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RANDO POROSK

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Press

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the original publications referred to in the text by Roman numerals (I–III):

I. **Porosk R**; Terasmaa A; Mahlapuu R; Soomets U and Kilk K. Metabolomics of the Wolfram syndrome 1 gene (*Wfs1*) deficient mice. OMICS 2017; 21(12):721–732

II. **Porosk R**; Kilk K; Mahlapuu R; Terasmaa A and Soomets U. Glutathione system in Wolfram syndrome 1-deficient mice. Mol Med Rep 2017; 16(5):7092–7097

III. Eskla KL; **Porosk R;** Reimets R; Visnapuu T; Vasar E; Hundahl CA and Luuk H. Hypothermia augments stress response in mammalian cells. Free Radic Biol Med 2018; 121:157–168

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My contribution to the above papers is as follows:

Publication I: Designed and wrote the animal study application, performed most of the experiments, conducted data analysis and wrote/co-wrote the manuscript.

Publication II: Designed and wrote the animal study application, performed most of the experiments, conducted data analysis and wrote/co-wrote the manuscript.

Publication III: Performed the measurement of total and oxidized glutathione concentration in different cell lines.

Other publications:

IV. Ainelo, A; **Porosk, R**; Kilk, K; Rosendahl, S; Remme, J and Hõrak, R. Pseudomonas putida responds to the toxin GraT by inducing ribosoome biogenesis factors and repressing TCA cycle enzymes. Toxins 2019 (*Accepted*)

V. Nõmm M; **Porosk R**; Pärn P; Kilk K; Soomets U; Kõks S and Jaakma Ü. In vitro culture and non-invasive metabolic profiling of single bovine embryos. Reprod Fertil Dev 2018; 1.10.1071/RD1746

VI. Aug A; Altraja S; Kilk K; **Porosk R**; Soomets U and Altraja A. E-cigarette affects the metabolome of primary normal human bronchial epithelial cells. PLoS One 2015; 10(11):e0142053

VII. Meitern R; Sild E; Kilk K; **Porosk R** and Hõrak P. On the methodological limitations of detecting oxidative stress: effects of paraquat on measures of oxidative status of greenfinches. J Exp Biol 2013; 216(14):2713–21

ABBREVIATIONS

ADH	Antidiuretic hormone
AGE	Advanced glycation end product
ANOVA	Analysis of variance
ARE	Antioxidant response element
BCAA	Branched chain amino acids
CHOP	CCAAT-enhancer-binding protein homologous protein
DI	Diabetes insipidus
DIDMOAD	Diabetes insipidus, Diabetes Mellitus, Optic Atrophy, Deafness
DM	Diabetes mellitus
DTNB	5,5-Dithiobis-(2-nitrobenzoic acid)
ER	Endoplasmic reticulum
Ero1-α	Endoplasmic reticulum oxidoreductin-α
GCL	Glutamate cysteine ligase
GCLc	Glutamate cysteine ligase catalytic subunit
GCLm	Glutamate cysteine ligase modified subunit
GLP-1	Glucagon-like peptide-1
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthetase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HZ	Wolfram syndrome 1 gene deficient mouse, heterozygous ($Wfs1^{+/-}$)
Keap1	Kelch-like ECH-associated protein 1
KO	Wolfram syndrome 1 gene deficient mouse, homozygous ($Wfs1^{-/-}$)
LC-MS	Liquid chromatography mass-spectrometry
MGO	Methylglyoxal
MRM	Multiple reaction monitoring
NAC	N-acetyl-L-cysteine
Nrf2	Nuclear factor-E2 related factor 2 transcription factor
OA	Optic atrophy
OxS	Oxidative stress
PCA	Principal component analysis
RCS	Reactive carbon species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

SEM	Standard error of mean
TNB	5-Thio-2-nitrobenzoic acid
UPF1	L-Tyr(Me)-L-γ-Glu-L-Cys-Gly
UPF17	L-Tyr(Me)-L-Glu-L-Cys-Gly
WFS1	Wolfram syndrome 1 gene in human
Wfs1	Wolfram syndrome 1 gene in mouse
WS	Wolfram syndrome 1
WT	Wild-type mouse (*Wfs1*$^{+/+}$)
XBP1	X-box binding protein 1

1. INTRODUCTION

Oxidative stress is defined as a disturbance in the balance between the production of oxidative stressors (pro-oxidants, free radicals) and antioxidants with a shift towards pro-oxidants which may lead to potential harmful processes. Glutathione (GSH) is a predominant non-enzymatic low molecular weight antioxidant in eukaryotic cells (thiol-containing tripeptide) helping to control redox status.

Diabetes generally has polygenic nature. It can be caused by multiple environmental and genetic factors or their combination. Diabetes can also be caused by defects in a single gene (5–10% of cases). This type of diabetes consists of a heterogeneous group of rare disorders. An ongoing list of genes/loci has been associated with monogenic diabetes and insulin resistance. Every single gene associated with monogenic diabetes expresses a distinct phenotype and clinical features. Genome-wide association studies have found that one of the genes associated with development of diabetes is Wolfram syndrome 1 gene (*WFS1*).

WFS1 encoding trans-membrane glycoprotein called wolframin is primarily located in the endoplasmic reticulum (ER). The exact biological function of wolframin is unknown, but it is postulated that given protein is a key factor in many specific interactions including post-translational modification and folding/ assembly of newly synthesized proteins (e.g. insulin), calcium storage, redox regulation and cell death. Previously it has been reported that pancreatic β-cells and neurons are sensitive to ER dysfunctions, most likely due to their high rates of protein synthesis (Fonseca et al. 2011). As a consequence of mutations in *WFS1,* it could lead to an elevated ER and oxidative stress level, activation of ER-stress associated cell death and therefore destruction of pancreatic β-cells.

The deficiency in *WFS1* gene causes Wolfram syndrome 1 (WS), which represents a valuable disease model currently available for studying the pathophysiology of endoplasmic reticulum (ER) stress, juvenile-onset diabetes and neurodegeneration. Over 200 mutations have been identified in WS patients and the majority of them are located in 8th exon (Piccinno et al. 2014). The *Wfs1*-deficient mouse generated in the University of Tartu has a deletion in 8th exon and serve as a relevant rodent model for studying WS and the role of ER and oxidative stress in its progression.

Metabolomics provides comprehensive investigation methods for profiling tissues or bodily fluids. Metabolites show more expeditious fluctuations in response to a physiological change than the changes in gene expression or protein production. Studying oxidative stress and metabolic profiling of *Wfs1*-deficient mice to find therapies aimed at reducing stress in patients or those at risk for developing diabetes. Also this might give new insight into the association between *Wfs1* and its biological functions and describing the phenotype-genotype connections of WS.

Mild therapeutic hypothermia is another condition, which is proposed to be related to oxidative and ER stress. Therefore, the main aim was to describe the role of ER and oxidative stress in WS and hypothermia conditions.

The metabolic profiling of *Wfs1*-deficient mouse revealed a broad spectrum of changes including indications of higher levels of glucose use, gluconeogenesis, and anaerobic glycolysis, especially in the early stages of the disease. In later stages of the WS, the energy demand is satisfied by intensified lipolysis. The analysis of glutathione system of *Wfs1*-deficient mouse revealed a decreased concentration of GSH and alterations in the activity of glutathione reductase and peroxidase. The administration of antioxidative UPF peptides improved the glutathione status mainly in the liver and heart tissue. Finally, the antioxidative defense system was upregulated in hypothermia conditions by similar pathway as UPF peptides, which activate Nrf2 and synthesis of GSH.

2. REVIEW OF LITERATURE

2.1. Wolfram syndrome 1

2.1.1. Wolfram syndrome 1

Wolfram syndrome 1 (WS) (OMIM 222300) is a rare autosomal recessive neurodegenerative disease, which is also known and characterized by DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy and Deafness) (Strom et al. 1998). The prevalence of WS is 1:160 000 in UK and 1:770 000 in North America (Kinsley et al. 1995; T. G. Barrett et al. 1995). It was first reported by Wolfram and Wagener in 1938 who found four siblings with optic nerve atrophy and juvenile diabetes mellitus (Wolfram & Wagener 1938). Diabetes mellitus is typically the first outcome of WS, diagnosed around age 6, followed by a loss of color vision and peripheral vision at age 11. Other common manifestations are diabetes insipidus, urinary tract problems, neurological manifestations and sensorineural deafness (all affecting around 70% of patients). Urinary tract problems include obstruction of ducts between the kidneys and bladder, disrupted urination (also controlling the urine flow), high-capacity atonal bladder, and bladder sphincter dyssynergia. Deafness can range in severity – deafness at birth to mild hearing loss, which worsens over time. Neurological manifestations are commonly bulbar dysfunction, ataxia and brain stem atrophy. The latter is also a prominent cause of death with central apnea at age 25–49 years (T. G. Barrett et al. 1995; Barrett & Bundey 1997). Approximately 60% of homozygous WFS1 carriers have psychiatric disorder and heterozygotes have 26-fold higher likelihood of psychiatric hospitalization, primarily for depression (Swift et al. 1990; Swift & Swift 2000).

