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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
anti-WoN	antibody against the N-terminus
AR	autosomal-recessive inheritance pattern
BGC	blood glucose concentration
CD	cytoplasmic droplets (morphological characteristic of sperm)
CISD2	CDGSH Iron Sulfur Domain 2 (Wolfram syndrome 2 gene in human)
CNS	central nervous system
DI	diabetes insipidus
DM	diabetes mellitus
DM1	diabetes mellitus type 1
ER	endoplasmic reticulum
FSH	follicle stimulating hormone
GH	growth hormone
GWA	genome wide association
HbA1c	hemoglobin A1c
HFD	high frequency hearing loss/deafness
IGF-1	insulin-like growth factor 1
IPGTT	intraperitoneal glucose tolerance test
LFSNHL	low-frequency sensorineural hearing loss
MRI	magnetic resonance imaging
mRNA	messenger RNA (ribonucleic acid)
OA	optic atrophy
PC2	prohormone convertase 2
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
UPR	unfolded protein response
T4	thyroxine
TSH	thyroid stimulating hormone
WFS1	Wolfram syndrome 1 gene in human
WFS1	Wolfram syndrome 1 related protein coded by *WFS1* gene in human
Wfs1	Wolfram syndrome 1 gene in any species other than human
Wfs1	Wolfram syndrome 1 related protein coded by *Wfs1* gene in any species other than human
Wfs1KO	*Wfs1* knock-out mice
WS	Wolfram syndrome
WSD	Wolfram syndrome related diabetes
wt	wild-type (without the mutation) mice
XBP1	X-box-binding protein 1

1. INTRODUCTION

Wolfram syndrome (WS), also known as DIDMOAD syndrome, was first described by Wolfram and Wagener in 1938. It is an autosomal recessive disorder usually diagnosed in childhood when non-autoimmune type I diabetes occurs with optic atrophy, cranial diabetes insipidus, and sensorineural deafness (Wolfram and Wagener, 1938; Barrett and Bundey, 1997; Smith *et al.*, 2004). Other abnormalities related to this syndrome are dilated renal outflow tracts, multiple neurological abnormalities, and various neurological and psychiatric disorders (Peden *et al.*, 1986; Barrett *et al.*, 1995; Barrett and Bundey, 1997; Medlej *et al.*, 2004). Wolfram syndrome is caused by a mutation in the *WFS1* gene on chromosome 4p16 (Collier *et al.*, 1996). This gene is responsible for encoding wolframin, a glycoprotein of the endoplasmic reticulum, although the function of the wolframin protein is not fully understood (Hofmann *et al.*, 2003; Fonseca *et al.*, 2005; Riggs *et al.*, 2005). There is growing evidence that Wfs1 plays an important role in the pathogenesis of endoplasmic reticulum (ER) stress and apoptosis (Fonseca *et al.*, 2005; Riggs *et al.*, 2005; Yamada *et al.*, 2006).

The exact mechanism of development of the diabetes related to WS is not yet known. Some data has shown that the onset of diabetes tends to occur earlier in boys than in girls (Barrett and Bundey, 1997; Hardy *et al.*, 1999; Smith *et al.*, 2004). The development of diabetes has been associated with increased ER stress and apoptosis in pancreatic beta cells, leading to insufficient insulin secretion (Ishihara *et al.*, 2004; Riggs *et al.*, 2005). However, no studies have measured the concentration of proinsulin, an insulin precursor, in plasma. Some data indicate that prohormone convertase 2 (PC2) is absent in some patients with WS, and disturbances in converting prohormones into biologically active forms (e.g. proinsulin to insulin) may therefore exist (Gabreels *et al.*, 1998). In addition, recent genetic association studies have also indicated the role of *Wfs1* in the development of type 2 diabetes (Sandhu *et al.*, 2007; Franks *et al.*, 2008; Sparso *et al.*, 2008), in which the leading mechanism in the development of diabetes is insulin resistance.

There is data to suggest that one of the manifestations of WS is growth hormone deficiency (Barrett *et al.*, 1995; Barrett and Bundey, 1997; Koks *et al.*, 2009), which is thought to be the reason for growth retardation in patients with WS, but there is a lack of longitudinal growth data that would describe the possible growth failure.

As yet there has been no data regarding the fertility of patients with WS. Previous studies have described anterior pituitary dysfunction (Medlej *et al.*, 2004) and, in male patients, the presence of primary gonadal atrophy and hypergonadotropic hypogonadism (Peden *et al.*, 1986; Barrett and Bundey, 1997; Medlej *et al.*, 2004). As far as we know, the role of the Wfs1 gene in fertility has not been studied.

There are some brief reports showing impaired thyroid function in patients with WS, but the incidence is not known (Tranebjaerg *et al.*, 1993; Hildebrand *et al.*, 2008; Yan *et al.*, 2013). Most published cases lack information about thyroid function. The *Wfs1* gene is highly expressed in the thyroid tissue of mice (Kõks S, 2008) and moderately in the thyroid tissue of adult humans (De Falco *et al.*, 2012).

The recent review paper by Cai et al. showed that many neurodegenerative diseases, such as Alzheimer's, Parkinson's, or Huntingdon's disease, can affect the metabolism in these patients through different mechanisms, such as changes in weight, glucose metabolism, or leptin levels, etc. (Cai *et al.*, 2012). Patients with WS, a disease that is also considered to be a neurodegenerative disorder, may therefore also have an increased risk for disturbed metabolism. To our best knowledge, there have been no studies about the energy metabolism in patients with WS or its animal model, Wfs1-deficient mice.

Mice lacking the *Wfs1* gene (Wfs1KO) were created at the Laboratory of Physiology, University of Tartu (Luuk *et al.*, 2009). This animal model of WS is useful for studying the functions of various endocrine systems affected in WS, including fertility, energy metabolism, the development of diabetes, and other endocrine problems.

The aims of our study were to determine whether the fertility of Wfs1KO male mice is reduced, and, if so, to explore possible reasons. We wanted to describe the development of diabetes and investigate the secretion of insulin and proinsulin in the organism instead of cell culture. In addition, we aimed to describe the development of possible growth failure, to study the reasons for that, and to investigate the energy metabolism of these mice.

2. REVIEW OF THE LITERATURE

2.1. Wolfram syndrome

Wolfram syndrome (WS; OMIM 222300) is a rare, autosomal recessive (AR), neurodegenerative and progressive disease first described by Wolfram and Wagener in 1938 (Wolfram and Wagener, 1938). The acronym "DIDMOAD" (diabetes insipidus, diabetes mellitus, optic atrophy and deafness) is frequently used to describe the clinical features of WS (Barrett and Bundey, 1997). Although cranial diabetes insipidus (DI), sensorineural hearing loss, renal tract, and neurological abnormalities are seen in the majority of patients, only juvenile-onset diabetes mellitus (DM) and bilateral optic atrophy (OA) are necessary to make the diagnosis (Barrett *et al.*, 1995; Collier *et al.*, 1996; Farmer *et al.*, 2013).

Insulin-dependent non-autoimmune diabetes usually occurs as the initial manifestation during the first decade of life (average 6 years). The onset of DM tends to occur earlier in boys than it does in girls (Barrett and Bundey, 1997; Hardy *et al.*, 1999; Smith *et al.*, 2004). Bilateral OA presents at an average age of 11 years (Barrett and Bundey, 1997; Rigoli *et al.*, 2011). In the second decade of life, sensorineural deafness develops in two-thirds of the patients and also partial cranial DI that usually responds well to intranasal or oral desmopressin (Barrett and Bundey, 1997; Rigoli *et al.*, 2011). Most of the renal tract abnormalities (incontinence, recurrent urinary infections, and neuropathic bladder) develop early in the third decade (Barrett and Bundey, 1997; Rigoli *et al.*, 2011). Neurological symptoms (cerebellar ataxia, peripheral neuropathy, central apnea, hemiparesis) and psychiatric illnesses (depression, psychosis, organic brain syndrome) are present in the fourth decade of life (Swift *et al.*, 1990; Barrett and Bundey, 1997), although a recent study showed that early brain development is also affected (Hershey *et al.*, 2012). Other complications include primary gonadal atrophy with reduced fertility (Barrett *et al.*, 1995), gastrointestinal dysmotility causing constipation or diarrhea, and respiratory symptoms (central apneas) (Barrett and Bundey, 1997). Life span is calculated to be 30–40 years, and death usually occurs by respiratory failure due to respiratory centre atrophy or asphyxia by food aspiration (Barrett *et al.*, 1995; de Heredia *et al.*, 2013). Full characterisation of all clinical features of WS is difficult because the number of patients in the majority of reports is small (Ganie and Bhat, 2009). The largest cohort of patients with WS is described in the paper by de Heredia et al. (2013). Clinical features of 392 WS patients have been described in this study (see Table 1).

Table 1. The clinical features of WS patients. Minimum, maximum, mean, median (2nd quartile), standard deviation (SD) and percentage of all patients (n=392) presenting the clinical feature indicated (Table modified from (de Heredia *et al.*, 2013)).

Clinical feature	Min (years)	Median (years)	Mean (years)	Max (years)	SD (years)	% of patients (n=392)
Diabetes mellitus	1	6	7.7	50	6.4	98.21%
Optic atrophy	2	11	12.4	53	7.0	82.14%
Diabetes insipidus	1.5	13	14.7	72	8.4	37.76%
Hearing defects	1	14	15.4	60	9.1	48.21%
Urological defects	1	20	20.0	46	9.7	19.39%
Neurological defects	2	23	23.2	53	12.3	17.09%
Deceased	10	27	29.8	48	11.4	7.40%

Wolfram syndrome is rare, and the prevalence varies from 1 in 100,000 in North America (Fraser and Gunn, 1977) to around 1 in 770,000 individuals in the UK (Barrett and Bundey, 1997). According to the same nationwide study, the prevalence in children was found to be 1 in 500,000 and the carrier rate was estimated to be 1 in 354 individuals (Barrett and Bundey, 1997). The highest published prevalence of WS is 1 in 68,000 in Lebanon, which is probably due to the high rate of consanguinity in this region (Medlej *et al.*, 2004). Studies of around 400 patients with WS have been published up to 2013 (de Heredia *et al.*, 2013).

The cause of WS is mutation in the *WFS1* gene that produces a protein called wolframin. This was discovered in 1998. The gene is mapped to human chromosome 4p16.1 and is comprised of 8 exons spanning 33.4 kb of genomic DNA (Inoue *et al.*, 1998; Strom *et al.*, 1998). Wolfram syndrome is a very heterogenic disease, with more than 200 known mutations in the *WFS1* gene, which complicates the establishment of a clear genotype-phenotype correlation (de Heredia *et al.*, 2013). In addition to *WFS1*, WS can be also caused by mutations in the *CISD2* (also known as *ZCD2*) gene (Chen *et al.*, 2010). This disease is called Wolfram syndrome type 2 (WS2). Mutations in the *CISD2* gene have been mapped to the chromosome 4q22–24 (Amr *et al.*, 2007). Wolfram syndrome 2 is similar to WS characterised by juvenile-onset diabetes mellitus, optic atrophy, high-frequency sensorineural hearing loss, urinary tract dilatation, and hypergonadotropic hypogonadism (Tranebjaerg *et al.*, 1993; El-Shanti *et al.*, 2000; al-Sheyyab *et al.*, 2001; Amr *et al.*, 2007). In addition, patients with WS2 may present with severe gastrointestinal ulceration and bleeding (Amr *et al.*, 2007). Presence of diabetes insipidus has not been described in WS2 (Tranebjaerg *et al.*, 1993). This thesis is purely about WS caused by mutations in the *WFS1* gene, sometimes also called Wolfram syndrome type 1.

2.2. The clinical picture of Wolfram syndrome

2.2.1. Diabetes

Wolframin (WFS1), a product of the *WFS1* gene, is highly expressed in the pancreas of humans (Inoue *et al.*, 1998) as well as in rodents (Hofmann *et al.*, 2003; Fonseca *et al.*, 2005; Kõks S, 2008). WFS1 possibly helps to fold a protein precursor of insulin (proinsulin) into the mature form of the hormone (Fonseca *et al.*, 2005; Rigoli *et al.*, 2011). The expression of *WFS1* is much greater in pancreatic islet cells than in the exocrine cells. In addition to the β-cells where the expression of *WFS1* is the highest, expression is also found in δ-cells (Hofmann *et al.*, 2003; Fonseca *et al.*, 2005). WFS1 deficiency leads to a progressive loss of β-cells, impaired glucose tolerance, and cell cycle progression, accompanied by the activation of unfolded protein response pathways and higher susceptibility to apoptosis through endoplasmic reticulum (ER) stress (Ishihara *et al.*, 2004; Riggs *et al.*, 2005; Yamada *et al.*, 2006; Rigoli *et al.*, 2011). The reason for WS-related diabetes (WSD) is thought to be due to the insulinopenia that is secondary to the degeneration of the β-cells (Medlej *et al.*, 2004). Post-mortem reports have shown selective loss of β-cells (Karasik *et al.*, 1989). Animal studies also suggest possible defective insulin secretion in addition to β-cell loss (Ishihara *et al.*, 2004).

Diabetes is usually the first manifestation that occurs in patients. Studies on a large cohort of WS patients indicate that the average onset of WSD tends to be at 6 years (Barrett *et al.*, 1995; Kinsley *et al.*, 1995; Medlej *et al.*, 2004). Rohayem *et al* (2011) has shown that WSD tends to occur significantly earlier than type 1 diabetes (DM1) (5.4 vs. 7.9 years respectively, $p<0.001$) (Rohayem *et al.*, 2011). There is strong evidence that the onset of WSD tends to occur earlier in boys than it does in girls (Hardy *et al.*, 1999; Smith *et al.*, 2004; Marshall *et al.*, 2013). The course of WSD tends to be milder than in DM1, with less microvascular complications. Patients with WS rarely develop ketoacidosis and they have lower daily insulin requirements and lower haemoglobin A1c (HbA1c) compared to patients with DM1, indicating better glycaemic control in WSD (Peden *et al.*, 1986; Barrett *et al.*, 1995; Kinsley *et al.*, 1995; Cano *et al.*, 2007a). Patients with WSD have shown remarkably long partial remission periods (>8 years) and measurable C-peptide levels, even 8 years after the onset of diabetes (Fishman and Ehrlich, 1986; Rohayem *et al.*, 2011). However, after patients with WSD have started insulin treatment, the risk for severe hypoglycaemia increases and remains significantly higher than in patients with DM1 (37% vs. 7.9%, $p<0.001$) (Rohayem *et al.*, 2011). DM1 related autoantibodies are almost never detectable in diabetic patients with WS (Kumar, 2010). The main clinical differences distinguishing the diabetes in WS and DM1 are shown in table 2.

Table 2. Differences between WS and type 1 DM (Modified from (Kumar, 2010)).

Characteristic	WS	Type 1 DM
– Ketoacidosis at presentation[a]	3%	30%
– Insulin requirement[b]	Satisfactory control with less intensive regimen	Intensive regimen
– Other features of WS	Yes	No
– HLA subtype[d]	HLA-DR2 (44.4%)	Mainly HLA-DR3 and HLA-DR4 (HLA-DR2 in only 6.77%)
– Presence of antibodies	Rare reports[g]	93%[c]
– Coarse of complications influenced by glycaemic control[a]	No	Yes
– Diabetic retinopathy (after 15 years)[b]	35%	90%
– Diabetic nephropathy[b]	8%	27%
– Median age of death	30 years[a]	50 years[e]
– Cause of death[a]	Neurological disorder, urological abnormalities, infection	Myocardial infarction or coronary artery disease
– Recurrence risk in next pregnancy	AR inheritance: 25%	3–6%[f]

[a]Kinsley BT *et al*. (Kinsley *et al.*, 1995)
[b]Cano A *et al*. (Cano *et al.*, 2007a)
[c]Glastras SJ *et al*. (Glastras *et al.*, 2005)
[d]Vendrell J *et al*. (Vendrell *et al.*, 1994)
[e]Raymond NT *et al*. (Raymond *et al.*, 1995)
[f]Nelson Textbook of Pediatrics (Alemzadeh R, 2007)
[g]Nakamura A *et al*. (Nakamura *et al.*, 2006)

Many relatives of WS patients have diabetes (Fraser and Gunn, 1977), which suggests that the heterozygote carriers of *WFS1* may contribute to the genetic heterogeneity of diabetes (Barrett, 2001). Ohata *et al.* (1998) has also presented a large Japanese family with WS, and showed that the obligate carriers of *WFS1* had increased risk of DM (Ohata *et al.*, 1998).

