studies in murine as well as human material, but the mechanistic link is complex and hardly understood[332,333]. In this context, the amount of circulating glucose is crucial for the downstream effects on beta-cells. At high concentrations and prolonged exposure, glucose initiates pro-inflammatory pathways with a decline in beta-cell mass, whereas moderate concentrations and short-term administration might stimulate beta cell proliferation and growth[334,335]. Transferred to our data, although the islet proliferative potential was similar in both age groups, prolonged hyperglycemic conditions induce apoptosis in young mice, whereas moderate hyperglycemia seemed to stimulate further proliferation in animals at middle age. On a molecular level, several

intracellular signaling pathways have been elucidated, contributing to glucose-induced beta-cell proliferation in humans and rodents, such as the activation of ChREBP transcription factor[333]. However, our data revealed reduced *Mlxipl* mRNA levels and increased *Mlx* expression as well as unchanged ChREBP protein expression, suggesting no association between moderate hyperglycemia and ChREBP activation in islets of middle-aged NZO mice. In this context, TXNIP protein levels were unchanged and even reduced at the end of the study, preventing the induction of its pro-apoptotic downstream mechanisms, indicated by consistent *Txn2* mRNA expression as well as caspase-3 and PARP protein levels. Moreover, *Txn1* mRNA expression was increased in response to the CRD intervention, suggesting that the redox potential as well as the anti-oxidative defense of the TXN pathway was maintained in islets of NZO mice at middle age, similar to young mice that might contribute to the maintenance of functional beta-cells.

Besides ChREBP signaling, the activation of mTOR (Mammalian target of rapamycin), LKB1 (Liver kinase B1) and AMPK (AMP-activated protein kinase) pathways as well as Ca^{2+}-signaling have been shown to induce beta cell proliferation in response to increasing glucose concentrations by several cell cycle activators, such as A, E, D cyclins and CDKs, as reviewed by Bernal-Mizrachi et al[333]. In rodents, cyclin D2 seems to play a particular role in cell cycle progression of beta-cells, because heterozygous cyclin D2 knockout causes glucose intolerance and the progression of type 2 diabetes[205]. Moreover, increased cyclin D2 levels mediate beta-cell proliferation under moderate hyperglycemia in C57BL/6J mice associated with unchanged islet number, beta-cell size and death[336]. In contrast, the relevance of cyclin D2 in human islets is not finally clarified since they showed only minor cyclin D2 expression[332,337]. Our microarray-based transcriptomics revealed no changes in the regulation of cyclin D2 in response to moderate hyperglycemia in islets of middle-aged NZO mice (data not shown). Nevertheless, GO-term enrichment analysis indicated enhanced potential for cell cycle entry and progression. Interestingly, this is comparable with the results found in young diabetes-resistant B6-*ob/ob* mice on the same dietary regimen, published recently by Kluth and colleagues[294]. However, our NZO mice exhibited moderate hyperglycemia, whereas B6-*ob/ob* mice revealed only a transient increase in circulating glucose compensated by elevated islet size and area in response to diet-induced metabolic stress conditions, as shown in a previous study by the same group. The authors further revealed that proliferation was associated with increased expression of *Cdk2* and

cyclin A2, resulting in G1 to S phase transition[293]. In accordance with this, our transcriptomic data also revealed an upregulation in cyclin A2 transcriptional expression (data not shown). Thus, the identification of relevant cell cycle activators associated with beta-cell proliferation is of high importance to mechanistically explain the maintenance of beta-cells in middle-aged NZO mice under metabolic stress.

Another potential player in this scenario might be the TCF19 (Transcription factor 19). Basically, TCF19 was associated with type 1 diabetes, but also observed in obese mouse models, correlating with islet proliferation[338,339]. Moreover, Krautkramer et al. proposed recently an important role of TCF19 in type 2 diabetes. Deficiency of this transcription factor in INS-1 cells results in impaired proliferation by reduced expression of various cell cycle genes with simultaneous increase in ER stress-mediated apoptosis, indicating its role as transcriptional regulator of beta-cell mass[340]. Due to the validation of major differential expressed transcripts by mRNA profiling, we found that *Tcf19* expression was exclusively elevated in middle-aged NZOs in response to the CRD, suggesting its potential role in the ada ptive proliferative response on metabolic stress conditions.