WS is caused by mutations in the Wolfram syndrome 1 gene (*WFS1*) that encodes a transmembrane glycoprotein called wolframin. It is ubiquitously expressed with the highest levels in heart, brain, pancreatic beta-cells, placenta and lungs (Inoue et al. 1998). Human wolframin consists of 87% same amino acids as its mouse homolog (*Wfs1*). *Wfs1* has nine central transmembrane domains with a cytoplasmic N- and luminal C-terminus. N-glycosylation is essential for its biogenesis and stability (Hofmann et al. 2003). Wolframin is primarily located in the endoplasmic reticulum (ER) which has many roles including post-translational modification and folding/assembly of newly synthesized proteins (e.g. insulin). Perturbations such as *Wfs1*-deficiency cause imbalances between these processes leading to accumulation of misfolded or unfolded proteins, which in turn leads to ER stress and cell dysfunction. The expression of *Wfs1* in mouse pancreatic islets is upregulated during glucose-induced insulin secretion (Fonseca et al. 2005). It has been shown that *Wfs1* regulates a key transcription factor involved in ER stress signaling, ATF6α, (Yoshida et al. 1998) and intracellular Ca^{2+} homeostasis (Takei et al. 2006; Lu et al. 2014). Zatyka *et al.* found that WFS1 interacts with Na^{+}/K^{+} ATPase beta-1

subunit, which is important for the maturation and this interaction may contribute to the pathology seen in WS (Zatyka et al. 2007; Zatyka et al. 2015).

2.1.2. DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic atrophy, Deafness)

The major diagnostic criteria for WS are diabetes mellitus and optic atrophy (both < 16 years). In addition to the first, the minor criteria are diabetes insipidus, sensorineural deafness, neurological signs (ataxia, neuropathy), renal tract abnormalities, a loss of function mutation in WFS1 and/or family history of WS (Figure 1). The minimum required for diagnosis are 2 major or 1 major plus 2 minor criteria. Commonly the patients also have hypogonadism, absence of type 1 diabetes auto-antibodies, bilateral cataracts, psychiatric disorder and/or gastrointestinal disorders (Maleki et al. 2015).

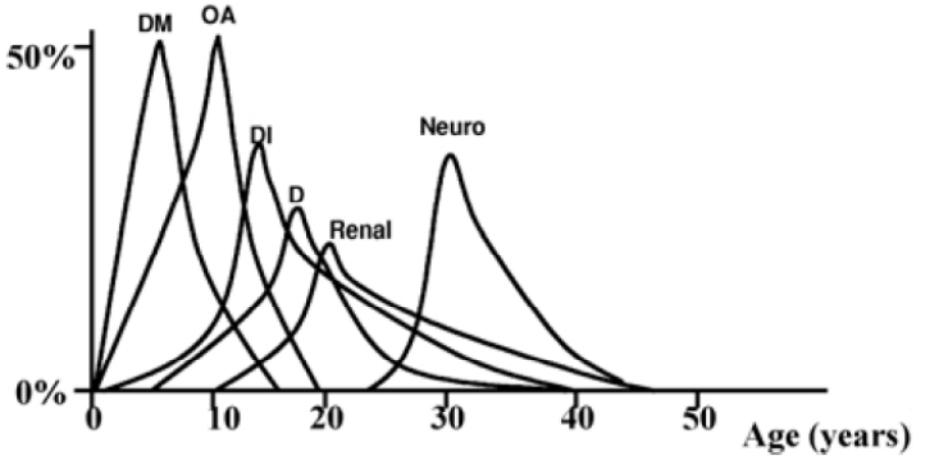


Figure 1. The median age of onset of the complication in Wolfram syndrome by Minton *et al.* (Minton et al. 2003). DM – diabetes mellitus; OA – optic atrophy; DI – diabetes insipidus; D – deafness; Renal – renal tract complications; Neuro – neurological complications.

Diabetes Mellitus

Diabetes mellitus (DM) is usually the first manifestation to occur. The mean age of DM has been reported to be 5 ± 4 years (Rohayem et al. 2011). It is caused by insulinopenia and leading to degeneration of pancreatic β-cells. Wolframin is highly expressed in the pancreas (mainly in islet β-cells) and it may help to fold proinsulin into insulin (Fonseca et al. 2005). This leads to hyperglycemia which exceeds the renal threshold for reabsorption. The deficiency of insulin changes the energy metabolism into catabolism of proteins and fats. Proteolysis

increases the usage of amino acids for gluconeogenesis, and together with lipolysis, tends to induce negative energy balance, which sum up as weight loss.

Diabetic ketoacidosis is also a key feature in DM patients, but patients affected with WS have approximately 10-fold lower activation of ketogenic machinery compared with DM patients (Garcia-Luna et al. 1988). Autoantibodies usually found in case of type I diabetes are absent in almost all WS patients (Maltoni et al. 2016). In addition, patients with WS have smaller glycemic variability compared to DM and this may be associated with persistent residual insulin secretion (Zmyslowska et al. 2015). *Wfs1*-deficient mice have a progressive β-cell loss, impaired glucose tolerance and activated unfolded protein response (UPR) in ER (Ishihara et al. 2004).

Optic Atrophy

Frequently apparent optic atrophy (OA) is the second major criteria for WS. It usually occurs after DM compilations at early stages of life (around age 11). Common findings are progressive ophthalmologic symptoms, constriction of visual fields, declined color vision and visual activity with clear indications to diabetic retinopathy (Fishman & Ehrlich n.d.). It has also been proposed that retinal thinning is a marker of disease progression in patients with WS (Zmyslowska et al. 2017). Wolframin is located primarily in retinal ganglion cells, photoreceptors, cells in inner nuclear layer and in glial cells in the proximal portion of the optic nerve (Schmidt-Kastner et al. 2009).

Diabetes Insipidus

Diabetes insipidus (DI) is caused by the deficiency of antidiuretic hormone (ADH). It leads to excessive urination due to an inability of the kidneys to resorb water from urine. In patients of WS it results mainly from disorder of hypothalamus and appears often in 2nd to 3rd decades (70% of patients). It has been shown that *Wfs1* expression is widely distributed in the normal mouse brain during postnatal development (Kawano et al. 2009). Loss of vasopressin-producing neurons and a defect in vasopressin precursor processing in the hypothalamus cause the WS-associated DI (Scolding et al. 1996).

Deafness

Wfs1 is expressed in the cochlear cells and it is possible that wolframin contributes to development and maintenance of cells in the auditory system. Sensorineural hearing loss occurs often in 2nd to 3rd decades probably due to the disruption of calcium homeostasis or membrane trafficking essential for hearing (Cryns et al. 2003). The severity can range from deafness beginning at birth to mild hearing loss in adolescence that worsens over time. Commonly the high frequencies are affected first and progresses relatively slowly (Karzon & Hullar 2013).

Other complications

In addition to DIDMOAD, several WS patients display neurological complications, psychiatric disorders and urinary tract abnormalities. More than half of WS patients have neurological manifestations such as problems with balance and coordination (ataxia), central apnea related to brain stem atrophy, dysfunctions and/or death of neurons, psychiatric disorders include anxiety, depression, dysphagia and mood swings (T G Barrett et al. 1995; Urano 2016). Up to 90% of WS patients have urinary tract problems. Most common are high-capacity atonal bladder, obstructions of the ducts between the kidneys and the bladder, disrupted urination and difficulty controlling urine flow (T G Barrett et al. 1995).

2.1.3. *Wfs1*-deficient animal models

Mouse models are important tools in medical research for understanding of disease mechanism and development of treatments. For rare diseases they are especially important because there is very small population of patients to evaluate the effect of therapeutic compounds in clinical studies. It is necessary to design relevant animal models for studying human diseases. Over 200 mutations have been identified in WS patients and the majority of them are located in the 8th exon (Piccinno et al. 2014). *Wfs1*-deficient mice generated in the University of Tartu has the 8th exon at the C-terminal end replaced by NLS-LacZ-Neo expression cassette, whereas the N-terminal domain of the wolframin remains functional. Mice used in the experiments are F2 hybrids with a [(129S6/SvEvTac × C57BL/6) × (129S6/SvEvTac × C57BL/6)] genetic background (Luuk et al. 2009).

Previous research about *Wfs1*-deficient mouse has shown that *Wfs1*-deficient animals display down-regulation of Gabra1 and Gabra2 genes, subunits of GABA(A) receptors in the frontal cortex and temporal lobe (Raud et al. 2009). Male mice have impaired fertility due to changes in sperm morphology and reduced number of spermatogenic cells and they have higher risk in developing diabetes, because of the disturbances in converting proinsulin to insulin (Noormets et al. 2009; K. Noormets et al. 2011). The most recent studies have shown that the WFS1 deficiency induces ER stress, leading to inositol 1,4,5-trisphosphate receptor dysfunction and disturbed cytosolic Ca^{2+} homeostasis (Cagalinec et al. 2016). Several antidepressants have stronger effects on *Wfs1*-deficient mice and selective serotonin reuptake inhibitors could be the most suitable for the management of WS-induced diabetes (Reimets et al. 2016). A study of transcriptome of pancreatic islets in *Wfs1*-deficient mice showed lower islet and insulin content (Ivask et al. 2016).

In addition to our *Wfs1*-deficient mouse model, Ishihara *et al* and Riggs *et al* have developed their own WS mouse models. Ishihara *et al* created a full body knock-out by inserting a neomycin-resistance gene into the 2nd exon of the

Wfs1 gene and Riggs *et al* used *Cre* recombinase under the control of insulin promoter and *loxP* sequences flanking the 8th exon of the *Wfs1* gene (Ishihara et al. 2004; Riggs et al. 2005).

