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MAARJA TOOTS

Pharmacological challenge in rodent models of Wolfram syndrome with emphasis on diabetic phenotype

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Pharmacological challenge in rodent models of Wolfram syndrome with emphasis on diabetic phenotype

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Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

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Supervisors: Mario Plaas, PhD, Head of Laboratory Animal Centre,
Laboratory Animal Centre,
Institute of Biomedicine and Translational Medicine,
University of Tartu, Estonia

Eero Vasar, MD, PhD, Professor, Department of Physiology,
Institute of Biomedicine and Translational Medicine,
University of Tartu, Estonia

Reviewers: Tambet Tõnissoo, PhD, Associate Professor,
Institute of Molecular and Cell Biology,
University of Tartu, Estonia

Kalle Kilk, PhD, Senior Research Fellow,
Department of Biochemistry,
Institute of Biomedicine and Translational Medicine,
University of Tartu, Estonia

Opponent: Margit Mahlapuu, PhD, Associate Professor,
University of Gothenburg, Gothenburg, Sweden

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Contribution of the author:

I The author participated in designing the study, performed the animal experiments and biochemical analyzes (jointly with R. Reimets), wrote the manuscript, and handled the correspondence.

II The author extracted Langerhans islets for gene expression analyzes, extracted RNA, carried out quantitative real-time PCR from islets (jointly with T. Koppel), measured Langerhans islet mass from histological slices (jointly with M. Plaas and A. Terasmaa), and participated in writing the manuscript.

III The author participated in designing the study and performing the animal experiments, prepared the histological slices and measured Langerhans islet mass from histological slices (jointly with K. Seppa), performed biochemical analyzes, extracted Langerhans islets for gene expression analyzes, extracted RNA, carried out quantitative real-time PCR from islets, counted axons from electron microscopy slices (jointly with T. Jagomäe), and wrote the manuscript.

ABBREVATIONS

Ach	–	Acetylcholine
ANOVA	–	Analysis of variance
Atf4	–	Activating transcription factor 4 gene
Bax	–	Bcl-2-like protein 4 gene
Bcl2	–	B-cell lymphoma 2 gene
BSA	–	Bovine serum albumin
Carb	–	Carbachol, Carbamoylcholine chloride
Ca^{2+}	–	Calcium ion
cAMP	–	Cyclic adenosine monophosphate
cDNA	–	Complementary deoxyribonucleic acid
DNA	–	Deoxyribonucleic acid
DPP-4	–	Dipeptidyl peptidase-4 protein
ER	–	Endoplasmic reticulum
GIP	–	Glucose-dependent insulinotropic peptide
Glc	–	D-(+)-glucose
GLP-1	–	Glucagon-like peptide-1
GLP-1R	–	Glucagon-like peptide-1 receptor
GPCR	–	G-protein coupled receptor
Grp78	–	Binding immunoglobulin protein (BiP) gene
GSIS	–	Glucose stimulated insulin secretion
GWAS	–	Genome-wide association studies
HET	–	*Wfs1* mutation heterozygous carrier
Hprt1	–	hypoxanthine phosphoribosyltransferase 1 gene
i.p	–	Intraperitoneal injection
IPGTT	–	Intraperitoneal glucose tolerance test
IP10	–	Interferon gamma-induced protein 10 gene
IP3	–	Inositol trisphosphate
ITT	–	Insulin tolerance test
Ki67	–	Antigen KI-67 gene
KO	–	*Wfs1* mutation homozygous animal
KO Lira	–	*Wfs1* exon 5 knock-out rat on liraglutide treatment
KO Sal	–	*Wfs1* exon 5 knock-out rat on saline (0.9% NaCl) treatment
mRNA	–	Messenger ribonucleic acid
M3R	–	Muscarinic receptor 3
PB	–	Phosphate buffer
PFA	–	Paraformaldehyde
PKC	–	Protein kinase C
qRT-PCR	–	Quantitative real-time PCR
s.c	–	Subcutaneous injection
SEM	–	Standard error of the mean
T2D	–	Type 2 diabetes mellitus
WFS1	–	Wolframin1 gene in humans

Wfs1 – Wolframin1 gene in mice and rats
WFS1 – Wolframin1 protein
WS – Wolfram syndrome
WS2 – Wolfram syndrome 2
WT – Wild-type mouse/rat
WT Lira – Wild-type rat on liraglutide treatment
WT Sal – Wild-type rat on saline (0.9% NaCl) treatment
Xbp1 – X-box binding protein 1 gene
ZNF – Zinc-finger nuclease

INTRODUCTION

Wolfram syndrome (WS) is an autosomal recessive disorder, caused by mutations in Wolframin1 (*WFS1*) gene. WS is characterized by diabetes mellitus, diabetes insipidus, optic nerve atrophy, deafness and neural degeneration (Barrett & Bundey, 1997; Barrett *et al*., 1995; Inoue *et al*., 1998).

The first symptom to manifest in patients with WS is non-autoimmune diabetes, resulting from apoptosis of insulin secreting beta cells (Barrett *et al*., 1995; Urano, 2016). Onset of diabetes is dependent of the type of mutation in *WFS1* gene: earliest onset of diabetes with complete loss of function mutations, later onset with partial or minor loss of function mutations (Rohayem *et al*., 2011). Diabetes is usually followed by loss of vision due to optic nerve atrophy, diabetes insipidus, endocrine and neurological problems (Barrett *et al*., 1995).

WS prevalence is estimated between 1 in 770 000 in UK (Barrett *et al*., 1995) and 1 in 100 000 in North America (Fraser & Gunn, 1977). However, the carrier frequency is approximately 1 in 354 in UK (Barrett *et al*., 1995) and 1 in 100–200 in USA (Swift *et al*., 1991). In addition to rare *WFS1* mutations, causing autosomal dominant diabetes (Bonnycastle *et al*., 2013), genome-wide association studies (GWAS) have found that the carriers of heterozygous *WFS1* mutation have an increased risk for development of type 2 diabetes (Cheurfa *et al*., 2011; Franks *et al*., 2008; Han *et al*., 2010; Sandhu *et al*., 2007). Type 2 diabetes mellitus is an expanding health problem all over the world, counting for more than 90% of diabetes cases (DeFronzo *et al*., 2015). Type 2 diabetes is caused by combination of lifestyle choices and genetic predisposition, leading to decreased insulin sensitivity and secretion (DeFronzo *et al*., 2015; Olokoba *et al*., 2012). Therefore, deeper knowledge of WFS1 function is useful not only for patients suffering from rare WS, but also for patients suffering from more common disorders associated with the development type 2 diabetes. For example, significant amount of patients suffering from major psychiatric disorders (schizophrenia, bipolar disorder and major depressive disorder) develop during the course of disease severe insulin resistance and glucose intolerance (Mezuk *et al*., 2008; Vancampfort *et al*., 2016). Moreover, all these psychiatric disorders are associated with the mutations of *WFS1* gene (Koido *et al*., 2005; Swift *et al*., 1998).

Currently there is no cure or medications to delay the progression of WS, therefore treatment is mainly focused on relieving the symptoms (Urano, 2016), until death in the third decade of life (Barrett *et al*., 1995). Therefore, the main objectives of the current thesis were to identify new treatment strategies and possible drug candidates for the treatment of WS. For the development of treatment strategies, both *Wfs1* exon 8 knock-out mice and new *Wfs1* exon 5 knock-out rats were used.

Insulin secretion is modulated by many hormones and neurotransmitters including acetylcholine, the major neurotransmitter of the peripheral para-sympathetic nervous system. Beta cell M3 muscarinic receptors play a key role in maintaining proper insulin release and glucose homeostasis (Duttaroy *et al*.,

2004; Gautam *et al*., 2006). Therefore, the first aim of the current thesis was to describe the insulin secretion in one of the mouse models of WS and to assess the possibility to normalize insulin secretion by the activation of M3 receptors. The results confirm the changes in insulin secretion in both homozygous and heterozygous *Wfs1* mutation carriers. However, pharmacological activation of M3 receptors with carbachol is able to activate insulin release from beta cells and normalize blood glucose concentration in genetically modified mice. Therefore, activation of M3 or possibly other GPCR-s that use similar intracellular signaling cascades is a promising treatment strategy in diabetes patients with *WFS1* mutations.

Unlike human WS patients, none of the current mouse models exhibit overt diabetes with basal hyperglycemia (Ishihara *et al*., 2004; Noormets *et al*., 2011; Riggs *et al*., 2005), therefore there are some physiological limits in using them in drug discovery experiments. In an attempt to generate a model that better mimics the development of diabetes in human patients, a new Wfs1 deficient rat model was created and validated as a model of WS. In the current thesis, the description of development of diabetic phenotype in Wfs1 deficient rats is presented. Results show that progressing decrease in Langerhans islet mass, most likely due to increased endoplasmic reticulum stress, leads to development of insulin dependent diabetes. This is the first rodent model of WS to exhibit overt diabetes and clear progression of most of the WS symptoms.

GLP-1 (glucagon-like peptide-1) receptor agonists have been accepted as a promising class of anti-diabetic drugs, shown to regulate glucose homeostasis, metabolism, and neural survival directly on tissues expressing GLP-1 receptors, and indirectly through neuronal and endocrine pathways (Bockaert & Pin, 1999; Persaud, 2017). The GLP-1 agonist Exendin-4 was shown to reduce endoplasmic reticulum stress in cardiac myocytes (Younce *et al*., 2013); early intervention with the GLP-1 receptor agonist liraglutide improved glucose tolerance and reduced beta cell apoptosis in prediabetic Goto-Kakizaki rats (Luo *et al*., 2013). Also, acute treatment with the GLP-1 agonist exenatide has shown a promising anti-diabetic effect in Wfs1 knock-out mice (Sedman *et al*., 2016).

After describing a clear evolution of the diabetic phenotype in Wfs1 deficient rats, an attempt to prevent the development of diabetes in these rats was taken by pharmacological means. For that purpose, treatment with GLP-1 receptor agonist liraglutide was started before the onset of first WS symptoms. As described in current thesis, 4.5 months of preventive chronic treatment with liraglutide was able to preserve normal glucose tolerance and inhibit the loss of Langerhans islet mass, by improving the overall islet health and hormone secretion.

As mentioned above, there is no cure or medications to delay the progression of WS (Urano, 2016). In the current study, a GLP-1 analogue displays a clear therapeutic effect to prevent or considerably hinder the development of diabetic symptoms in Wfs1 deficient rats shown for the first time. We believe that early diagnosis of WS and treatment with GLP-1R agonists may prove to also be effective in human medicine by significantly improving and prolonging the life of WS patients.

1. REVIEW OF LITERATURE

1.1. Blood glucose regulation

Glucose is used by tissues as the preliminary source of energy. While most tissues are also able to use alternative sources of energy (lipids, proteins), with a normal diet, neurons rely solely on glucose. Because of that, hypoglycemia can result in neuronal death, altered consciousness, coma, and death (Yamada *et al*., 2005). While even short-term low blood glucose levels can be life threatening, long-term hyperglycemia also causes neurological problems and damages many other tissues. To keep blood glucose levels in a specific narrow range, hormones and other signaling molecules are constantly at work, to increase or decrease blood glucose levels (Guemes *et al*., 2016).

Blood glucose is mainly increased by two processes: absorbance of glucose from the intestine after a meal and release of glucose stored in the liver. Glucose in the liver is stored as polysaccharide glycogen. Between meals, hormone glucagon functions as a signaling molecule to release stored glycogen through the process of glycogenolysis in response to the lowering of blood glucose level. Glucose absorbed during a meal or released from the liver increases blood glucose levels, thereby activating insulin secretion from pancreatic beta cells. Insulin stimulates glucose pick-up by cells, thereby lowering blood glucose levels (Elrick *et al*., 1956; Vranic *et al*., 1975).

1.2. Insulin secretion

Langerhans islets of the pancreas are micro-organs, consisting of several different cell types. Most important and numerous are glucagon secreting alpha cells (up to 20% of islet cells) and insulin secreting beta cells (more than half of islet cells) (Elayat *et al*., 1995).

Insulin is synthesized in beta cells as precursor protein pre-pro-insulin. Pre-pro-insulin is cleaved, folded and added three disulfide bonds and then cleaved again to form insulin and C-peptide (Patzelt *et al*., 1978; Huang *et al*., 1995). Insulin and C-peptide are then stored in secretory granules and co-secreted in equimolar concentrations (Katz & Rubenstein, 1973). In response to glucose, insulin is first secreted in substantial amounts during a short period of time; afterwards, lower level of insulin is secreted during a longer second phase (Henquin, 2000).

Glucose is the most important regulator of insulin secretion from pancreatic beta cells. For glucose stimulated insulin secretion (GSIS), glucose is transported into pancreatic beta cells and metabolized, introducing a cascade of events, leading to an influx of Ca^{2+} through voltage operated Ca^{2+} channels and the activation of exocytosis machinery (Henquin, 2000; Milner & Hales, 1967).

Insulin secretion can be further potentiated or inhibited by different hormones and neurotransmitters via their effect on cytosolic Ca^{2+} and cAMP levels (Figure 1). This secondary regulation is defective in individuals suffering from type 2 diabetes (Del Guerra *et al*., 2005).

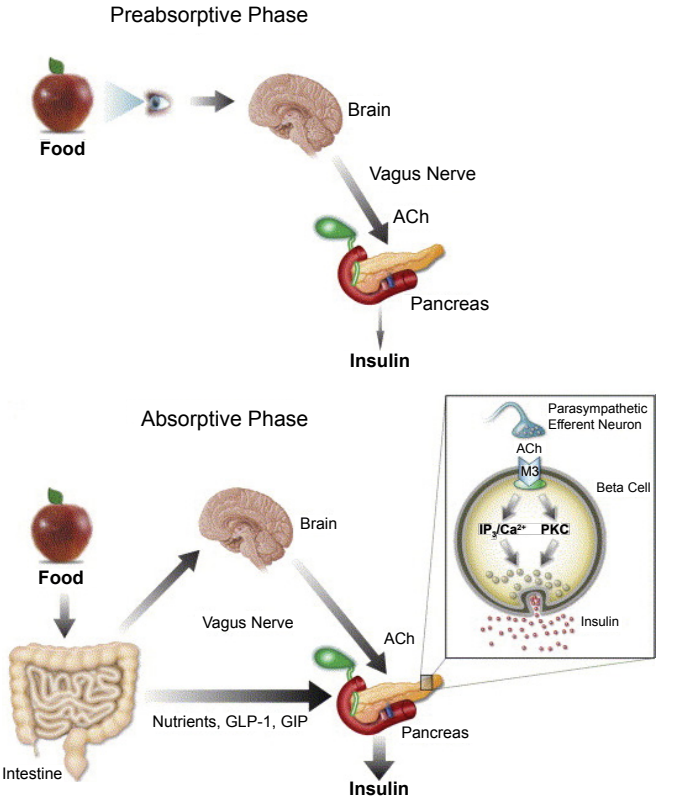

Figure 1. Insulin secretion stimulation by Gromada and Hughes, 2006; modified by Toots. Ach – acetylcholine; GLP-1 – Glucagon-like peptide-1; GIP – Glucose-dependent insulinotropic peptide; IP3 – Inositol trisphosphate; Ca^{2+} – Calcium ion; PKC – Protein kinase C.

First signals to prepare for the increased need for insulin are transmitted through para-sympathetic pathways in response to visual and olfactory stimuli. Acetylcholine, released from the vagal nerve, binds to muscarinic receptors on the surface of beta cells, thereby initiating insulin secretion and amplifying GSIS. In small amounts, there are various subtypes of muscarinic receptors present on beta cells, but muscarinic 3 receptor (M3R) is shown to be the most prominent in the regulation of insulin secretion. Mice lacking M3R have lower serum insulin levels and blunted insulin secretion in response to glucose administration. This effect is also apparent in heterozygous M3R deficient mice, further demonstrating the importance of M3R signaling in insulin secretion (Duttaroy *et al*., 2004).

Nutrients absorbed in the gut stimulate the release of incretin hormones. Incretin hormones are secreted by gut endocrine cells to potentiate insulin release after nutrient intake. Most important of these hormones are glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). Binding of these hormones to their respective receptors on beta cells amplifies GSIS (Parker *et al*., 2014; Reimann & Gribble, 2016).