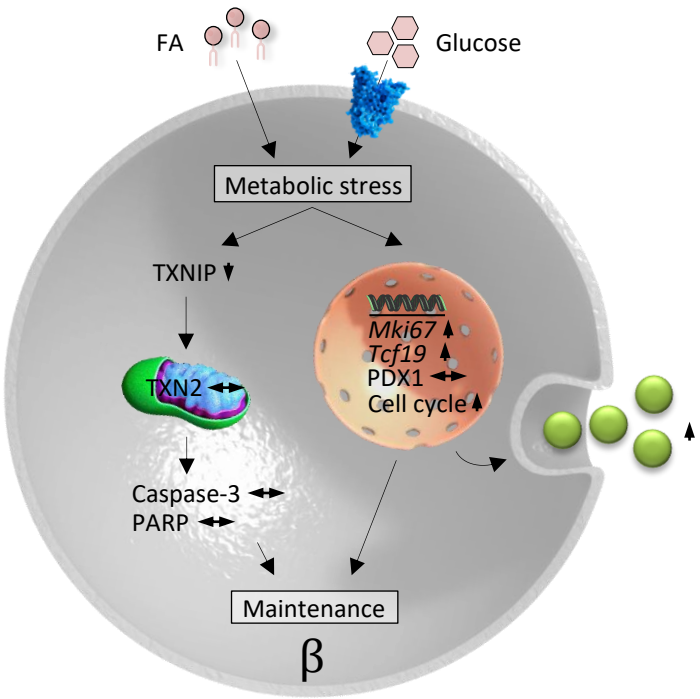

Figure 11 - Mechanisms of beta-cell maintenance in middle-aged NZO mice.
Potential mechanisms of metabolic stress-mediated maintenance of functional beta-cells in middle-aged NZO mice due to increased proliferation and presumably cell cycle progression, indicated by increased *Mki67* and *Tcf19* expression and sustained PDX1 levels. Moreover, TXNIP expression was reduced accompanied by unchanged *Txn2* expression (shown as TXN2), caspase-3 and PARP levels. Ki67 (Antigen identified by monoclonal antibody Ki67), NZO (New zealand obese), PARP (Poly(ADP-ribose)-polymerase), PDX1 (Pancreatic and duodenal homeobox 1), TCF19 (Transcription factor 19), TXN (Thioredoxin), TXNIP (Thioredoxin-interacting protein).

In addition to that, insulin resistance *per se* might be causative for beta-cell proliferation, as indicated by transplantation of wild-type islets into genetically insulin-resistant mice, showing increased proliferation without hyperglycemia[341,342]. In this context, serpin B1, a protein secreted by the liver, might be a potential regulator in this mechanism[343]. In turn, other studies revealed that beta-cell proliferation occurs before insulin resistance develops, as shown in C57BL/6J mice after short-term high-fat diet feeding[344,345]. In agreement with this, our data suggest that insulin resistance was existent earlier as proliferation was observed. Scientific attention has also been directed to the role of epigenetic regulation of beta cell proliferation. Intermediates of glycolysis or beta-oxidation, such as acetyl-coenzyme A, serve as co-substrates for several epigenetically active enzymes, driving proliferation[346,347]. This promising research area might be a target for future investigations. Because NZO mice were only middle-aged, hyperglycemia was moderate, and neither beta-cell dysfunction nor apoptosis was found in middle-aged NZO mice, the formation of AGEs and the generation of oxidative and nitrosative stress as well as the induction of cellular senescence were not addressed so far but should be examined in future experiments.

Taken together, middle-aged NZO mice showed only moderate hyperglycemia by increased proliferation and presumably elevated cell cycle progression accompanied by maintained redox potential that ensure beta-cell functionality and survival. However, it has to be considered that the susceptibility to diet-induced hyperglycemia was heterogeneous. While some animals exhibited only minor elevated circulating glucose, others revealed severe hyperglycemia combined with hyperinsulinemia at the end of the study. Thus, we cannot exclude that beta-cell dysfunction and loss occur after extending the CRD feeding period. Nevertheless, our data indicate that advanced age is not associated with adverse effects on beta-cell function, redox balance and survival in NZO mice under metabolic stress conditions.