In the recent years, Plaas *et al* have created a *Wfs1*-deficient rat model, which has the core symptoms of WS, including progressive glucose intolerance, hyperglycemia, glycosuria, optic atrophy and reduction of beta cell mass (Plaas et al. 2017). Furthermore, they have shown that treatment with liraglutide, the receptor agonist of glucagon-like peptide-1 (GLP-1), prevented the development of glucose intolerance, improved insulin secretion and reduced ER stress (Toots et al. 2018).

2.2. Endoplasmic reticulum and oxidative stress

2.2.1. Endoplasmic reticulum stress

ER stress is a situation in which misfolded proteins accumulate in the lumen of ER. The unfolded protein response (UPR) that follows upregulates survival related signaling and chaperone synthesis, and inhibits the synthesis of many other proteins (Walter & Ron 2011). Oxidative stress is considered to increase ER stress through the activity of oxidoreductin-1 and protein disulfide isomerases (Zeeshan et al. 2016; Delaunay-Moisan & Appenzeller-Herzog 2015). Protein folding depends on many factors other than chaperones; among these factors, proper reduction/oxidation (redox) ratio and glutathione (GSH) levels are directly and indirectly via GSH sensitive regulatory proteins involved in appropriate disulfide bridge formation (Ellgaard & Ruddock 2005). GSH is a thiol-containing tripeptide comprising γ-glutamate, cysteine and glycine, and its formation occurs in the cytosol and requires no folding compared with redox enzymes. ER stress is known to enhance GSH synthesis through the transcription factors cyclic AMP-dependent transcription factor 4 and nuclear factor erythroid 2-related factor 2 (Cullinan et al. 2003; Harding et al. 2003). Although the aim of UPR is cell survival, persistent stress may induce the cells to trigger apoptosis. It remains unknown how acute and chronic ER stress are managed by individual cells and organisms as a whole.

2.2.2. Oxidative stress and antioxidants

Oxidative stress (OxS) is an imbalance between the production of oxidative stressors (pro-oxidants, free radicals) and antioxidants with a shift towards pro-oxidants which may lead to potential harmful processes. In human body, pro-oxidants could be any factor that causes OxS (smoking, radiation, xenobiotics and drugs, excess of heavy metals) and diminish the capacity of antioxidants in the organism (Sies 1997). Free radicals are endogenous or exogenous short lived (unstable) and very reactive chemical compounds containing one or more

unpaired electrons, which induce damage to cells by passing the unpaired electron and resulting in oxidation of biological molecules (DNA, proteins and other macromolecules). The oxidation of biological molecules has been implicated in the pathogenesis of a wide variety of diseases. The most common stressors are reactive oxygen species (ROS) – superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2); reactive nitrogen species (RNS) – nitric oxide (NO), peroxynitrite ($ONOO^{\bullet}$) and reactive carbon species (RCS) – methylglyoxal (MGO). These compounds are continuously produced by the human metabolism and are not necessarily evil. Free radicals are important for activating different signaling pathways inside the cell, gene transcription, leukocyte adhesion, thrombosis, angiogenesis and they could also act as neurotransmitters or mediators of immunity (Fang et al. 2002).

Human body has different mechanisms to produce antioxidants for scavenging the deleterious effects of free radicals. The antioxidant defense system consists of enzymes, low molecular weight substances (glutathione, vitamin E, ascorbate, etc.) and blood albumin, which prevent the formation of free radicals, convert the existing stressors to less toxic form or delay the oxidation of a substrate. A major mechanism in the defense against OxS is activation of Nrf2-ARE (nuclear factor-E2 related factor 2 – antioxidant response element) signaling pathway which is essential in the detoxification and elimination of oxidants (Moi et al. 1994).

Nrf2 transcription factor is regulated by actin-associated Keap1 (Kelch-like ECH-associated protein 1) protein acting negative regulator and a binder of Nrf2 in the cytoplasm. In response to stress signals, activating of Nrf2 disrupts the association, releasing Nrf2 for translocation into the nucleus for the transcriptional activity (Itoh et al. 1999; Kensler et al. 2007). Many detoxifying endogenous antioxidant enzymes (for example glutathione peroxidase, super-oxide dismutase, catalase and glutamate cysteine ligase) are reported to be targets of Nrf2 activation (Itoh et al. 1997). Improving the antioxidant defense by up-regulating detoxification potential by listed enzymes, represent a new class of therapeutic strategy to prevent cell damage against inflammation and OxS that are also a key mediators in diabetes and its complications (Giacco & Brownlee 2010).

Aforementioned hormone GLP-1, which stimulates insulin secretion from pancreatic islets in a glucose-dependent manner, has also an antioxidative action reducing oxidative stress markers by activating Nrf2 (Guglielmi & Sbraccia 2017). In addition, it is well-known that activation of Nrf2 plays a significant role in the protection of pancreatic beta cells from oxidative stress and improves insulin sensitivity and glucose uptake (Uruno et al. 2013).

2.2.3. Oxidative stress and diabetes

Understanding the pathophysiology of global health burden DM is difficult because of its complex aetiology from a combination of genetic, epigenetic, environmental and lifestyle risk factors (Hu 2011). DM is defined by hyperglycemia in plasma, but it is definitely not simply a disorder of carbohydrate metabolism as alterations in proteins, lipids and amino acids induce the insulin resistance likewise (DeFronzo & Tripathy 2009).

It is a leading hypothesis that OxS is a common pathogenic factor leading to β-cell dysfunction, insulin resistance, impaired glucose tolerance and finally to DM (Ceriello 2004). Hyperglycemia can induce micro- or macrovascular damage to tissues through different pathways (Figure 2). Firstly, enhanced polyol pathway activity, where under hyperglycemic conditions blood sugar glucose is converted to polyalcohol sorbitol using NADPH as co-factor. Therefore intracellular NADPH and glutathione (GSH) are depleted and this leads to overproduction of ROS and a decrease in antioxidant defense. Sorbitol is further metabolized to fructose by sorbitol dehydrogenase, which leads to the inhibition of glyceraldehyde-3-phosphate dehydrogenase and the increase of triose phosphate level by extent usage of NADH (Brownlee 2001). Higher triose phosphate level promotes increased formation of advanced glycation end products (AGEs) by more intense synthesis of AGE precursors – MGO and diacylglycerol.

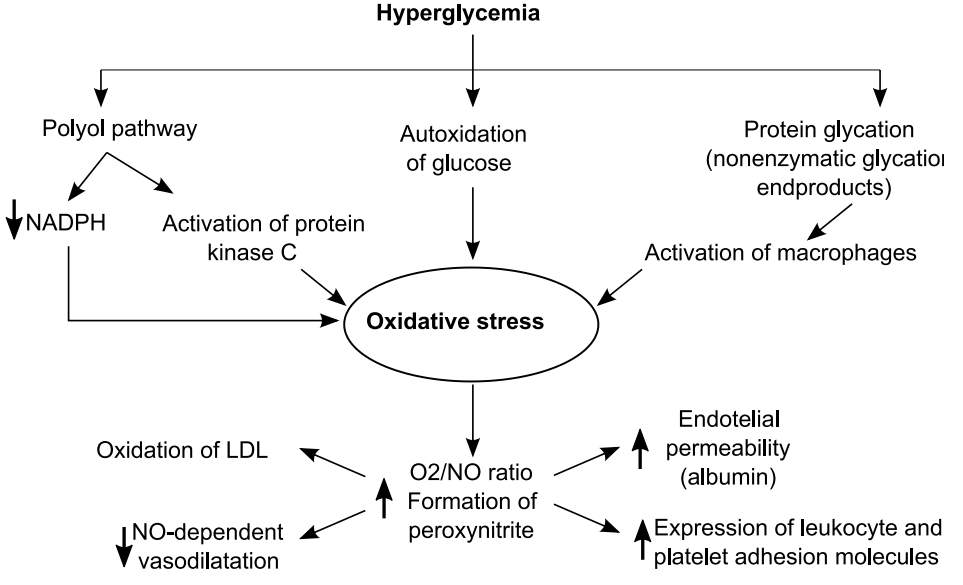


Figure 2. The relationship between oxidative stress and hyperglycemia by Vidigal *et al*. (de Carvalho Vidigal et al. 2012).

AGEs are non-enzymatic chemical modification to proteins, which perturb protein functions. MGO belongs to the group of RCS and can also be elevated by glucose autoxidation. MGO inhibits protein functions such as enzymatic activity or receptor-ligand interaction by modifying arginine residues in the protein active sites (Chetyrkin et al. 2011). This furthermore leads to activation of protein kinase C (PKC) isoforms, which is initiated by the concentration of cytokines, growth factors, endothelin-I, angiotensin II and also circulating free fatty acids (Robin et al. 2002).

Studying OxS under hyperglycemic conditions is very important, mostly to find therapies aimed at reducing OxS in patients or those at risk for developing diabetes.

2.3. Glutathione system

Glutathione (GSH) is a thiol-containing tripeptide consisting of γ-glutamate, cysteine and glycine (Figure 3). GSH is a predominant non-enzymatic low molecular weight antioxidant in eukaryotic cells, and is mainly distributed in cytosol. Liver is the main site for producing and exporting GSH (concentration up to 10 mM). The biosynthesis of GSH occurs in the two steps: firstly, glutamate cysteine ligase (GCL) catalyzes the formation of dipeptide γ-glutamylcysteine and then glycine is added by glutathione synthetase (GS) to generate GSH (Wu et al. 2004). GCL is a heterodimer with a 72-kDa catalytic subunit (GCLc) and 30-kDa modifying subunit (GCLm) (White et al. 2003). GSH acts as a potent scavenger of free radicals and other oxidant species in which it is oxidized by selenium-containing glutathione peroxidase (GPx) to oxidized glutathione (GSSG) and reduced back to GSH by glutathione reductase (GR) (Figure 4). The antioxidant capacity of cells is mostly described by the GSSG/GSH redox couple and is related to several pathological states, including neurodegenerative, cardiovascular and immune system diseases (Ballatori et al. 2009). Administration of GSH is not reasonable because of its degradation in the plasma and poor cellular uptake (Wendel & Cikryt 1980), but N-acetyl-L-cysteine (NAC) has been used for increasing level of amino acid cysteine which is need for the synthesis of GSH (Yim et al. 1994).