2.2.2. Optic atrophy

Generalised OA usually presents in all patients at the time of diagnosis of WS, but is rarely the presenting finding. However, there are some reports where OA presented before WSD (Chaussenot *et al.*, 2011). In Wolfram's original description, the two eldest siblings out of four developed poor vision at 6 and 8

years, respectively, and when examined 10 years later, acuity was reduced to the level where they could count only fingers (Wolfram and Wagener, 1938; Barrett *et al.*, 1997). The median age for presentation of OA is shown to be 11 years (range of 6 weeks to 19 years) (Barrett *et al.*, 1995). The presenting symptoms are decreasing visual acuity and loss of colour vision. Patients typically complain that "everything started to go grey" (Barrett *et al.*, 1997). It is shown that the loss of colour vision can be present and noticeable for patients long before the diagnosis of OA is made (median age for vision problems is 6 years) (Marshall *et al.*, 2013). Optic atrophy leads to perception of light and dark only, over a median of 8 years' duration (Barrett *et al.*, 1997).

The natural history and pathogenesis of OA in WS remain unclear so far. Magnetic resonance imaging (MRI) scans in 2 patients have shown widespread atrophic changes throughout the brain, including the cerebellum, optic nerves, and chiasm (Rando *et al.*, 1992). A few experimental studies have demonstrated that WFS1 is primarily localised in the retinal ganglion cells and in different cells of the optic nerve: in the inner nuclear layer, photoreceptors, and glial cells (Yamamoto *et al.*, 2006; Kawano *et al.*, 2008; Schmidt-Kastner *et al.*, 2009; Rigoli *et al.*, 2011). This could indicate that dysfunction of the *WFS1* in the ER or in the cell body can lead to optic nerve atrophy through deficits in protein synthesis and axonal transport (Rigoli *et al.*, 2011).

Other ophthalmologic findings reported in WS, but yet not confirmed as part of the phenotype, are cataracts (Hansen *et al.*, 2005), pigmentary retinopathy (Dhalla *et al.*, 2006) and nystagmus (Tranebjaerg *et al.*, 1993).

2.2.3. Deafness

Sensorineural deafness, mostly high frequency deafness (HFD), is usually thought to be the third manifestation that occurs in patients with WS during the second decade of life. A recent large overview of 392 patients with WS patients shows that in almost 50% of them HFD appeared as the fourth or fifth clinical manifestation (29% and 19.4%, respectively) (de Heredia *et al.*, 2013). Deafness is present in about 66% of individuals with WS. Deafness can present as a congenital form or progressively developing hearing impairment, while this sometimes may be mild (Tranebjaerg *et al.*, 1993). Median age of onset is 12.5 years (Barrett *et al.*, 1995). It is suggested that females with WS more frequently have hearing impairment than males (Pennings *et al.*, 2004) whereas Plantinga *et al.* did not find such a sex-related difference (Plantinga *et al.*, 2008).

According to the audiograms, hearing loss is slowly progressive and involves mostly the high frequencies (Pennings *et al.*, 2004; Plantinga *et al.*, 2008).

WFS1 is expressed in different cochlear cells, such as in inner and outer hair cells, lateral wall cells, spiral ganglion and vestibule cells, as well as in many supporting cells (Cryns *et al.*, 2003; Rigoli *et al.*, 2011). It is suggested that

WFS1 helps to maintain proper levels of calcium ions in the inner ear that are essential for hearing (Cryns *et al.*, 2003).

It is unknown why hearing loss occurs at a high frequency in WS and at a low frequency in another disease related to Wfs1. The latter is called *WFS1*-related low-frequency sensorineural hearing loss (LFSNHL) (Tranebjaerg *et al.*, 1993).

2.2.4. Diabetes insipidus

Central DI usually develops in the second decade of life, and is thought to arise as a result of disturbed processing of the vasopressin precursor (Gabreels *et al.*, 1998). The other mechanism suggested for developing DI is the degeneration of the supraoptic and paraventricular nuclei (Scolding *et al.*, 1996). It is shown that *WFS1* is highly expressed in the human hypothalamus and moderately in the mouse hypothalamus (Kõks S, 2008). Kawano *et al*. showed that in mouse supraoptic and paraventricular nuclei, *Wfs1* mRNA expression is relatively and constantly high during intrauterine development, but relatively low in postnatal life (Kawano *et al.*, 2009).

Neuroradiological and post-mortem reports of WS patients have shown an absence of normal T1 hyperintensity (so called bright spot) seen in the posterior pituitary lobe, a typical finding for central DI, along with gliosis and atrophy of the paraventricular and supraoptic nuclei of the hypothalamus (Galluzzi *et al.*, 1999; Pakdemirli *et al.*, 2005; Ito *et al.*, 2007; Boutzios *et al.*, 2011).

The prevalence of DI varies between 37% and 87%, depending on the studies of different patient cohorts (Barrett *et al.*, 1995; Kinsley *et al.*, 1995; Medlej *et al.*, 2004; de Heredia *et al.*, 2013; Marshall *et al.*, 2013). The average onset of DI for WS patients is shown to be from 10.5 (Marshall *et al.*, 2013) to 14 years (Barrett *et al.*, 1995). It seems that similarly with WSD, DI tends to occur earlier in male patients than in females (Marshall *et al.*, 2013). Central DI in WS responds well to vasopressin treatment (Barrett and Bundey, 1997; Smith *et al.*, 2004)

2.2.5. Neurological and psychiatric symptoms

Neurological and psychiatric complications are common in patients with WS. They present in 62% of patients and at a median age of 30 years (Barrett *et al.*, 1995). There are some studies that indicate that the onset of neurological symptoms occurs much earlier – between the first and second decade of life (Tranebjaerg *et al.*, 1993; Chaussenot *et al.*, 2011).

WFS1 is highly expressed in different parts of the brain in humans, as well as in rodents. In rats a high expression of *Wfs1* mRNA and protein in some areas of the limbic system, such as the amygdala, hippocampal region, olfactory tubercle, and superficial layer of the preform allocortex, has been shown

(Takeda *et al.*, 2001). In mice, *Wfs1* expression is highest in the amygdala, followed by the cerebral cortex, frontal cortex, dorsal striatum, and hippocampus. In humans *WFS1* expression in the brain and the parts of the brain is also higher than the average, with the highest levels in the prefrontal cortex, amygdala, caudate nucleus, hypothalamus, and thalamus (Kõks S, 2008).

Common neurological manifestations of WS patients are truncal ataxia causing unsteady gait and falls, startle myoclonus, reduced limb reflexes, nystagmus, dysarthria, central apnoea, loss of taste and smell, hemiparesis, loss of gag reflex, epilepsy, and neurogenic bladder (Cremers *et al.*, 1977; Rando *et al.*, 1992; Barrett *et al.*, 1995; Scolding *et al.*, 1996; Barrett and Bundey, 1997; Baz *et al.*, 1999; Medlej *et al.*, 2004). Neurological findings are progressive and result from general brain atrophy with brain stem and cranial nerve involvement (Barrett and Bundey, 1997; Pakdemirli *et al.*, 2005; Domenech *et al.*, 2006). MRI can show generalised brain atrophy that is most prominent in the cerebellum, medulla, and pons. In addition, reduced signal intensity of the optic nerves and the posterior part of the hypothalamus is seen (Barrett *et al.*, 1995). The correlation between the MRI findings and clinical picture is not always strong (Ito *et al.*, 2007). A recent study by Hershey *et al*. (2012) demonstrates abnormalities in the brainstem and cerebellum already in young patients that could indicate that *WFS1* also affects brain development and not only the later neurodegeneration (Hershey *et al.*, 2012).

25% of WS patients seem to be affected by episodes of severe depression, psychosis, as well as aggression. There is also a trend for patients with WS to show a higher suicide rate (Swift *et al.*, 1990; Sequeira *et al.*, 2003; Rigoli *et al.*, 2011). Swift *et al.* (1998) have suggested that the prevalence of *WFS1* heterozygous carriers is approximately 1% in the general population, and the carriers have a 7-fold increased risk of hospitalisation for psychiatric diseases (Swift *et al.*, 1998).

2.2.6. Reproductive system

Studies of reproductive function in patients with WS are currently lacking. Some patients are affected by anterior pituitary dysfunction. In male patients the presence of gonadal atrophy, as well as hypergonadotropic or hypogonadotropic hypogonadism have been described (Cremers *et al.*, 1977; Homan and MacKay, 1987; Barrett *et al.*, 1995; Barrett and Bundey, 1997; Simsek *et al.*, 2003; Medlej *et al.*, 2004; Rigoli *et al.*, 2011). In 2013, Haghighi *et al*. first described two male patients from one family carrying a novel mutation (p.Asp211Asn, p.Gln486*) who had been able to father children (Haghighi *et al.*, 2013). In females ovarian function seems to be normal, only with abnormalities in the menstruation cycle (Medlej *et al.*, 2004), and successful pregnancies with unaffected children have been reported (Davidson *et al.*, 1993).

WFS1 is highly expressed in human reproductive organs: expression is highest in the testes, particularly in germ cells, but also in the prostate and uterus. In mice the expression rate of *Wfs1* in reproductive organs is mild, with the highest expression in ovaries and uterus, followed by lower expression in the testes and prostate (Kõks S, 2008).

According to present knowledge, the role of the *Wfs1* gene in fertility in WS has been not investigated.

2.2.7. Growth

Short stature is quite common in patients with WS (Hofmann *et al.*, 1997a; Simsek *et al.*, 2003; Medlej *et al.*, 2004; Ganie *et al.*, 2011). Medlej *et al.* (2004) reported pituitary dysfunction in their study of Lebanese patients with WS, probably of hypothalamic origin. The most common abnormality in this study was growth hormone (GH) deficiency, followed by adrenocorticotropic hormone (ACTH) deficiency (Medlej *et al.*, 2004). Soliman *et al.* (1995) reported two girls with isolated GH deficiency and short stature (Soliman *et al.*, 1995).

Despite the fact that short stature is common in patients with WS, the exact mechanism for impaired growth remains unclear.

2.2.8. Energy metabolism and thyroid function

There is no data about the energy metabolism of patients with WS. There are only a few studies showing impaired thyroid function in patients with WS (Smith *et al.*, 2004; Hildebrand *et al.*, 2008; Yan *et al.*, 2013), but the incidence of this problem is not known (Tranebjaerg *et al.*, 1993). The *Wfs1* gene is highly expressed in the thyroid tissue of mice (Kõks S, 2008), and moderately in the thyroid tissue of adult humans (De Falco *et al.*, 2012).

2.3. *WFS1* gene and it's protein wolframin

Wolfram syndrome was initially thought to be caused by mitochondrial mutations, but these have been reported only in some very few cases (Bundey *et al.*, 1992; Rotig *et al.*, 1993; Barrientos *et al.*, 1996; Hofmann *et al.*, 1997b). In 1998 a nuclear gene for WS, *WFS1*, was discovered and mapped to chromosome 4.16.1 by linkage studies (Inoue *et al.*, 1998; Strom *et al.*, 1998).

In humans the *WFS1* gene is located in the short arm of the 4th chromosome (4p16.1) and in mice on the long arm of the 5th chromosome (5qB3). There is an 83% overlap in the nucleotide sequence of the *WFS1* gene and 87% overlap in

the amino acid sequence of its protein wolframin in humans and mice (Strom *et al.*, 1998).

WFS1 has eight exons; the first exon is noncoding and comprises 33.4 kbp on chromosome 4p16.1 (Inoue *et al.*, 1998). The 8th exon is the largest (2.8 kb), containing about 60% of the whole protein-coding sequence of the *WFS1* gene, and the majority of the mutations described in WS are located in this exon (Cryns *et al.*, 2003).

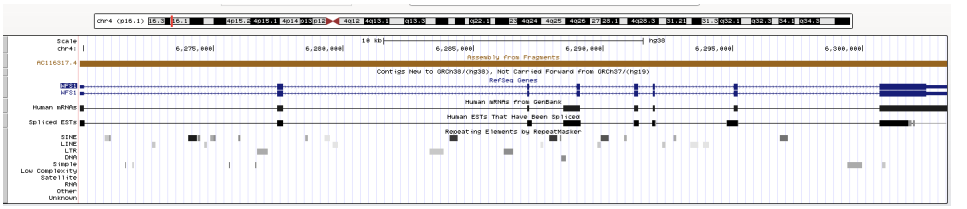

Figure 1. Structure of the *WFS1* gene (http://genome.ucsc.edu).

WFS1 encodes the 890-amino-acid protein called wolframin. The protein has nine transmembrane segments across the ER membrane and an $N_{cytoplasm}/C_{ER\ lumen}$ membrane topology that forms homomeric complexes of 400 kDa under physiological conditions (Inoue *et al.*, 1998; Takeda *et al.*, 2001; Hofmann *et al.*, 2003).

2.3.1. WFS1 functions

The exact function of wolframin (Wfs1) is not known. It seems to have a significant role in the transportation of proteins, lipids, and many other materials – particularly Ca^{2+} ions – into different parts of the cell by the endoplasmic reticulum (Osman *et al.*, 2003; Yamada *et al.*, 2006). Mutations in the *WFS1* gene lead to the production of a wolframin protein that has reduced or absent function. This leads to a case in which calcium levels within cells are not properly regulated, and to the development of dysfunction of the endoplasmic reticulum (ER). Wolframin is localised in ER, where it is folded and modified to obtain the necessary structure for normal functioning. Wolframin seems to modulate Ca^{2+} the endoplasmic reticulum does not have enough functional wolframin, it causes ER stress, impairs cell cycle progression, and triggers the apoptotic pathway, most likely through impairment of Ca^{2+} homeostasis (Collier, 2008). Depending on the cells, it leads to the development of different clinical features of WS.

Studies of Wfs1-deficient pancreatic β-cells have shown impairments of glucose-stimulated insulin secretion and cell cycle progression by activation of ER stress/unfolded protein response (UPR) pathways and enhanced susceptibility to apoptosis (Ishihara *et al.*, 2004; Riggs *et al.*, 2005; Yamada *et al.*, 2006). *WFS1* appears also to have a function in the survival of neurons (Gharanei *et al.*, 2013).

Early studies on the role of the *Wfs1* gene in the development of diabetes focused mostly on beta cell survival (Sandhu *et al.*, 2007). Normal *Wfs1* seems to protect beta cells, as mice with a disrupted *Wfs1* gene exhibit beta cell loss (Yamada *et al.*, 2006). This phenotype has been thought to result from the activation of ER stress (Ueda *et al.*, 2005). ER stress is the response to the overload in ER luminal conditions (e.g. increased secretory activity). ER stress activates a signalling cascade that attempts to restore a favourable folding environment. The activated response is UPR. UPR is a molecular transduction system that monitors the protein folding capacity of the ER and signals cell responses that attempt to maintain folding capacity and prevent a build-up of unproductive and potentially toxic protein products (transient inhibition of protein synthesis, etc.). If UPR is not sufficient to deal with the stress conditions, apoptotic cell death is initiated (Lai *et al.*, 2007). The UPR consists of three main signaling systems initiated by three prototypical ER localized stress sensors – IRE1, PERK, and ATF6. The Wfs1 protein has proven to be related to the IRE1 and PERK pathways (Kakiuchi *et al.*, 2006; Yamada *et al.*, 2006). *Wfs1* induces expression of XBP1 (X-box-binding protein 1), which is the major initiator for UPR target genes (binds to the ER stress response element) (Kakiuchi *et al.*, 2006; Yamada *et al.*, 2006). While it is clear that the Wfs1 protein is closely related to ER stress and increased expression of *Wfs1* protects cells from ER-stress-induced death, the molecular mechanism of this action is not at all clear (Yamada *et al.*, 2006).

Taking into consideration the most common symptoms of WS patients, pancreatic β-cells and neurons seem to be most affected from the loss of WFS1 function.