Summary and Conclusion

The increasing age of worldwide population is a major contributor for the rising prevalence of major pathologies and disease, such as type 2 diabetes, mediated by massive insulin resistance and a decline in functional beta-cell mass, highly associated with an elevated incidence of obesity[93,177,331,348]. Thus, the impact of aging under physiological conditions and in combination with diet-induced metabolic stress on characteristics of pancreatic islets and beta-cells, with the focus on functionality and structural integrity, were investigated in the present dissertation.

Primarily induced by malnutrition due to chronic and excess intake of high caloric diets, containing large amounts of carbohydrates and fats, obesity followed by systemic inflammation and peripheral insulin resistance occurs over time, initiating metabolic stress conditions[104,349]. Elevated insulin demands initiate an adaptive response by beta-cell mass expansion due to increased proliferation, but prolonged stress conditions drive beta-cell failure and loss[40,350]. Aging has been also shown to affect beta-cell functionality and morphology, in particular by proliferative limitations[184,202]. However, most studies in rodents were performed under beta-cell challenging conditions, such as high-fat diet interventions[329,331]. Thus, in the first part of the thesis (**publication I**), a characterization of age-related alterations on pancreatic islets and beta-cells was performed by using plasma samples and pancreatic tissue sections of standard diet-fed C57BL/6J wild-type mice in several age groups (2.5, 5, 10, 15 and 21 months).

Aging was accompanied by decreased but sustained islet proliferative potential as well as an induction of cellular senescence. This was associated with a progressive islet expansion to maintain normoglycemia throughout lifespan. Moreover, beta-cell function and mass were not impaired although the formation and accumulation of AGEs occurred, located predominantly in the islet vasculature, accompanied by an induction of oxidative and nitrosative (redox) stress.

The nutritional behavior throughout human lifespan; however, is not restricted to a balanced diet. This emphasizes the significance to investigate malnutrition by the intake of high-energy diets, inducing metabolic stress conditions that synergistically with aging might amplify the detrimental effects on endocrine pancreas. Using diabetes-prone NZO mice

aged 7 weeks, fed a dietary regimen of carbohydrate restriction for different periods (young mice - 11 weeks, middle-aged mice - 32 weeks) followed by a carbohydrate intervention for 3 weeks, offered the opportunity to distinguish the effects of diet-induced metabolic stress in different ages on the functionality and integrity of pancreatic islets and their beta-cells (**publication II, manuscript**).

Interestingly, while young NZO mice exhibited massive hyperglycemia in response to diet-induced metabolic stress accompanied by beta-cell dysfunction and apoptosis, middle-aged animals revealed only moderate hyperglycemia by the maintenance of functional beta-cells. The loss of functional beta-cell mass in islets of young mice was associated with reduced expression of PDX1 transcription factor, increased endocrine AGE formation and related redox stress as well as TXNIP-dependent induction of the mitochondrial death pathway. Although the amounts of secreted insulin and the proliferative potential were comparable in both age groups, islets of middle-aged mice exhibited sustained PDX1 expression, almost regular insulin secretory function, increased capacity for cell cycle progression as well as maintained redox potential.

The results of the present thesis indicate a loss of functional beta-cell mass in young diabetes-prone NZO mice, occurring by redox imbalance and induction of apoptotic signaling pathways. In contrast, aging under physiological conditions in C57BL/6J mice and in combination with diet-induced metabolic stress in NZO mice does not appear to have adverse effects on the functionality and structural integrity of pancreatic islets and beta-cells, associated with adaptive responses on changing metabolic demands. However, considering the detrimental effects of aging, it has to be assumed that the compensatory potential of mice might be exhausted at a later point of time, finally leading to a loss of functional beta-cell mass and the onset and progression of type 2 diabetes.

The polygenic, diabetes-prone NZO mouse is a suitable model for the investigation of human obesity-associated type 2 diabetes. However, mice at advanced age attenuated the diabetic phenotype or do not respond to the dietary stimuli. This might be explained by the middle age of mice, corresponding to the human age of about 38-40 years, in which the compensatory mechanisms of pancreatic islets and beta cells towards metabolic stress conditions are presumably more active.