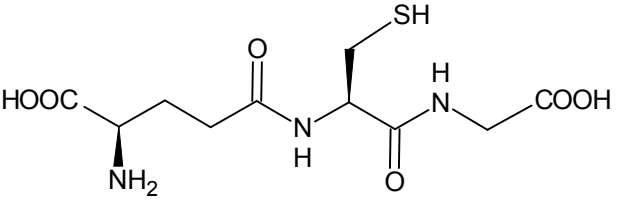


Figure 3. Glutathione (L-γ-Glu-L-Cys-Gly).

In addition to being the most principal cellular antioxidant, GSH has s wide spectrum of biofunctions. Antioxidant function is conducted via non-enzymatic reactions with free radical or being a co-factor in GPx (Cnubben et al. 2001). By executing the antioxidant activity, GSH regulates important redox systems (GSH/GSSG and NADPH/$NADP^+$), which are major determinants of cell homeostasis. These redox ratios are involved in the modulation of redox-sensitive proteins, regulation of cell growth, differentiations, apoptosis and stress factors (Jefferies et al. 2003). GSH acting as a nucleophile detoxifies electrophilic compounds in cooperation with glutathione S-transferase, an enzyme conjugating the thiol group of GSH to the xenobiotics (Dickinson & Forman 2002). Post-translational S-glutathionylation is another important biofunction of GSH, where protein sulfhydryl groups are protected by GSH (Ghezzi 2005). Additional biofunctions of GSH are NO transport and storage, amino acid transport via γ-glutamyltrasferase, synthesis of proteins, nucleic acids and prostaglandins and restoration of the antioxidant capacity of vitamins E and C (Ballatori et al. 2009).

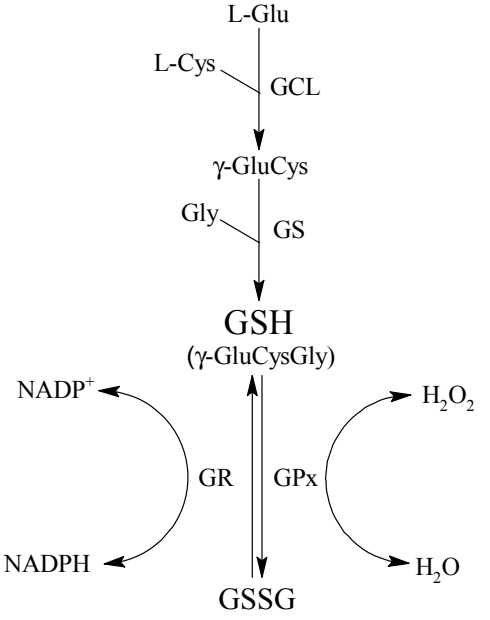
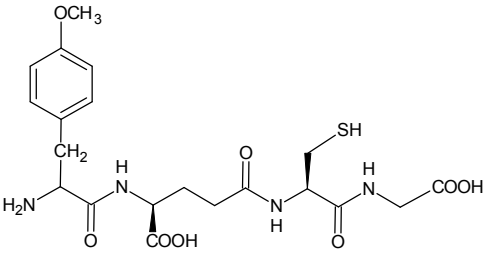


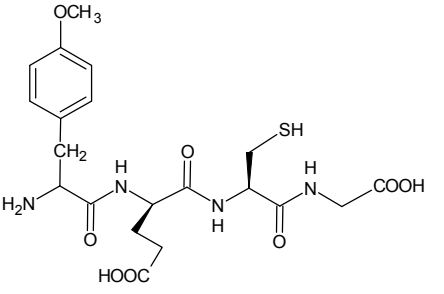
Figure 4. The synthesis and redox cycle of glutathione.
GCL – glutamate cysteine ligase;
GPx – glutathione peroxidase;
GR – glutathione reductase;
GS – glutathione synthetase;
GSH – reduced glutathione;
GSSG – oxidized glutathione.

2.4. UPF peptides and N-acetyl-L-cysteine

Several GSH analogues have been designed and synthesized to increase the GSH level. Our research group has previously created a library of novel GSH analogues (Ehrlich et al. 2007). Two of them, UPF1 (L-Tyr(Me)-L-γ-Glu-L-Cys-Gly) and UPF17 (L-Tyr(Me)-L-Glu-L-Cys-Gly) were used in this study (Figure 5). These tetrapeptides have an O-methyl-L-tyrosine residue added in the N-terminus of GSH molecule to increase the hydrophobicity and antioxidant properties. UPF17 contains α-glutamyl moiety while UPF1 has γ-glutamyl moiety similarly to GSH. We have previously shown that these peptides were up to 500-fold more effective hydroxyl radical scavengers *in vitro* compared to GSH itself (Ehrlich et al. 2007). Moreover, UPF17 and UPF1 have been shown to be non-toxic for K562 cells (200 μM) and for the primary culture of cerebellar granule cells (100 μM) (Ehrlich et al. 2007; Põder et al. 2004). UPF1 has shown protective properties in oxidative stress status of myocardial stunning and in global brain in ischemia/reperfusion model of Wistar rats (Põder et al. 2004; Kals et al. 2008).



UPF1 [L-Tyr(Me)-L-γ-Glu-L-Cys-Gly]



UPF17 [L-Tyr(Me)-L-Glu-L-Cys-Gly]

Figure 5. Peptides UPF1 and UPF17.

Thiol-containing N-acetyl-L-cysteine (NAC) is widely used as a mucolytic agent and as a precursor for L-cysteine, which is in turn a precursor for GSH. NAC can be used by cells in two different ways: NAC is hydrolyzed to cysteine and transported by Na^+-dependent alanine-serine-cysteine transport system to the cells or free NAC can straightly enter a cell and release cysteine for synthesis of GSH (Bannai 1984). By protecting the loss of GSH in several multifactorial diseases, NAC is considered as potential therapeutic agent for example in multiple sclerosis (Stanislaus et al. 2005), Huntington´s disease (Sandhir et al. 2012), Alzheimer´s disease (Adair et al. 2001; Clark et al. 2010) and type II diabetes (Ozkilic et al. 2006). In type II diabetes, the beneficial effects and mechanisms of NAC on insulin resistance have been associated to its antioxidative or anti-inflammatory properties and its role on signaling pathways or apoptosis (El Midaoui et al. 2008; Diniz et al. 2006; Shoelson et al. 2007).

2.5. Hypothermia and stress

Mild therapeutic hypothermia (28–32 °C) is used as a clinical treatment to reduce damage to several tissues including heart, kidneys and liver (Ostadal et al. 2013; Testori et al. 2011). The mechanism has been proposed to be the reduction of oxidative stress (Dohi et al. 2013). It is accomplished by increased activity of superoxide dismutase, GPx, glutathione S-transferase and altered xanthine oxidase activity (Hackenhaar et al. 2017). Additionally, hypothermia can also trigger the full activation of unfolded protein response and disrupt the cell secretory pathway (Fujita 1999) resulting in apoptosis through CCAAT-enhancer-binding protein homologous protein (CHOP) activation and increased endoplasmic reticulum oxidoreductin-α (Ero1-α) expression (Tajiri et al. 2004; Poone et al. 2015).

Wolfram syndrome 1 is tightly related to ER stress. *Wfs1*-deficient mice (9–12 months old) have lower body temperature accompanied with lower food and water consumption. Moreover, they have higher oxygen consumption and carbon dioxide and heat production compared to WT mice (Ehrlich et al. 2016).

2.6. Metabolomics

It is postulated that the functional status of a biological system is reflected in the pattern of metabolites in biological fluids or tissues, which is termed as the metabolome (Pauling et al. 1971). The advantages of metabolomics compared to other "omics" is the close linkage to phenotype, since the metabolites reflect dynamic processes that have been already performed or were happening at the moment of sample collection. Metabolomics is widely used for the detection and quantification of all or selected groups of endogenous and exogenous small-molecule metabolites (<1500 Da) measured in a biological sample (Fiehn 2002). The methods of metabolomics provide comprehensive investigation of metabolome from body fluids such as plasma, urine or tissues. Metabolites show more expeditious fluctuation in response to a physiologic change than the changes in gene expression or protein production and analysis of the metabolites may detect association between the genes and their functions. The endogenous metabolism for humans and mammalians is believed to involve a few thousands of metabolites.

Most metabolomics studies can be divided into targeted and untargeted approaches. Targeted metabolomics is focused on a predetermined specified list or class of metabolites that are being investigated. The use of isotope-labeled internal standards allow clear identification and quantification of analytes, therefore targeted analysis usually result in high sensitivity and accurate detection of metabolites. On the contrary, untargeted metabolomics is directed to detect as many metabolites as possible, followed by identification of metabolites using databases based on known or predicted spectral patterns. Data

analysis and visualization (for example principal component analysis – PCA) can be used to classify phenotypes based on metabolite pattern.