2.3.2. *Wfs1* expression

The first information concerning expression of the *Wfs1* gene comes from the cloning studies by Inoue *et al*. and Strom *et al*. (Inoue *et al.*, 1998; Strom *et al.*, 1998). In 1998, Inoue and his colleagues performed Northern blot from the panel of RNA of adult human tissues and found *WFS1* expression in basically all analysed tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas). The expression was equally strong in all studied tissues with the weakest signals from the liver and skeletal muscle. The exocrine tissue of the pancreas had a weaker signal than the islets (Inoue *et al.*, 1998; Kõks S, 2008). Strom *et al.* analysed the tissue distribution of the *WFS1* gene. They found the strongest signal in the heart, followed by the brain, placenta, lung, and pancreas. Weak signal was present in the liver, skeletal muscle, and kidney (Strom *et al.*, 1998; Kõks S, 2008). Thus, the very first studies already showed that *WFS1* has a very widespread expression in the human organism, which probably explains the very variable clinical picture of WS.

Hofmann *et al*. studied the distribution of *Wfs1* with immunohistochemical methods analysing *Wfs1* expression in different mouse tissues (Hofmann *et al.*, 2003). In their study, *Wfs1* was expressed in all tissues studied. The highest expression levels were found in the brain, pancreas, heart, and muscle; followed by the liver, the lowest levels were seen in the kidneys and spleen. Analysis of different mammalian cell lines with anti-WoN antibodies revealed the highest expression in insulinoma cells and much lower expression in human fibroblasts and neuroblastoma cells (Hofmann *et al.*, 2003; Kõks S, 2008).

The primary focus of attention in Wfs1 protein research has been on its function in different parts of the brain, in pancreas islet cells, and in different cells of the eyes and ears. This is not surprising, because different brain regions, as well as the pancreas, eyes, and ears, express Wfs1 protein in high levels and are the main affected organs in WS. In the mouse brain, the *Wfs1* gene expression level is higher in brain structures related to emotions, learning, and memory (Luuk *et al.*, 2008): very strong expression of *Wfs1* is detectable in the central extended amygdala and ventral striatum, followed by the hippocampal region, prefrontal cortex, and proisocortical areas.

Strong *Wfs1* expression has been evident in the retina and in the optic nerve (Yamamoto *et al.*, 2006). Several studies suggest that mutation in the *Wfs1* gene can influence survival and function of both retinal ganglion cells and glial cells in the optic nerve (Takeda *et al.*, 2001; Yamamoto *et al.*, 2006; Kõks S, 2008). *WFS1* is expressed in different cochlear cells, such as in the inner and outer hair cells, lateral wall cells, spiral ganglion, and vestibule cells, but also in many supporting cells (Cryns *et al.*, 2003; Rigoli *et al.*, 2011). It is suggested that WFS1 helps to maintain proper levels of calcium ions in the inner ear that are essential for hearing (Cryns *et al.*, 2003; Kõks S, 2008).

2.3.3. Mutations of *WFS1* in Wolfram syndrome

Genetic analyses in patients with WS have identified a wide spectrum of mutations in the *WFS1* gene (Tranebjaerg *et al.*, 1993; Inoue *et al.*, 1998; Strom *et al.*, 1998; Hardy *et al.*, 1999; Khanim *et al.*, 2001; Cryns *et al.*, 2003; Smith *et al.*, 2004; Hansen *et al.*, 2005; Cano *et al.*, 2007b; Hildebrand *et al.*, 2008; de Heredia *et al.*, 2013). In WS patients the mutations in the *WFS1* gene are distributed across the length of the coding region in exon 8, and include deletions, insertions, nonsense, and missense mutations (Khanim *et al.*, 2001). Mutations in *WFS1* are deleterious for protein expression (Hofmann *et al.*, 2003; Guo *et al.*, 2011; de Heredia *et al.*, 2013). Glycosylation sites are identified as being important for protein stability (Hofmann *et al.*, 2003). A region that targets unfolded WFS1 to degradation (degron) has also been found (Guo *et al.*, 2011; de Heredia *et al.*, 2013). Many of the missense mutations are located in the C-terminal hydrophilic part of the protein (Hardy *et al.*, 1999). More than 200 variations in WFS1 have been described in patients with WS,

which complicates the establishment of clear phenotypic-genotypic correlation (de Heredia *et al.*, 2013).

De Heredia and his colleagues have recently published a comprehensive review of all published mutations of *WFS1* in the last 15 years (de Heredia *et al.*, 2013). To ease the phenotype-genotype correlations, the mutations were classified into different types based on their predicted effect on *WFS1* expression. Accordingly, three types of mutations were determined. Type I mutations lead to complete depletion of the WFS1 protein due to the activation of nonsense-mediated decay, including nonsense and frameshift mutations producing stop codons before exon 8. Type II includes mutations that lead to complete degradation of WFS1 by keeping the degron of WFS1 functional. This group includes all missense mutations except those between amino acids 671–700, and those nonsense mutations producing a stop codon after p.Trp700. The third group, type III, includes mutations leading to the expression of a defective or shorter WFS1 protein. In this group are nonsense mutations that introduce a stop codon after exon 8 and before p.Trp700, frameshift mutations introducing a stop codon after exon 8, and missense mutations in amino acids 671–700 (de Heredia *et al.*, 2013).

De Heredia *et al.* detected 178 different mutations in 337 patients. The mutations are distributed all along the protein with no major hotspots. They concentrate mainly in transmembrane domains and glycosylation sites. Only six mutations were present in more than 5% of patients: c.2649delC (p.Phe884Serfs*68), c.1230_1233del (p.Val412Serfs*29), c.409_424dup (p.Val142Glyfs*110), c.2119G→A (p.Val707Ile), c.1362_1377del (p.Tyr454*), and c.1243_1245del (p.Val415del). According to the above-mentioned classification, 5.62% of the mutations were assigned to type I, 55.6% to type II, and 38.2% to type III (de Heredia *et al.*, 2013).

To study whether different mutation types have a role in the earlier onset of the clinical features, de Heredia *et al*. classified the patients into different classes by their genotype. Class A includes genotypes where no WFS1 protein was produced. This class was further subdivided into three subclasses by the mechanism of WFS1 depletion: subclass A1 due to *WFS1* mRNA degradation, subclass A2 due to mRNA and protein degradation, and subclass A3 due to WFS1 protein degradation. Class B includes genotypes with reduced expression of a defective WFS1 protein and class C those with expression of a defective WFS1 protein. Out of 337 patients, there were 51.9% in class A, 19% in class B, and 29.1% in class C. The distribution of the genotypes differed between the countries. Patients in class C showed earlier onset of most clinical features of WS. Patients in class A1 had earlier onset of DM, in contrast to patients in class A2 who developed earlier DI and OA. The progression rate showed to be faster in patients in class C and slowest in class A1 patients (de Heredia *et al.*, 2013).

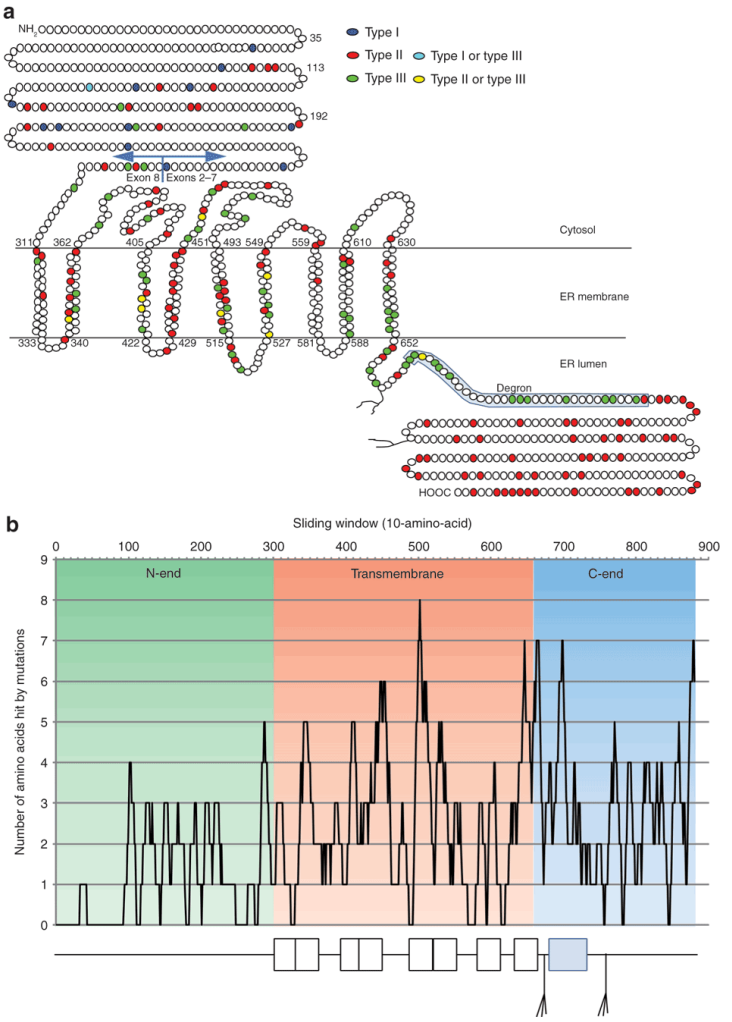

Figure 2. Mutations distribution in *WFS1* (figure from de Heredia *et. al* 2013 with permission)

a) Mutation position. Position and type of each mutation are shown in a schematic model of WFS1 protein. The position of the degron (amino acids 671–700), the first amino acid encoded by exon 8 (p.Lys287), the two glycosylation sites and amino acid positions at ER membrane contents are indicated. Amino acid positions can have more than one mutation and have a different color only if affected by different mutation types. Color code identifies mutation types at that amino acid position: blue – type I mutation, red – type II, green – type III, cyan – type II or III, yellow – type I or II.

b) Mutation distribution. The number of mutations is calculated for a 10-amino-acid sliding window along the protein. White boxes below the chart indicate windows including the transmembrane domains; blue box indicates windows including the degron. Glycosylation sites are also indicated at their approximated locations (de Heredia *et al.*, 2013).

2.3.4. *WFS1* and other diseases

There appear to be several diseases that are related to the different mutations in the *WFS1* gene. Some case series have reported a higher prevalence of DM, sensorineural hearing loss, or psychiatric disorders among the relatives of WS patients (Scolding *et al.*, 1996; Ohata *et al.*, 1998; Swift *et al.*, 1998).

Several investigators have found that mutations in the *WFS1* gene are a common cause of autosomal dominant low-frequency sensorineural hearing loss (LFSNHL) (Bespalova *et al.*, 2001; Young *et al.*, 2001; Cryns *et al.*, 2003; Fukuoka *et al.*, 2007). This is remarkable because in WS the hearing loss is mostly at high frequencies (Collier, 2008). With few exceptions, all mutations causing LFSNHL are located in the fifth intracellular domain, whereas hearing loss in WS is mostly due to inactivating mutations that are spread throughout the entire coding region of the *WFS1* gene (Cryns *et al.*, 2003; Collier, 2008).

WFS1 has also been associated with diabetes mellitus (Sandhu *et al.*, 2007; Collier, 2008). Sandhu *et al*. (2007) included *WFS1* in a panel of 84 genes related to β-cell function, and found a positive association with *WFS1* SNPs in three type 2 diabetes populations from the UK, and one of Ashkenazi Jews. This indicates that *WFS*1 appears to be a significant genetic cause of type 2 diabetes (Sandhu *et al.*, 2007; Collier, 2008). Genome-wide association (GWA) studies have shown that heterozygote mutations of *WFS1* have also been related to DM2 (Cheng *et al.*, 2013). In the case of DM1 the data is ambivalent: earlier studies have linked a DM1 locus in the region of *WFS1* (Larsen et al., 2004) and pointed out also some polymorphisms in *WFS1* that could be related to the DM1 (Awata *et al.*, 2000). Later studies have not found any association between WS and DM1 (Collier, 2008).

Heterozygote carriers of *WFS1* gene mutations, mainly the first line relatives of WS patients, are supposed to be at increased risk for psychiatric disease and suicidality, and need frequent hospitalisation due to psychiatric problems (Swift *et al.*, 1998)

2.4. Wfs1-deficient mouse models

To study the role of Wfs1, several mouse models of Wfs1 deficiency have been created. So far, three different Wfs1-deficient mouse models have been independently developed by different research groups, one of them by the scientists from the University of Tartu, Estonia.

2.4.1. Japanese model (Ishihara group)

The first Wfs1-deficient mouse model reported in the literature was created by Ishihara and colleagues in 2004 (Ishihara *et al.*, 2004). Their mouse model is a so-called “full knock-out” mode; namely, exon 2 of the *Wfs1* gene has been

deleted in these mice. It is important that *Wfs1* mRNA is still detectable in these mice. The mice were normal in appearance, growth, and fertility and showed distribution by Mendelian genotypic rules. Studies showed that these mice had decreased insulin secretion and high blood sugar levels in response to the glucose tolerance test. Progressive loss of pancreatic β-cells that was caused by apoptosis and ER stress resulting in severe impairment of glucose homeostasis was shown only in the 129SVEV x C57BL/6 F2 genetic background. The findings were more severe in male mice compared to female mice. While the mice were backcrossed to the C57BL/6 F5 background, the overt diabetes was no longer present. This finding indicates that this background protects mice against diabetes in the case of Wfs1 deficiency. To reduce the phenotypic variations, only F5 generation males were used in further experiments (Ishihara *et al.*, 2004).

In the other study, Kato and colleagues used the same mice in the behavioural experiments (Kato *et al.*, 2008). All the mice they used were males backcrossed to the C57BL/6 background for at least 8 generations. The most notable finding was that the homozygous Wfs1-deficient mice displayed an overall tendency for lower social interaction. The results support the impression that Wfs1-deficient mice have subtle impairments in behavioural activation in demanding situations (Kato *et al.*, 2008).

2.4.2. University of California model (Riggs group)

The second model was created in 2005 by Riggs and colleagues (Riggs *et al.*, 2005). Their model is a conditional *Wfs1* exon 8 knock-out mouse. The mice are in the 129SVJ genetic background with a pancreatic-specific deletion of exon 8 of the *Wfs1* gene. The mice are reported to be fertile and distributed by Mendelian rules (Riggs *et al.*, 2005). These male mice are used in glucose metabolism investigations and are shown to develop progressive glucose intolerance, insulin deficiency, and DM2-like features by 4 months of age. Their body weight is significantly lower than in wild-type controls. The findings of Riggs et al. show that these mice have a significantly smaller mass of insulin-producing pancreatic β-cells, which is caused by increased apoptosis and ER stress. Most of the disturbances became significantly evident by the age of 6 months (Riggs *et al.*, 2005). In relation to the mice investigated by Ishihara *et al.* (2004), mice from Riggs *et al*. showed earlier development of metabolic disturbances.

2.4.3. The University of Tartu model (Kõks group)

The third model of Wfs1-deficient mice was generated at the University of Tartu in 2005. In these conventional Wfs1 knock-out mice, exon 8 in the Wfs1 has been deleted. Wfs1-deficient mice were generated by targeting construct to

replace most of the coding region of the *Wfs1* gene in all tissues. This is different from the Riggs model, where Wfs1 was deleted only in the pancreas. A more detailed description of the methodology for generating this animal model is given in the Methods section.

The rationale behind the strategy for generating Wfs1 mutant mice at the University of Tartu was to delete the majority of the coding sequence forming the mouse genome. This approach ensures that Wfs1 protein is not being produced or only a truncated form is produced. We deleted 2/3 of the protein coding sequence and fused it with the lacZ gene. New fusion protein is easily detectable in histochemical analysis and we used this mouse for detailed expression mapping of the Wfs1 gene. Moreover, when comparing our animal data to the animal data from other labs, we can conclude that our animals have a more severe phenotype than mutant mice developed by others. Taken together, the Wfs1 mutant mouse we developed is a more precise model for Wolfram syndrome than the model developed in other labs.

Before our work, these mice were mainly used to describe the behavioural phenotype of Wfs1-deficient mice and the distribution of Wfs1 protein in the brain.

3. AIMS OF THE STUDY

1. To determine whether the fertility of Wfs1-deficient (Wfs1KO) male mice is reduced and, if so, to explore the possible reasons
2. To compare the morphology of testes and sperm between Wfs1KO and wt mice
3. To investigate the sex differences in longitudinal changes of blood glucose concentration and weight of Wfs1KO mice.
4. To compare plasma proinsulin and insulin levels between Wfs1KO and wt mice
5. To investigate energy metabolism and thyroid function in Wfs1KO mice.