Zusammenfassung und Schlussfolgerung

Das steigende Alter der Weltbevölkerung ist ein wesentlicher Faktor für die zunehmende Prävalenz bedeutsamer Pathologien und Krankheiten, wie dem Typ-2-Diabetes, der durch eine massive Insulinresistenz und eine Abnahme der funktionellen Beta-Zellmasse hervorgerufen wird und in hohem Maße mit einem verstärkten Auftreten von Fettleibigkeit assoziiert ist. Daher wurde in der vorliegenden Dissertation der Einfluss der Alterung unter physiologischen Bedingungen und in Kombination mit ernährungsbedingtem, metabolischem Stress auf die Eigenschaften von Langerhans-Inseln und Beta-Zellen, mit dem Schwerpunkt auf Funktionalität und strukturelle Integrität, untersucht.

Primär induziert durch Fehlernährung infolge des chronischen und übermäßigen Konsums von kalorienreichen Diäten, die große Mengen an Kohlenhydraten und Fetten enthalten, kann Adipositas, gefolgt von systemischen Entzündungen und peripherer Insulinresistenz, im Laufe des Lebens entstehen und metabolische Stresszustände auslösen. Daraus resultiert ein erhöhter Insulinbedarf, der eine adaptive Reaktion durch die Vergrößerung der Beta-Zellmasse infolge gesteigerter Proliferation auslöst. Längere Stressbedingungen führen hingegen zu Schäden an und Verlust von Beta-Zellen. Es wurde zudem gezeigt, dass das Altern die Funktionalität und Morphologie von Beta-Zellen, insbesondere durch proliferative Limitationen, beeinflusst. Die meisten Studien in Nagetieren wurden jedoch unter erhöhten Stressbedingungen für Beta-Zellen, beispielsweise durch die Fütterung von Hochfett-Diäten, durchgeführt. Deshalb wurde im ersten Teil der Arbeit (**Publikation I**) eine Charakterisierung von altersbedingten Veränderungen auf die Langerhans-Inseln und Beta-Zellen unter Verwendung von Plasmaproben und Pankreasgewebeschnitten von C57BL/6J-Wildtyp-Mäusen verschiedener Altersgruppen (2,5; 5; 10; 15 und 21 Monate), die mit einer Standarddiät gefüttert wurden, durchgeführt.

Das Altern ging mit einem reduzierten, jedoch anhaltenden Proliferationspotential der Langerhans-Inseln sowie einer Induktion der zellulären Seneszenz einher. Dies war mit einem fortschreitenden Wachstum der Langerhans-Inseln verbunden, um eine Normoglykämie während der gesamten Lebensdauer aufrechtzuerhalten. Zudem wurden die Beta-Zell-Masse und die Funktionalität nicht beeinträchtigt, obwohl eine Bildung und

Akkumulation von AGEs, die vorwiegend im Gefäßsystem der Langerhans-Inseln lokalisiert und von einer Induktion von oxidativem und nitrosativem (redox) Stress begleitet war, auftrat.

Das Ernährungsverhalten während der gesamten Lebensspanne ist jedoch nicht auf eine ausgewogene Ernährung beschränkt. Dies unterstreicht die Bedeutung der Untersuchung von Fehlernährung durch die Einnahme energiereicher Diäten, wodurch metabolische Stresszustände induziert werden, die synergistisch mit dem Altern schädigende Effekte auf das endokrine Pankreas verstärken können. Verwendet wurden 7 Wochen alte, zur Entwicklung von Typ-2-Diabetes neigende NZO-Mäuse, die unterschiedlich langen kohlenhydratrestriktiven Fütterungsperioden (junge Mäuse - 11 Wochen, Mäuse mittleren Alters - 32 Wochen), gefolgt von einer 3-wöchigen Kohlenhydratintervention ausgesetzt waren. Dadurch konnten die Auswirkungen von ernährungsbedingtem metabolischem Stress auf die Funktionalität und Integrität von Langerhans-Inseln und deren Beta-Zellen in verschiedenen Altersstufen untersucht werden (**Publikation II, Manuskript**).