2.7. Summary of literature

Endoplasmic reticulum and oxidative stress are tightly related to hypothermia and several pathologies including Wolfram syndrome, which is systemic and affects several organs throughout the body. Metabolomics provide comprehensive investigation methods for profiling tissues or bodily fluids. Studying oxidative stress and metabolic profiling of *Wfs1*-deficient mice under hyperglycemic conditions is substantial to find therapies (such as antioxidants) aimed at reducing stress. Also this might give new insight of the association between the *Wfs1* and its functions, and describing the phenotype-genotype connections and detect early biomarkers for complications of WS and diabetes. Therefore the disturbances of glutathione system as the main indicator of oxidative stress was studied in *Wfs1*-deficiency and hypothermia conditions.

3. AIMS OF THE STUDIES

The purpose of this study is oriented to the oxidative stress status and metabolic profiling in *Wfs1*-deficient mouse model and in hypothermia-conditions. In addition, the aim was to evaluate the effect of antioxidative therapies targeted to reduce the stress in *Wfs1*-deficient mouse model.

The specific aims of the study were as follows:

1. Describe the metabolic profile of *Wfs1*-deficient mouse model in several organs related to pathology of Wolfram syndrome.
2. Describe the antioxidative glutathione system in several organs of *Wfs1*-deficient mouse model.
3. Evaluate the effect of antioxidants administered to the *Wfs1*-deficient mouse model on glutathione system in several organs. (Unpublished data)
4. Evaluate the effect on glutathione level in hypothermia-induced conditions of different cell lines.

4. MATERIALS AND METHODS

4.1. Study subjects/materials

4.1.1. Animals (Papers I and II)

The animal experiments in papers I and II were carried out in accordance with European Communities Directive (86/609/EEC) from the Estonian National Board of Animal Experiments (permission number 36, 23.07.2014). During the study, mice were housed in groups of 8–9 under standard laboratory conditions: 12 h light/dark cycle with free access to chow diet and water. Male 2- and 6-months old wild-type (*Wfs1*$^{+/+}$, WT) and *Wfs1*-deficient (heterozygous *Wfs1*$^{+/-}$, HZ and homozygous *Wfs1*$^{-/-}$, KO) were used. WS is known to be a progressive disease and therefore the study animals in the study are at different age to describe the mild and more severe stage of pathologies. A brief review of the detailed generation, breeding and genotyping analysis of *Wfs1*-deficient mice (8th exon at the C-terminal end replaced by NLS-LacZ-Neo expression cassette) can be seen in the review of literature (Chapter 2.1.3). Each experimental group consisted of 8 animals.

4.1.2. Cell lines (Paper III)

HeLa cells (CCL-2) (ATCC, United Kingdom) and mouse embryonic fibroblasts (MEFs) (Millipore, USA) were used for the measurement of total and oxidized glutathione. HeLa cells were cultured in low glucose minimum essential medium (Capricorn Scientific, Germany) supplemented with 10% fetal bovine serum (PAN Biotech, Germany), 1 × penicillin/streptomycin at 37 °C in a 5% CO_2 and the switched to 32 °C incubator in 5% CO_2 during hypothermia experiment.

4.1.3. Antioxidants (Additional)

Wfs1-mice were given i.p. injections of UPF1 peptides (0.1 mM) and NAC for 5 days (1 mg/kg) and 0.9% NaCl was used as vehicle. The introduction to UPF peptides and NAC can be seen in the review of literature (Chapter 2.4).

4.1.4. Materials (All papers)

In paper II, DC Protein Assay was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) and Glutathione Assay, Glutathione Peroxidase Assay and Glutathione Reductase Assay kits were from Cayman Chemical Company (Ann Arbor, USA). The GSH/GSSG-Glo™ Assay (V6612) used in paper III was purchased from Promega, USA.

All other chemicals used throughout the studies were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

4.2. Methods

4.2.1. Peptide synthesis (Additional)

UPF peptides were synthesized manually by solid phase peptide synthesis using Fmoc-chemistry as described previously (Ehrlich et al. 2007). The purity of peptides was > 99% as demonstrated by HPLC using reversed-phase column (Jupiter 5μm C18 300Å, 250 × 21.20 mm) and the peptides were identified with MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass-spectrometry (Voyager DE Pro, *Applied Biosystems*).

4.2.2. Tissue/bodily fluids collection (Papers I and II)

After the treatment of UPF peptides, NAC or saline, the mice were euthanized by manual cervical dislocation. Urine samples were collected outside the cage with the aid of pipette the day before. Trunk blood was collected after decapitation immediately *post mortem* and liver, heart, pancreas and kidney tissues were collected, perfused with ice-cold saline, snap frozen in liquid nitrogen and stored at –80 °C until processing.

4.2.3. Sample preparation (Papers I and II)

For mass-spectrometry in paper I, the widely used hydrophobic-hydrophilic phase extraction protocol for homogenization of tissues was used as slightly modified (Beckonert et al. 2007). For the extraction of metabolites, frozen samples were weighed and 4 ml/g of LC-MS grade methanol and 0.85 ml/g of water was added before homogenization. The samples were homogenized by ultrasound homogenizator (Bandelin Sonopuls, Germany) and followed by adding 4 ml/g of chloroform and 2 ml/g of water. The samples were mixed and centrifuged for 15 min 1000 × g at 4 °C, which allowed the mixture to settle into two layers (upper hydrophilic and lower lipophilic phase). Proteins of the hydrophilic phase and the samples of urine and trunk blood were precipitated with 75% methanol and centrifuged for 15 min at 21250 × g 4 °C. All procedures were done on ice.

For the measurement of GSH level and the activity of GR and GPx in paper II, the tissue samples (15–250 mg) were homogenized in 0.1 M phosphate buffer (1:10 w/v; pH 7.4) and centrifuged for 15 min at 10,000 × g 4 °C. Supernatants were collected and immediately aliquoted for the measurement of total GSH (tGSH) or the enzymatic activity of GR or GPx. For the measurement of tGSH and GSSG, proteins were precipitated with 10% metaphosphoric acid (1:1 w/v) to avoid interference owing to particulates and sulfhydryl groups in the assay.

4.2.4. Mass-spectrometry (Paper I)

The samples were randomized and supernatants were evaluated on QTRAP 3200 mass spectrometer (*AB Sciex*). Samples were analyzed for 5 min in isocratic flow of 0.05 ml/min of methanol and 0.1% of formic acid for the lipophilic phase (tissues) and binary flow 0.025 ml/min of water and methanol, followed by 0.05 ml/min of methanol and 0.1% formic acid for the hydrophilic phase (tissues, urine and trunk blood). The full spectra (mass-to-charge ratio from 50 to 1500) was obtained in positive and negative enhanced mass scan mode. The ionspray voltage, declustering and entrance potential were 4500 V, 20 V and 10 V, respectively, and respective negative voltages were applied for negative scan mode.

Multiple reaction monitoring (MRM) was performed for all metabolites in targeted analysis. The concentration of purines and pyrimidines were measured using the method described by la Marca *et al.* (la Marca et al. 2006). Amino acids and acylcarnitines were analyzed as butylesters and the sample preparation was performed using the method described by Matern *et al*. (Matern 2008). Technical details of LC-MS conditions for amino acids, acylcarnitines and hydroxy acids have been reported previously (Zagura et al. 2015). For this, 50 µl of labelled internal standards (Cambridge Isotopes Inc, USA) in methanol were added to 10 µl of sample. After 20 min of incubation, the samples were centrifuged at 4 °C and 21250 × g for 15 min and the supernatant was evaporated. After evaporation, the extracts were butylated using 60 µl butanol/HCl at 65 °C for 15 min and the samples were again evaporated and dissolved in 100 µl of acetonitrile/H_2O/formic acid (50:50:0.025, respectively). Fifteen µl of the sample was injected into MS/MS. Acylcarnitines were analysed as precursors of m/z 85 ion and amino acids were analyzed by MRM scan with the following transitions: [$^2H_3$]Leu 191/89, [$^2H_2$]Orn 191/72, [$^2H_3$]Met 209/107, [$^2H_4$]Ala150/48, [$^2H_5$]Phe 228/126, [$^2H_6$]Val 182/80, [$^2H_4$,^{13}C]Arg236/75, [$^2H_2$]Cit 234/115, [$^2H_3$]Glu 263/87, [$^{13}C_6$]Tyr 244/142, [^{15}N, ^{13}C]Gly 134/78, [$^2H_3$]Asp 249/147, Orn 189/70, Arg 231/70, Gly 132/76, Cit 232/113, Ala 146/44, Asn 189/144, Asp 246/144, Cys 206/104, Gln 203/84, Glu 260/84, His 212/110, Leu+Ile 188/86, Lys 203/84, Met 206/104, Phe 222/120, Pro 172/70, Ser 162/60, Thr 176/74, Trp 261/244, Tyr 238/136, Val 174/72 and hydroxy-Pro 189/87. Ionization was performed at 4500 V and 400 °C, declustering potential was set to 40 V and collision energy to 38 V. For hydroxy acid analysis, 5 µl plasma was mixed with 35 µl (500 µM [$^2H_4$]succinic acid and [$^2H_4$]mallonic acid in methanol). The samples were centrifuged for 15 min at 10 000 × g and 20 µl was injected. An HILIC (Luna 5 µm HILIC 200 A, 150 × 3 mm^2; Phenomenex, Torrance, CA, USA) column was used with a flow rate of 0.2 ml/min^{-1} and the eluents used were: A – 5 mM ammonium formate in water and B – 5 mM ammonium formate in methanol. The gradiential was 5 min isocratic 95% eluent B, gradiental decline to 5% eluent B within 15 min and 5 isocratic flow of 5% eluent B. MRM transitions in negative polarization mode were [$^2H_4$]succinic acid 121/77, [$^2H_4$]mallonic acid 106/59, citrate 191/111, α-oxoglu-

tarate 145/101, pyruvate 87/43, succinate 117/73, mallonic acid 103/41, β-hydroxybutyrate 103/59 and oxaloacetate 131/87. Ionization was performed at –4500 V and 200 °C, declustering potential was set to –20 V and collision energy from –10 to –30 V.