4. MATERIALS AND METHODS

4.1. Animals

In accordance with the European Communities Directive (86/609/EEC), the Estonian National Board of Animal Experiments granted permission (No. 86, 28.08. 2007) for the animal experiments described in this study. Mice were housed under standard laboratory conditions on a 12-hour light/dark cycle (lights on at 07:00 hours) with free access to food and water.

In all the experiments the Wfs1KO mice and their wt littermates were used. The mice were generated by targeting construct to replace most of the coding region of the *Wfs1* gene (Figure 3). For that the 8.8 kb BamHI restriction fragment from the PAC clone 391-J24 (RPCI21 library, MRC UK HGMP Resource Centre, UK) was subcloned into a pGem11 cloning plasmid (Promega, Madison, WI). The 3.7-kb NcoI fragment was replaced with an in-frame NLS-LacZ-Neo cassette. This resulted in the deletion of amino acids 360–890 in the Wfs1 protein and a fusion between the Wfs1 1–360 fragment and LacZ. This construct was inserted into W4/129S6 embryonic stem (ES) cells (Taconic, Hudson, NY) at the Biocenter of the University of Oulu (http://www.biocenter.oulu.fi). Colonies resistant to G418 and ganciclovir were screened for homologous recombination by polymerase chain reaction (PCR) by using the recombination-specific primers NeoR1 5'GACCGCTATCAGGACA TAGCG3' and Wfs1_WTR1 5'AGGACTCAGGTTCTGCCTCA3'. The PCR product was sequenced to verify that homologous recombination took place, and injected ES clone 8A2 into C57BL/6 blastocysts. The invalidation of *Wfs1* gene was verified by mRNA expression analysis and we confirmed the lack of Wfs1 transcript in homozygous Wfs1 mutant mice (Koks *et al.*, 2009).

According to the Mouse Genome Database (http://www.informatics.jax.org/), the official designation of this strain is Wfs1tm1Koks. In order to avoid the "congenic footprint" effect (Schalkwyk *et al.*, 2007) the animals were bred into two different backgrounds and only mice with the isogenic 129S6 background were used.

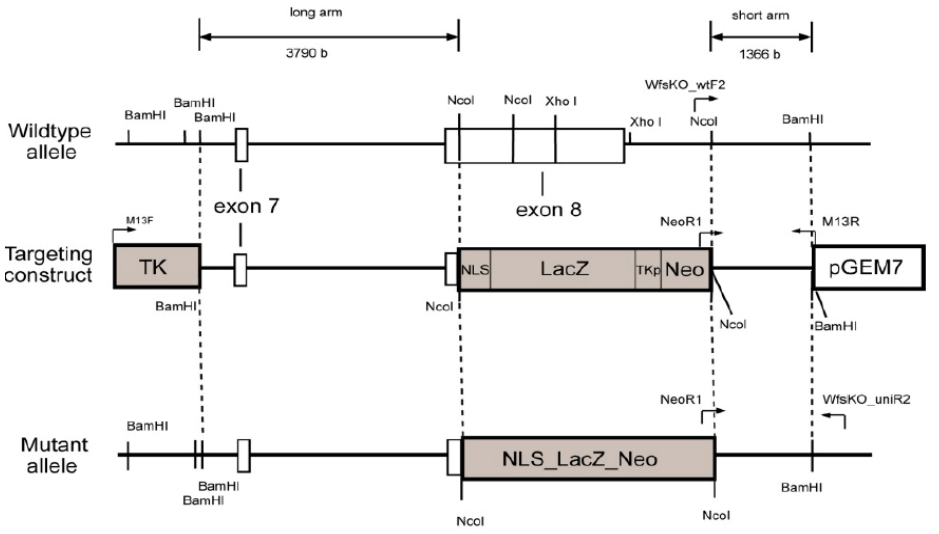

Figure 3. The strategy of making Wfs1 knock-out mouse. Exon 8 in the Wfs1 gene was replaced with NLS-LacZ-Neo expression cassette (Luuk *et al.*, 2008)

4.1.1. Fertility in Wfs1KO male mice (Paper 1)

In the fertility study we used 13 Wfs1KO, 13 wild type (wt) male mice and 156 wt female mice. All mice were 8–12 weeks old. At the end of the fertility study the male mice (both Wfs1KO and wt) were used to measure sex hormone levels. Eleven adult male Wfs1KO and 12 wt male mice were used for sperm morphology and motility study. Three Wfs1KO and 3 wt male mice were used to examine testes histology.

4.1.2. Growth and development of diabetes in Wfs1KO mice (Paper 2)

In Study 2, we used 21 male Wfs1KO, 21 male wild type (wt), 21 female Wfs1KO and 21 female wt mice. At the beginning of the study all mice were 9 weeks old. The mice were observed until the age of 32 weeks. All mice were used in weight analysis and blood glucose measurements. After the longitudinal study different hormones (not all in the same mouse) were measured from blood of these animals: 20 wt mice and 20 Wfs1KO mice were used for plasma insulin measurements, 19 wt and 19 Wfs1KO mice for proinsulin, 10 wt and 10 Wfs1KO mice for C-peptide levels.

4.1.3. Energy metabolism and thyroid function in Wfs1KO mice (Paper 3)

For the measurements of different metabolic parameters 16 male mice (8 Wfs1KO and 8 wt) and 16 female mice (8 Wfs1KO, 8wt) at the age of 8–10 weeks were used. For hormone measurements altogether 52 Wfs1KO mice (20 for thyroid hormones and 32 for leptin) and 57 wt mice (20 for thyroid hormones and 37 for leptin) were used. All mice were 14–20 weeks old. For the thyroid histology studies additional 5 Wfs1KO mice (3 males and 2 females) and 7 wt mice (4 males and 3 females) at the age of 16–20 weeks were used.

4.2. Description of experiments

4.2.1. Fertility study (Paper 1)

All male mice, both wt and Wfs1KO, were housed in one cage with two wt female mice each. Every morning the females were checked for the presence of vaginal plugs, an indication that sexual activity had taken place. If a vaginal plug was present, the female was taken away from the cage and placed in another one. If there was no vaginal plug after three days, the mice were separated and two other females were introduced to the males. This was done three times. Thus, at the end of the period, six females had been introduced to each male mouse (6 x 13 x 2). Each week, every female was weighed and if they had gained weight they were transferred to a single cage, where they delivered their pups. The fertility rate and the size of the litter were counted for each male.

4.2.2. Blood glucose measurements (Paper 2)

At the beginning of the study, all mice were 9 weeks old. Until 20 weeks of age, non-fasting blood glucose concentration (BGC) from the tail vein was measured weekly between 9 a.m. and 11 a.m. using a portative glucometer. After 20 weeks of age, BGC was measured once every 2 weeks. At 30 weeks of age, an intraperitoneal glucose tolerance test (IPGTT) was conducted. After 16 h of fasting, animals were weighed and a basal glucose level (Glc 0') was measured from the tail vein. The solution of glucose (20 % Glc in 0.9 % NaCl) was injected intraperitoneally (2 g of glucose / kg). Blood glucose levels were measured 60 and 120 min after the injection (Glc 60’ and Glc 120'). The procedure was carried out according to the simplified IPGTT protocol from the European Mouse Phenotyping Resource of Standardised Screens database (http://empress.har.mrc.ac.uk/).

4.2.3. Longitudinal growth study (Paper 3)

At the same time as the blood glucose measurements were made, animals were weighed using an electronic scale with accuracy of 0.1 g. The following characteristics were calculated:

- The maximal weight gain between the maximal weight during the experiment and the weight at 9 weeks of age.
- The absolute weight gain between the weight at 32 weeks of age and the weight at 9 weeks of age and
- The weight loss between the maximal weight and weight at 32 weeks of age.

4.2.4. Study of energy metabolism (Paper 3)

Mice were at first separated from their littermates and housed in single cage for 3 weeks. After this adaption period mice were studied alone in a special metabolic cage (TSE Systems) for 48 hours. These 48-hour studies were repeated three times separated from each other by a one-week break in a single cage. All the recordings were done during the last 48-hour period in metabolic cage. Mean food and water intake per body weight (g/g and ml/g), mean O_2 consumption (ml/h/kg), CO_2 production (ml/h/kg) and heat production (kcal/h/kg) were recorded. Movement counts over 48 hours (cnts/48 hrs) of each mouse in 3 different directions (X-axis, Y-axis and Z-axis i.e. reared ups) were recorded. The mean horizontal (X- and Y-axis) and vertical movements (Z-axis) were recorded.

4.3. Serum hormone measurements

Blood samples were taken from mice after they were sacrificed by cervical dislocation. Samples were centrifuged for 15–20 minutes at 1000 – 2000xg, 4°C. Serum or plasma was removed and samples analysed by different ELISA kits: USCNLIFE (China) kit for serum testosterone, follicle-stimulating hormone (FSH), proinsulin, thyroid stimulating hormone (TSH) and for serum thyroxine (T4); Chrystal Chem Inc (USA) for plasma insulin; R&D Systems (USA) kit for plasma leptin and Biovendor (Germany) for plasma C-peptide measurements. All procedures were preformed according to the user manual of every specific kit. The optical density of the wells was determined with the ELISA reader SUNRISE (Tecan, Switzerland).

4.4. Histological and morphological studies

4.4.1. Motility and morphology of sperm (Paper 1)

Sperm were obtained from the cauda epididymides of mature male mice previously sacrificed by cervical dislocation. Two hundred spermatozoa per male were analyzed, totalling 2200 spermatozoa in the Wfs1KO group and 2400 in the control group. Sperm motility was observed and recorded by CASA (SpermVision™, Minitübe, Germany). The percentage of motile spermatozoa and straight-line motile spermatozoa was calculated. Sperm morphology was studied on wet preparations made from formal-saline fixed samples, under phase-contrast microscope at 1000x magnification. The sperm head morphology, sperm tail morphology and the presence of cytoplasmic droplets was studied using the methodology described by Kawai *et al*. (Kawai *et al.*, 2006). Using sperm head morphology, the percentage of spermatozoa with abnormal sperm heads, including triangular, collapsed and hammer heads or with a hairpin at the neck, was calculated. Using sperm tail morphology, sperm tails were classified into three categories: straight tail, proximal bent tail and distal bent tail including angled and hairpin forms. The percentage of every form was calculated. The percentage of spermatozoa with none, light-type or heavy-type cytoplasmic droplets (CD) was also calculated.

4.4.2. Histology of testes (Paper 1)

The structure of both testes of three wild-type mice and three Wfs1KO mice were analyzed (totalling 12 testes). Samples were fixed in 10% buffered formalin and embedded in paraffin according to routine methods. Specimens were cut at 4µm thickness and stained with hematoxylin and eosin for examination by light microscopy. Specific cell counts were performed in each testicle in the seminiferous epithelium of five round-shaped seminiferous tubules, i.e. cells were counted in 10 tubules per mouse. Two independent, blind observers performed the cell counts.

4.4.3. Histology of thyroid glands (Paper 3)

Histology of thyroid gland was studied in 5 Wfs1KO mice (3 males and 2 females) and 7 wt mice (4 males and 3 females). All mice were 16–20 weeks old. Mice were sacrificed and their thyroid glands were separated. Tissue samples of thyroid gland were fixed in 10% buffered formalin solution and embedded in paraffin with vacuum infiltration processor according to standard methods. Specimens were cut at four-µm thickness and stained using the hematoxylin-eosin and van Gieson methods for examination by light microscopy. The extent of morphological changes in the thyroid glands was evaluated by two independent observers in a blinded fashion.

4.5. Statistical analysis

All data was analysed using the statistical software package, SAS version 9.1 (SAS Institute Inc, Cary, North Carolina, USA) or GraphPad InStat version 3.0b statistical software package. The Chi-square test or Fisher's Exact Test (when expected values were <5%) was used to compare the fertility rates. The 2-sample t-test or the Welch 's t-test was used to analyse the differences in various parameters between genotypes and gender. Mean ± SEM are shown. P-values < 0.05 were considered statistically significant.

The standard curve for analysing the ELISA samples was created by a 4-parameter logistic curve fit using GraphPad Prism version 4.0b.

5. RESULTS

5.1. Fertility in male Wfs1KO mice (Paper I)

As a result of the fertility study we found out that the pregnancy rate in female mice mated with Wfs1KO males tended to be lower than in those mated with wt males: 15/78 (19%; 95%CI 11.5–30.0) vs. 25/78 (32%; 95%CI 22.9–43.6), p=0.1. We noticed that in the Wfs1KO group there was one male who had pups with four out of the six females. This was more than 3 SD above the group's mean. When this male and his six females were excluded from the analysis, the pregnancy rate in females mated with Wfs1KO males was significantly lower than the pregnancy rate in the control group: 11/72 (15%; 95%CI 7.9–25.7) vs. 25/78 (32%; 95%CI 21.9–43.6). P < 0.05; (Figure 4).

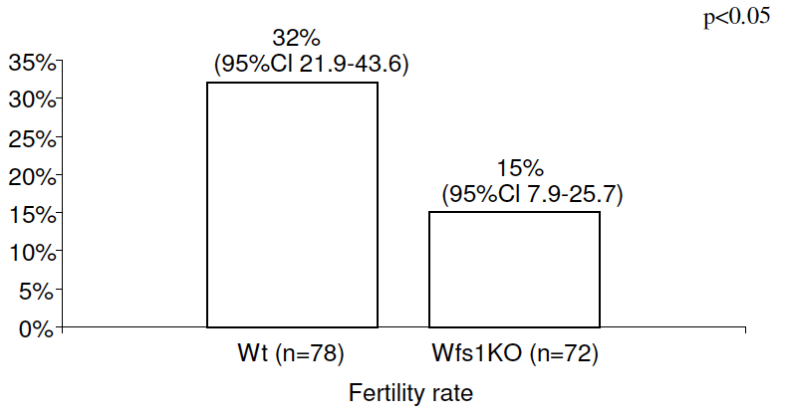

Figure 4. The fertility rate in percentages, with 95% CI in brackets, in 72 female wt mice mated with Wfs1KO (n=12) male and in 78 female wt mice mated with wt male mice (n=13).

In analysing the fertility of Wfs1KO males, we found that five out of 13 of them did not have any litter, whereas all 13 of the control males had at least one litter (p < 0.05). There was no significant difference in the litter size: 5.7±0.5 pups in the Wfs1KO group vs. 6.6±0.5 pups in the wt group. When analysing the occurrence of vaginal plugs we found that out of the six female mice who were mated with one male mouse, vaginal plugs occurred in 1.1±0.2 females in the Wfs1KO group, compared to their occurrence in 2.5±0.4 females out of six in the wt group (p<0.05).

To find the reason for the altered fertility of male Wfs1KO mice, we conducted a study to investigate sperm motility and morphology. Sperm motility was not affected in Wfs1KO mice. Surprisingly, the mean percentage of motile sperm was even higher in the Wfs1KO mice than in the wt mice, whereas no statistical differences were observed in the percentage of straight motility (Table 3). The sperm morphology study showed that Wfs1KO males

had fewer proximal bent tails than wt males, but had fewer abnormal sperm heads than wt males (Table 3, Figure 5). The sperm of Wfs1KO mice also tended to have more cytoplasmic droplets, both light-type and heavy-type, but the difference was not statistically significant (Table 3, Figure 5).

Table 3. Sperm morphology in male mice according to Kawai *et al*. (Kawai *et al.*, 2006). Percentage of spermatozoa (out of 200) having the specific characteristic (mean ± SEM). CD – cytoplasmic droplets.