Interessanterweise zeigten junge NZO-Mäuse eine massive Hyperglykämie als Reaktion auf den ernährungsbedingten, metabolischen Stress was von Beta-Zelldysfunktion und Apoptose begleitet war. Tiere mittleren Alters zeigten hingegen nur eine moderate Hyperglykämie durch den Erhalt funktioneller Beta-Zellen. Der Verlust funktioneller Beta-Zellmasse in jungen Mäusen war mit einer verminderten Expression des PDX1-Transkriptionsfaktors, einer erhöhten endokrinen AGE-Bildung und damit verbundenem Redox Stress sowie einer TXNIP-abhängigen Induktion des mitochondrialen Apoptosewegs verbunden. Obwohl die Mengen an sekretiertem Insulin sowie das Proliferationspotential in beiden Altersgruppen vergleichbar waren, zeigten die Langerhans-Inseln der Mäuse im mittleren Alter eine anhaltende PDX1-Expression, eine nahezu reguläre Insulinsekretionsfunktion, eine erhöhte Kapazität für das Fortschreiten des Zellzyklus sowie einen Erhalt des Redoxpotentials.

Die Ergebnisse der vorliegenden Arbeit zeigen einen Verlust funktioneller Beta-Zellmasse bei jungen, diabetogenen NZO-Mäusen, der durch ein Redox-Ungleichgewicht und die Induktion apoptotischer Signalwege verursacht wurde. Im Gegensatz dazu scheint das Altern unter physiologischen Bedingungen bei C57BL/6J-Mäusen und in Kombination mit ernährungsbedingtem metabolischem Stress bei NZO-Mäusen keine nachteiligen Auswirkungen auf die Funktionalität und strukturelle Integrität von Langerhans und Beta-

Zellen zu haben, was mit adaptiven Reaktionen auf wechselnde Stoffwechsel-anforderungen assoziiert war. In Anbetracht der negativen Auswirkungen der Alterung muss jedoch berücksichtigt werden, dass das Kompensationsverhalten von Mäusen zu einem späteren Zeitpunkt erschöpft sein könnte, was schließlich zu einem Verlust der funktionellen Beta-Zellmasse und dem Auftreten und Fortschreiten von Typ-2-Diabetes führt.

Die polygene, zu Typ-2-Diabetes neigende NZO-Maus ist ein geeignetes Modell für die Untersuchung von mit Adipositas-assoziiertem Typ-2-Diabetes beim Menschen. Mäuse im fortgeschrittenen Alter zeigten jedoch einen verminderten diabetischen Phänotyp oder reagierten nicht auf die diätetischen Reize. Dies könnte durch das mittlere Alter der Mäuse erklärt werden, das dem menschlichen Alter von etwa 38 bis 40 Jahren entspricht, in dem die Kompensationsmechanismen von Langerhans-Inseln und Beta-Zellen gegenüber metabolischen Stressbedingungen möglicherweise aktiver sind.

Outlook

A better understanding of beta-cell dysfunction and apoptosis prior to and during the development of type 2 diabetes, dependent on potent risk factors, such as aging and nutrition, will directly affect future directions of disease treatment. Therefore, the present doctoral thesis was performed to improve current knowledge of age-related alterations in combination with diet-induced metabolic stress on pancreatic islet and beta-cell functionality and integrity. Since TXNIP appeared to mediate the loss of functional beta-cell mass in young hyperglycemic NZO mice, the relevance of this protein in human beta-cell apoptosis in the context of type 2 diabetes should be investigated in further studies. Moreover, the elevated TXNIP expression in response to hyperglycemia was not mediated by the transcription factor ChREBP, but is potentially connected with an activation of its homolog MondoA. Future investigations on this transcription factor should follow to explain the underlying mechanisms of increased TXNIP expression in NZO mice, potentially identifying a novel role of MondoA in murine pancreatic beta-cells. Based on the finding that aging combined with diet-induced metabolic stress had no adverse effects on pancreatic islets and beta-cells of NZO mice presumably due to adaptive proliferation, leading to beta-cell maintenance, the identification of pivotal cell cycle activators should be one major target for future experiments. Moreover, NZO mice did not develop the diabetic phenotype at advanced age in response to the used dietary regimen, as described also for other mouse models. However, since older adults represent the fastest growing segment of diabetes patients, the development and establishment of further suitable models for the investigation of type 2 diabetes in aging should be in the focus of future research. The gained knowledge could help to design novel treatments and therapies to prevent the increasing prevalence and incidence of type 2 diabetes as well as reduce the growing public health burden in our aging society.