4.2.5. Measurement of intracellular glutathione (Papers II and III)

In paper II, the concentration of glutathione was measured using a commercial glutathione assay kit (*Cayman Chemicals*), which utilizes optimized enzymatic GR recycling method first described by Tietze (Tietze 1969). Briefly, the thiol group of GSH reacts with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) resulting in formation of yellow-colored 5-thio-2-nitrobenzoic acid (TNB) and mixed disulfide of GSH and DTNB. The latter is reduced by GR to recycle the GSH and produce extra TNB. The production of total TNB is directly proportional to the concentration of GSH in the sample. The quantification of GSSG is accomplished by first derivatizing GSH with 2-vinylpyridine and needs to be analyzed separately. The samples were measured at 412 nm spectrophotometrically (*Sunrise Tecan*).

The concentration of total and oxidized glutathione in paper III was measured using the commercial GSH/GSSG-Glo™ Assay, which is a luminescence-based system, where GSH-dependent reaction leads to production of luciferin and finally the activity of luciferase is dependent on the amount of GSH. The concentration of GSSG is measured in parallel as reduced glutathione is blocked by a specific reagent. Luminescence was measured using the Concert™ Triad microplate reader (*Dynex Technologies*). This luminescence-based method is more sensitive compared to the spectrophotometrical assay used in paper II.

Data were analyzed using GraphPad Prism version 5.0.0 for Windows (*GraphPad Software*). The results are presented as the mean ± standard error of the mean (SEM). The comparisons between groups were made using the Student's *t*-test.

4.2.6. Measurement of the activity of glutathione reductase and peroxidase (Paper II)

The overexpression of mRNA or protein does not necessarily result in an increase in activity, therefore in paper II the activity of GPx and GR was measured (both with a commercial *Cayman Chemicals* assay kit). GR catalyzes the NADPH-dependent reduction of GSSG to GSH and therefore maintains adequate level of cellular GSH. GPx catalyzes the reduction of hydrogen peroxide to protect the cell from oxidative stress and uses GSH as the ultimate electron donor. The assay measures GR activity by the rate of NADPH oxidation and GPx activity indirectly by coupled reaction with GR (Ursini et al. 1985; Carlberg & Mannervik 1985). The samples were measured at 340 nm

spectrophotometrically (*Sunrise Tecan*). Data were analyzed using GraphPad Prism version 5.0.0 for Windows (*GraphPad Software*). The results are presented as the mean ± standard error of the mean (SEM). The comparisons between groups were made using the Student's *t*-test.

4.2.7. Data analysis (all papers)

In paper I, the spectral signals of samples were binned with the resolution of 1 Da and normalized to the mean intensity of the spectra. Principal component analysis (PCA) of full mass spectra was used to detect and illustrate the genotype- and/or age-dependent variances. One- and two-way analysis of variance (ANOVA) for univariate factor analysis was used to compare the mean differences between the *Wfs1* genotypes and/or age. All statistical analyses were performed with R version 3.2.2 (*The R Foundation for Statistical Computing*).

In papers II and III, the data were analyzed using GraphPad Prism version 5.0.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). The results are presented as the mean ± standard error of the mean (SEM). Comparisons between groups were made using one-way analysis of variance followed by Tukey's test or Student´s *t*-test in paper III. $P<0.05$ was considered to indicate a statistically significant difference.

5. RESULTS

5.1. Untargeted metabolomics

Wfs1-deficient mice have been genotyped and phenotyped in behavioral studies (Luuk et al. 2009). The most obvious difference in phenotype is retarded growth of KO mice. This, and the fact that growth hormone production is increased in the growth retarded mice, have raised the question if more specific metabolic pathways could be behind the *Wfs1*-deficient mice phenotype and WS pathology in general. Anxiety-like behaviour is increased and therefore serotonergic, dopaminergic and adrenergic signaling in brain have been characterized with significant changes in neurotransmitter systems (Reimets et al. 2016; Visnapuu et al. 2013). Thyroid axis and the metabolic activity measured by O_2 consumption and CO_2 production has not been found to depend on genotype at 3 months of age (Noormets et al. 2014).

Firstly, for an overview, principal component analysis (PCA) was performed to visualize the variance between the metabolome of *Wfs1*-deficient and WT mice. In the first principal component, mice were separated mainly by age, but not by genotype (Figure 6). The next components showed levels of efficiency in separating genotypes. This result may, however, be biased due to patch effect from the 4 month time difference between the analysis of younger and older animals.

Factor loading comparisons revealed that the metabolic profiles of liver and pancreas were the highest contributors to component 1 (i.e. aging effects) (Table 1). The next 8 principal components after the first were all related to genotype effects, meaning that the genotype causes changes, which cannot be described by a single or a few pattern changes in the global metabolic profiles. While some principal components are specific to one tissue (e.g. component 2 is based on the metabolic profile of pancreas mainly), others (e.g. components 4, 5, 7, 8, and 9) hint for metabolic processes that occur in several or all tissues.

One-way analysis of variance with post-hoc Tukey HSD test was used to determine the significantly changed m/z values in untargeted metabolic profiles of different ages and genotypes. At 2 months of age, the lowest number of statistically different (level $p<0.05$) metabolites was found between WT and HZ. Knock-out mice had 392 (1.4% of all metabolome) and 357 (1.2%) statistically different signals from WT and HZ, respectively (Figure 7A). At 6 months the differences between KO and WT had increased to 1319 signals (4.6%), while KO and HZ differed by 600 signals (2.1%). Aging caused the highest number of significantly ($p<0.05$) altered metabolites in liver, kidney and heart. In these tissues 18–38% of the metabolic profile was altered at $p<0.05$ significance and 2–11% with Bonferroni corrected significance threshold ($p<10^{-6}$).The extent of metabolic changes due to age were similar for all genotypes in liver, heart, urine and trunk blood, but pancreas and kidney showed significantly (χ^2 test; $p<10^{-10}$) more changes due to age in KO and HZ animals compared with WT mice (Figure 7B).

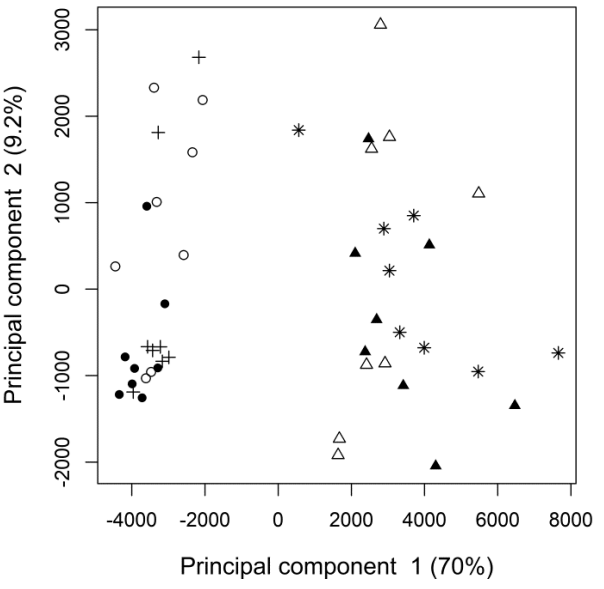


Figure 6. Principal component analysis of *Wfs1*-deficient mice, with components 1 and 2 as the highest separation with age and genotype, respectively. In parenthesis in axis titles is the percentage of total variance that the component describes. Each dot represents an array of metabolic profiles from liver, kidney, heart, trunk blood, urine and pancreas. The symbols are as follows: 2-month old mice: filled circle – knock-out, cross – heterozygote, circle – wild-type; 6-month old mice: triangle point up – knock-out, star – heterozygote, filled triangle point up – wild-type. (Porosk et al. 2017)

Table 1. The key characteristics of the top nine components explaining >95% of total variance in untargeted metabolic profiling. (Porosk et al. 2017)

Principal compo-nent	% of total variance	Stronger association with:	Relative importance of tissue profiles in respective principal component					
			Blood	Heart	Kidney	Liver	Pancreas	Urine
1	70	age	1%	6%	2%	49%	42%	0%
2	9.2	genotype	0%	4%	1%	4%	90%	0%
3	6.3	genotype	1%	2%	2%	90%	5%	0%
4	4.5	genotype	0%	2%	1%	47%	50%	0%
5	1.9	genotype	0%	39%	42%	7%	11%	1%
6	1.1	genotype	0%	78%	2%	1%	17%	1%
7	0.95	genotype	1%	14%	31%	33%	19%	2%
8	0.75	genotype	7%	9%	5%	37%	39%	3%
9	0.62	genotype	5%	25%	26%	20%	22%	2%

While the untargeted metabolic profiling helps to understand which tissues are affected the most, targeted analysis of specific metabolites is necessary to identify the role of specific metabolic pathways.