1.2.1. Glucagon-like peptide-1

Incretin hormone glucagon-like peptide-1 (GLP-1) is mainly secreted in response to meal ingestion by intestinal epithelial endocrine L-cells. In L-cells, pro-glucagon is cleaved to glicentin, oxyntomodulin, GLP-1, GLP-2, and intervening peptide 2. GLP-1 affects different cells in manners, still being identified. In Langerhans islets, GLP-1 does not only increase insulin and decrease glucagon secretion in glucose dependent manner, but also regulates beta cell glucose sensitivity, proliferation and apoptosis. Outside pancreas, GLP-1 increases hepatic glucose disposal and glycogen synthesis by muscles, therefore decreasing blood glucose levels (Campbell & Drucker, 2013).

1.3. Type 2 diabetes

Type 2 diabetes (T2D) is the most common form of diabetes mellitus. While type 1 diabetes results from autoimmune destruction of insulin secreting beta cells, type 2 diabetes develops as a result of the combined effect of lifestyle choices and genetic predisposition, leading to decreased insulin sensitivity and secretion. The main lifestyle factor in T2D development is obesity, with inactivity, alcohol consumption and environmental toxins also having contributing roles (Hu *et al*., 2001; Wild *et al*., 2004). In addition to environmental factors, there is also a strong genetic component in the development of T2D, as having relatives with T2D significantly increases a person's risk of also developing T2D. Genome wide association studies (GWAS) have revealed several genes associated with T2D. These genes include for example several transcription factors, subunits for voltage gated K-channel, and cation transporters (Han *et al*., 2010; McCarthy, 2010).

Different functions of these genes also reflect the complicated etiology of T2D. While T2D is generally characterized with insulin resistance, declining insulin secretion, and eventual pancreatic beta cell failure, the severity and progression of the disease is very different between patients (Ahlqvist *et al*., 2018; Fujioka, 2007). This heterogeneity was recently, to some extent, explained by a Swedish research group, identifying five clusters of patients with diabetes, which had different patient characteristics and risk of diabetic complications (Ahlqvist *et al*., 2018). In their analysis, no genetic loci was associated with all clusters, and future GWAS studies might be able to further stratify the genetic background in these clusters.

Currently there are several different medications used in the treatment of T2D, including dipeptidyl peptidase-IV inhibitors, GLP-1 analogues, hypoglycemic agents, insulin sensitizers, and glucagon-receptor antagonists (Nathan *et al*., 2009). If T2D truly consists of several types of diabetes with different genetic causes, knowing more about the function of implicated genes may help to develop new drugs and to assign better drug regime according to patients' specific etiology.

1.4. Wolframin protein

Wolframin1 (WFS1) is a transmembrane protein, mainly located in endoplasmic reticulum (ER), but also found in secretory granules in pancreatic beta cells (Hatanaka *et al*., 2011; Philbrook *et al*., 2005). It has nine transmembrane segments and N_{cyt}/C_{lum} topology (Hofmann *et al*., 2003). Wolframin is highly expressed in different brain tissues, heart, lungs, placenta and pancreas (Inoue *et al*., 1998). In pancreas, Wolframin expression is present in beta cells of Langerhans islet, but not in other islet cells or cells outside islets (Ishihara *et al*., 2004). Human *WFS1* gene was identified already in 1998 (Strom *et al*., 1998), but the precise function of WFS1 protein is still unknown. So far, most of the research indicates WFS1 involvement in ER stress response and ER Ca^{2+} accumulation (Takei *et al*., 2006; T. Yamada *et al*., 2006), as Ca^{2+} concentration in ER increases in WFS1 over-expressing cells and is reduced in WFS1 knockdown cells (Takei *et al*., 2006).

1.5. Wolfram syndrome

Defects in *WFS1* gene lead to autosomal recessive disorder Wolfram syndrome (WS). WS is characterized by diabetes mellitus, diabetes insipidus, optic nerve atrophy, deafness and neural degeneration (Barrett & Bundey, 1997; Barrett *et al*., 1995; Inoue *et al*., 1998). Currently there is no cure to WS and treatment is mainly focused on relieving the symptoms (Urano, 2016), until death in the third decade of life (Barrett *et al*., 1995).

First symptom to manifest in patients with WS at the mean age of six years is diabetes mellitus, resulting from progressive beta cell loss (Barrett *et al*., 1995). This is most likely due to disruption of intracellular Ca^{2+} regulation, leading to ER stress and apoptosis (Urano, 2016). Diabetes mellitus is usually followed at around 11-years-of-age with the loss of color vision and peripheral vision due to optic atrophy. Sensorineural deafness, diabetes insipidus, urinary tract problems and neurological problems follow, with different frequency and severity among patients. WS causes pleomorphic suffering. There are several neurological problems associated with WS, including autonomic neuropathy, headache, ataxia, dysphagia, and brain stem atrophy. A number of patients suffer from psychiatric problems, especially from depression (Swift *et al*.,

1991). Central apnea due to brain stem atrophy is one of the main causes of death in WS patients (Urano, 2016).

1.6. WFS1 and type 2 diabetes

WFS1 is one of the few genes that have been shown to cause monogenic autosomal dominant diabetes (Bonnycastle *et al*., 2013). Even though these dominant *WFS1* mutations are rare, genome-wide association studies (GWAS) have also shown that the carriers of heterozygous *WFS1* mutation have an increased risk for development of type 2 diabetes (Cheurfa *et al*., 2011; Franks *et al*., 2008; Han *et al*., 2010; Sandhu *et al*., 2007). While WS prevalence is estimated to 1 in 770 000 in UK (Barrett *et al*., 1995) and 1 in 100 000 in North America (Fraser & Gunn, 1977), the carrier frequency is approximately 1 in 354 in UK (Barrett *et al*., 1995) and 1 in 100–200 in USA (Swift *et al*., 1991). The exact mechanism involved in this predisposition is not known, but considering WFS1 role in Ca^{2+} dynamics, ER stress response, and insulin maturation, heterozygous defective *WFS1* may lead to beta cell dysfunction (Bonnycastle *et al*., 2013; Urano, 2016).

1.7. Previously existing Wfs1 deficient animal models

There are currently three different mouse models of Wfs1 deficiency, created by three separate workgroups.

Ishihara *et al* created a full body Wfs1 knock-out by inserting a neomycin-resistance gene into the second exon of the *Wfs1* gene. This resulted in a mouse model, where WFS1 function is lost or severely impaired (Ishihara *et al*., 2004). This mouse model shows severe glucose intolerance due to deficit in insulin secretion, while insulin tolerance remains unchanged. Isolated islets from these mice exhibited increased apoptosis both in response to glucose and inducers of ER stress. Compared to wild-type mice, Wfs1 deficient mice insulin content was decreased already at two-weeks of age and beta cell mass was decreased at two months of age (earliest time-points observed). In [(129Sv×B6)×B6]F2 hybrid background, these mice started to exhibit increase in basal glucose levels in non-fasted state. As this result had very big variance, *Wfs1* mutation was backcrossed to B6 background, where the increase in basal blood glucose level disappeared. Therefore, B6 background probably diminishes the severity of symptoms from the lack of functional WFS1 protein.

Riggs *et al* created Wfs1 knock-out mice using Cre recombinase under the control of insulin promoter and loxP sequences flanking exon 8 of the *Wfs1* gene (Riggs *et al*., 2005). This resulted in mice lacking functional WFS1 in pancreatic beta cells, but left WFS1 function intact in other tissues. Fasting blood glucose levels in most of these mice did not differ from wild-type mice, with the exception of a few animals that developed fasting hyperglycemia by 24 months

of age. Similarly to mice created by Ishihara *et al*, beta cell specific knock-out of *Wfs1* resulted in progressive glucose intolerance, reduced insulin secretion, increased apoptosis of beta cells and a decrease in beta cell mass at 24 months of age, but not at 12 months of age.

Luuk *et al* created whole body Wfs1 knock-out by replacing the majority of eighth exon with in-frame NLSLacZNeo cassette (Luuk *et al*., 2009). This resulted in functional WFS1 deficiency, as approximately 60% of total *Wfs1* gene coding sequence was replaced. Wfs1 deficient mice had lower body weight, and similarly to previous models, lower insulin secretion and severe glucose intolerance already at two months of age, but did not exhibit significant increase in fasted blood glucose levels until a slight increase at 24 months of age (Noormets *et al*., 2011).

1.8. Concluding remarks

WFS1 protein has a strong impact on the development of diabetes in humans with Wolfram syndrome, but also in people carrying one defective copy of the gene. WFS1 importance in insulin secretion and beta cell survival is further demonstrated by different mouse models, which also develop glucose intolerance. However, while human patients with WS develop insulin dependent diabetes at an average age of six years, current mouse models seem to not exhibit overt diabetes, thus indicating a need for a model, better imitating the progression of human condition.

Beta cell expressed GPCR ligands regulate cell apoptosis, ER stress, Ca^{2+} homeostasis and insulin secretion. All these aspects seem to be affected in Wfs1 deficiency, therefore, the ligands interacting with GPCR-s on beta cells are possible new targets to tackle the problems resulting from defective function of WFS1 protein.

2. AIMS OF THE STUDY

The purpose of this study was to create and characterize a new rat model of Wolfram syndrome, possibly better mimicking the human condition, and to assess the effectiveness of insulin secretion stimulating ligands to reverse and prevent insulin secretion deficits in Wfs1 deficient rodent models.

The specific aims of the study were as follows:

1. Describe the insulin secretion in homozygous and heterozygous Wfs1 deficient mouse model and evaluate muscarinic-receptor agonist carbachol ability to normalize insulin secretion in Wfs1 deficient mice.
2. Generate a new Wfs1 deficient rat model and characterize the progression of diabetes in this model.
3. Evaluate the effect of GLP-1 receptor agonist liraglutide to prevent the development of glucose intolerance in rat model of WS.
4. Evaluate the feasibility of rat model of Wolfram syndrome.

3. MATERIALS AND METHODS

3.1. Animals

3.1.1. Mice (Paper I)

The animal experiments described in first study were performed according to the permission from the Estonian National Board of Animal Experiments (No. 37, 14.08.2014 and No. 55, 14.05.2015) and in accordance with the European Communities Directive of September 2010 (2010/63/EU). Generation of Wfs1 exon 8 knockout mice has been described by Luuk *et al* (Luuk *et al*., 2008). In brief, majority of 8th exon of *Wfs1* was replaced with in-frame NLSLacZNeo cassette, resulting in functional Wolframin1 knock-out. All studies were performed in 2 months old male F2 [(129S6/SvEvTac × 129S6/SvEvTac) × (129S6/SvEvTac × 129S6/SvEvTac] mice. 2-month-old mice were chosen, as in this age, all animals had developed glucose intolerance, but still had enough beta cells for the stimulatory effect of carbachol. Average weight of animals was 22.9±0.3 grams for WT mice, 23.2±0.3 grams for heterozygous and 19.2±0.3 grams for homozygous Wfs1-deficient mice. Altogether 180 mice were used. Breeding and genotyping were conducted in the Department of Biomedicine and Translational Medicine, University of Tartu. Mice were housed in groups of seven to eight at 20±2°C under 12/12-h light/dark cycle (lights on at 07:00 hours) with free access to standard food pellets and water. All animal experiments were carried out between 10:00 and 15:00. Wfs1-deficient homozygous and heterozygous mice were always used in parallel with their WT littermates and the animals were randomly divided between the experimental groups (Toots *et al*., 2018 (1)).

3.1.2. Generation of Wfs1 deficient rat line (Paper II)

Rat *Wfs1* exon 5-specific zinc-finger nucleases (ZNFs) and microinjection-ready mRNA were obtained from Sigma-Aldrich (CSTZFN-1KT lot: 02091202 MN). The Rat *Wfs1* exon 5 DNA (deoxyribonucleic acid) sequence and ZFN binding and cutting sites are described in Figure 2 (Plaas *et al*., 2017).

AGAAGTGGCTACCCAGGGATATGACCCTTGCATATGATGAGACCCCAAGCCCTGTGATCCCTGCGAACAGTGATAACCC
AGGATCCCGAGCTCCTATACCCAGTGGGCTACAGGGCAGGACGACTCAGCAGCCCTGGCTTCCAATCCTCTCTGATCTT
ACAGATGGAGGGGCGCAGCCAGGCCCCGTCCCCAAGTCcctgcaGAAGCAGAGGCGCATGCTGGAGCGTCTAGTGAGCA
GTGAATGTGAGTATGTCCACCCTGTGAGTATGTCCACCCTGCGCTGCAGTCCTGCTACCTGCTTCCCGCACACCAC**CCA
GCACCATACCCACTCTT**

Figure 2. Zink finger nuclease (ZFN) design and cutting site. Genotyping primers are in bold and underlined, ZFN binding site in red, ZFN cut site in lower case red, and blue indicates the start and end of exon 5 of the rat *Wfs1* gene. Plaas *et al*., 2017, modified by Toots.

Sprague-Dawley rats (Crl: CD(SD) rats CD® IGS, Charles River Laboratories) were housed in standard cages and maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. 4- to 5-week-old embryo donors were superovulated by injection with 20 units of pregnant mare serum gonadotropin (PMSG Sigma Cat. no. G-4877), and 48 h later, just before mating, rats were injected with 50 units of human chorionic gonadotropin (hCG Sigma Cat. no. CG-5). Fertilized egg cells were harvested a day later in M2 medium (Sigma Cat. No. M7167), and cells where incubated in KSOM medium (Specialty Media, Cat. #MR 121-D). For embryo manipulations, ZFN mRNA was injected into the pronucleus of fertilized eggs. The final concentration of each ZFN mRNA was 2.5 ng/µl. For the synchronization of female recipients, rats were injected with 40 µg of LH-Rh 72 h before mating them with vasectomized males. Female rats in the proestrus phase were mated with vasectomized studs at day 0 to induce pseudo-pregnancy. On the following day (day 1), the mated females where inspected for copulatory plugs. Thereafter, microinjected egg cells were transferred to the oviduct of pseudopregnant Sprague-Dawley recipients (max of 40 embryos per female) (Plaas *et al*., 2017).

Mutations in target region were detected using PCR analysis amplify a 333-bp region surrounding the target site. The resulting PCR products were pyro-sequenced. The primer set for the PCR genotyping analysis was: rwfs_zf_genoR1 (5'-AAG AGT GGG TAT GGT GCT GG-3') and rwfs_zf_genoF1 (5'-AGA AGT GGC TAC CCA GGG AT-3'). In the mutation detection assay, we found that founder line 232 had 2 bands, indicating a possible large deletion in exon 5 of the *Wfs1* gene. Both of the PCR products were extracted from the agarose gel and analyzed via DNA pyrosequencing separately (Plaas *et al*., 2017).

To verify the *Wfs1* mutation on transcription level, total RNA was extracted individually from the hearts of WT and WFS1ex5-KO232 (later marked as Wfs1 KO) rats using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized using poly(T)18 oligonucleotides and SuperScript™ III Reverse Transcriptase (Invitrogen, USA). Primers for cDNA genotyping and pyrosequencing were as follows: Wfsex4F (5'-TCA CTT CTG AGA ATG AGG CCG-3') and Wfsex7R (5'-ATG AGG GCG TTG ATG TGA TGG-3') (Plaas *et al*., 2017).

3.1.3. Rats (Papers II–III)

Breeding and genotyping were performed at the Laboratory Animal Centre, University of Tartu. For the study of progression of diabetes in rat model of WS, 1- to 14-month-old male homozygous Wfs1-deficient and wild-type (WT) littermate control rats were used. For the liraglutide study, 2- to 7-month-old and 5-month-old male homozygous Wfs1-deficient and WT littermate control rats were used. The animals were housed in cages in groups of 2–4 animals per cage under a 12 h light/dark cycle (lights on at 7 a.m.). Rats had unlimited access to food and water except during testing. Sniff universal mouse and rat

maintenance diet (Sniff #V1534) and reverse osmosis-purified water were used. Experiments were performed between 9 a.m. and 5 p.m. Permission for this study was given by the Estonian National Board of Animal Experiments (No. 54, 23th of February 2015 and No 103, 22nd of May 2017) in accordance with the European Communities Directive of September 2010 (2010/63/EU) (Plaas *et al*., 2017; Toots *et al*., 2018 (2)).