Characteristic	*Wfs1KO (n=11)*	*WT (n=12)*	*p-value*
Motility	78.0 ± 9.3%	70.0 ± 11.6%	0.04
Straight motility	66.0 ± 11.7%	58.0 ± 15.4%	0.08
Sperm without CD	57.0 ± 16.3%	68.9 ± 18.7%	0.07
Light CD	30.5 ± 10.9%	22.5 ± 13.3%	0.07
Heavy CD	17.6 ± 6.1%	8.4 ± 6.2%	0.09
Straight tail	53.1 ± 5.0%	50.2 ± 5.7%	0.1
Proximal bent tail	14.4 ± 4.1%	21.5 ± 4.4%	0.0003
Distal bent tail	32.5 ± 7.6%	28.2 ± 5.9%	0.07
Hairpin at the neck	9.7 ± 2.3%	9.7 ± 2.6%	0.5
Abnormal head	22.7 ± 5.9%	31.5 ± 12.1%	0.02

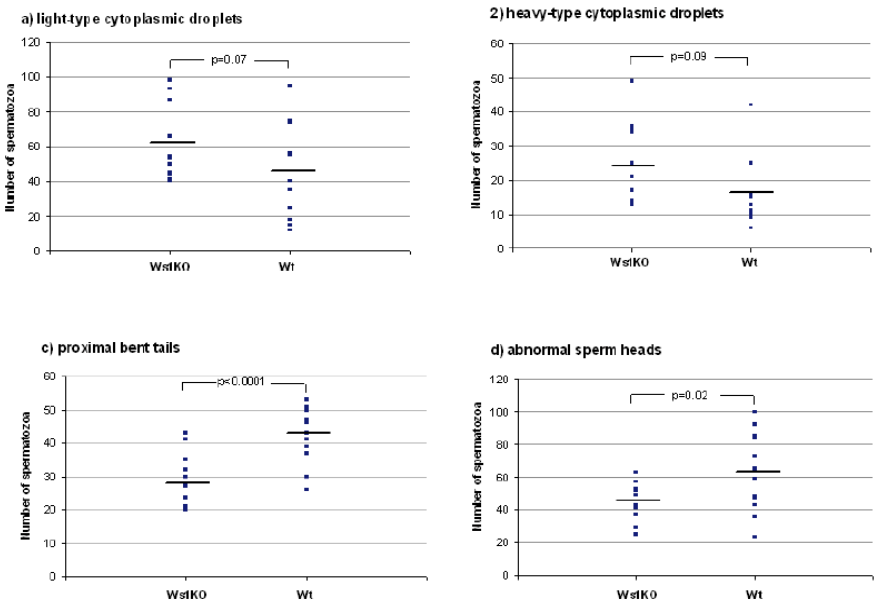

Figure 5. Four most important abnormalities in sperm morphology: a) light-type cytoplasmic droplets; b) heavy-type cytoplasmic droplets; c) proximal bent tails and d) abnormal sperm heads. The number of spermatozoa (out of 200) of each mouse having the specific characteristic is shown in dots. The mean number of the group is shown with a bold line.

Testes histology showed the organized architecture of the seminiferous epithelium of the seminiferous tubules seen in wt mice was lost in Wfs1KO mice (Figure 6).

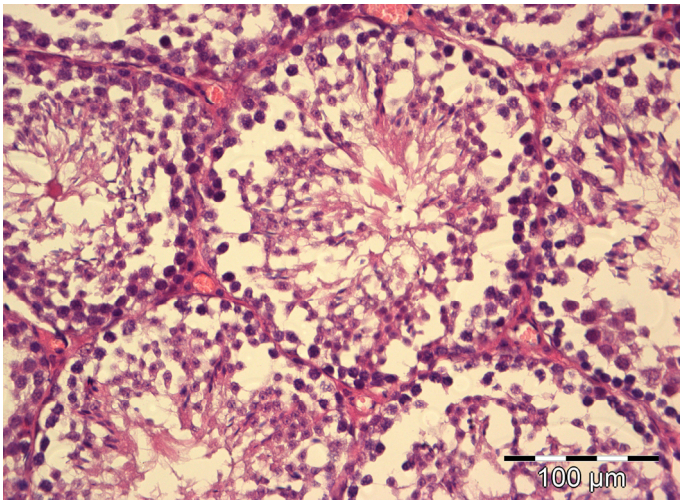

Figure 6. Normal seminiferous epithelium of the seminiferous tubules in a wild-type mouse.

Contrary to the wild-type mice, the lumina of the seminiferous tubules in Wfs1-deficient mice have a typically irregular contour or the lumen may even be obliterated. Accumulation of eosinophilic luminal content is seen. Furthermore, several segments of these tubules have no spermatogenic cells at all (Figure 7).

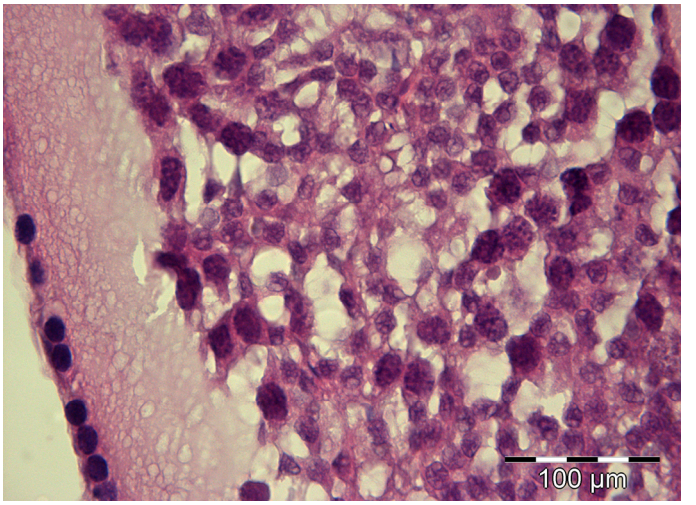

Figure 7. Altered structure of the seminiferous epithelium in a Wfs1KO mouse.

The seminiferous epithelium of Wfs1-deficient mice has reduced numbers of Spermatogonia and Sertoli cells, resulting in reduced sperm production (Table 4). There were no significant differences between the number and structure of Leydig cells between wt and Wfs1KO mice.

Table 4. The number of cells (mean and ± SEM) in the seminiferous epithelium of seminiferous tubules in wt and Wfs1KO mice. SG – spermatogonia, PSC – primary spermatocytes, SSC – secondary spermatocytes. *p<0.05

Group	*SG*	*PSC*	*SSC*	*Spermatids*	*Sperms*	*Sertoli cells*
Control (n=3)	38.12 ± 4.88	54.33 ± 1.46	2.13 ±1.15	134.07 ± 10.63	35.4 ± 9.62	9.17 ± 1.65
Wfs1 (n=3)	23.92 ± 4.91*	47.23 ± 12.07	0 ± 0	109.97 ± 26.42	13.28 ± 6.95*	6.35 ± 0.81*

When measuring the serum concentrations of sex hormones in male Wfs1KO mice we found that serum testosterone and FSH concentrations in Wfs1KO males (12.0 ± 0.5 nmol/l and 5.6 ± 0.1 mIU/ml respectively) did not differ significantly from those in wt males (11.5 ± 0.7 nmol/l and 5.8 ± 0.1 mIU/ml respectively).

5.2. Growth in Wfs1KO mice (Paper 2)

At 9 weeks of age, when the experiment started, the male Wfs1KO mice were already smaller than the male wt mice (18.69 ± 0.42 g vs. 21.9 ± 0.51 g, $p < 0.0001$); no such difference was observed in females at this point in time. The female Wfs1KO mice became statistically lighter than the wt mice from 16 weeks of age. The average maximum weight and the average maximum weight gain over the study period in male Wfs1KO mice were significantly lower than in wt males (23.00 ± 0.39 vs. 30.13 ± 0.53 g; $p < 0.0001$ and 4.36 ± 0.36 vs. 8.27 ± 0.30 g, $p < 0.0001$ accordingly). A similar pattern was seen in females: the maximum weight and the maximum weight gain over the study period were smaller in Wfs1KO mice than in wt females (19.10 ± 0.34 vs. 22.17 ± 0.31 g, $p < 0.0001$ and 2.73 ± 0.19 g vs. 5.39 ± 0.24 g; $p < 0.0001$ accordingly). Wfs1KO mice started to lose weight during the final weeks of the study: the average weight loss was 2.02 ± 0.32 g in male Wsf1KO mice and 1.55 ± 0.15 g in female Wfs1KO mice. The weight loss between the sexes was not statistically different. Figure 8 shows the weight profiles of the mice.

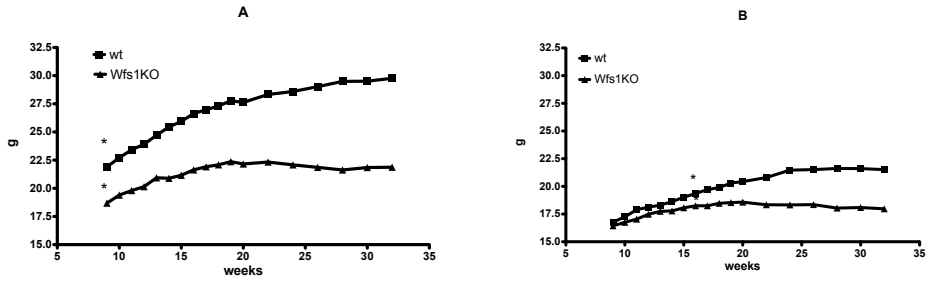

Figure 8. The weight curve in male (a) and female (b) Wfs1KO (shown in triangles) and wt (shown in squares) mice. The first time-point from the beginning of the study at which the difference between the groups became statistically significant ($p < 0.05$) is marked with a *.

5.3. Sex differences in the development of diabetes in Wfs1KO mice (Paper 2)

From 24 weeks of age the mean BGC in Wfs1KO males became significantly higher than in wt males and continued to rise further. At 32 weeks of age the average BGC in male Wfs1KO mice was 9.40 ± 0.60 mmol/l compared to 7.90 ± 0.20 mmol/ l in wt males ($p < 0.05$). The opposite effect was seen in females: at 32 weeks of age the mean BGC in Wfs1KO mice was lower than in wt mice (6.67 ± 0.20 vs. 7.49 ± 0.19 mmol/l, $p < 0.05$) (Figure 9). The IPGTT performed at the age of 30 weeks showed that both male and female Wfs1KO mice responded with a significantly higher increase in blood sugar levels than wt mice, indicating impaired glucose tolerance or possible diabetes already, particularly in males (Table 6).

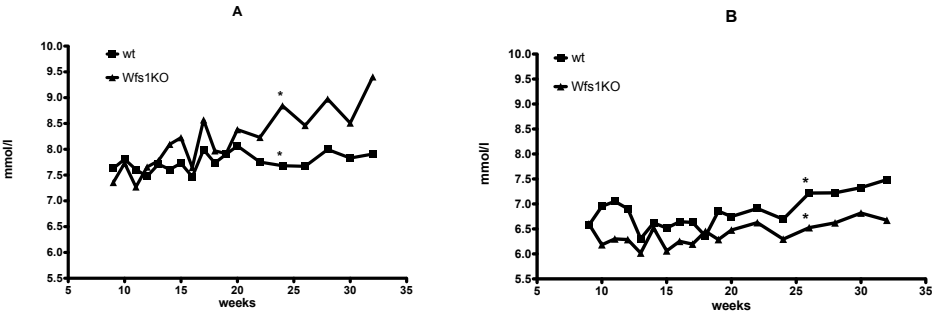

Figure 9. The mean blood glucose concentration in male (a) and female (b) Wfs1KO (shown in triangles) and wt (shown in squares) mice. The first time-point from the beginning of the study at which the difference between the groups became statistically significant ($p < 0.05$) is marked with a *.

Table 6. The results of the IPGTT of WFS1KO and wt mice of both sexes at the age of 30 weeks.

Time	*Wfs1KO male mmol/l*	*Wt male mmol/l*	*Wfs1KO female mmol/l*	*Wt female mmol/l*
0 min	4.96 ± 0.20	4.53 ±0.19	4.26 ± 0.26	3.72 ± 0.17
60 min	20.18 ± 0.93[a]	11.72 ± 0.73	17.06 ± 1.07[b]	10.49 ± 0.72
120 min	14.79 ± 0.61[a]	7.48 ± 0.29	11.0 ± 0.94[c]	6.82 ± 0.41

[a]$p<0.0001$ vs. wt males; [b]$p<0.0001$ vs. wt females; [c]$p<0.05$ vs. wt females.

Male Wfs1KO mice had significantly lower plasma insulin levels than wt males (57.78 ± 1.80 ng / ml vs. 69.42 ± 3.06 ng / ml; $p < 0.01$). The mean insulin level in male Wfs1KO mice was also significantly lower than in Wfs1KO females (70.30 ± 4.42 ng / ml; $p < 0.05$.) Interestingly, the highest mean plasma proinsulin level was seen in the male Wfs1KO group (Table 6), but the differences were not statistically significant. However, the Wfs1KO male mice had relatively more proinsulin compared to the insulin levels. The proinsulin/ insulin ratio in Wfs1KO males (0.09 ± 0.02) was significantly higher than in wt males (0.05 ± 0.01; $p = 0.05$) and in Wfs1KO females (0.04 ± 0.01; $p < 0.05$). The difference in proinsulin/insulin ratio between the females was not statistically significant. Plasma C-peptide concentration was measured only in male mice; it was significantly reduced in Wfs1KO mice compared to wt mice (55.3 ± 14.0 pg / ml vs. 112.7 ± 21.9 pg / ml; $p < 0.05$).

Table 6. Mean (± SEM) plasma insulin (ng/ ml), proinsulin (ng/ ml) – concentration and proinsulin/insulin ratio in different groups. Statistically significant differences between the groups are marked with superscript. [a]$p < 0.01$ vs. wt males; [b]$p < 0.05$ vs. female Wfs1KO; [c]$p = 0.05$ vs. wt males.

Hormone	*Wfs1KO males*	*wt males*	*Wfs1KO females*	*wt females*
Insulin	57.78 ±1.80[a, b]	69.42 ± 3.06	70.30 ± 4.42	64.36 ± 4.09
Proinsulin	5.20 ± 1.11	3.37 ± 0.87	2.80 ± 0.73	3.16 ± 0.30
Proinsulin/insulin	0.09 ± 0.02[b,c]	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.004

5.4. Energy metabolism and thyroid function in Wfs1KO mice (Paper 3)

Mean O_2 consumption, CO_2 or heat production in Wfs1KO mice did not differ from those in wt mice. However, there were significant gender differences in both genotypes: all three parameters were higher in females. Mean food intake per body weight was significantly lower in Wfs1KO males than in wt males (0.09 ± 0.01 vs. 0.16 ± 0.02 g/g; $p < 0.05$). Wfs1KO males were lighter than

their wt mates already at the beginning of the experiment and lost much more weight during the 48-hour trial in cage than wt males (Table 6). There were no differences in horizontal activity between the groups in male mice, but Wfs1KO females reared up more frequently (vertical activity) compared to wt female mice (199.8 ± 63.5 vs. 39.3± 24.7 cnts/48 hours; $p < 0.05$) indicating more curiosity in this group. Mean characteristics of energy metabolism, food intake and physical activity are shown in Table 7.

Table 7. Mean ± SEM values of different parameters measured during the metabolism study. Statistically significant differences between the groups are marked with superscript. Abbreviations: VO_2 – O_2 consumption; VCO_2 – CO_2 production; heat – energy produced. [a]$p < 0.05$ vs. wt females; [b]$p < 0.05$ vs. wt males. [c]$p < 0.05$ vs. Wfs1KO males.

Parameter	***Wfs1KO females (n=8)***	***wt females (n=8)***	***Wfs1KO males (n=8)***	***wt males (n=8)***
VO_2 (ml/h/kg)	3410 ± 127.00[c]	3186 ± 61.21[b]	2806 ± 82.43	2721 ± 117.30
VCO_2 (ml/h/kg)	2925 ± 151.30[c]	2850 ± 109.80[b]	2328 ± 89.49	2376 ± 131.5
Heat (kcal/h/kg)	16.67 ± 0.66[c]	15.71 ± 0.35[b]	13.62 ± 0.41	13.62 ± 0.41
Weight start (g)	17.21 ± 0.67[a]	20.28 ± 0.74	20.98 ± 0.51[b]	23.74 ± 0.96
Weight end (g)	15.29 ± 1.03[a]	18.98 ± 1.17	16.69 ± 0.40[b]	20.19 ± 0.60
Weight loss (%)	11.80 ± 3.00[c]	6.84 ± 3.27	20.43 ± 0.41[b]	16.07 ± 0.86
Drink per g (ml/g)	0.30 ± 0.04[c]	0.29 ± 0.05	0.09 ± 0.01	0.09 ± 0.03
Food per g (g/g)	0.19 ± 0.03[c]	0.26 ± 0.03	0.09 ± 0.01[b]	0.16 ± 0.02
Horizontal activity (cnts/48h)	48802 ± 9106	29576 ± 3360	34204 ± 6567	28415 ± 5539
Vertical activity (cnts/48h)	199.8 ± 63.46[a]	39.26 ± 24.71	86.50 ± 22.73	111.0 ± 36.20

Male Wfs1KO mice had significantly lower levels of plasma leptin than wt males (3.37 +/– 0.40 vs 5.82 +/– 0.71 ng/ml; $p < 0.01$) whereas no such difference was seen in females (Figure 10).