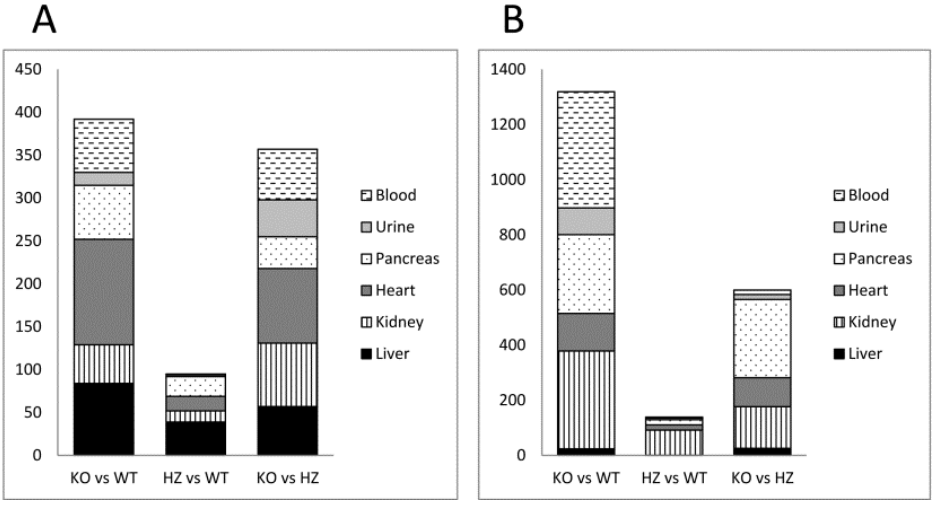


Figure 7. Number of statistically significant ($p<0.05$) changed metabolites in trunk blood, urine, pancreas, heart, kidney and liver tissue within *Wfs1* knock-out (KO), heterozygous (HZ) and wild-type (WT) 2- and 6-month old mice (Figures A and B, respectively). (Porosk et al. 2017)

5.2. Targeted metabolomics

Subsequently, targeted analysis of metabolites was carried out. The most significant changes due to *Wfs1* deficiency were found in glucose and amino acid metabolism (see Paper 1, table 2). Changes in individual organic and fatty acid levels were more subtle, although as discussed in discussion section, even small changes in individual compounds may be physiologically relevant if viewed in the context of metabolic pathways.

5.2.1. Glucose utilization

Energy metabolism depends on glucose utilization, gluconeogenesis and keto-genesis. Therefor the levels of hexoses, phosphohexoses and lactate were measured in several tissues and bodily fluids of mice. The most significant were that at 6 months of age the KO mice had the highest blood hexose levels and in the younger KO mice the hexoses in urine were increased (Figure 8). Moreover, lactate levels were increased in insulin sensitive tissues of the 2 months old KO and in older HZ animals.

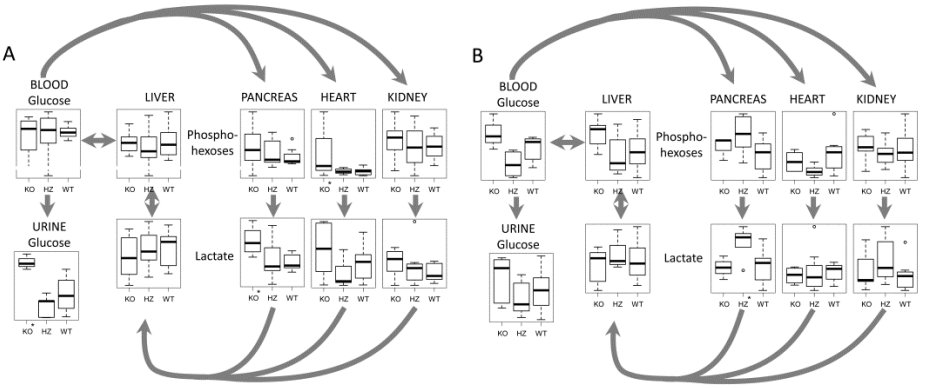


Figure 8. A simplified overview of glucose metabolism in *Wfs1*-deficient (KO – knock-out, HZ – heterozygous, WT – wild-type) mice. In blood and urine glucose was not modified, in parenchymatous tissues phosphohexoses were more relevant as glycolysis starting points. Lactate is the endpoint of glucose metabolism under anaerobic conditions, but is converted back to glucose in liver. A) 2-month old mice, B) 6-month old mice. (Porosk et al. 2017)

5.2.2. Other energy sources

Untargeted metabolic profiles from chloroform extract of tissues with negative ionization were used to evaluate the relative abundance of neutral lipid species. There was a remarkable increase in triglyceride levels in pancreas and heart of young KO animals compared with WT and signs of lipolysis in older animals in the same tissues (Figure 9).

Additionally, the levels of hydroxybutyrate and acetoacetate (ketone body), acyl- (acetyl-, propionyl-, butyryl-, palmitoyl- and stearoyl-) and free carnitines along with branched chain amino acids (BCAA) were measured by targeted metabolomics approach. The levels of long chain acylcarnitines were increased or remained unchanged in the KO mice compared to WT animals. Short-chain acyl- and free carnitine levels were not affected by the *Wfs1*- deficiency. From the BCAA, we found that Val and Leu/Ile were decreased the most in the liver of the KO mice between the genotypes. The concentration of acetoacetate was not changed significantly in the tissues of the KO mice.

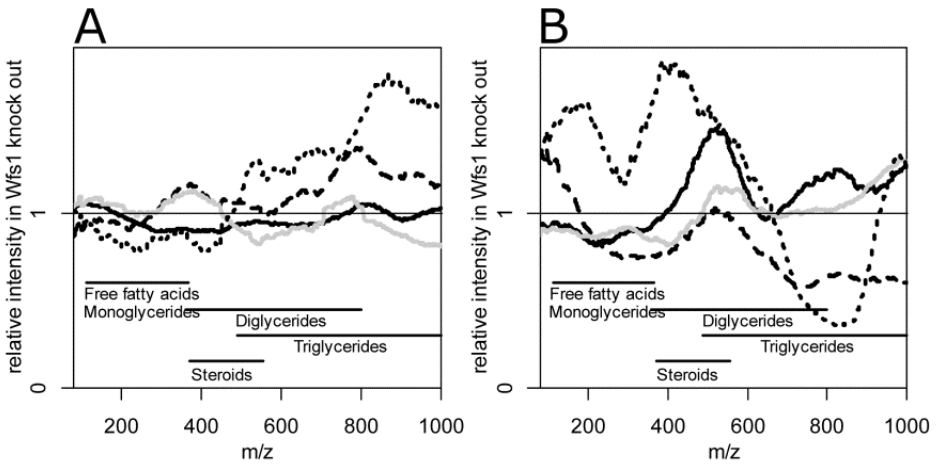


Figure 9. The relative change in negative ionization mass spectra (untargeted global metabolic profiling) from chloroform extract of *Wfs1* knock-out tissues in comparison to wild-type. Liver – solid black line, kidney – solid gray line, heart – dashed line, pancreas – dotted line. The regions of tri-, di- and monoglycerides as well as steroids are given. The free fatty acids are grouped with monoglycerides. A) 2-month old mice, B) 6-month old mice. The signal is smoothed by moving average approach with 25 Da range. (Porosk et al. 2017)

5.2.3. Protein metabolism

For the analysis on protein metabolism, the levels of several free amino acids and intermediates from amino acid metabolism were measured (see Section 4.2.4). We found elevated levels of Ala, Arg, Asn, Gly, Leu+Ile, hydroxyl-Pro, Pro and Thr in the heart tissue of the younger KO mice. In the liver, where to the urea cycle mainly takes place, only Cit did not show a tendency to decrease in the KO mice. We also found the increased levels of free amino acids in the pancreatic tissue of HZ mice. In the urine hippuric acid was increasingly excreted by the KO mice. Also significant changes of Pro and hydroxyl-Pro were found.

5.2.4. Uric acid and cysteine metabolism

The genotype effects on the levels of purine and pyrimidine nucleobases and nucleosides were found to be highly variable between tissues. The level of uric acid was significantly increased in trunk blood and kidney of 6-month old KO mice (Figure 10). At younger age the same genotype, however, had a significant reduction of uric acid production in liver. Additionally, uric acid was non-significantly decreased in trunk blood and increased in urine at young age. Decreased hypoxanthine and inosine levels under hyperuremic condition were

detected and ascorbic acid level was decreased in the heart of older KO mice. Homocysteine, the amino acid related to glutathione system and oxidative stress status, was decreased in the pancreas at young age, but Cys or Met levels were not disturbed by genotype.

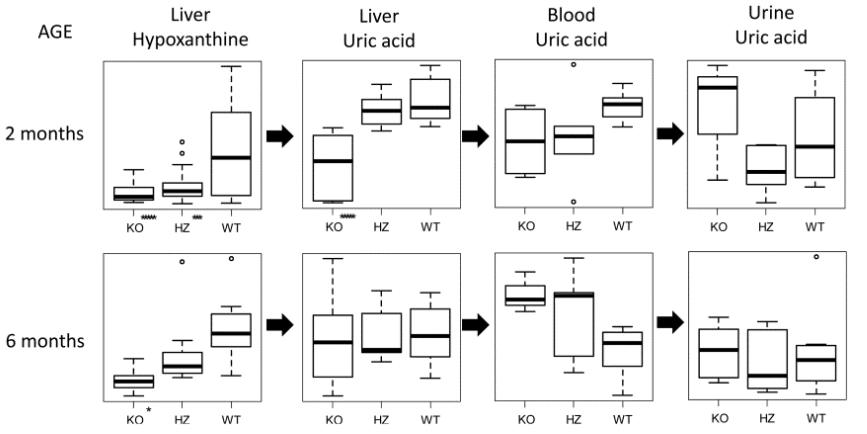


Figure 10. Hypoxanthine and uric acid levels in *Wfs1*-deficient (KO – knock-out, HZ – heterozygous, WT – wild-type) mice. (Porosk et al. 2017)

5.3. Oxidative stress and glutathione system

The concentration of total glutathione and the activities of glutathione peroxidase and reductase were measured in the heart, liver, kidneys and pancreatic tissue of KO, HZ and WT mice. Additionally, the concentration of total and oxidized glutathione was measured in HeLa, wild-type and Nrf2 KO mouse embryonic fibroblasts.