3.2. Animal experiments

3.2.1. Intraperitoneal glucose tolerance test (IPGTT) in mice (Paper I)

For IPGTT, food was removed 90 min prior to the beginning of experiment. 90 min fasting was used, as it was sufficient to allow blood glucose levels to equalize while having smaller effect on metabolic parameters, observed with longer fasting (Agouni *et al*., 2010; Andrikopoulos *et al*., 2008; Palou *et al*., 2008). Basal levels of blood glucose were determined from the tail vein by needle puncture, using commercial glucose meter (Accu-Check Performa, Roche). After that, mice were given i.p. injection according to their body weight with a) 2 g/kg glucose; b) 0.02 mg/kg carbachol (carbamoylcholine chloride, muscarinic receptor agonist); or c) 2 g/kg glucose + 0.02 mg/kg carbachol. 5, 10 or 30 minutes later animals were euthanized, blood samples collected for insulin measurement and blood glucose levels determined. Carbachol (SigmaAldrich) and D-(+)-glucose (SigmaAldrich) were both dissolved in saline (0,9% NaCl; B. Braun). Carbachol concentration 0.02 mg/kg was chosen as it corresponds to 1 μmol/kg dose and approximately 10 μM concentration in blood, showing strong effects in previously reported experiments (Gautam *et al*., 2007; Johnson *et al*., 2005; Renuka *et al*., 2006; Toots *et al*., 2018 (1)).

3.2.2. Intraperitoneal glucose tolerance test (IPGTT) in rats (Papers II–III)

Animals were deprived of food for 3 h before and during the experiment; water was available throughout the experiment. D-Glucose (Sigma-Aldrich) was dissolved in 0.9% saline solution (20% w/vol) and administered intraperitoneally at a dose of 2 g/kg of body weight. Blood glucose levels were measured at the indicated time points from the tail vein using a handheld glucometer (Accu-Check Go, Roche, Germany). Blood samples were drawn from the tail vein immediately before and 30 min after glucose administration for further analyses (Plaas *et al*., 2017; Toots *et al*., 2018 (2)).

3.2.3. Subcutaneous insulin tolerance test (ITT) (Papers II–III)

An insulin tolerance test was performed in the same manner as the glucose tolerance tests except that human insulin (1 unit/kg s.c., Lantus Solostar) was administered instead of glucose (Plaas *et al*., 2017; Toots *et al*., 2018 (2)).

3.2.4. 8-day repeated liraglutide treatment (Paper III)

In this experiment, 5-month-old rats were used. After the first IPGTT test, the rats received 0.4 mg/kg liraglutide (Novo Nordisk, Denmark, solution made in 0.9% NaCl) subcutaneously for 8 days. Injections of 1 ml/kg volume were administered once a day between 8 and 11 a.m. The second IPGTT was performed 4.5 hours after the last liraglutide injection (Toots *et al*., 2018 (2)).

3.2.5. Chronic liraglutide treatment (Paper III)

The rats were 2 months old at the beginning of the experiment. After the first IPGTT test, the rats were randomly allocated into the liraglutide or control group. The liraglutide group animals received 0.4 mg/kg liraglutide (Novo Nordisk, Denmark) and the control group animals a 0.9% saline solution (vehicle) subcutaneously. Injections of 1 ml/kg volume were made once a day between 8 and 11 a.m. (or immediately after a glucose/insulin tolerance test). Rats were weighed once a week. Glucose tolerance and insulin tolerance tests were performed 24 hours after the previous liraglutide/saline injection. After the last IPGTT, rats were equally allocated for subsequent histological/quantitative real-time PCR analysis according to their IPGTT results (Toots *et al*., 2018 (2)).

3.3. Biochemical and gene expression analyses

3.3.1. Urine glucose concentration measurements (Paper II)

Urine from non-fasted rats was collected during the light phase for urine glucose levels measurement and determined using standardized procedures at the United Laboratories of Tartu University Hospital (Plaas *et al*., 2017).

3.3.2. Insulin, C-peptide, and Glucagon measurements (Papers I–III)

For serum separation, blood was allowed to clot, centrifuged for 15 min at 2000x g, and stored in –80 ^{o}C until further analysis. Serum insulin levels were measured using an ultra-sensitive mouse insulin ELISA kit (CrystalChem cat#90080) or, an ultra-sensitive rat insulin ELISA kit (CrystalChem cat#

90060), C-peptide levels were measured using rat C-peptide ELISA (CrystalChem cat# 90055), and glucagon levels were measured using rat glucagon ELISA (CrystalChem cat# 81519), according to the manufacturer's instructions (Plaas *et al*., 2017; Toots *et al*., 2018 (1); Toots *et al*., 2018 (2)).

3.3.3. Gene expression analyses from Langerhans islets (Papers II–III)

3.3.3.1. Langerhans islet isolation

Islets of Langerhans were isolated as has been described previously (Carter *et al*., 2009). In brief, 0.9 mg/ml collagenase (Sigma-Aldrich, #C7657) solution was injected into the common bile duct of euthanized animals; inflated pancreases were collected and tissues enzymatically dispersed. The majority of exocrine tissue was removed by gradient separation in Histopaque solution (Sigma-Aldrich). Islets of Langerhans were collected by hand from remaining exocrine tissue under a stereo microscope (Plaas *et al*., 2017; Toots *et al*., 2018 (2)).

3.3.3.2. RNA extraction and cDNA synthesis

RNA from islets of Langerhans was isolated using Direct-zol RNA MiniPrep (Zymo Research), according to the manufacturer's protocol. First-strand cDNA was synthesized using random hexamers and SuperScript™ III Reverse Transcriptase (Invitrogen, USA) (Plaas *et al*., 2017; Toots *et al*., 2018 (2)).

3.3.3.3. Xbp1 splicing

Xbp1 splicing was analyzed using rat *Xbp1*-specific PCR as has been described (Yusta *et al*., 2006). In brief, PCR was performed with primers 5'-AAA CAG AGT AGC AGC ACA GAC TGC-3' and 5'-TCC TTC TGG GTA GAC CTC TGG GAG-3'. The program for amplification was: 15 min 95 ^{o}C; 30 × (20 sec 95 ^{o}C; 45 sec 60 ^{o}C; 60 sec 72 ^{o}C); 10 min 72 ^{o}C. PCR products were separated on 3% agarose gel for approximately 5.5 hours, 145 mV, and images obtained using Uvidoc imaging system. Integrated density levels were measured using ImageJ software. *Xbp1* spliced from the whole *Xbp1* was calculated and normalized to their expression in WT (Paper II) or WT saline-treated (Paper III) rats (Plaas *et al*., 2017; Toots *et al*., 2018 (2)).

3.3.3.4. Quantitative real-time PCR

Gene expression was analyzed using quantitative real-time PCR (qRT-PCR) with TaqMan Gene Expression Assays (Table 1; Thermo Fisher Scientific), and Taqman Gene Expression Mastermix (Thermo Fisher Scientific). *Wfs1* assay binds to exon 1 and 2 boundary, therefore also recognizes mutant *Wfs1* lacking

exon 5. Relative quantification was performed using the $2^{-\Delta Ct}$ method, with *Hprt1* (has been shown to have stable expression levels) as an internal control. All gene expressions were done in three parallels to minimize possible errors and results were normalized to their expression in WT (Paper II) or WT saline-treated (Paper III) rats (Plaas *et al*., 2017; Toots *et al*., 2018 (2)).

Table 1. Taqman assays used

Gene symbol	Paper	Assay ID
Grp78 (Bip)	II, III	Rn00565250_m1
Glp1R	III	Rn00562406_m1
Wfs1	III	Rn00582735_m1
Atf4	III	Rn00824644_g1
Bax	III	Rn01480161_g1
Caspase1	III	Rn01515235_m1
Caspase3	III	Rn00563902_m1
Bcl2	III	Rn99999125_m1
IP10	III	Rn01413889_g1
Ki67	III	Rn01451446_m1
Hprt1	II, III	Rn01527840_m1

Grp78 (Bip) – 78 kDa glucose-regulated protein; *Glp1R* – Glucagon-like protein-1 receptor; *Wfs1* – Wolframin1; *Atf4* – activating transcription factor 4; *Bax* – Bcl-2-associated X protein; *IP10* – interferon gamma-induced protein 10; *Ki67* – antigen KI-67; *Hprt1* – hypoxanthine-guanine phosphoribosyltransferase

3.4. Immunohistochemistry (Paper II)

Rats were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and dexmedetomidine (500 µg/kg) and were then perfused transcardially with 4% paraformaldehyde (PFA, Sigma-Aldrich) in 0.1 M phosphate buffer (PB, pH 7.4). Pancreases were dissected and further fixed in the same fixative overnight at 4 °C. Tissues were cryoprotected in 30% sucrose (AppliChem)/0.1 M PB solution until they sank and were then frozen at –80 °C until further use. Sections (40 µm thick) were cut using a cryomicrotome (Microm HM-560) and collected on Superfrost Polysine Slides (Thermo Scientific). After washing with phosphate buffered saline (PBS) for 10 min, sections were permeabilized with 0.2% Triton X-100 (Naxo, Tartu, Estonia)/PBS solution for 40 min. Sections were further incubated in a blocking solution containing 5% donkey serum/1% bovine serum albumin (BSA, Sigma-Aldrich)/PBS for 2 h at room temperature. Primary and secondary antibodies were diluted in 0.1% Tween-20/1% BSA/ PBS. Sections were incubated with primary antibodies for 12 h at 4 °C and were then washed with PBS for 1 h. Sections were incubated with the appropriate

secondary antibody at room temperature for 2 h. After subsequent washes with PBS (1 h), cell nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich) at a 1:2000 dilution in 0.1% Tween-20/PBS for 15 min and further washed with PBS. Sections were mounted in Vectashield mounting medium (Vector Laboratories Inc.) and covered with a 0.17-mm coverslip (Deltalab). Images were taken with an Olympus FV-1000 (Olympus) confocal microscope or Olympus BX51 Fluorescence Microscope and annotated with Adobe Photoshop CC (Adobe Systems Incorporated) (Plaas *et al*., 2017).

Primary antibodies and their dilutions were as follows: rabbit anti-XPB1 (1:500, Abcam Cat# ab 37151, RRID:AB_778942), rabbit anti-BiP (1:200, Cell Signalling Technology Cat# 3177P, RRID:AB_10828008). Secondary antibody dilution was: FITC AffiniPure donkey anti-rabbit (1:1000, Jackson ImmunoResearch Lab., 711-095-152, RRID:AB_2315776) (Plaas *et al*., 2017).

Histological analyses were performed using material from 3 to 7 animals per genotype from each age group. Negative controls were made without primary antibody. Representative images for each group are shown. Fluorescence intensity measurements of ER stress markers were performed using ImageJ software (NIH, Bethesda, MD, USA). Specifically, 8-bit images from optical sections approximately 5 µm thick were converted to greyscale, and intensity values were measured as the mean pixel value in the islets of Langerhans (Plaas *et al*., 2017).

3.5. Langerhans islet measurements (Papers II–III)

Beta cell mass was estimated as has been described previously (Iglesias *et al*., 2012). In brief, rats were perfused, their pancreases dissected, and excess fat removed. The weight of each pancreas was recorded, and the tissue was processed for histological analysis as described earlier. 40-µm-thick sections were cut at intervals of 400 µm (Plaas *et al*., 2017; Toots *et al*., 2018 (2)).

Sections were washed with PBS for 3x5 min, permeabilized with 0.2% Triton X-100 (Naxo, Estonia)/PBS solution for 30 min, incubated in 0.5% H_2O_2/PBS for 1 h, and blocked in 5% donkey serum/1% bovine serum albumin (BSA, Sigma-Aldrich)/PBS for 1 h. Primary and secondary antibodies were diluted in 0.1% Tween-20/1% BSA/PBS. Sections were incubated with mouse anti-insulin antibody (1:800, Cell Signaling Technology Cat# 8138S RRID:AB_10949314) for 1 h and washed with PBS for 3x10 min, followed by incubation with donkey anti-mouse peroxidase conjugated antibody (1:2000, Rockland Cat# 610-703-002 RRID:AB_219700) for 30 min and washed with PBS 3x5 min. Sections were incubated in 0.025% diaminobenzidine (Sigma Aldrich)/0.005% H_2O_2/0.05% CoCl2 (Sigma Aldrich)/PBS for 15 min and washed with water. Dried sections were mounted using PeRtEx (HistoLab) and covered with 0.17 mm coverslip (Deltalab). Images were taken using a Leica SCN 400 slide scanner at 20x magnification. The images obtained were

analyzed using the ImageJ software. Langerhans islet mass for each animal was estimated by dividing the total islet area by the total pancreas area, and the obtained relative islet area was multiplied by the weight of the pancreas to estimate Langerhans islet mass (Plaas *et al*., 2017; Toots *et al*., 2018 (2)).

3.6. Statistical analysis (Papers I–III)

The data are presented as the mean ± SEM and were compared using factorial or repeated measures ANOVA (after positive Shapiro-Wilks normality test) followed by unequal N HSD post hoc tests. The data were analyzed using version 8 of the Statistica software (Statistica, USA). $p<0.05$ was considered statistically significant.

4. RESULTS AND DISCUSSION

4.1. Insulin secretion in Wfs1 deficient mice (Paper I)

4.1.1. Early response insulin secretion in Wfs1 deficient mice

Prior to meal consumption, neuroendocrine signals prepare beta cells for the increased need for insulin secretion. Acetylcholine released by vagus nerve binds to muscarinic receptors on beta cells to release insulin and to amplify GSIS. Muscarinic agonists have been previously shown to initiate insulin secretion in diabetic models, an effect that is abolished by muscarinic antagonist atropine (Ahren *et al*., 1997; Guenifi *et al*., 2001). None of these agonists are specific to one muscarinic or nicotinic receptor subtype. However, studies investigating acetylcholine effect on pancreatic beta cells and insulin secretion have revealed the central role of M3R (muscarinic receptor 3), as M3R deficient mice do not exhibit increased glucose stimulated insulin secretion after treatment with muscarinic agonist oxotremorine (Duttaroy *et al*., 2004). It should be taken into account that a small proportion of the activity of muscarinic agonists may be related to other muscarinic receptor subtypes and even to nicotine receptors, but the direct effect and even expression of other receptors than M3 is still debated upon (Duttaroy *et al*., 2004; Toots *et al*., 2018 (1); Yoshikawa *et al*., 2005).

While previous reports have described lower insulin levels in Wfs1 KO mice, we aimed to further study the changes in insulin secretion dynamics in Wfs1 deficient mice (Noormets *et al*., 2011). For this, WT, Wfs1 mutation heterozygous, and Wfs1 mutation homozygous mice were injected with 2 g/kg glucose and blood samples were collected at different time-points.

Similarly to previous reports (Noormets *et al*., 2011), our findings confirmed the significant impairment in glucose stimulated insulin secretion in Wfs1 deficient mice (Figure 3). Glucose injection caused strong hyperglycemia in Wfs1 KO mice, explained by the lack of insulin secretion in response to glucose. Heterozygous mice blood glucose levels did not differ from that of WT mice, but their insulin levels stayed elevated for longer, indicating changes in insulin secretion dynamics. This coincides with heterozygous *WFS1* mutation carriers having increased risk for the development of type 2 diabetes, most likely due to changes in beta cell function, represented by altered insulin secretion (Cheurfa *et al*., 2011; Franks *et al*., 2008; Han *et al*., 2010; Sandhu *et al*., 2007; Toots *et al*., 2018 (1)).

To further study the changes in early insulin secretion and possible changes in response to muscarinic stimulation, mice were administrated 0.02 mg/kg carbachol. Carbachol activates muscarinic receptors present on the surface of beta cells (Duttaroy *et al*., 2004).

While carbachol did not change the blood glucose levels compared to baseline measurements, it had a significant effect on insulin release (Figure 4). In WT mice, there was a five-time increase in insulin secretion during 5 to 10

minutes after injection. In both homozygous and heterozygous Wfs1 deficient mice, this early response of insulin secretion was approximately half of that seen in WT mice. This experiment further revealed the changes in heterozygous mice insulin secretion, first observed during GSIS. Regulation of insulin secretion by hormones and/or neurotransmitters (e.g. acetylcholine effect through M3 receptors on beta cell) is defective in people with type 2 diabetes (Del Guerra *et al*., 2005). Human GWAS studies have revealed that heterozygous Wfs1 mutation carriers have increased risk for development of type 2 diabetes (Cheurfa *et al*., 2011; Franks *et al*., 2008; Han *et al*., 2010; Sandhu *et al*., 2007). This correlates with experiments on Wfs1 deficient mice, where psychiatric drugs amitriptyline and desipramine, that block M3 receptor as a side-effect, caused stronger increase of blood glucose levels in *Wfs1* heterozygous mice than in WT mice and hyperglycemia in *Wfs1* homozygous mice (Reimets *et al*., 2016; Toots *et al*., 2018 (1)).