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Appendix

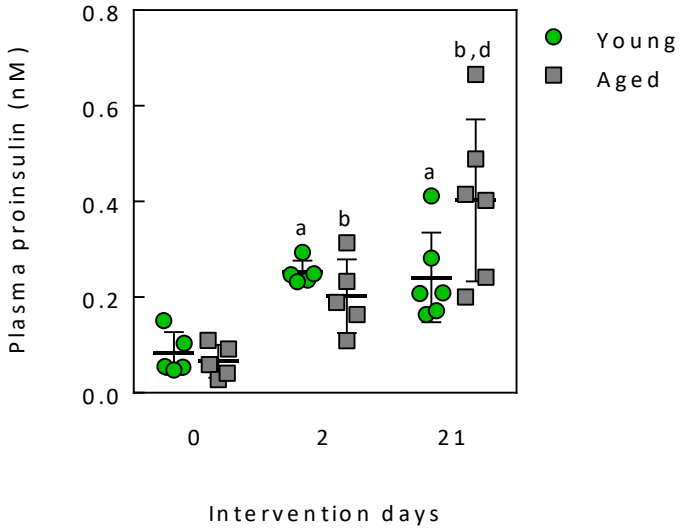

Figure 12 - Appendix 1.
Plasma proinsulin levels of young (green) and aged (grey) NZO at indicated time points (n = 5). Data are represented as mean ± SD. Statistical significance was assessed by Two-way ANOVA with Sidak´s multiple comparison test, $^{a,b,d}p < 0.05$ (a = significant to young 0, b = significant to aged 0, d = significant to aged 2).

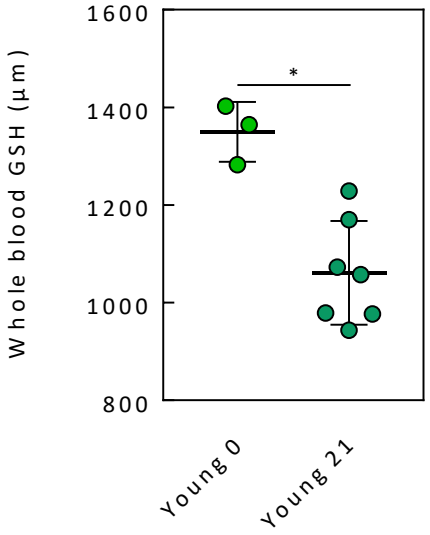

Figure 13 - Appendix 2.
Whole blood GSH levels of young NZO mice before (light green) and after 21 days (dark green) of the CRD intervention (n = 3-7). Data are represented as mean ± SD. Statistical significance was assessed by Mann-Whitney-U-Test (unpaired), $^{*}p < 0.05$.

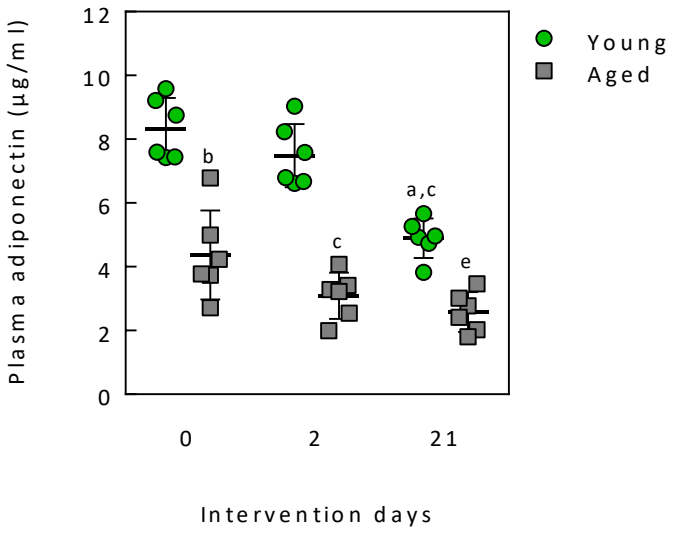

Figure 14 - Appendix 3.
Plasma adiponectin levels of young (green) and aged (grey) NZO at indicated time points (n = 6). Data are represented as mean ± SD. Statistical significance was assessed by Two-way ANOVA with Sidak´s multiple comparison test, $^{a,b,c,e}p < 0.05$ (a = significant to young 0, b = significant to aged 0, c = significant to young 2, e = significant to young 21).