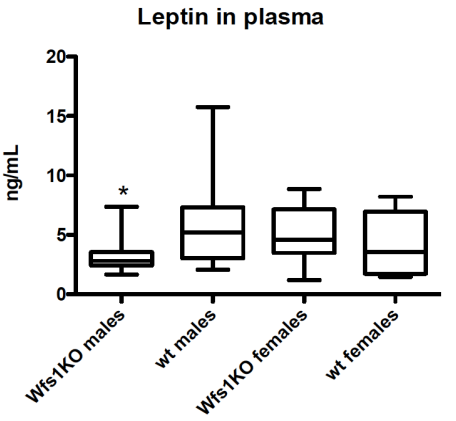

Figure 10. The levels of leptin in plasma of Wfs1KO and wt mice (both male and female). Lower plasma leptin level in Wfs1KO males compared to wt males is shown with a star ($p<0.05$).

Mean plasma TSH and T4 levels in both sexes did not differ between the Wfs1KO and wt groups (Figure 11), but surprisingly, female mice had lower mean plasma T4 concentration than the males both in the Wfs1KO group (40.8 +/– 4.4 vs. 113.8 +/– 20.5 ng/ml; $p < 0.01$) as well as in the wt group (39.2 +/– 3.5 vs. 131.0 +/– 18.2 ng/ml; $p<0.0001$; Figure 11).

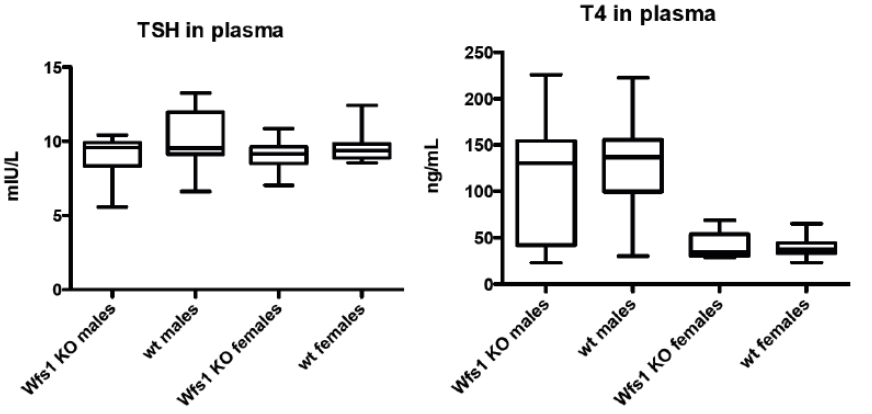

Figure 11. The levels of plasma thyroxine and TSH of Wfs1KO and wt mice (both male and female).

We also examined the histological architecture of the thyroid glands. In Wfs1KO females the follicles were larger, all filled with colloid. Epithelial cells and their nuclei were extremely flat; the mean number of epithelial cells per follicle was 10.5 and large amount of interstitium was seen (Figure 12B). In wt females very small follicles filled with colloid were dominating, orientation of

epithelial cells was irregular, and the mean number of epithelial cells per follicle was 10.2 (Figure 12A).

In Wfs1KO males thyroid follicles were relatively small filled with homogeneous colloid; the mean number of epithelial cells per follicle was 13.2. Large amount of interstitium was noted (Figure 12D). In comparison in wt males there were large spherical follicles filled with colloid. Epithelial cells of follicles were regular; the mean number of epithelial cells per follicle was 15.1. Small amount of interstitium was noted (Figure 12C).

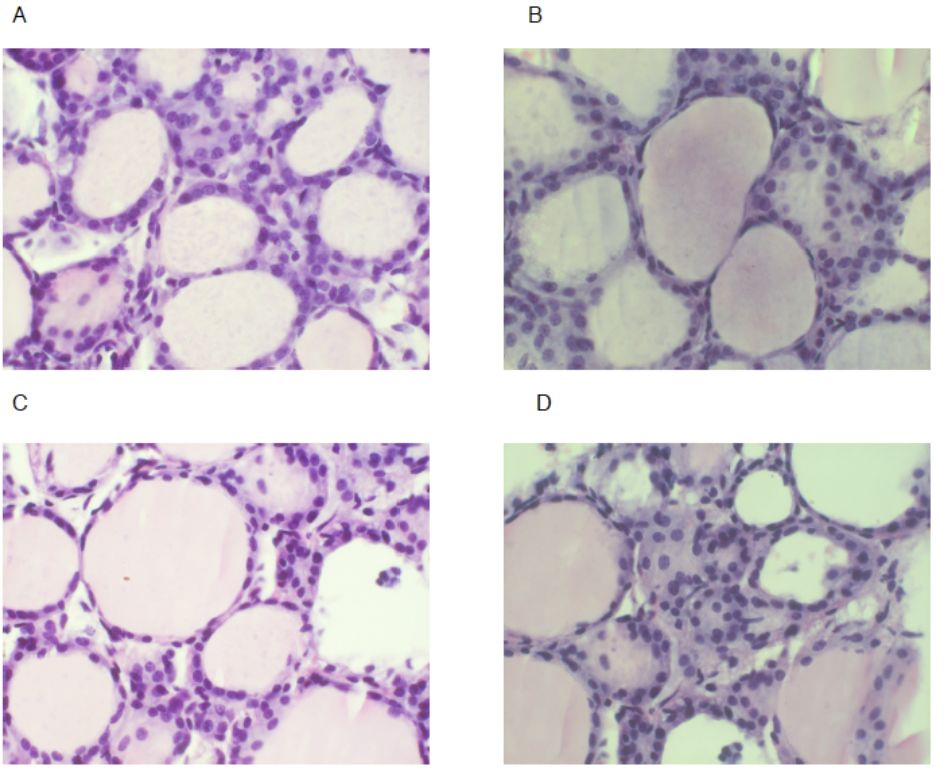

Figure 12. A) Normal histology of thyroid tissue of female wt mice. H&E, original magnification 580x. B) Altered histology of thyroid tissue of female Wfs1KO mice: Thyroid follicles with flat epithelial cells are seen. H&E, original magnification 580x. C) Normal histology of thyroid tissue of male wt mice. H&E, original magnification 580x. D) Altered histology of thyroid tissue of male Wfs1KO mice: Thyroid follicles with varying size and increased amount of interstitium are seen. H&E, original magnification 580x.

Although we could not do the statistical analysis between the groups, the mean number of epithelial cells per follicle was higher in males compared to females in both groups.

6. DISCUSSION

6.1. Fertility of the male *Wfs1*-deficient mice (Paper I)

We have shown for the first time that male Wfs1KO mice have reduced fertility compared to wt male mice. It is known that primary hypogonadism may occur in male patients with WS (Peden *et al.*, 1986; Barrett *et al.*, 1995; Barrett and Bundey, 1997; Medlej *et al.*, 2004), but there have been no data about the fertility of patients with WS. One reason for this may be the few patients available with whom to carry out such a study, or the fact that other clinical symptoms, such as diabetes mellitus and vision or hearing impairments, dominate and precede the fertility problems.

Consequently, we investigated whether Wfs1KO male mice have reduced fertility and, if so, their possible causes. The pregnancy rate of wt female mice was lower when mated with Wfs1KO male mice than when mated with wt males. All wt males used in our study gave at least one litter with one female out of six, whereas only eight Wfs1KO males out of 13 produced litters, indicating impaired fertility in male Wfs1KO mice.

In order to examine the possible mechanisms causing reduced fertility, we examined the sperm of the same males used in the fertility study. Having studied the motility and morphology of the sperm in the Wfs1KO and wt groups, it was shown that the motility of spermatozoa in Wfs1KO mice was not impaired and was even slightly better than in the wt animals; however, many changes in sperm morphology were found. The most statistically significant difference was that the sperm of Wfs1KO mice contained fewer spermatozoa with proximal bent tails than the sperm of wt mice. The in vitro fertilisation rate in mice has been shown to be positively correlated to proximal bent tails, but negatively to heavy-type CD and distal bent tails. These last two characteristics were higher in Wfs1KO mice compared to wt mice, but the difference did not reach statistical significance ($p < 0.09$ and 0.07, respectively). We did find that Wfs1KO males had fewer abnormal sperm heads; however, the impact of an abnormal sperm head on fertility has been found to be much smaller than the impact of differently bent tails or cytoplasmic droplets, as seen in the study by Kawai *et al.* where a high percentage of abnormal sperm heads was associated with a relatively good fertilisation rate (Kawai *et al.*, 2006).

Testicular histology showed a normal pattern of seminiferous epithelium in the tubules of wt mice, which was lost in Wfs1KO mice. Several segments of seminiferous tubules in Wfs1KO mice had no spermatogenic cells at all. The number of spermatogonia and Sertoli cells essential for effective spermatogenesis was decreased, leading to reduced sperm production. Thus, both sperm morphology and quantity is affected in Wfs1KO mice; however, the number and structure of Leydig cells responsible for testosterone synthesis did

not different between the Wfs1KO and wt mice. Normal testosterone levels in the two groups also confirmed this.

The mechanism by which wolframin deficiency may cause impaired fertility and changes in sperm morphology and quantity is not clear. One possible explanation may be through increased endoplasmic reticulum (ER) stress, which has been shown to cause progressive cell loss in pancreatic β-cells in Wfs1-deficient mice (Ishihara *et al.*, 2004; Ueda *et al.*, 2005; Yamada *et al.*, 2006). ER stress enhances Wfs1 gene expression and Wfs1-deficient mice are more susceptible to ER stress-induced apoptosis than wild-type mice (Ishihara *et al.*, 2004).

Wfs1 is also expressed in the testes of mice (Kõks S, 2008), so it is possible that increased ER stress in the testes of Wfs1KO mice may cause changes in spermatogenesis. Further studies, including electron microscopic studies of the endoplasmic reticulum of spermatozoa and the epithelium of seminiferous tubules in Wfs1KO mice, are necessary to confirm or reject our hypothesis.

Another possible reason for impaired fertility in Wfs1KO mice may be due to changes in the hypothalamic-pituitary axis. It is known that patients with WS have disturbed anterior pituitary function (Medlej *et al.*, 2004), and we ourselves have been shown changes in the gene expression levels of GH, POMC, and NPY in different parts of the brain (Koks *et al.*, 2009). Therefore, we also measured serum FSH and testosterone levels in these mice. We chose these two hormones because they both play an important role in spermatogenesis; however, we did not find any differences in their levels. Unfortunately, we did not have enough serum to measure LH, but it is unlikely that serum LH concentrations would have been different due to comparable serum testosterone concentrations as well as the number and structure of Leydig cells. This observation suggests that it is unlikely that altered gonadotrophin levels would cause the impaired fertility in these mice. As the number of Sertoli cells was significantly lower in the Wfs1KO group, inhibin B should be measured in further studies.

We cannot exclude the possibility that part of the impaired fertility in Wfs1KO male mice may be explained by different sexual behaviours. It is known that patients with WS frequently have psychiatric problems (Swift *et al.*, 1990) and therefore may have defects in sexual behaviour. The occurrence of vaginal plugs in female mice was significantly lower when mated with Wfs1KO males, in comparison to wt males. However, the occurrence of vaginal plugs is not a very reliable method, since the plug lasts only couple of hours and can easily be missed. Specially designed studies with video recording are necessary to clarify whether sexual behaviour in Ws1KO mice is different from wild-type mice.

6.2. Growth retardation (Paper 2)

We studied weight in Wfs1KO mice longitudinally from the age of 9 weeks until the age of 32 weeks. Over the last weeks of this study period, both female and male Wfs1KO mice significantly lost weight (8.4% and 8.7%, respectively). These types of longitudinal studies have also been carried out before (Ishihara *et al.*, 2004; Riggs *et al.*, 2005); however, in previous studies mice did not present with such severe weight loss. In one study, male knock-out mice grew similar to the wild-type mice until the age of 24 weeks (Riggs *et al.*, 2005), whereas in our case male Wfs1KO mice were statistically smaller already at the beginning of the study. The present study used only mice with the isogenic 129S6 background and the study lasted until 32 weeks of age. Thus, these differences in genomic background and shorter duration of the experiment may explain the differences in the weight pattern in mice between our study and the study by Ishihara *et al*. (Ishihara *et al.*, 2004) and Riggs *et al*. (Riggs *et al.*, 2005).

6.3. Sex related differences in developing of diabetes in Wfs1KO mice (Paper 2)

This is the first study to describe detailed sex-related longitudinal changes in BGC in Wfs1KO mice – an animal model for WS. Changes in blood glucose levels were studied from 9 weeks of age, at which time mice are considered young adults. Over the next 21 weeks, both male and female Wfs1KO mice developed impaired glucose tolerance according to the IPGTT results at 30 weeks of age. As there are no specific criteria for diabetes in mice, the results of IPGTT could already suggest possible overt diabetes, particularly in males, as significant hyperglycaemia with accompanied weight loss was seen only in male Wfs1KO mice. Their genetic background can explain the relatively mild phenotype of diabetes in these mice. Ishihara *et al*. (Ishihara *et al.*, 2004) showed that the development and severity of diabetes in Wfs1KO mice depends on the mouse's genetic background: in the F2 generation of B6 and 129 hybrids, 60% of mice developed overt diabetes at 36 weeks of age. Further back-crossing to B6 weakened diabetic phenotype in these mice, although that study only used male mice, as females had previously shown to have a milder phenotype (Ishihara *et al.*, 2004). Riggs *et al*. (Riggs *et al.*, 2005) also used only male mice to study the blood glucose levels in Wfs1KO mice. They used a mouse model with conditional deletion of Wfs1 in beta-cells. These knock-out males had significantly higher BGCs starting from the 24th week of age, similar to our study. The present study used only mice with the isogenic 129S6 background and the study lasted until 32 weeks of age. Thus, these differences in genomic background and shorter duration of the experiment may explain the differences in the phenotype of diabetes in male mice between our study and the

study by Ishihara *et al*. (Ishihara *et al.*, 2004) and Riggs *et al*. (Riggs *et al.*, 2005).

In the present study, only male Wfs1KO mice developed the non-fasting hyperglycaemia at 24 weeks of age. In classical rodent models of beta-cell failure, oestrogens at physiological concentrations protect pancreatic beta-cells against lipotoxicity (Pick *et al.*, 1998), oxidative stress (Contreras *et al.*, 2002; Eckhoff *et al.*, 2003; Eckhoff *et al.*, 2004; Le May *et al.*, 2006), and apoptosis (Pick *et al.*, 1998; Contreras *et al.*, 2002), while 17-β–estradiol administration to rodents produces islet hypertrophy (Zhu *et al.*, 1998), increases pancreas insulin concentration, and enhances insulin secretion (Alonso-Magdalena *et al.*, 2008). The prevalence of diabetes, especially diabetic syndromes with insulin deficiency, is lower in premenopausal women, suggesting that the female hormone 17-β-estradiol protects pancreatic beta-cell function (Liu and Mauvais-Jarvis, 2010).

Some data indicate that the onset of diabetes in humans with WS tends to start earlier in boys than in girls (Hardy *et al.*, 1999; Smith *et al.*, 2004), which suggests that the sex difference in the development of diabetes also exists in humans with WS.

In *Wfs1*-deficient mice, the development of diabetes has been associated with increased endoplasmic reticulum stress and apoptosis in pancreatic beta-cells, leading to insufficient insulin secretion (Ishihara *et al.*, 2004). Thus, the oestrogen receptors present in beta-cells can enhance islet survival (Liu *et al.*, 2009). They also improve islet lipid homeostasis (Louet *et al.*, 2004) and insulin biosynthesis (Alonso-Magdalena *et al.*, 2008).