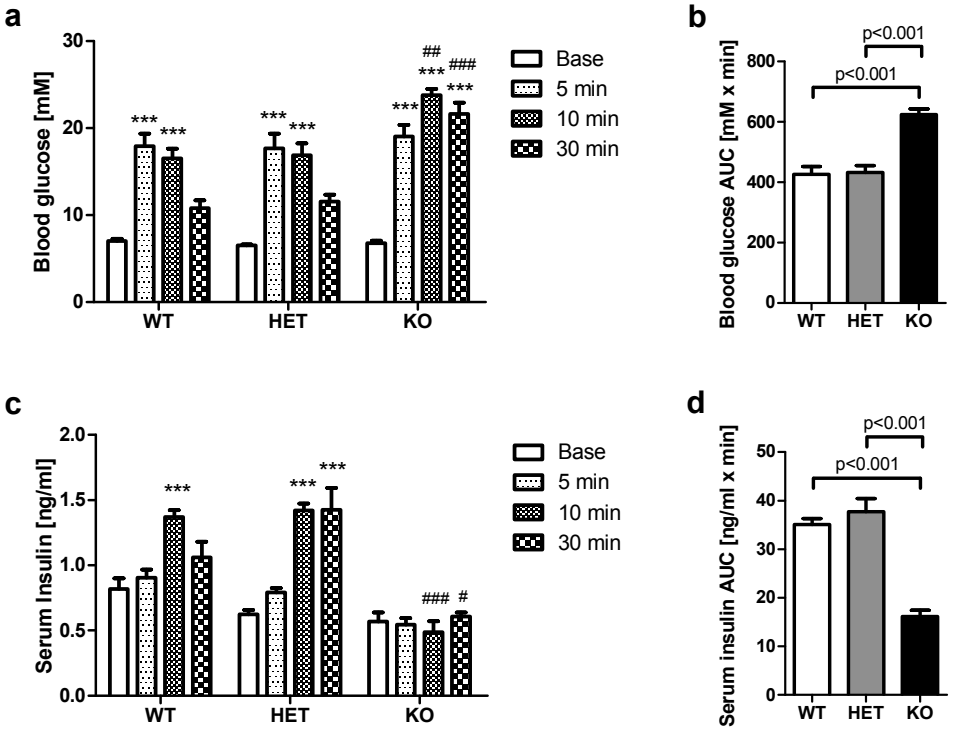

Figure 3. Response to 2 g/kg glucose injection. Blood glucose **(a)** levels 5, 10 or 30 minutes after administration and corresponding area under curve **(b)**. Serum insulin **(c)** levels 5, 10 or 30 minutes after administration and corresponding area under curve **(d)**. *** $p < 0.001$ compared to baseline level of same genotype. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to WT in same time-point. WT – wild-type mice; HET – heterozygous Wfs1 mutation carrier; KO – homozygous Wfs1 mutation carrier. n=5–7. Toots *et al*., 2018 (1), modified by Toots.

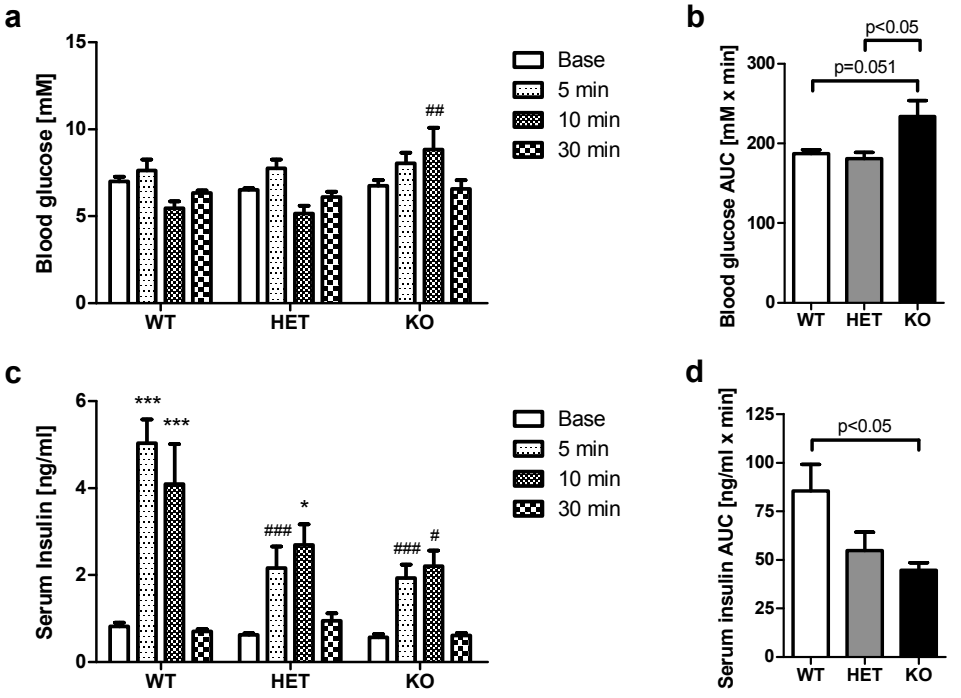

Figure 4. Response to 0.02 mg/kg carbachol injection. Blood glucose **(a)** levels 5, 10 or 30 minutes after administration and corresponding area under curve **(b)**. Serum insulin **(c)** levels 5, 10 or 30 minutes after administration and corresponding area under curve **(d)**. * $p < 0.05$, *** $p < 0.001$ compared to base level of same genotype. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to WT in same time-point. WT – wild-type mice; HET – heterozygous Wfs1 mutation carrier; KO – homozygous Wfs1 mutation carrier. n=5–7. Toots *et al*., 2018 (1), modified by Toots.

4.1.2. Carbachol and glucose co-administration to Wfs1 deficient mice

As carbachol administration was able to release insulin from homozygous Wfs1 deficient mice beta cells, we further investigated whether co-administration of carbachol with glucose is able to prevent hyperglycemia in KO mice.

Co-administration of carbachol and glucose increased blood glucose levels in all genotypes (Figure 5a). The blood glucose level in KO animals was somewhat higher at the time-point 10 minutes compared to other genotypes, but long-lasting hyperglycemia that existed in KO mice administrated only glucose, had normalized after 30 minutes (Figure 3a and 5a) (Toots *et al*., 2018 (1)).

Furthermore, co-administrated KO mice blood glucose profile was similar to WT mice receiving glucose (Figure 6a). This normalization of glycemic control was explained by the fact that even though early response serum insulin levels in co-injected WT and HET mice were significantly higher than in KO mice (Figure 5c), KO mice were able to secrete insulin in levels similar to WT mice receiving only glucose (Figure 6b) (Toots *et al*., 2018 (1)).

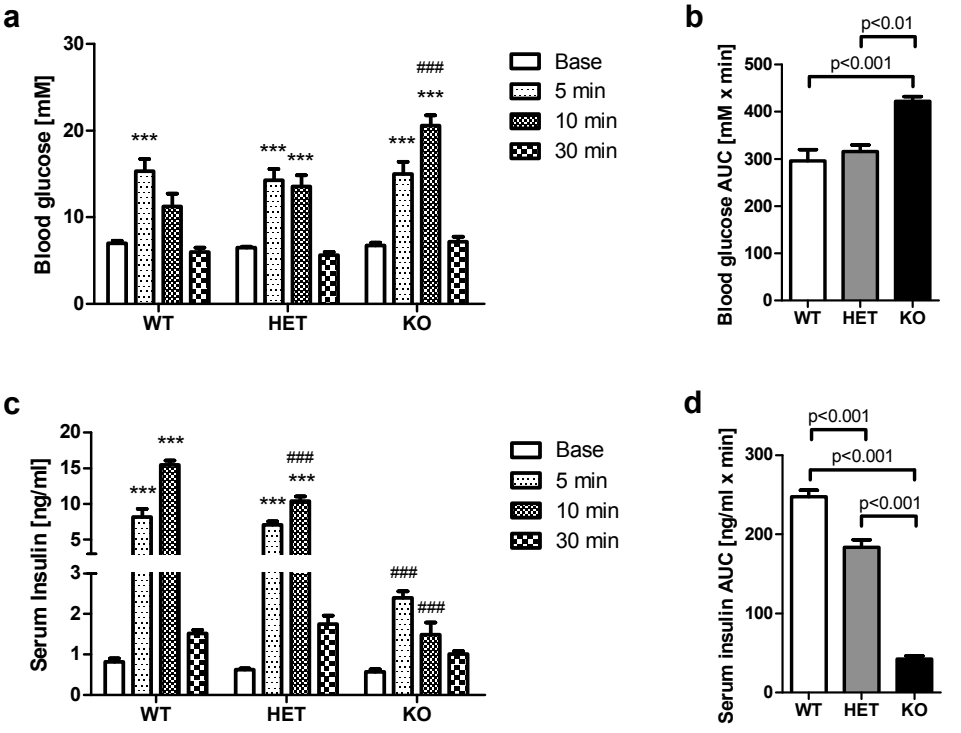

Figure 5. Response to 0.02 mg/kg carbachol and 2 g/kg glucose co-injection. Blood glucose **(a)** levels 5, 10 or 30 minutes after administration and corresponding area under curve **(b)**. Serum insulin **(c)** levels 5, 10 or 30 minutes after administration and corresponding area under curve **(d)**. *** $p < 0.001$ compared to baseline level of same genotype. ### $p < 0.001$ compared to WT in same time-point. WT – wild-type mice; HET – heterozygous Wfs1 mutation carrier; KO – homozygous Wfs1 mutation carrier. n=5–7. Toots *et al*., 2018 (1), modified by Toots.

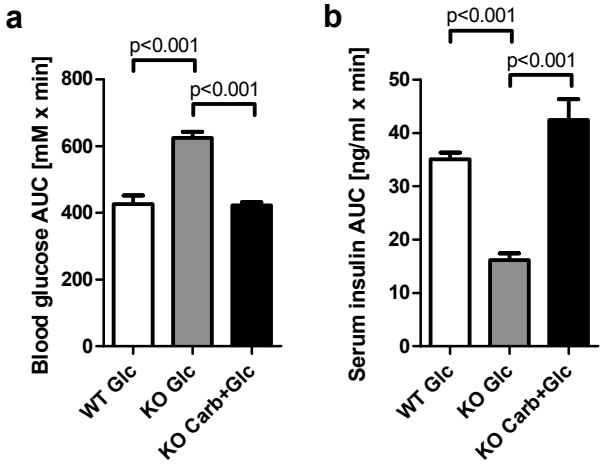

Figure 6. WT and KO mice response to 2 g/kg glucose or 0.02 mg/kg carbachol and 2 g/kg glucose co-injection. Blood glucose **(a)** and insulin **(b)** area under curve during 30 minutes after injection. WT – wild-type mice; KO – homozygous Wfs1 mutation carrier. n=5–6. Toots *et al*., 2018 (1), modified by Toots.

4.1.3. Conclusion

Wfs1 deficiency causes glucose intolerance as a result of decreased insulin secretion. This deficit is also apparent in heterozygous Wfs1 deficient mice who do not exhibit glucose intolerance. These changes in heterozygous mice may explain the increased risk for development of type 2 diabetes in human *WFS1* mutation carriers. Activation of M3 receptors on beta cell by carbachol was able to initiate insulin secretion from beta cells and normalize blood glucose control in Wfs1 deficient mice.

Carbachol inability to distinguish between M3R and other types of muscarinic/nicotinic receptors limits its use in human diabetes treatment. However, selective M3R agonists and possibly other ligands, able to activate same pathways in beta cell, are a promising way to release insulin and to reverse glucose intolerance resulting from Wfs1 deficiency (Toots *et al*., 2018 (1)).

4.2. Generation and characterization of new Wfs1 deficient rat model (Paper II)

4.2.1. Generation of rat line without exon 5 of *Wfs1* gene

While human patients of WS develop insulin dependent diabetes at a young age (average age 6 years), none of the three mouse models of WS created to this date, exhibit consistently developing overt diabetes (Ishihara *et al*., 2004; Noormets *et al*., 2011; Riggs *et al*., 2005; Urano, 2016). In hopes of creating a model better mimicking the human condition diabetes and other symptoms of WS, as well as to make live imaging of neural degeneration more precise, a rat model of Wfs1 deficiency was created.

Using zinc-finger technology, three different Wfs1 mutant rat lines were created. Genomic sequencing revealed that two of the lines (Wfs1-ex5-KO232 and Wfs1-ex5-KO266) had an in frame deletion in exon 5 of the *Wfs1* gene and the third line (Wfs1-ex5-INS244) had an in frame substitution in exon 5 of the *Wfs1* gene. This substitution is predicted to result in amino-acids 224–226 (LQK) being substituted with YCMNTI in the WFS1 protein (Plaas *et al*., 2017).

Rats from all three lines exhibited glucose intolerance at 7 months of age (Figure 7a-c), further demonstrating that glucose intolerance effect was most likely not an off-target effect. Both of the strains with deletion in *Wfs1* gene exhibited lower body mass compared to WT littermates at 7 months of age, while the *Wfs1* mutation line with insertion exhibited higher body mass, compared to WT littermates (Figure 7d-f). Mutation of *Wfs1* in Wfs1-ex5-KO232 rats was verified by pyrosequencing cDNA, which confirmed a deletion of exon 5 of the rat *Wfs1* gene (Figure 8a). This deletion is predicted to result in a loss of 27 aa from the WFS1 protein sequence (aa 212-238) and a substitution of serine to alanine at position 239 (Figure 8b) in Wfs1-ex5-KO232 rats. The

Wfs1-ex5-KO232 line (in further studies Wfs1 KO or KO) was characterized and used (males only) in the subsequent experiments, but all 3 Wfs1 mutant lines are currently being maintained for future studies (Plaas *et al*., 2017).

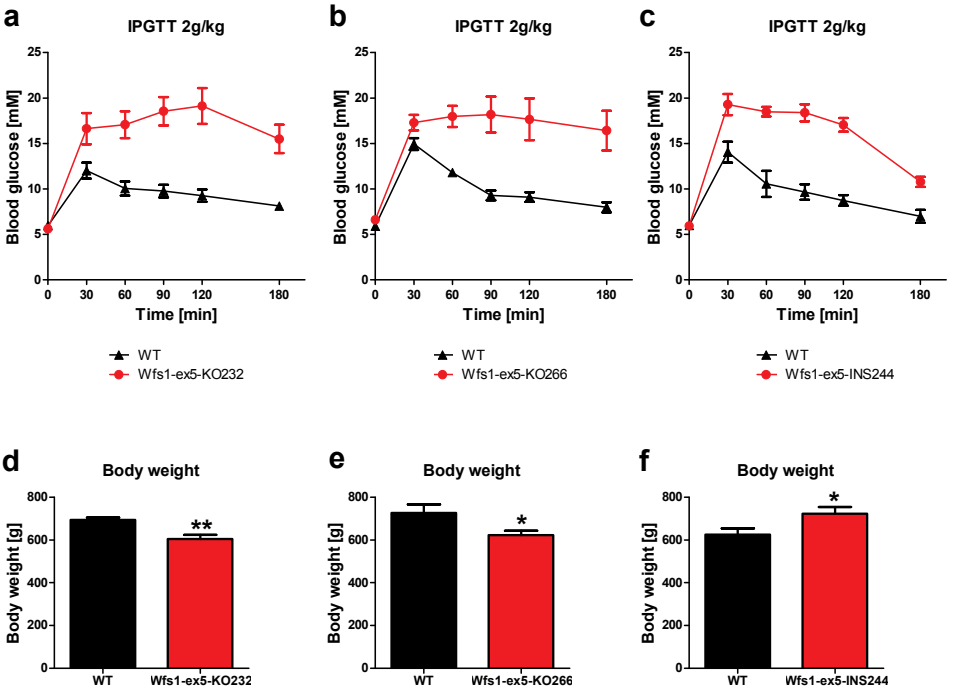

Figure 7. Glucose tolerance and body weight of three different Wfs1 mutant rat-lines at 7 months of age (males). (a, b, c) All Wfs1 mutant rats display glucose intolerance at this age. **(d, e)** Wfs1-ex5-KO232 and Wfs1-ex5-KO266 rats are lighter than wild-type littermates. **(f)** Wfs1-ex5-INS244 rats are heavier than littermates. Student's t-test; *p < 0.05, ** < 0.01 between genotypes. The data are presented as mean ± SEM, n=4 –7. Plaas *et al*., 2017, modified by Toots.

a

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Wfs1 WT          AGAAGTGGCTACCCAGGGATATGACCCTTGCATATGATGAGACCCCAAGCCCTGTG
Wfs1-ex5-KO232   AGAAGTGGCTACCCAGGGATATGACCCTTGCAT----------------------

Wfs1 WT          ATCCCTGCGAACAGTGATAACCCAGGATCCCGAGCTCCTATACCCAGTGGGCTACA
Wfs1-ex5-KO232   -------------------------- del 184bp -------------------