List of publications

1. **“Happily (n)ever after: Aging in the context of oxidative stress, proteostasis loss and cellular senescence.”**
   Annika Höhn, Daniela Weber, Tobias Jung, Christiane Ott, Martin Hugo, Bastian Kochlik, **Richard Kehm**, Jeannette König, Tilman Grune and José-Pedro Castro
   Redox Biol. 2017 Apr;11:482-501.

2. **"Cyt/Nuc," a Customizable and Documenting ImageJ Macro for Evaluation of Protein Distributions Between Cytosol and Nucleus.”**
   Tilman Grune, **Richard Kehm**, Annika Höhn and Tobias Jung
   Biotechnol J. 2018 May;13(5).

3. **“Age-related oxidative changes in pancreatic islets are predominantly located in the vascular system.”**
   Richard Kehm, Jeannette König, Kerstin Nowotny, Tobias Jung, Stephanie Deubel, Sabrina Gohlke, Tim Julius Schulz and Annika Höhn
   Redox Biol. 2018 May;15:387-393.

4. **“Endogenous advanced glycation end products in pancreatic islets after short-term carbohydrate intervention in obese, diabetes-prone mice.”**
   Richard Kehm, Jana Rückriemen, Daniela Weber, Stefanie Deubel, Tilman Grune and Annika Höhn
   Nutr Diabetes. 2019 Mar 11;9(1):9.

5. **"Metformin attenuates the onset of non-alcoholic fatty liver disease and affects intestinal microbiota and barrier in small intestine."**
   Annette Brandt, Angélica Hernández-Arriaga, **Richard Kehm**, Victor Sánchez, Cheng Jun Jin, Anika Nier, Anja Baumann, Amélia Camarinha-Silva and Ina Bergheim
   Sci Rep. 2019 Apr 30;9(1):6668.

6. **"Age-dependent maintenance of pancreatic beta-cells is mediated by Thioredoxin-interacting protein (Txnip) in obese, diabetes-prone mice under glucolipotoxic stress."**
   Richard Kehm, Markus Jähnert, Jeannette König, Stefanie Deubel, Mandy Stadion, Wenke Jonas, Annette Schürmann, Tilman Grune and Annika Höhn
   Prepared for submission

Scientific contributions

"The role of aging and senescence on pancreatic β-cell function and proliferation" (Poster)

Richard Kehm, Oliver Kluth, Annette Schürmann, Tilman Grune and Annika Höhn

Society of Free Radical Research (SFRR)-Europe

June 2017, Berlin, Germany

"Old NZO mice are protected against the carbohydrate-induced loss of β-cells" (Poster)

Richard Kehm, Oliver Kluth, Annette Schürmann, Tilman Grune and Annika Höhn

German center for diabetes research (DZD) workshop

October 2017, Munich, Germany

"The impact of age-related alterations on pancreatic islets" (Poster, poster price - "Antioxidants")

Richard Kehm, Daniela Weber, Annika Höhn and Tilman Grune.

Society of Free Radical Research (SFRR)-Europe, SFRR-E Summer School 2018

September 2019, Spetses, Greece

"Aged New Zealand Obese (NZO) mice are protected against diet-induced loss of beta-cells" (Poster)

Richard Kehm, Oliver Kluth, Annette Schürmann, Tilman Grune and Annika Höhn

54th European Association for the Study of diabetes (EASD) Annual Meeting

October 2019, Berlin, Germany

Curriculum Vitae

Die Seite 13 (Curriculum Vitae) enthält persönliche Daten. Sie ist deshalb nicht Bestandteil der Online-Veröffentlichung.

Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation ohne fremde Hilfe und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe. Die Arbeit wurde keiner anderen Prüfungsbehörde vorgelegt.

Nuthetal, den _____

Richard Kehm