The data about gender differences related to diabetes in humans are conflicting. Some human data indicate that females are intrinsically more insulin-resistant than males, possibly because of specific sex-linked gene expression and the resulting differences in metabolic control (Mittendorfer, 2005). On the other hand, despite having lower fat mass, the prevalence of diabetes and early abnormalities of glucose metabolism is higher in men than in women (Kuhl *et al.*, 2005; Macotela *et al.*, 2009).

In different animal models of glucose intolerance, insulin resistance, and diabetes, male mice usually show a stronger phenotype than females (Clark *et al.*, 1983; Zierath *et al.*, 1997; Li *et al.*, 2000; Macotela *et al.*, 2009). The mean plasma insulin level in our male Wfs1KO mice was significantly lower than in wt mice, similar to the study by Ishihara *et al*. (Ishihara *et al.*, 2004) and Riggs *et al*. (Riggs *et al.*, 2005). Unfortunately, their studies did not include female mice.

We also measured plasma proinsulin level in these mice and found that the mean proinsulin level tended to be higher in male Wfs1KO mice than wt mice. The increased proinsulin/insulin ratio in male mutant mice indicates that there may be problems converting proinsulin to insulin. This was later confirmed by Hatanaka *et al.* (2011), who found that Wfs1-null islets have impaired proinsulin processing, resulting in an increased circulating proinsulin level

(Hatanaka *et al.*, 2011). There are data indicating that prohormone convertase 2 (PC2) is absent in some WS patients (Gabreels *et al.*, 1998), which means that disturbances converting prohormones into biologically active forms (such as proinsulin to insulin) may exist. The other reason may be impaired acidification of insulin granules required before their exocytosis from β-cells (Hatanaka *et al.*, 2011). As the clinical picture and changes in BGC were more pronounced in males, their plasma C-peptide levels were also measured. These measurements found decreased C-peptide levels in male mutant mice, which suggests that in WS there may be also a defect in insulin processing that through increased endoplasmic reticulum stress and apoptosis in pancreatic beta-cells in the long-term leads to insulin deficiency. This process is probably much less rapid than the autoimmune pancreatic beta-cell destruction seen in type 1 diabetes; however, the fact that C-peptide was only measured in males means it is not possible to reach any conclusions regarding the sex differences in plasma C-peptide concentrations.

The study showed an interesting sex-related phenotype of diabetes in Wfs1KO mice. Male Wfs1KO mice developed overt diabetes with decreased plasma insulin levels – a phenotype seen more in Type 1 diabetes – whereas female knock-out mice developed impaired glucose tolerance with relatively high insulin levels – a phenotype more similar to Type 2 diabetes. The relatively high insulin level in Wfs1KO females may also explain their relatively low BGC. Recently, variants in the Wfs1 gene have been shown to be associated with Type 2 diabetes (Sandhu *et al.*, 2007; Franks *et al.*, 2008; Sparso *et al.*, 2008). Some medications used for treating Type 2 diabetes have also been found to be effective in preventing pancreatic beta-cell apoptosis in Wfs1KO mice (Akiyama *et al.*, 2009).

6.4. Energy metabolism and thyroid function (Paper 3)

This is the first study to describe the energy metabolism and thyroid function in Wfs1KO mice – an animal model for WS.

Patients with neurodegenerative disorders such as Alzheimer's, Parkinson's, or Huntington's disease have impaired glucose metabolism, increased insulin resistance, and abnormal appetite regulation (Cai *et al.*, 2012). Patients with Huntington's disease show severe weight loss in spite of having high caloric intake (Morales *et al.*, 1989; Trejo *et al.*, 2004; Cai *et al.*, 2012), and they also have higher energy expenditure compared to controls (Pratley *et al.*, 2000; Stoy and McKay, 2000; Gaba *et al.*, 2005; Cai *et al.*, 2012). The same has been shown in patients with Parkinson's disease (Cai *et al.*, 2012). Abnormally low leptin levels have been shown in patients with Alzheimer's disease (Olsson *et al.*, 1998; Power *et al.*, 2001). Increasing evidence suggests that there is a link

in Huntingdon's and Parkinson's disease between the progression of the disease and metabolic dysfunction (Cai *et al.*, 2012).

Several recent studies have demonstrated that therapies targeted to restore metabolic homeostasis may also improve cognitive and motor function, as well as increase lifespan in patients with Alzheimer's or Huntington's disease (Watson *et al.*, 2005; Martin *et al.*, 2009). Therefore, it is also very important to know the metabolism in Wolfram syndrome, another neurodegenerative disorder.

In this study, we focused on energy metabolism and thyroid function; however, we did not find such a difference in the energy metabolism of Wfs1KO mice compared to wt mice.

We analysed our mice for 48 hours in metabolic cages where O_2 consumption and CO_2 and energy production were measured, while vertical and horizontal motor activity and food and water consumption were registered. These cages have been used successfully in many animal studies to describe the energy metabolism in mice (Mauer *et al.*, 2010; Jung *et al.*, 2013).

In our study, Wfs1KO mice were smaller than wt mice. Wfs1KO mice also lost more weight than the wt mice during the 48-hour period, possibly due to lower food intake. Male Wfs1KO mice ate less than wt mice, which may be due to the emotional stress caused by an unfamiliar environment. Female mice showed their insecurity in the unusual environment by rearing up more often than their wt littermates. Impaired behavioural adaption to stress in Wfs1KO mice has been shown before (Luuk *et al.*, 2009), and it is therefore the likely mechanism for that increased weight loss. The same study also showed that the exposure to stress in female Wfs1KO mice induced a remarkable elevation of plasma corticosterone level compared to the wt littermates (Luuk *et al.*, 2009).

The other possible explanation as to why we could not see the differences in energy metabolism (We expected to see a hypermetabolic state similar to Huntington's (Aziz *et al.*, 2008; Goodman *et al.*, 2008) or Parkinson's disease (Levi *et al.*, 1990; Markus *et al.*, 1992)) in Wfs1KO mice is the age of our animals; namely, our animals were relatively young: 11–13 weeks in the metabolic cage study and 14–20 weeks at the time of blood sampling for hormone measurements and thyroid tissue removal for the histology study. However, as we have shown, overt diabetes was not yet present at this age in these mice (Noormets *et al.*, 2011), though it is the diabetes that develops first in patients with WS out of many clinical problems they have (de Heredia *et al.*, 2013). Thus, it is likely that if we had used older mice, the likelihood of discovering disturbances in energy metabolism would have been higher.

As expected, mean plasma leptin level in male Wfs1KO mice was significantly lower than in wt mice, as they probably also had lower fat mass – an important determinant of leptin level. This, however, is purely speculation, as we did not measure the length in these mice to calculate their BMI – a marker of body fat mass. In patients with Huntington's disease, another neurodegenerative disease, low leptin levels have been found, suggesting

negative energy balance (Popovic *et al.*, 2004). On the other hand, female Wfs1KO mice also had lower body weight than wild-type females, but they did not show such a decline in serum leptin levels suggesting some degree of leptin resistance. This resistance to leptin, a strong anorexigenic peptide, did not allow suppression of appetite, and the female knock-out mice ate more and in this way lost less weight than male Ws1KO animals in spite of their increased O2 consumption. No such resistance to leptin was seen in male Wfs1KO animals.

Sex-related differences seem to be common in Wfs1KO mice. We have shown that only the male Wfs1KO mice had impaired fertility, and only the male mice developed overt diabetes, whereas female Wfs1KO mice presented even very low glucose levels during fasting, followed by significant increase of blood sugar levels in IPGTT (Noormets *et al.*, 2011). We also found a sex-related difference in thyroid function in this study. It seems that males are more affected by the deletion of the *Wfs1* gene than females. There are also data from humans showing that the onset of diabetes, the first manifestation of WS, is earlier in boys than in girls (Barrett and Bundey, 1997; Hardy *et al.*, 1999; Smith *et al.*, 2004).

As *Wfs1* is expressed in thyroid tissue both in mice and in humans (Kõks S, 2008; De Falco *et al.*, 2012), we also investigated thyroid function and morphology. We did not find that *Wfs1* deficiency had any effect on thyroid function in these mice; however, to our great surprise, we found significantly lower plasma thyroxine levels in females in both groups compared to the male animals. McLachlan *et al*. have described the level of thyroxine as differing greatly between the mouse strains (McLachlan *et al.*, 2011), but in our case all mice studied were of the same background, and therefore the sex difference in thyroid function could not be explained by this factor. A gender difference in thyroxine levels has been noticed before: according to the Mouse Phenome Database (http://phenome.jax.org), thyroxine levels differ between the sexes greatly, depending on the background of different mice. Pohlenz *et al.* (Pohlenz *et al.*, 1999) also showed similar sex-related differences in TSH levels within one mouse strain. It is probably for these reasons that frequently only female mice are used to describe thyroid function in mice.

The histology of thyroid tissue also showed some differences between sexes, with a higher mean number of epithelial cells per follicle in male compared to female mice and more flattened epithelial cells in Wfs1KO females. A lower number of epithelial cells could lead to decreased thyroid function characterised by low thyroxine level, a picture seen in our female mice.

Further studies are necessary to clarify the precise clinical significance of these changes in thyroid morphology.

7. CONCLUSIONS

1. The fertility of Wfs1KO male mice is impaired compared to wt male mice. The reason for this is the changes in sperm morphology and in the morphology of the testes. Sperm motility was not affected in Wfs1KO mice.
2. Wfs1KO males have fewer proximal bent tails and fewer abnormal sperm heads than wt males, and both findings tend to be associated with lower in vitro fertilisation rates. Testicular histology showed a loss of normal seminiferous epithelium in the tubules of Wfs1KO mice and decreased number of spermatogonia and Sertoli cells, leading to reduced sperm production.
3. There are severe sex-related differences in the development of growth failure and diabetes. Growth failure in male Wfs1KO mice is already present from birth, whereas in females it develops during the first months of life. Severe glucose intolerance developed in both sexes, but overt diabetes with low plasma insulin levels occurs only in male Wfs1KO mice, whereas female Wfs1KO mice are even hypoglycaemic with relatively high insulin levels.
4. For the first time, we showed that one of the reasons for developing WS related diabetes is not the insulin deficiency itself, but the impairment in converting proinsulin to active insulin. A proof for that is the increased proinsulin/insulin ratio in male Wfs1 mice, confirmed later by other investigators.
5. The energy metabolism did not differ significantly between Wfs1 KO and wt mice; however, we found that female mice had lower mean plasma T4 concentration than the males, both in the Wfs1KO as well as in the wt group. Our data also suggest some degree of leptin resistance in female Wfs1KO mice, which needs to be confirmed in further studies.

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SUMMARY IN ESTONIAN

Diabeedi kujunemine, fertiilsuse ja energia ainevahetuse häired Wfs1 puudulikkusega hiirel kui Wolframi sündroomi loommudelil

Wolframi sündroom (WS) on tuntud ka akronüümi DIDMOAD (*Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy and Deafness*) nime all. Antud sündroomi kirjeldasid esmakordselt Wolfram ja Wagener 1938. aastal. See on autosoom-retsessiivse pärandumismustriga haigus, mille peamisteks kliinilisteks avaldusteks on varases lapseeas algav 1. tüüpi diabeet, nägemisnärvi atroofia, magediabeet ja sensorineuraalne kuulmislangus (Wolfram ja Wagener, 1938; Barrett ja Bundey, 1997; Smith jt, 2004). Lisaks võivad esineda ka urotrakti väärarendid, mitmed erinevad neuroloogilised ning psühhiaatrilised probleemid (Peden jt, 1986; Barrett jt, 1995; Barrett ja Bundey, 1997; Medlej jt, 2004).

Wolframi sündroomi põhjuseks on *WFS1* geeni mutatsioon, mis paikneb 4. kromosoomi lühikeses õlas (4p16) (Collier jt, 1996). Seni on kirjeldatud ligi 200 erinevat geenimutatsiooni (de Heredia jt, 2013). *WFS1* geen toodab volframiini. See on endoplasmaatilise retiikulumi (ER) koosseisus olev glükoproteiin, mis omab olulist rolli ER stressi ja raku apoptoosi väljakujunemisel (Fonseca jt, 2005; Riggs jt, 2005; Yamada jt, 2006).

Esimeseks kliiniliseks avalduseks WS korral on diabeet. Diabeet tekib WS haigetel keskmiselt 6-aastaselt (Barrett jt, 1995; Kinsley jt, 1995; Medlej jt, 2004), poistel pisut varem kui tüdrukutel (Hardy jt, 1999; Smith jt, 2004; Marshall jt, 2013). Võrreldes 1. tüüpi diabeediga esineb Wolframi sündroomiga patsientidel vähem mikrovaskulaarseid komplikatsioone, ketoatsidoosi ja ka insuliini vajadus on väiksem (Peden jt, 1986; Barrett jt, 1995; Kinsley jt, 1995; Cano jt 2007a).

Täpne diabeedi tekkemehhanism WS korral ei ole teada. Seda on seostatud ER stressiga, mis põhjustab pankrease β-rakkude apoptoosi ja sellest tuleneva insuliini puudulikkuse (Ishihara jt, 2004; Riggs jt, 2005). On teada ka, et WS-ga patsientidel võib esineda prohormooni konvertaasi (PC) puudulikkust, mille tulemusel ei toodeta inaktiivsetest hormoonide eelvormidest bioloogiliselt aktiivseid hormoone (Gabreels jt, 1998). Meile teadaolevalt ei ole seni proinsuliini (insuliini eellashormooni) taset WS korral uuritud.

Sageli kaasub WS-ga lühike kasv (Hofmann jt, 1997a; Simsek jt, 2003; Medlej jt, 2004; Ganie jt, 2011), mille üheks põhjuseks on leitud kasvuhormooni puudulikkus (Barrett jt, 1995; Barrett ja Bundey, 1997; Kõks jt, 2009). Siiani puudub info WS-ga patsientide fertiilsusest. Varasemalt on kirjeldatud häirunud hüpofüüsi funktsiooni (Medlej jt, 2004) ja meeste puhul ka primaarset gonaadide atroofiat koos hüpergonadotroopse hüpogonadismiga (Peden jt, 1986; Barrett ja Bundey, 1997; Medlej jt, 2004). Meile teadaolevalt ei ole siiani uuritud *WFS1* geeni rolli fertiilsuse mõjutamisel.

Patsientidel, kellel esineb WS, on kirjeldatud häirunud kilpnäärme funktsiooni, kuid tegemist on vaid üksikute kirjeldustega (Tranebjaerg jt, 1993; Hildebrand jt, 2008; Yan jt, 2013).

Hiljuti avaldatud andmed on näidanud, et erinevate neurodegeneratiivsete haiguste (nt Alzheimeri tõbi, Parkinsoni tõbi või Huntingtoni tõbi) korral häirub patsientidel ainevahetus, kujunevad kaaluprobleemid, veresuhkru ja leptiini ainevahetuse häired jms (Cai jt, 2012). Kuna WS on peetud ka neurodegeneratiivseks haiguseks, võiksime oodata sarnaseid ainevahetusprobleeme ka antud haigusega patsientidel.

Tartu Ülikoolis on loodud WS loommudel – Wfs1 puudulikkusega hiir.

Uurimistöö eesmärgid

Antud uurimistöö eesmärgiks oli välja selgitada, kas isaste Wfs1 puudulikkusega hiirte (Wfs1KO) viljakus võrreldes tervete hiirtega on muutunud ja kui see on nii, siis püüda leida selle põhjusi. Lisaks soovisime kirjeldada diabeedi kujunemist vanuselises ja sugude lõikes ning mõõta neil loomadel proinsuliini ja insuliini taset. Soovisime kirjeldada ka võimaliku kasvuhäire kujunemist, leida selle põhjusi ning uurida energia ainevahetust Wfs1 puudulikkusega hiirtel.

Uuringu metoodika ja katseloomad

Uuringutes kasutati Tartu Ülikooli teadlaste poolt loodud Wfs1-puudulikkusega hiiri. Kasutati ainult homosügootseid hiiri ja nende metsik-tüüpi (ilma mutatsioonita – wt) pesakonnakaaslasi. Kokku kasutati käesoleva töö läbiviimiseks 142 Wfs1KO hiirt ja 306 metsik-tüüpi hiirt. Loomad asusid konstantsetes laboritingimustes, kus oli 12-tunnine pimeduse ja valguse tsükkel. Kõigil hiirtel oli ööpäevaringselt vaba juurdepääs veele ja toidule. Kõik loomadega läbiviidud protseduurid olid kooskõlas EU direktiiviga 86/609/EEC ja nende läbiviimiseks oli olemas Eesti Loomkatsekomisjoni luba (number 86, 28.08.2007).