Wfs1 WT          GGGCAGGACGACTCAGCAGCCCTGGCTTCCAATCCTCTCTGATCTTACAGATGGAG
Wfs1-ex5-KO232   ------------------------------------------------------

Wfs1 WT          GGGCGCAGCCAGGCCCCGTCCCCAAGTCctgcaGAAGCAGAGGCGCATGCTGGAGC
Wfs1-ex5-KO232   -------------------------------------------------GCTGGAGC

Wfs1 WT          GTCTAGTGAGCAGTGAATGTGAGTATGTCCACCCTGTGAGTATGTCCACCCTGCGC
Wfs1-ex5-KO232   GTCTAGTGAGCAGTGAATGTGAGTATGTCCACCCTGTGAGTATGTCCACCCTGCGC

Wfs1 WT          TGCAGTCCTGCTACCTGCTTCCCGCACACCACCCAGCACCATACCCACTCTT
Wfs1-ex5-KO232   TGCAGTCCTGCTACCTGCTTCCCGCACACCACCCAGCACCATACCCACTCTT
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b

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                 Glu Gln Asp Gly Gly Ala Gln Pro Gly Pro Val Pro Lys Ser Leu Gln
Wfs1 WT          GAA CAG GAT GGA GGG GCG CAG CCA GGC CCC GTC CCC AAG TCC CTG CAG
Wfs1-ex5-KO232   GAA CAG G-------- del 81 bp ----------------------------------
                 Glu Gln

                 Lys Gln Arg Arg Met Leu Glu Arg Leu Val Ser Ser Glu Ser Lys Asn
Wfs1 WT          AAG CAG AGG CGC ATG CTG GAG CGT CTA GTG AGC AGT GAA TCC AAG AAC
Wfs1-ex5-KO232   ----------------------------------------------------CC AAG AAC
                                                                    Ala Lys Asn
```

Figure 8. Wfs1-ex5-KO232 mutation in rats. (**a)** DNA Sequence of exon 5 of the *Wfs1* deficient rat line 232. Rat line Wfs1-ex5-KO232 lost 184 bp (17,833 – 18,017) in the *Wfs1* gene, including 55 bp in exon 5; blue indicates the start and end of exon 5. **(b)** Comparison of cDNA and protein sequences from wild-type (WT) and Wfs1-ex5-KO232 rat lines. Line Wfs1-ex5-KO232 has lost all nucleotides from coding exon 5 of the *Wfs1* gene. According to cDNA sequencing and ORF analysis, the resulting strain had lost exon 5 in the *Wfs1* gene. Protein sequence is predicted from cDNA analysis; deletion of 55 bp from exon 5 of the rat *Wfs1* gene did not result in a frame-shift mutation. Thus, there is a loss of 27 amino acids (from coding exon 5 of the *Wfs1* gene) and a new GCC codon (coding A – alanine) at the junction of exon 4 and exon 6 in the Wfs1-ex5-KO232 rats (marked red). Plaas *et al*., 2017, modified by Toots.

4.2.2. Development of diabetes in Wfs1 KO rats

4.2.2.1. Glucose tolerance, insulin secretion and insulin sensitivity

To characterize the development of glucose intolerance and diabetes in Wfs1 KO rats, we assessed their response to glucose challenge at different ages. Results revealed that similarly to human patients who do not develop diabetes until around 6-years-of-age (Urano, 2016), until 2 months of age, KO rat's glucose tolerance and GSIS were indistinguishable from WT rats (Figure 9g-h). After that, Wfs1 KO rats began to show increasing glucose intolerance, decreased basal insulin levels, and diminished glucose stimulated insulin secretion, culminating in diabetes with basal blood glucose levels around 20 mM at 13 months of age (Figure 9). Therefore, Wfs1 KO rats are the first WS animal model with overt diabetes (Plaas *et al*., 2017).

Before the strong increase in basal blood glucose levels, KO rats exhibited glycosuria already at 10 months of age (Figure 9c). This coincides with the changes in bodyweight (Figure 9a), as KO rat's bodyweight was indistinguishable from WT until 3 months of age, remained slightly lower than WT after that, and started to decrease around the same time as their urine glucose levels increased (Plaas *et al*., 2017).

As glucose intolerance may also result from decreased tissue responsiveness to insulin, 3- and 11-month-old WT and Wfs1 KO rats were subjected to insulin tolerance tests (Figure 9e-f). As with mouse models, there were no differences between WT and KO rats, regardless of age (Plaas *et al*., 2017).

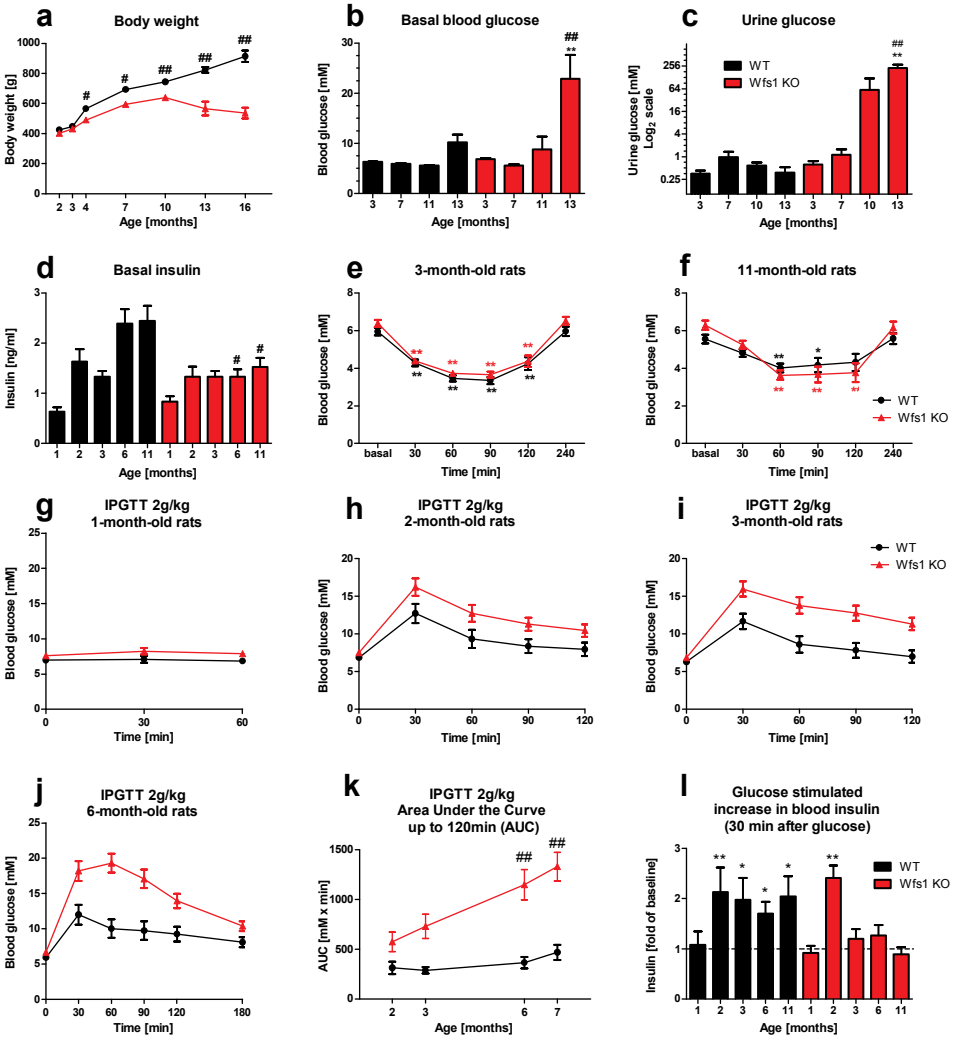

Figure 9. Development of diabetes mellitus in Wfs1 KO rats. (a) Wfs1 KO animals are slightly lighter than wild-type (WT) rats of similar ages and begin to lose weight after 10 months of age. **(b)** Basal blood glucose levels were similar for both genotypes up to 11 months of age; thereafter, Wfs1 KO rats develop hyperglycemia. **(c)** Wfs1 KO rats developed glycosuria after 10 months of age. **(d)** Insulin levels were lower in older Wfs1 KO animals compared to levels in WT animals of the same age. For the insulin tolerance tests (ITTs), human insulin was administered (1 U/kg, s.c.), and blood glucose levels were measured at the indicated time points. There were no genotype-associated changes in insulin sensitivity in either **(e)** young or **(f)** old animals. For intraperitoneal glucose tolerance tests (IPGTTs), blood glucose levels were measured after administration of glucose (2 g/kg i.p.). **(g, h)** Glucose tolerance was similar for both genotypes at 1 and 2 months. **(i)** At 3 months of age, Wfs1 KO rats showed a slight glucose intolerance compared with WT rats, which was exacerbated at **(j)** 6 months. **(k)** Area under the curve for IPGTT results at different ages. **(l) G**lucose-stimulated increases in blood insulin levels (relative to baseline) 30 minutes after glucose administration, Wfs1 KO animals showed a defect in insulin secretion after 3 months of age. The data were compared using two-way ANOVAs followed by unequal N HSD post hoc tests; #$p < 0.05$, ## < 0.01 between genotypes, *$p < 0.05$ **, $p < 0.01$ within genotype (vs baseline and vs 3 months age). The data are presented as the mean ± SEM, n = 6–8. Plaas *et al.*, 2017, modified by Toots.

4.2.2.2. Langerhans islet mass

Wfs1 KO rats exhibited a decrease in glucose tolerance and insulin secretion, but no changes in insulin sensitivity. As previous reports have shown decreased beta cell mass in Wfs1 mouse models (Ishihara *et al*., 2004; Riggs *et al*., 2005), we quantified Langerhans islet size and mass from WT and KO rats at different ages (Plaas *et al*., 2017).

At 3 months of age, Wfs1 KO and WT rats had similar islet mass, even though the number of small islets in WT rats was higher (Figure 10a, d). By 7 months of age, WT rat's islet mass had increased, but KO rats had started to lose islet mass, resulting in KO animals only having about half of the islet mass of WT rats (Figure 10b, e). This explains the KO rat's strong glucose intolerance and deficit in insulin secretion at that age. By 14 months of age, Langerhans islets had virtually disappeared in KO rats (Figure 10c, f), verifying the loss of islet mass to be, at least for most part, the cause of diabetes mellitus in Wfs1 KO rats (Plaas *et al*., 2017).

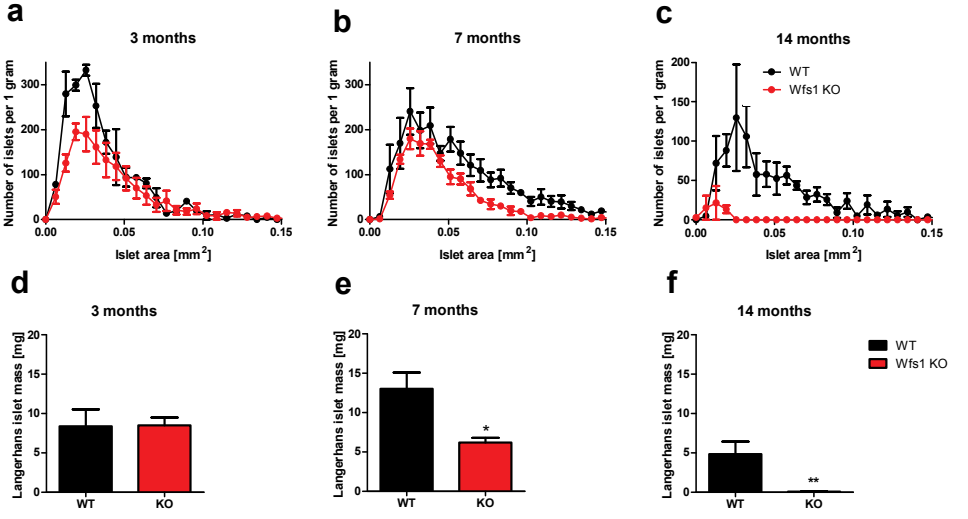

Figure 10. Langerhans islet mass in Wfs1 KO rats. Size distribution of islets of Langerhans in rats at **(a)** 3, **(b)** 7 and **(c)** 14 months of age. Islet mass in rats at **(d)** 3, **(e)** 7 and **(f)** 14 months of age. The data were compared using one-way ANOVAs followed by unequal N HSD post hoc tests; *p < 0.05, ** < 0.01 between genotypes. The data are presented as the mean ± SEM, n = 2–6. Plaas *et al*., 2017, modified by Toots.

4.2.2.3. ER stress markers in Langerhans islets

WFS1 function has been associated with regulating ER stress, as cells lacking functional WFS1 and Langerhans islets isolated from Wfs1 deficient mice both exhibit enhanced ER stress response (Yamada *et al*., 2006). Therefore, the loss in islet mass might be due to increased ER stress. The mRNA expressions of ER stress markers *Xbp1* splicing and *BiP* were evaluated in isolated Langerhans islets. While there were no differences between KO and WT rat's *BiP* levels either at 3 or 7 months of age (Figure 11m, o), *Xbp1* splicing was increased already in 3-month-old KO rat's islets when compared to WT (Figure 11n), and this difference was further exuberated by 7 months of age (Figure 11p) (Plaas *et al*., 2017).

Quantification of BiP and XBP1 from histological slices did not reveal increased ER stress in KO animal's islets at 3 months of age (Figure 11i-j), but showed strong increase in levels of both ER stress markers in 7-month-old KO rat's Langerhans islets, compared to WT islets (Figure 11k-l) (Plaas *et al*., 2017).

These results indicate that ER stress may have a critical role in Wfs1 KO rat's loss of Langerhans islet mass and resulting diabetes mellitus (Plaas *et al*., 2017).

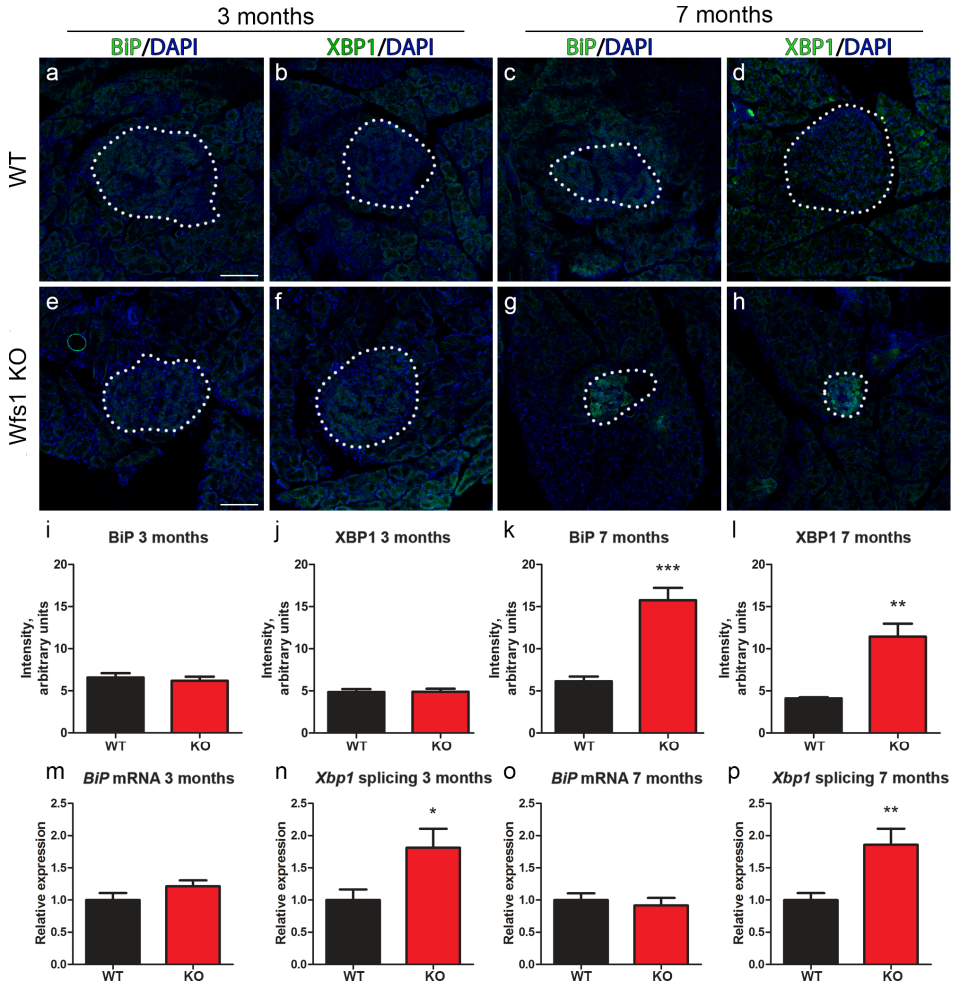

Figure 11. Expression of XBP1 and BiP in islet of Langerhans. Immunofluorescence analysis of endoplasmic reticulum (ER) stress markers **(a, e, c, g)** BiP and **(b, f, d, h)** XBP1 in islets of Langerhans (dotted lines) from 3- and 7-month-old rats. At 3 months, the expression of **(a)** BiP and **(b)** XBP1 in control rats and **(e, f)** Wfs1 KO rats was comparable. By 7 months, the expression of **(c)** BiP and **(d)** XBP1 remained at basal level in WT rats, whereas the expression of these ER stress markers was clearly elevated in **(g, h)** the islets of Langerhans of Wfs1 KO rats. **(i – l)** Quantification of signal intensity of ER stress markers. Levels of **(k)** BiP and **(l)** XBP1 were increased in islets of Langerhans from 7-month-old Wfs1 KO rats. **(m, n, o, p)** mRNA analysis of ER stress markers *BiP* and spliced *Xbp1* in lysates of isolated islets of Langerhans. Expression level of *BiP* was not altered in **(m)** 3-month-old or **(o)** 7-month-old Wfs1 KO rats compared to expression in wild-type rats. *Xbp1* splicing was increased in **(n)** 3-month- and **(p)** 7-month-old Wfs1 KO rats compared to expression in WT rats. The data were compared using one-way ANOVAs followed by unequal N HSD post hoc tests; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ between genotypes. The data are presented as the mean ± SEM, n = 4 to 9. Cell nuclei (blue) were counterstained with DAPI. Scalebar: 100 µm. Plaas *et al.*, 2017, modified by Toots.

4.2.2.4. Conclusion

Rat model of Wolfram syndrome exhibits clear progression of diabetic symptoms (Figure 12) with normal Langerhans islet mass, insulin secretion and glucose tolerance at early age. In time, their insulin secretion decreases due to loss in beta cell mass (as indicated by reduction in Langerhans islet mass), most likely as a result from ER stress. This leads to glucose intolerance that culminates with diabetes mellitus, basal hyperglycemia and glycosuria (Plaas *et al*., 2017).

With the progression of symptoms, Wfs1 KO rat presents a new model to evaluate the effectiveness of drugs to possibly slow the progression of both Wolfram syndrome, and diabetes mellitus resulting from loss of beta cells due to ER stress (Plaas *et al*., 2017).

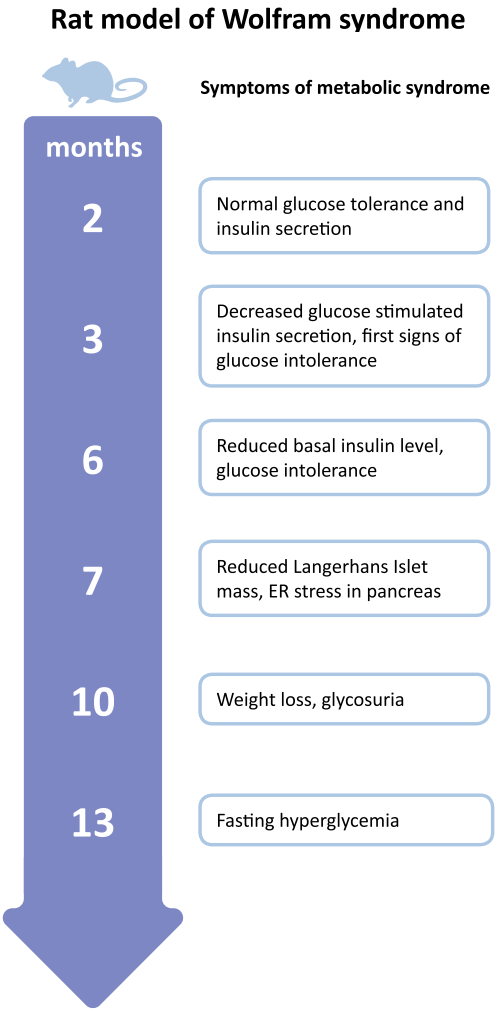

Figure 12. Development of metabolic symptoms in Wfs1 KO rat. Toots *et al*., 2018 (2), modified by Toots.

4.3. Treatment of Wfs1 deficient rats with liraglutide (Paper III)

4.3.1. Liraglutide effect on glucose intolerant Wfs1 KO rats

GLP-1 regulates directly through GLP-1R and indirectly through neuronal and endocrine pathways different physiological processes (Bockaert & Pin, 1999). The precise ways, GLP-1 affects cells, are still being identified. Among other things, GLP-1 increases insulin secretion in glucose dependent manner, regulates cell apoptosis, proliferation, and decreases ER stress (Bouskine *et al*., 2009; Persaud, 2017; Sargsyan *et al*., 2016; Wang *et al*., 2004). Acute treatment with GLP-1 receptor agonist exenatide has shown blood glucose lowering effect in Wfs1 KO mice (Sedman *et al*., 2016). To see if a similar effect can be seen in Wfs1 KO rats, 5-month-old Wfs1 KO and WT rats were subjected to glucose tolerance test before, and after 8 days of chronic liraglutide (GLP-1 receptor agonist) treatment, with last injection 4.5 hours before the experiment (Toots *et al*., 2018 (2)).

As expected, unlike WT rats, 5-month-old naïve Wfs1 KO rats exhibited long-lasting hyperglycemia, in response to glucose ($p<0.001$) (Figure 13a, b). 8-day treatment with liraglutide was able to normalize glucose tolerance in KO rats, decreasing area-under-curve to similar level with WT rats (Figure 13b) (Toots *et al*., 2018 (2)).

Serum insulin and C-peptide levels were significantly lower in KO rats than in WT rats, measured 30 minutes after glucose challenge (Figure 13c-d). There was no statistical difference in Wfs1 KO rats 30 min after glucose injection insulin or C-peptide levels before and after liraglutide treatment. However, liraglutide treatment decreased basal levels of both peptides. Taken this into consideration, KO rats had no increase in response to glucose before treatment, but after liraglutide treatment, their 30 min post glucose levels were two times higher than basal levels. Even though there was no statistical change in secretion before and after treatment, this change compared to baseline level indicates improved insulin secretion. Therefore, short-term treatment with GLP-1 agonist liraglutide normalizes glucose tolerance in Wfs1 KO rats, most likely by increasing GSIS (Toots *et al*., 2018 (2)).

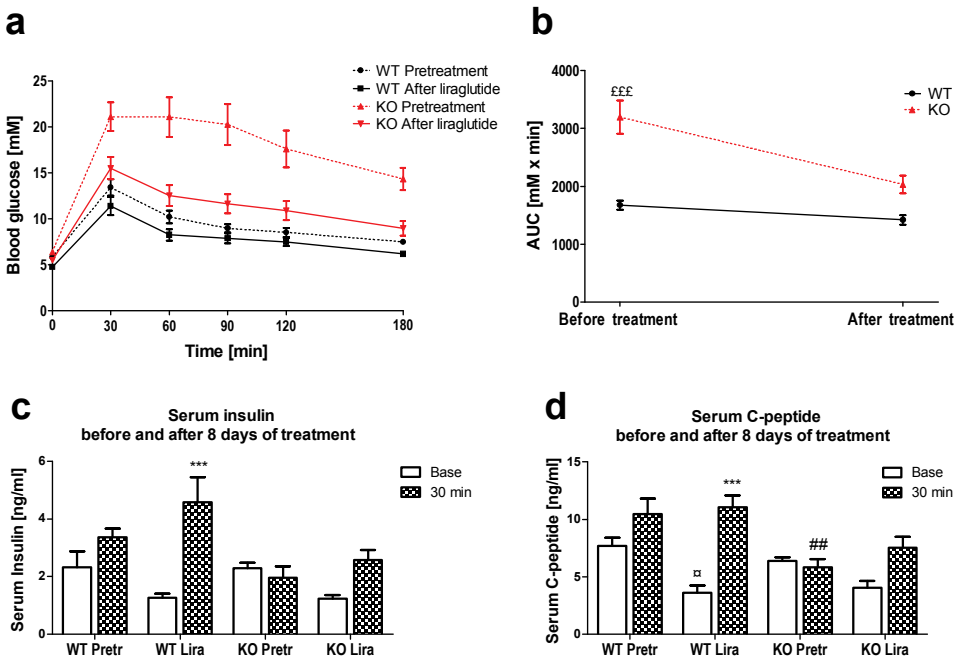

Figure 13. Repeated liraglutide effect on glucose intolerant 5-month-old Wfs1 KO rats. (a) IPGTT (2g/kg) before and after 8-day chronic administration of 0.4 mg/kg liraglutide to 5-month-old male Wfs1 KO and control rats. (**b)** Decrease in IPGTT (2g/kg) Area under curve before and after chronic liraglutide treatment for 8 days. Glucose-stimulated increases in blood **(c)** insulin and **(d)** C-peptide levels before and 30 minutes after glucose administration. The data were compared using repeated measures ANOVA followed by unequal N HSD post hoc tests; £££ $p<0.001$ compared to WT AUC during same IPGTT. *** $p<0.001$ compared to baseline of the same genotype during the same IPGTT. ## $p<0.01$ compared to WT animals in the same timepoint during the same IPGTT. ¤ $p<0.05$ compared to WT saline animals in the same timepoint during the first IPGTT. The data are presented as the mean ± SEM, n=6–8. Toots *et al*., 2018 (2), modified by Toots.

4.3.2. Preventive treatment with liraglutide

Previous experiments showed that Wfs1 KO rats exhibit increased ER stress in Langerhans islets, which may also be the cause for the decrease in islet mass and development of glucose intolerance in KO rats. GLP-1 agonist exendin-4 has been shown to reduce ER stress in cardiac myocytes (Younce *et al*., 2013), and GLP-1 receptor agonist liraglutide has been shown to decrease beta cell apoptosis in pre-diabetic Goto-Kakizaki rats (Luo *et al*., 2013). As short-term chronic treatment is able to restore glucose tolerance and insulin secretion in 5-month-old Wfs1 rats, we aimed to see, if starting pretreatment with liraglutide before the onset of symptoms, could decrease the ER stress and possibly apoptosis of beta cells, and therefore, postpone or protect against development of glucose intolerance in Wfs1 KO rats (Toots *et al*., 2018 (2)).

4.3.2.1. Glucose tolerance

We started the experiment with 2-month-old KO rats, whose blood glucose profile after glucose challenge was indistinguishable from WT littermates (Figure 14b). During the 19-week long experiment, the glucose tolerance profile of Wfs1 KO rats receiving liraglutide remained similar to that of their WT littermates, while Wfs1 KO rats in control group developed glucose intolerance (Figure 14b-e), also demonstrated by stable increase of area-under-curve ($p<0.001$) (Figure 14f). One week before the last IPGTT, rats' insulin sensitivity was also tested, revealing no differences between genotypes or treatment-groups (Figure 14i) (Toots *et al*., 2018 (2)).