1. Viljakuse uuring (1. artikkel)

Kaheksa- kuni kaheteistkümnenädalased isased hiired (13 Wfs1KO ja 13 wt) pandi ühte puuri kokku kahe emase wt hiirega. Hiired viibisid ühes puuris kolm ööpäeva või kuni emashiirtel oli sedastatav vaginaalse korgi olemasolu (indikaator paaritumisest). Ühe isase juurde pandi uuringu vältel kuus erinevat emashiirt. Emased hiired kaaluti iganädalaselt ja kaalutõusu omavad hiired (eeldatavasti tiined hiired) asetati üksikpuuridesse, kus nad poegisid. Viljakusemäär (mitu emast hiirt suutis üks isane viljastada) ja poegade arv pesakonnas registreeriti iga isashiire kohta.

2. Veresuhkru mõõtmised (2. artikkel)

Uuring teostati üheksanädalaste hiirtega (igas grupis 21 hiirt: mõlemast soost Wfs1KO ja wt hiired). Kuni 20. elunädalani mõõdeti glükomeetriga hiirte sabaveenist iganädalaselt hommikune veresuhkru tase (paastu ei rakendatud). Alates 20. elunädalast mõõdeti veresuhkrut iga kahe nädala tagant. 30. elunädalal viidi hiirtel läbi intraperitoneaalne glükoosi tolerantsuse test. Selleks olid hiired 16 tundi söömata. Seejärel nad kaaluti ning mõõdeti nende veresuhkru tase (0‘). Hiirtele manustati intraperitoneaalselt glükoosilahust (arvestuslikult 2g glükoosi kilogrammi kehamassi kohta). 60 ja 120 minuti järel mõõdeti veresuhkru tasemeid uuesti (vastavalt 60’ ja 120').

3. Kasvamise hindamine (2. artikkel)

Paralleelselt veresuhkru taseme mõõtmistega hiired ka kaaluti. Nende andmete põhjal arvutati välja:

– maksimaalne kaaluiive uuringu raames
– kaaluiive 9. – 32. elunädalani
– vahe maksimaalse kaalu ja 32. elunädala kaalu vahel.

4. Energia ainevahetuse hindamine (3. artikkel)

Hiired eraldati puurikaaslastest ja paigutati kolmeks nädalaks üksikpuuridesse. Kohanemisperioodi järgselt paigutati hiired üksi spetsiaalsesse metaboolsesse puuri. 48 tunni möödudes viidi hiired taas oma kodupuuridesse (üksikpuur) ja võimaldati neile ühe nädala pikkune taastumisperiood. Sellist harjutamist teostati kolmel korral. Viimase metaboolses puuris viibimise ajal mõõdeti järgmised energia ainevahetuse parameetrid: toidu- ja veetarve, keskmine hapnikutarve, soojuse ja süsihappegaasi produktsioon ning looma liikumised kolmes erinevas suunas (X-, Y- ja Z-teljel).

5. Vereseerumi hormoonide mõõtmised (1.; 2.; ja 3. artikkel)

Veri hormoonuuringuteks võeti hiirtelt peale nende eutaniseerimist. Vereproovid tsentrifuugiti, eraldati plasma ja proove analüüsiti erinevate ELISA kittide abil. Kõik tehnilised protseduurid teostati vastavalt iga individuaalse kiti kasutusjuhendile. Proovide optiline tihedus mõõdeti ELISA lugeri abil.

6. Histoloogilised ja morfoloogilised uuringud (1. ja 3. artikkel)

6.1. Sperma morfoloogia ja testiste histoloogia (1. artikkel)

Uuringus kasutati spermat, mis oli eraldatud eelnevalt eutaniseeritud isahiirte munandimanuste sabaosast. Ühe isashiire kohta analüüsiti 200 spermatosoidi. Spermatosoidide liikuvust hinnati ja salvestati spetsiaalse mikroskoobi abil

(CASA) ning arvutati liikuvate ja otse liikuvate spermide osa kõigist spermidest. Lisaks uuriti fikseeritud proove mikroskoobi all ja hinnati spermatosoidide morfoloogilisi tunnuseid. Hinnatavate tunnuste aluseks võeti Kawai 2006. a artiklis kasutatud metoodika (Kawai jt, 2006).

Lisaks eelpooltoodule hinnati kolme Wfs1KO ja kolme wt isashiire testiseid. Proovid fikseeriti parafiinis ja lõike hinnati valgusmikroskoobi all. Hindamine viidi läbi kahe hindaja poolt.

6.2. Kilpnäärmete histoloogia (3. artikkel)

Kilpnäärmete histoloogia hinnati viiel Wfs1KO hiirel ja seitsmel wt hiirel. Kõik hiired olid 17–20 nädalat vanad. Hiired eutaniseeriti ja kilpnäärmed prepareeriti. Koed fikseeriti formaliinis ja säilitati parafiiniblokkides. Lõigud värviti hematoksüliin-eosiini ja van Gieson'i meetodeid kasutades ning morfoloogiat hinnati valgusmikroskoobi all. Hinnangud on teostatud kahe objektiivse hindaja poolt.

Uurimistöö peamised tulemused

Esimeses uuringus leidsime, et Wfs1KO isashiirtega paaritatud emashiirte tiinestumismäär oli madalam kui wt isashiirtega paaritatud emaste oma (15% vs. 32%, $p<0.05$). Kolmeteistkümnest Wfs1KO isashiirest viis ei suutnud viljastada mitte ühtegi kuuest emasest, samas kui kontrollgrupis oli igal isashiirel vähemalt üks pesakond ($p<0.05$). Pesakonna suurustes statistilisi erinevusi Wfs1KO ja wt isaste vahel ei esinenud. Ilmnes, et ka vaginaalseid korke (mis annab võimaluse hinnata aset leidnud paaritumist) oli Wfs1KO isashiirtega koos elanud emashiirtel tunduvalt vähem kui wt isashiirtega paaritatud emastel.

Sperma liikuvus ei ole Wfs1KO isashiirtel häirunud. Ilmnes, et Wfs1KO isashiirte spermidel on vähem proksimaalseid keerde ja vähem ka ebahariliku morfoloogiaga spermi päid, mis on mõlemad seotud suhteliselt madala sperma viljastamisvõimega. Wfs1KO isashiirte spermidel esines ka rohkem tsütoplasma tilgakesi, mis on samuti seotud madala sperma viljastumisvõimekusega (Kawai jt, 2006). Antud erinevus ei olnud siiski võrreldes wt-grupiga statistiliselt oluline.

Testiste histoloogiline uuring tõi esile seemnetorukeste epiteeli tavapärase ehituse puudumise Wfs1KO hiirtel. Samuti esines seemnetorukeste epiteelil oluliselt vähem spermatogoone ja Sertoli rakke, mis võib viia vähenenud spermatosoidide produktsioonini.

Suguhormoonide (testosterooni ja FSH) tasemes ei esinenud erinevusi Wfs1KO ja wt isashiirte gruppide vahel.

Teises uuringus hindasime hiirte longitudinaalset kasvu ja veresuhkru väärtusi erinevas vanuses. Leidsime, et Wfs1KO isashiired olid oma wt pesakonnakaaslastest tunduvalt kergemad juba uuringu algul (üheksanädalaselt),

kuid emashiirtel kujunes kaaluvahe välja alles 16 nädala vanuselt. Samuti hakkasid Wfs1KO hiired uuringu viimastel nädalatel kaotama oma kehamassi. Veresuhkru väärtustes tekkis statistiline erinevus 24. elunädalal, kui Wfs1KO isashiirte veresuhkur tõusis oluliselt kõrgemaks kui wt hiirtel. 32. nädalal oli keskmine veresuhkru tase isastel Wfs1KO hiirtel 9.4 mmol/l võrrelduna 7.9 mmol/l wt isastel hiirtel ($p<0.05$). Üllatuslikult leidsime, et emashiirtel esinesid vastupidised muutused: emastel Wfs1KO hiirtel olid veresuhkru tasemed madalamad kui wt hiirtel. Sellele vaatamata ilmnes nii Wfs1KO isas- kui emashiirtel 30. elunädalal väljendunud glükoosi tolerantsi häire. Kuigi hiirtel puuduvad kindlad kriteeriumid diabeedi diagnoosimiseks, oli isashiirtel tõenäoliselt tegemist juba väljakujunenud diabeediga.

Isaste Wfs1KO hiirte insuliini tase oli tunduvalt madalam kui wt isasloomadel ja Wfs1KO emasloomadel. Samas ilmnes, et Wfs1KO isasloomadel esines kõrgem proinsuliini taseme ja proinsuliini/insuliini suhe kui wt isastel. Need tulemused näitavad, et diabeedi tekkepõhjuseks Wfs1KO hiirtel pole mitte niivõrd primaarselt insuliini defitsiit, kuivõrd võimetus konverteerida mitteaktiivset proinsuliini aktiivseks insuliiniks.

Kolmanda uuringu raames mõõtsime erinevaid energia ainevahetuse parameetreid. Leidsime, et WfsKO hiirte energia ainevahetus ei erinenud oluliselt wt hiirte omast. Küll olid aga olulised erinevused sugude vahel. Emaste hiirte hapnikutarve oli kõrgem kui isashiirtel, samuti produtseerisid nad võrreldes isashiirtega rohkem süsihappegaasi ja soojust. Need muutused olid sarnased mõlema genotüübi siseselt. Wfs1KO isashiired sõid oma kehakaalu kohta tunduvalt vähem ja kaotasid eksperimendi vältel tunduvalt rohkem oma kehakaalus kui wt isased. Isastel Wfs1KO hiirtel leiti madalam plasma leptiini tase kui wt hiirtel. Emasloomadel sellist erinevust ei leitud, mis võib viidata võimalikule leptiini resistentsusele Wfs1KO emashiirtel.

Järeldused

1. Wfs1KO isashiirte viljakus, võrreldes wt isashiirtega, on langenud. See on tingitud muutustest sperma ja testiste morfoloogias.
2. Wfs1KO isashiirte spermidel on vähem proksimaalselt keerdunud sabasid ja ebaharilikke päid, mis mõlemad seostuvad madalama viljastumisvõimega. Wfs1KO isashiirte seemnetorukestel puudub tavapärane epiteel ning esineb spermatogoonide ja Sertoli rakkude vähenemine, mis võib põhjustada langenud sperma produktsiooni.
3. Kasvuhäire ja diabeedi kujunemisel esinevad tõsised sugudevahelised erinevused. Kasvuhäire on Wfs1KO isashiirtel väljendunud juba sünnil, kusjuures emashiirtel kujuneb see alles esimeste elukuude jooksul. Mõlemast soost Wfs1KO hiirtel esineb väljendunud glükoosi tolerantsuse häire, kuid väljendunud diabeet koos madala plasma insuliini tasemega kujuneb ainult isashiirtel.

4. Leidsime esmakordselt, et üheks põhjuseks, miks Wfs1KO isashiirtel kujuneb diabeet, ei ole primaarsena mitte insuliini defitsiitsus, kuivõrd võimetus konverteerida mitteaktiivset proinsuliini aktiivseks insuliiniks. Selle leiu kinnituseks on ka hiljem teiste uurimisgruppide poolt avaldatud andmed tõusnud proinsuliini/insuliini tasemete kohta.
5. Energia ainevahetuses ei esine olulisi erinevusi Wfs1KO ja wt hiirte vahel. Küll aga leidsime, et emastel hiirtel, nii Wfs1KO kui ka wt grupis, on madalam plasma türoksiini tase kui isashiirtel. Meie tulemused viitavad ka võimalikule leptiini resistentsusele Wfs1KO emasloomadel, mille tõestamine vajab aga täiendavaid uuringuid.

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– My fellow in life and also in my residency studies in the last few years has been a great friend of mine – dr Stella Lilles. Her everlasting positivity and laughter has been a great motivation in times when no sun was shining behind the clouds. She has been the one I can rely on and I appreciate it greatly.
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when I have not had enough time for him. He has been the greatest father to my precious daughter and I love him for that very much. I would not have ever found the strength to finish this work without his everlasting support and belief in me. I feel obligated for my daughter Karola because I have not had enough time for her that she has needed during my studies. But she has been the meaning of my continuing and finishing the thesis regardless. With her love, passion and endless laughter she has motivated and encouraged me. As my appreciation for her love and support this thesis is dedicated to her.

PUBLICATIONS

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Education

2010 – …	University of Tartu, Faculty of Medicine, residency in Paediatrics (since 2013 Paediatric Neurology)
2007 – 2014	University of Tartu, Faculty of Medicine, PhD studies
2001 – 2007	University of Tartu, Faculty of Medicine
1997 – 2001	The German Gymnasium of Kadriorg
1989 – 1997	Õismäe Grammar School

Professional employment

2010 – …	Children's Clinic of Tartu University Hospital, Resident physician
2004 – 2007	Anaesthesiology and Intensive Care Clinic of Tartu University Hospital, Assistant intensive care nurse
2002	Neonatology ward of Tallinn Children's Hospital, Assistant nurse

Special courses

2007	ABC course of Animal Experiments, Helsinki University, Helsinki, Finland (2-weeks course)
2013	6th Eilat International Educational Course: Pharmacological Treatment of Epilepsy, Jerusalem, Israel (1-week course)
2013	Basic Gaucher Training 2013, Amsterdam, The Netherlands (2-days course)
2013	Epilepsy Genetics Workshop and the Meeting of Young Researchers in Epileptology 2013, Sde Boker, Israel (4-days course)
2014	EFNS/ENS Spring School for young Neurologists 2014, Stare Splavy, Czech Republic (4-days course)

Scientific work

5 publications and 12 oral or poster presentations at international scientific conferences (including annual ESPE conferences).

The topics of my research activity have been endocrine disturbances in a Wfs1-deficient mouse model of Wolfram syndrome and genetics of epilepsy.

I am a member of Estonian Paediatric Association, Estonian Doctors Association, International Child Neurology Association and a board member of Estonian Child Neurology Association.

List of publications

- **Noormets, K**.; Kõks, S.; Ivask, M.; Aunapuu, M.; Arend, A.; Vasar, E.; Tillmann. V (2014). Energy metabolism and thyroid function of mice with deleted wolfram (Wfs1) gene. Experimental and Clinical Endocrinology of Diabetes
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2007	"Loomkatsete ABC" Helsingi Ülikool, Helsingi, Soome (2-nädalane koolitus)
2013	6. Eilati rahvusvaheline täiendkoolitus "Epilepsia farmakoloogiline ravi" Jeruusalem, Iisrael (1-nädalane koolitus)
2013	"Gaucheri tõve baaskoolitus 2013" Amsterdam, Holland (2-päevane koolitus)
2013	"Epilepsia geneetika õpituba ja noorte epieptoloogide teaduskohtumine 2013", Sde Boker, Iisrael (4-päevane kursus)
2014	"EFNS/ENS noorte neuroloogide kevadkool 2014", Stare Splavy, Tšehhi vabariik (4-päevane koolitus)

Teadustegevus

5 teadusartiklit ja 12 suulist või posterettekannet rahvusvahelistel teaduskonverentsidel (muuhulgas ka ESPE – Euroopa Laste-endokrinoloogide Seltsi iga-aastastel konvrentsidel).

Minu peamisteks uurimisteemadeks on olnud endokriinhäired Wolframi sündroomi loommudelil – Wfs1 puudulikkusega hiirel ja epilepsia geneetika.

Olen Eesti Lastearstide Seltsi, Eesti Arstide Liidu, Rahvusvahelise Lasteneuroloogide Seltsi liige ning Eesti Lasteneuroloogide Seltsi juhatuse liige.

Publikatsioonide loetelu

- **Noormets, K**.; Kõks, S.; Ivask, M.; Aunapuu, M.; Arend, A.; Vasar, E.; Tillmann. V (2014). Energy metabolism and thyroid function of mice with deleted wolfram (Wfs1) gene. Experimental and Clinical Endocrinology of Diabetes
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