4.3.2.2. Langerhans islet mass

As shown before, KO rats receiving saline injections had almost two-fold lower Langerhans islet mass than WT rats in saline group ($p<0.01$) (Figure 14g). Similarly to previous reports, liraglutide decreased islet mass in WT rats (Figure 14g) (Ellenbroek *et al*., 2013; Mondragon *et al*., 2014). However, liraglutide seemed to have the opposite effect in Wfs1 KO rats, most likely by inhibiting decrease in beta cell mass seen in previous experiments (Figure 14g) (Toots *et al*., 2018 (2)).

Liraglutide treatment is accompanied by decrease in body weight (Figure 14a). Taking this into account, Langerhans islet mass/body weight ratio was unchanged upon liraglutide treatment in WT rats. However, saline-treated Wfs1 KO rats had significantly lower Langerhans islet mass/body mass ratio ($p<0.05$) than saline-treated WT rats and this difference was eliminated under liraglutide treatment, bringing Wfs1 KO rats' Langerhans islet mass/body mass ratio similar to that of WT rats (Figure 14h) (Toots *et al*., 2018 (2)).

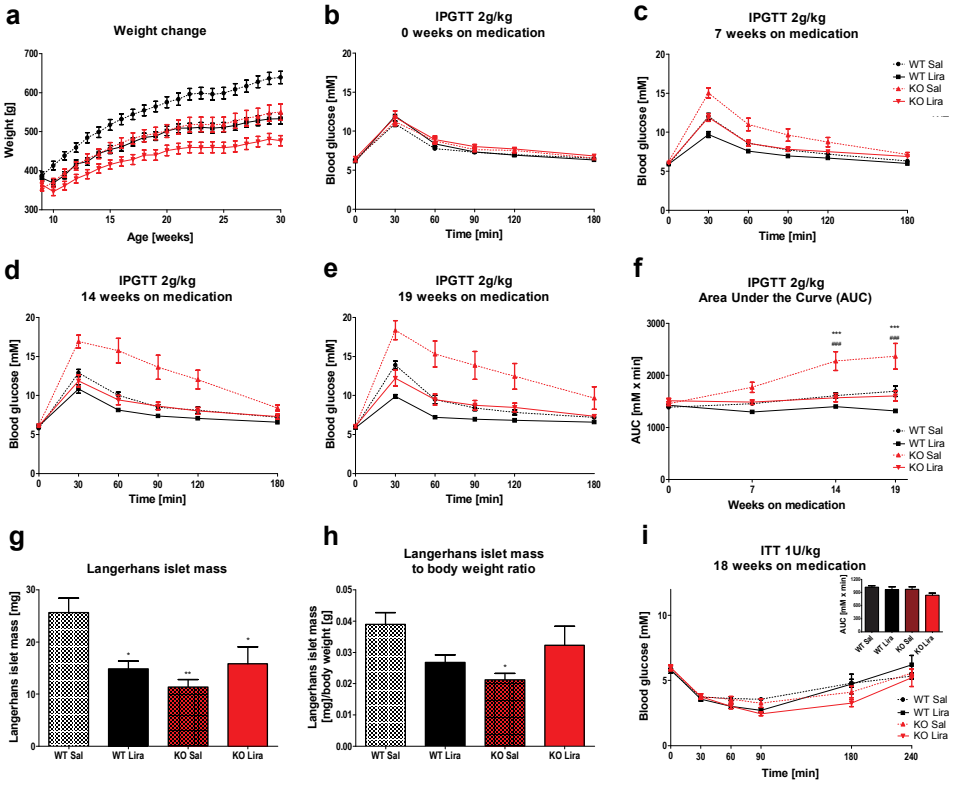

Figure 14. Development of glucose intolerance over 19 weeks of liraglutide treatment. (a) Weight change over 19 weeks of liraglutide administration. Blood glucose profile after glucose challenge during liraglutide treatment **(b)** before, **(c)** 7 weeks, **(d)** 14 weeks and **(e)** 19 weeks after the beginning of liraglutide treatment. **(f)** Area under curve analyses for IPGTT results at different timepoints. **(g)** Langerhans islet mass and **(h)** Langerhans islet mass/body weight ratio after 19 weeks of liraglutide treatment. **(i)** Insulin tolerance test with 1U/kg human insulin after 18 weeks of liraglutide treatment. The data were compared using repeated measures ANOVA or one-way ANOVA followed by unequal N HSD post hoc tests; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to (same-age) WT saline-treated animals. ### $p<0.001$ compared to same-age Wfs1 KO liraglutide-treated animals. The data are presented as the mean ± SEM, n=12–15 (weight and IPGTT), n=6–8 (ITT), n=4–6 (islet measurements). Toots *et al*., 2018 (2), modified by Toots.

4.3.2.3. Insulin, C-peptide and glucagon secretion

During 19 weeks, we saw no change is insulin or C-peptide concentration, measured 30 minutes after glucose injection (Figure 15a-e). However, liraglutide decreased basal insulin and C-peptide levels (Figure 15d-e). When compared to basal levels, liraglutide-treated Wfs1 KO rats showed a twofold increase in both insulin and C-peptide levels 30 minutes after stimulation, while saline-treated Wfs1 KO rats' insulin and C-peptide levels did not change from the basal level in response to glucose. While insulin is rapidly removed from circulation by the liver, the half-life of C-peptide is at least 20 minutes (Canas *et al*., 1995; Katz &

Rubenstein, 1973). Therefore, we can presume that C-peptide concentration measured 30 minutes after glucose challenge reflects the combined secretion of first- and second-phase insulin during the whole stimulatory period. These results indicate constant low-level insulin secretion from Wfs1 KO rats that is only slightly elevated by glucose challenge. Liraglutide treatment appears to correct that deficit by enhancing the first phase of insulin secretion. In addition to enhancement in insulin secretion, GLP-1R agonists have also been shown to reduce pro-insulin:insulin ratio (this ratio is elevated in subjects with type 2 diabetes), possibly as a result of reduced ER stress and therefore, improved proinsulin processing (Degn *et al*., 2004). Increased pro-insulin:insulin ratio has also been shown in mice lacking functional WFS1 (Noormets *et al*., 2011). Improper pro-insulin processing may also explain the lack of blood glucose lowering effect of depolarizing medication in Wfs1 deficient mice (Sedman *et al*., 2016). As both insulin and C-peptide ELISAs used in current study may also cross-react with pro-insulin (according to our inquiry to the manufacturer), the improved glycemic control may also in part result from properly processed insulin after liraglutide treatment. These results are in accordance with those obtained from Wfs1 deficient mice, whose blood glucose levels during IPGTT are significantly lowered after treatment with the GLP-1 analogue exenatide, an effect not accompanied by increased serum insulin concentration 30 minutes after stimulation (Sedman *et al*., 2016). Similar results were recently also obtained on one of the Wfs1 deficient mouse models, whose blood glucose control and insulin secretion were improved following both acute and chronic treatment with GLP-1R agonist exenatide. However, they did not see changes in pancreatic islet mass, most likely due to shorter treatment protocol (4 weeks) (Kondo *et al*., 2018). Also, administration of exenatide to Wolfram syndrome 2 (WS2, CISD2 mutation) patient lowered daily insulin dose 70% and improved glycemic control (Danielpur *et al*., 2016). Knock-down of CISD2 by shRNA in INS-1 cells decreased insulin secretion in response to glucose or KCl by 30% and this effect was also reversed by exenatide treatment (Danielpur *et al*., 2016). The similar response in these WS and WS2 models indicates the effectiveness of GLP-1 analogues, probably resulting from the same molecular mechanism (Toots *et al*., 2018 (2)).

Glucagon levels in liraglutide treated Wfs1 KO rats were significantly lower than in saline treated rats ($p<0.05$) (Figure 15f). The change in first-phase insulin secretion may also be at least partially responsible for the change in glucagon secretion, as intra-islet insulin, secreted from beta cells, is necessary for glucagon secretion inhibition (Greenbaum *et al*., 1991; Starke *et al*., 1987; Xu *et al*., 2006). Also, both liraglutide and GLP-1 have been shown to decrease glucagon levels (Harder *et al*., 2004; Zander *et al*., 2002). Exenatide experiments with Wfs1 KO mice did not reveal any differences in glucagon secretion, but this might be due to a shorter treatment protocol or the fact that Wfs1 KO mice do not exhibit such strong progression of glucose intolerance over time (Sedman *et al*., 2016). Disturbances in suppression of mealtime glucagon concentration are also present in type 2 diabetes patients, whose hepatic glucose production contributes to hyperglycemia (Bagger *et al*., 2014; Junker *et al*.,

2016). Changes in both insulin (as indicated by C-peptide levels) and glucagon secretion in Wfs1 KO rats are corrected by liraglutide treatment, indicating improved beta and alpha cell sensitivity and function (Toots *et al*., 2018 (2)).

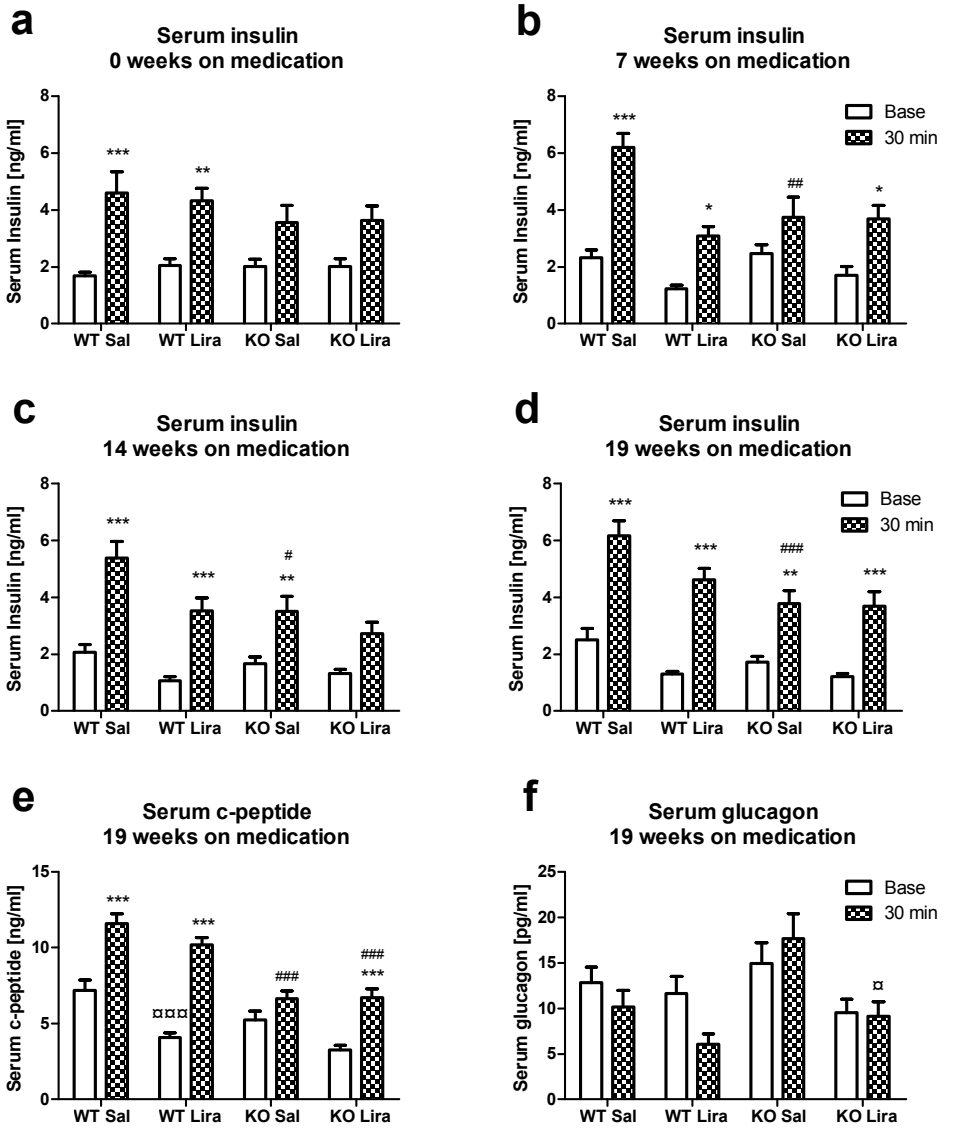

Figure 15. Serum insulin, C-peptide, and glucagon levels before and 30 minutes after glucose administration. (a-d) Insulin levels after various time on liraglutide treatment, **(e)** C-peptide and **(f)** glucagon levels after 19 weeks on liraglutide treatment. The data were compared using repeated measures ANOVA followed by unequal N HSD post hoc tests; * p <0.05, ** p<0.01, *** p<0.001 compared to the baseline of the same animals; # p <0.05, ## p<0.01, ### p<0.001 compared to WT animals of same treatment at the same timepoint; ¤ p <0.05, ¤¤¤ p<0.001 compared to Saline treatment of the same GT at the same timepoint. The data are presented as the mean ± SEM, n=12–15. Toots *et al*., 2018 (2), modified by Toots.

4.3.2.4. Gene expression analyses from Langerhans islets

Improvement in Langerhans islet mass and hormone secretion indicates overall better health of Langerhans islets. To assess the health of islet cells and ER stress in islets, we performed gene expression analyses from Langerhans islets, isolated from rats after 19 weeks on saline or liraglutide treatment (Toots *et al*., 2018 (2)).

Analyses revealed no significant changes in *Wfs1* expression (Figure 16a). However, *GLP-1 receptor* expression in Langerhans islets was decreased by 30% in Wfs1 KO animals, compared to WT ($p<0.001$). Downregulation of GLP-1 receptor in beta cells has also been observed in diabetic patients and animal models of diabetes (Shu *et al*., 2009; G. Xu *et al*., 2007). Chronic treatment with liraglutide had a slight increasing effect on *GLP-1* receptor mRNA levels in both genotypes (Figure 16b) (Toots *et al*., 2018).

In saline treated group, KO rats had a higher proliferation marker *Ki67* expression than WT rats ($p<0.05$) (Figure 16f). Even though decline in Langerhans islet mass did not occur in liraglutide-treated Wfs1 KO rats, Langerhans islet cell proliferation had significantly declined in both genotypes ($p<0.05$), indicating that the main effect in improved Langerhans islet mass was not caused by increased proliferation in Wfs1 KO rats. These results are in accordance with those obtained from pre-diabetic Goto-Kakizaki rats, whose proliferation marker Ki67 levels had also declined in response to liraglutide treatment (Luo *et al*., 2013). In humans, beta cell proliferation is very low after the first few years of life, and treatments increasing proliferation in rodents are unlikely to cause the same in human beta cells (Wang *et al*., 2015). Therefore, as liraglutide's positive effect appears not to be caused by increased proliferation, it remains a promising treatment strategy for human WS patients (Toots *et al*., 2018 (2)).

Unlike Luo *et al*, we did not see a significant effect of liraglutide on islet apoptosis (Figures 16g-k) (Luo *et al*., 2013). This might be due to the fact that Luo *et al*. used very young rats, whose beta cells are actively proliferating and a shorter treatment protocol (Finegood *et al*., 1995; Luo *et al*., 2013). Also, reduction in beta cell mass develops very slowly over time, as fasting hyperglycemia is apparent around 13 months of age, and even then, most likely there is some small portion of beta cells left (Plaas *et al*., 2017). Moreover, we are analyzing whole islets, making it more difficult to detect differences only apparent in beta cells. In healthy rats, 20–35% islet cells are not beta cells and this ratio is even higher in aging Wfs1 KO rats (Kageyama *et al*., 2005), who lose beta cell mass. Therefore, it is possible that the difference in rate of apoptosis is so low, that we are not able to detect it (Toots *et al*., 2018 (2)).

The ER stress markers *Grp78* and *Xbp1* splicing were increased in Wfs1 KO rats compared to WT littermates. Liraglutide treatment decreased ER stress, regardless of genotype, resulting in similar levels in saline-treated WT and liraglutide-treated KO animals ($p<0.05$) (Figure 16c–d).

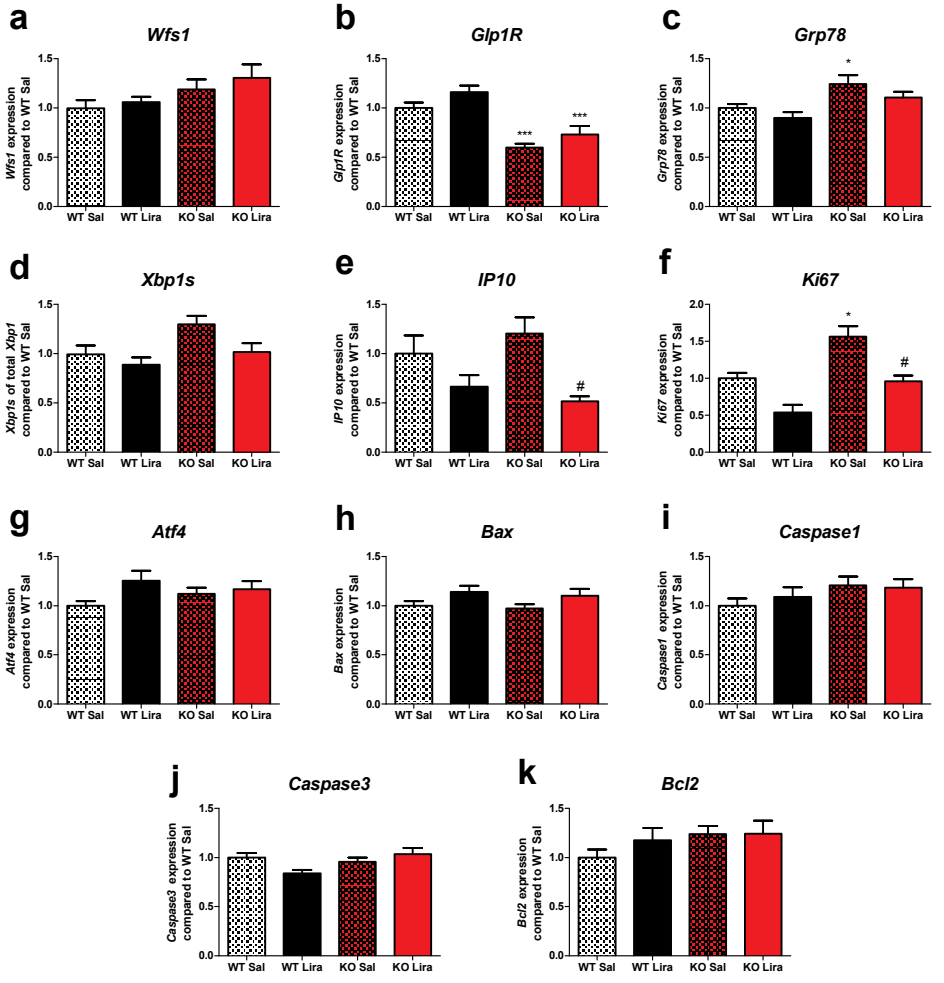

Figure 16. Langerhans islet gene expression analyses after 19 weeks of liraglutide treatment compared to WT saline group. Gene expression of **(a)** *Wolframin1*; **(b)** *GLP-1 receptor*; ER stress markers **(c)** *Grp78* and **(d)** *Xbp1* splicing; inflammation marker **(e)** *IP10*; proliferation marker **(f)** *Ki67*; apoptosis markers **(g)** *Atf4*, **(h)** *Bax*, **(i)** *Caspase 1*, **(j)** *Caspase 3,* and **(k)** *Bcl2*. The data were compared using factorial ANOVA followed by unequal N HSD post hoc tests; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to WT animals of the same treatment; # $p<0.05$, ## $p<0.01$ compared to saline animals of the same GT. The data are presented as the mean ± SEM, n=4–8. Toots *et al*., 2018 (2), modified by Toots.

Inflammatory marker *IP10* levels revealed no differences between genotypes, in neither saline nor liraglutide treatment group. However, liraglutide treatment had a significant decreasing effect on *IP10* expression in both genotypes ($p<0.05$) (Figure 16e). Inflammation has long been recognized as a contributing factor in the development of diabetes (Ehses *et al*., 2007; McDaniel *et al*., 1996), and both GLP-1 agonists and DPP-4 inhibitors have been shown to suppress inflammation in pancreatic islets and adipose tissue (Dobrian *et al*., 2011; Huang *et al*., 2015; Velmurugan *et al*., 2012). Taken together, these results indicate better overall health of islet cells (Toots *et al*., 2018 (2)).

4.3.3. Conclusion

Early chronic intervention with GLP-1 receptor agonist liraglutide decreased the loss of Langerhans islet mass in Wfs1 mutant rats, improved insulin and glucagon secretion control, and reduced ER stress and inflammation in Langerhans islets. Thus, treatment with GLP-1 receptor agonists is a promising strategy as a preventive treatment for human WS patients (Toots *et al*., 2018 (2)).

4.4. Feasibility of WS rat model

Currently existing Wfs1 deficient mouse models develop glucose intolerance at an early age, exhibit reduced insulin secretion, and reduction in beta cell mass. However, while human patients with WS develop insulin dependent diabetes at average age of six years, current mouse models seem to not exhibit overt diabetes (except for few occasional animals in different genetic backgrounds) (Ishihara *et al*., 2004; Riggs *et al*., 2005; Urano, 2016).

Wfs1 KO rat is the first WS animal model to show clear signs of progressing diabetic symptoms due to increased ER stress, leading to decrease in beta cell mass, culminating in overt diabetes. While human patients develop diabetes around 6-years-of-age, Wfs1 rats develop diabetes already around 1 year. This might be due to very fast metabolism of rats or, due to Sprague-Dawley male rats very fast gain of bodyweight, putting more strain on beta cells. Our preliminary experiments have shown that female Wfs1 KO rats develop diabetes later than male rats, possibly because of much slower weight gain or hormonal differences.

Wfs1 KO rats show increased glucagon secretion and reduced *GLP-1R* mRNA expression on islet cells. Both of these symptoms are also characteristic for type 2 diabetes patients (Bagger *et al*., 2014; Shu *et al*., 2009; Xu *et al*., 2007). As progression of diabetes in Wfs1 KO rats is slow and accompanied by symptoms present in type 2 diabetes, Wfs1 rat model can not only be used to study WS, but also presents a great model to test new diabetes drugs.

4.5. Future directions

Future experiments will most likely be performed using the new rat model, as rat offers much better model for long-term experiments (e.g. for repeated blood collection, more precise *in vivo* imaging). In current work, we started the experiment with 2-month-old rats and ended the experiment when strong hyperglycemia in response to glucose in Wfs1 KO saline group had developed. In future, it would be advised to perform lifelong experiments, starting few weeks after birth. This would also require bigger experimental groups, to analyze animals at different time-points. There are also newer GLP-1R agonists that would not require everyday injections and would reduce the stress to the animals.

WS consists of several different symptoms. In current work, only the first symptom – diabetes, was studied. GLP-1R agonists have also been shown to have neuroprotective properties, therefore, in next experiments, the effect of these medications to other symptoms of WS should be assessed.

Currently, patients reach the diagnosis of WS only after several symptoms have developed and the medical treatment is limited to managing the symptoms. As genetic testing is getting more and more available, in the future, it might be possible for children to get tested for *WFS1* mutations soon after birth. Pretreatment before the onset of symptoms might then provide these patients with the possibility to postpone the development of symptoms and to increase the amount of high quality years and increase life expectancy.

CONCLUSIONS

1. Wfs1 deficiency causes glucose intolerance in mice as a result of decreased insulin secretion. To a lesser extent, this deficit is also apparent in heterozygous Wfs1 deficient mice, even though they do not exhibit glucose intolerance. Activation of M3 receptors on beta cell by carbachol was able to initiate insulin secretion from beta cells and normalize blood glucose control in Wfs1 deficient mice. Therefore, selective M3R agonists and possibly other ligands, able to activate the same pathways in beta cell, are a promising way to release insulin and to reverse glucose intolerance, resulting from Wfs1 deficiency.
2. Using zinc-finger technology, three different Wfs1 mutant rat lines with mutations in exon 5 were created. Genomic sequencing revealed that two of the lines (Wfs1-ex5-KO232 and Wfs1-ex5-KO266) had an in frame deletion in exon 5 of the *Wfs1* gene and the third line (Wfs1-ex5-INS244) had an in frame substitution in exon 5 of the *Wfs1* gene. This substitution is predicted to result in amino-acids 224–226 (LQK) being substituted with YCMNTI in the WFS1 protein. All three rat lines exhibited glucose intolerance at 7 months of age, only line Wfs1-ex5-KO232 (in experiments marked as Wfs1 KO) males were used in subsequent experiments.

   Rat model of Wolfram syndrome exhibits normal Langerhans islet mass, insulin secretion and glucose tolerance at an early age. In time, their insulin secretion decreases due to loss in beta cell mass (as indicated by reduction in Langerhans islet mass) as a result from ER stress. This leads to glucose intolerance that culminates with diabetes mellitus, basal hyperglycemia and glycosuria. With progression of symptoms, Wfs1 KO rat presents a new model to evaluate the effectiveness of drugs to possibly slow the progression of both Wolfram syndrome, and diabetes mellitus resulting from loss of beta cells due to ER stress.
3. Treatment with GLP-1R agonist liraglutide was started at 2 months of age, when Wfs1 KO rat's glucose tolerance is indistinguishable from WT rats. Early chronic intervention decreased the loss of Langerhans islet mass in Wfs1 mutant rats, improved insulin and glucagon secretion control, and reduced ER stress and inflammation in Langerhans islets. Thus, treatment with GLP-1 receptor agonists is a promising strategy as a preventive treatment for human WS patients.
4. Similarly to human patients, Wfs1 KO rat is the first rodent model of WS to develop overt diabetes with basal hyperglycemia. With normal glucose homeostasis at an early age, Wfs1 KO rats show progressing diabetic symptoms due to increased ER stress, leading to decrease in beta cell mass, culminating in overt diabetes.

   Similarly to patients with type 2 diabetes, Wfs1 KO rats show increased glucagon secretion and reduced *GLP-1R* mRNA expression in islet cells. With these typical type 2 diabetes symptoms and slow progression of diabetes in Wfs1 KO rats, Wfs1 rat model can not only be used to study WS, but also presents a great model to test new diabetes drugs.

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SUMMARY IN ESTONIAN

Farmakoloogiline väljakutse diabeedi fenotüübi arengu pidurdamiseks Wolframi sündroomi näriliste mudelites

Wolframi sündroom (WS) on autosomaalne retsessiivne haigus, mida põhjustavad mutatsioonid Wolframin1 (*WFS1)* geenis. Esimese sümptomina avaldub patsientidel progresseeruvast beeta rakkude hävinemisest põhjustatud suhkrudiabeet. Suure tõenäosusega põhjustab rakkude hävinemist häiritud rakusisene Ca^{2+} taseme regulatsioon, mis viib suurenenud endoplasmaatilise retiikulumi stressi ning rakusurmani. Suhkrudiabeedile järgneb enamasti optilise närvi atroofiast põhjustatud nägemise halvenemine. Sensorineuraalne kurtus, magediabeet, kuseteede probleemid ja neuroloogilised probleemid järgnevad erineva sageduse ning raskusastmetega. WS-ga kaasneb erinevaid neuroloogilisi probleeme nagu neuropaatia, peavalud, ataksia, düsfaagia ning ajutüve atroofia. Lisaks kannatavad paljud patsiendid psühhiaatriliste probleemide all. Ajutüve atroofiast põhjustatud hingamisseiskus on WS patsientide peamiseks surma põhjuseks kolmekümnendates eluaastates.

Kui WS sagedus erinevates riikides on hinnanguliselt 1:100 000-1:770 000, siis heterosügootsete *WFS1* mutatsiooni kandjate sagedus on umbkaudu 1:100-1:354. Lisaks haruldastele *WFS1* mutatsioonidele, mis põhjustavad dominantset autosomaalset diabeeti, on ülegenoomsed assotsatsiooni uuringud leidnud, et heterosügootsetel *WFS1* mutatsiooni kandjatel on suurenenud risk teist tüüpi diabeedi tekkeks. Teist tüüpi diabeet on aina laiemini leviv globaalne terviseprobleem, hõlmates ligi 90% diabeedi juhtudest. Teist tüüpi diabeet on põhjustatud elustiili valikute ning geneetiliste riskide koostoimest, viies vähenenud insuliini tundlikkuse ning sekretsioonini. Seega aitaks parem arusaam WFS1 funktsioonist mitte ainult haruldast WS põdevaid patsiente, vaid ka inimesi, kes kannatavad selliste laialt levinud haiguste nagu teist tüüpi diabeet käes.

Hetkel ei ole olemas ravimeid, mis suudaksid aeglustada WS kulgu või seda ravida. Seetõttu on medikamentoosne ravi fokusseeritud sümptomite leevendamisele. Sellest tulenevalt oli käesoleva doktoritöö peamiseks eesmärgiks leida uusi ravi strateegiaid ning võimalikke ravimikandidaate, mis võiksid aidata Wolframi sündroomi patsiente.

Esimeses artiklis uurisime *Wfs1* mutatsioonist põhjustatud glükoosi talumatust ning insuliini sekretsiooni Wfs1 puudulikkusega hiirtes. Lisaks uurisime võimalusi normaliseerida insuliini sekretsiooni aktiveerides muskariini 3 retseptorit (M3R). Insuliini sekretsiooni mõjutavad paljud erinevad hormoonid ja virgatsained. Perifeerse parasümpaatilise närvisüsteemi virgatsaine atsetüülkoliin võimendab insuliini sekretsiooni beeta rakkudest pärast seondumist M3 retseptorile. Varasemad uuringud on näidanud, et selle mehhanismi kaudu on võimalik taastada insuliini sekretsioon näiteks geneetilises diabeedi mudelis, Goto-Kakizaki rotis. Sarnaselt varasematele katsetele leidsime, et Wfs1 puudulikkusega homosügootsetel hiirtel on oluliselt vähenenud glükoosi stimuleeritud insuliini sekretsioon, mis tingib glükoosi manustamisel hüperglükeemia. Lisaks

avastasime, et kuigi heterosügootsetel Wfs1 hiirtel ei esine glükoosi manustamise järel hüperglükeemiat, on nendel loomadel insuliini vabanemine häiritud. See langeb kokku suurenenud teist tüüpi diabeedi tekke võimalusega heterosügootsetel *WFS1* mutatsiooniga inimestel. M3R aktiveerimine, kasutades karbakooli, aitas homosügootsete hiirte beeta rakkudest vabastada insuliini ning normaliseeris sellega nende veresuhkru taseme. Seega on selektiivsed M3 agonistid ning teised ravimid, mis suudavad aktiveerida beeta rakkudes samu mehhanisme, lootustandvad vahendid Wfs1 defitsiidist põhjustatud insuliini sekretsiooni häirete ja glükoosi talumatuse raviks.

Erinevalt WS patsientidest ei tekki ühelgi hetkel olemasoleval WS hiiremudelil märkimisväärset diabeeti oluliselt suurenenud basaalse veresuhkruga. Seega on nende kasutamine leidmaks uusi ravimikandidaate piiratud. Teises artiklis püüdsime luua ja kirjeldada uut WS mudelit, mis oleks võimeline paremini jäljendama inimestel kujuneva diabeedi teket. Selle jaoks tehti tsinksõrmede tehnoloogiat kasutades kolm erinevat rotiliini, mutatsiooniga viiendas eksonis. Kolmest liinist kahel esines raaminihketa deletsioon ning ühel lühike raaminihketa insertsioon. Kõigil kolmel liinil oli tugev glükoosi talumatus juba 7 kuu vanuselt. Edasistes eksperimentides kasutasime hetkel ainult ühe liini isaseid loomi. Uue rotimudeli põhjalikum uurimine näitas, et kuni 2 kuu vanustel loomadel on metsiktüüpi pesakonnakaaslastele sarnane glükoosi tolerantsus, insuliini sekretsioon ning Langerhansi saarekeste mass. Edasi hakkab nende glükoosi tolerantsus ja insuliini sekretsioon progresseeruvalt halvenema, viies basaalse hüperglükeemia ja glükosuuriani 13 kuu vanuselt. Halvenev insuliini sekretsioon oli põhjustatud vähenevast Langerhansi saarte hulgast, mis omakorda oli põhjustatud suurenenud endoplasmaatilise retiikulumi stressist. Seega on Wfs1 puuduliku roti puhul tegemist esimese WS loommudeliga, millel tekkib progresseeruvate sümptomite tulemusel insuliinist sõltuv diabeet.

Kolmandas artiklis kasutasime progresseeruvate sümptomitega uut WS rotimudelit leidmaks uusi ravimikandidaate pidurdamaks WS sümptomite progressiooni. GLP-1 (glükagooni-sarnane peptiid-1) retseptori agonistid on uus paljutõotav klass diabeedi-vastaseid ravimeid. GLP-1 retseptori agonistid reguleerivad glükoosi homöostaasi, metabolismi ja neuronite elulemust nii kudedes, mis ekspresseerivad GLP-1 retseptoreid kui ka kaudselt läbi endokriinsete ja neuraalsete radade. GLP-1 agonistid vähendavad endoplasmaatilise retiikulumi stressi ning vähendavad beeta rakkude apoptoosi diabeedi-eelses Goto-Kakizaki rotis. Lisaks põhjustab akuutne ravi GLP-1 agonistiga antidiabeetilist efekti Wfs1 puudulikkusega hiires. Sellest tulenevalt alustasime Wfs1 puudulikkusega rottidel kroonilist ravi GLP-1 agonist liraglutiidiga enne haiguse sümptomite avaldumist, 2 kuu vanuselt. 19 nädala jooksul halvenes ravimit mitte saanud Wfs1 rottide glükoosi tolerantsus ja Langerhansi saarte mass oluliselt. Liraglutiidiga ravitud loomade glükoosi tolerantsus jäi metsiktüüpi loomadele sarnaseks. Liraglutiid oli võimeline pidurdama Wfs1 puudulike rottide Langerhansi saarekeste massi vähenemist tänu vähenenud endoplasmaatilise retiikulumi stressile. Lisaks parandas liraglutiid üldiselt Langerhansi saarekeste tervislikku

seisundit, mis väljendus vähenenud põletikumarkerite ekspressioonis ning paranenud insuliini ja glükagooni sekretsioonis.

Hetkel puuduvad ravimid peatamaks WS arengut. Antud töös näitasime GLP-1 analoogi arvestatavat terapeutilist efekti hoidmaks ära või aeglustamaks WS diabeedi sümptomite kujunemist. Varajane diagnoosimine ning ravi GLP-1 retseptori agonistidega võib osutuda paljulubavaks ravivõimaluseks inimmeditsiinis, aidates oluliselt parendada ja pikendada WS patsientide elu.

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ORIGINAL PUBLICATIONS

CURRICULUM VITAE

Name:	Maarja Toots (Loomets)
Date of birth:	September 16, 1989, Haapsalu
Citizenship:	Estonian
Address:	University of Tartu, Institute of Biomedicine and Translational Medicine, Department of Physiology, Ravila 19, Tartu 50411, Estonia
E-mail:	maarja.toots@ut.ee

Education:

2012–...	PhD, Neuroscience, University of Tartu
2011–2012	MSc, Biomedicine, University of Tartu
2008–2011	BS, Genetic engineering, University of Tartu
2002–2008	Haapsalu Wiedemann Gymnasium
1996–2002	Haapsalu Primary School

Career:

2016–2018	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Laboratory animal center; transgenetics specialist (1.00)
04.2016–09.2016	Icosagen AS; specialist (0.50)
2011–2015	Synlab Eesti; laboratory specialist (0.50)

Practical courses:

2017	"CRISPR Genome Editing: Cutting, Inserting & Deleting", Homerton College, University of Cambridge
2014	"Laboratory Animal Science II", Faculty of Medicine, Tartu University
2014	"Laboratory Animal Science I", Faculty of Medicine, Tartu University

Dissertations supervised:

2017	Tuuliki Koppel MSc, "The diabetic phenotype of Wfs1-deficient rat"

Publications:

Toots M, Seppa K, Jagomäe T, Koppel T, Pallase M, Heinla I, Terasmaa A, Plaas M, Vasar E. Preventive treatment with liraglutide protects against development of glucose intolerance in a rat model of Wolfram syndrome. Sci Rep. 2018 Jul 5;8(1):10183. doi: 10.1038/s41598-018-28314-z

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ELULOOKIRJELDUS

Nimi:	Maarja Toots (Loomets)
Sünniaeg:	September 16, 1989, Haapsalu
Kodakondsus:	Eestlane
Aadress:	Tartu Ülikool, Bio- ja Siirdemeditsiini instituut, Füsioloogia osakond Ravila 19, Tartu 50411, Eesti
E-post:	maarja.toots@ut.ee

Haridustee:

2012–...	PhD, Neuroteadused, Tartu Ülikool
2011–2012	MSc, Biomeditsiin, Tartu Ülikool
2008–2011	BS, Geenitehnoloogia, Tartu Ülikool
2002–2008	Haapsalu Wiedemanni Gümnaasium
1996–2002	Haapsalu Linna Algkool

Töökogemus:

2016–2018	Tartu Ülikool, Meditsiiniteaduste valdkond, Bio- ja Siirdemeditsiini instituut; Katseloomakeskus; transgeneetika spetsialist (1.00)
04.2016–09.2016	Icosagen AS; spetsialist (0.50)
2011–2015	Synlab Eesti; laborispetsialist (0.50)

Praktilised koolitused:

2017	"CRISPR Genoomi muutmine: lõikamine, sisestamine ja kustutamine", Homerton kolledž, Cambridge Ülikool
2014	"Katseloomakursus II", Meditsiiniteaduste valdkond, Tartu Ülikool
2014	"Katseloomakursus I", Meditsiiniteaduste valdkond, Tartu Ülikool

Juhendamised:

2017	Tuuliki Koppel MSc, " Wfs1-puuduliku roti suhkurdiabeedi fenotüübi kirjeldamine "

Publikatsioonid:

Toots M, Seppa K, Jagomäe T, Koppel T, Pallase M, Heinla I, Terasmaa A, Plaas M, Vasar E. Preventive treatment with liraglutide protects against development of glucose intolerance in a rat model of Wolfram syndrome. Sci Rep. 2018 Jul 5;8(1):10183. doi: 10.1038/s41598-018-28314-z

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