Kidneys. The 2-month-old KO mice exhibited a significantly lower level of tGSH concentration in the kidney tissue compared with tGSH concentration in the WT littermates (1.6-fold; $F_{2,15}=5.9$; $P<0.05$; Figure 11A). HZ 6-month-old mice exhibited a 1.2-fold higher concentration of tGSH in the kidney tissue compared to WT mice ($F_{2,19}=8.2$; $P<0.05$; Figure 11B). The level of GSSG was below the detection limit and could not be measured. GPx activity was 1.7-fold higher in 2-month-old KO mice compared with WT mice ($F_{2,21}=18.0$; $P<0.0001$; Figure 12A). GR activity was 1.4-fold higher in 2-month-old KO mice, but this was not indicted to be statistically significant (Figure 12B). GPx and GR activities in 6-month-old mice could not be measured due to their insufficient activity in the available amount of renal tissue.

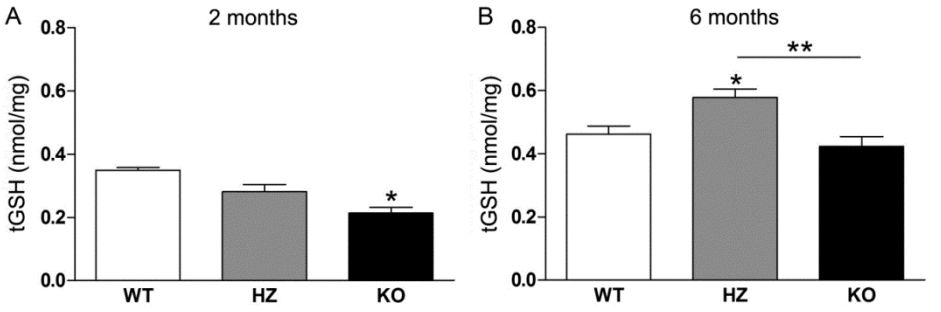


Figure 11. The concentration of total glutathione (tGSH) in **kidneys** of *Wfs1* wild-type (WT), heterozygous (HZ) and knockout (KO) 2- and 6-month (Figures A and B, respectively) old mice (n=8). Asterisk with the line indicates significance between HZ and KO and asterisk without a line significance compared to WT. Values are mean ± SEM. * $p < 0.05$; ** $p < 0.01$ (Porosk et al. 2017)

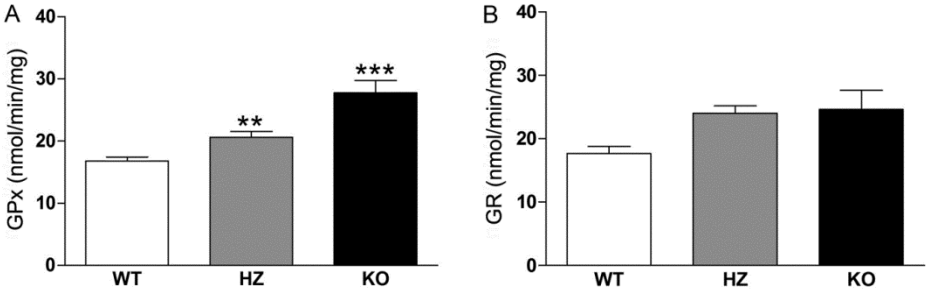


Figure 12. The activity of glutathione peroxidase (GPx, Figure A) and reductase (GR, Figure B) in **kidneys** of *Wfs1* wild-type (WT), heterozygous (HZ) and knockout (KO) 2-month old mice (n=8). Asterisk indicates significance compared to WT. Values are mean ± SEM. ** $p < 0.01$; *** $p < 0.001$ (Porosk et al. 2017)

Heart. Analyses on heart tissues identified slightly lower, albeit not statistically significant, levels of GSSG and GSH compared with WT. In 6-month-old KO mice, the GSH concentration was 1.5-fold lower ($F_{2,20}$=10.9; P<0.001; Figure 13) compared with WT littermates. GPx activity was slightly higher and GR activity lower in KO 2-month-old mice compared with WT mice, but these differences were not statistically significant. The GSSG/GSH ratio was 2-fold higher ($F_{2,20}$=4.9; P<0.05) in older and slightly higher in younger *Wfs1*-deficient mice compared with WT (Figure 14).

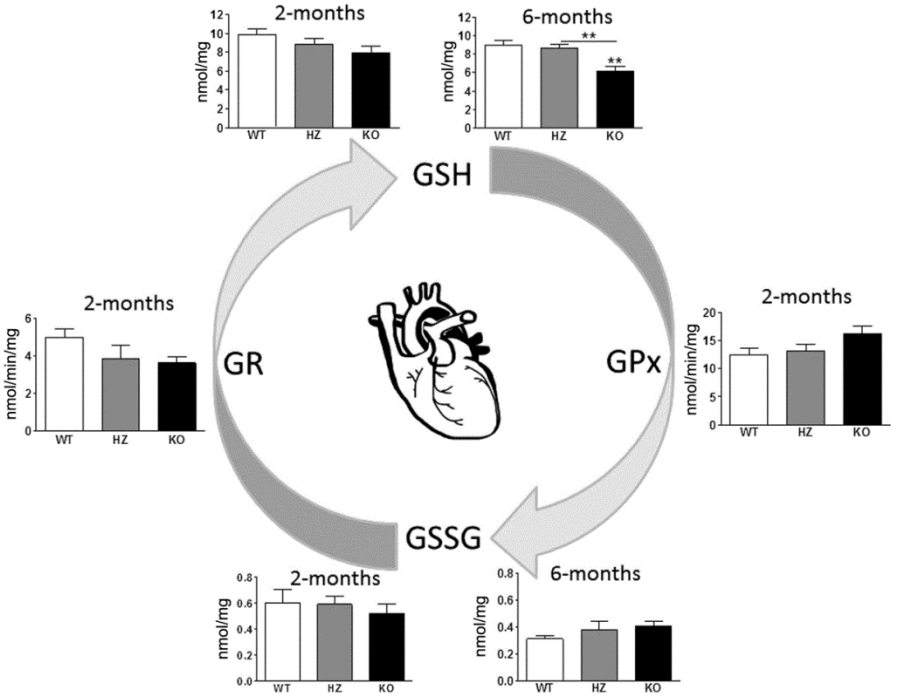


Figure 13. Schematic figure of glutathione system affected by knock-out of *Wfs1* in 2- and 6-month old mice in **heart** tissue. The x-axis corresponds to the concentration (nmol/mg protein) or the enzyme activity (nmol/min/mg protein). Asterisk with the line indicates significance between HZ and KO and asterisk without a line significance compared to WT. Values are mean ± SEM. ** $p < 0.01$; n=8. Abbreviations: GPx – glutathione peroxidase, GR – glutathione reductase, GSH – reduced glutathione, GSSG – oxidized glutathione, HZ – *Wfs1* heterozygous, KO – *Wfs1* knockout, WT – *Wfs1* wild-type. (Porosk et al. 2017)

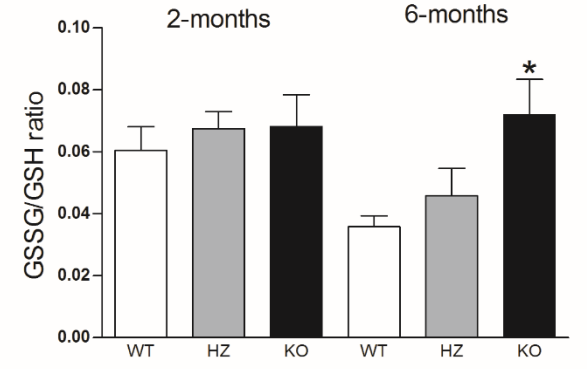


Figure 14. The glutathione redox ratio (GSSG/GSH) in the **heart** of 6-month old *Wfs1* wild-type (WT), heterozygous (HZ) and knockout (KO) mice (n=8). Asterisk indicates significance compared to WT. Values are mean ± SEM. * $p < 0.05$ (Porosk et al. 2017)

Liver. In the liver, there was a 1.1-fold higher level (not statistically significant) of GSH in 2-month-old KO mice and a 1.7-fold lower level of GSH in 6-month-old mice compared with WT littermates ($F_{2,21}$=4.2; P<0.05; Figure 15). GR activity was 1.6-fold lower in 2-month-old KO mice compared to WT mice ($F_{2,21}$=6.4; P<0.001). Notably, the activity of GPx (1.3-fold; $F_{2,21}$=5.6; P<0.05) and GR (1.6-fold; $F_{2,20}$=5.2; P<0.05) were significantly increased in 6-month-old KO and HZ mice compared with WT littermates. These data indicated more intensive usage of GSH by GPx in older mice, whereas the activity of GR is recovered.

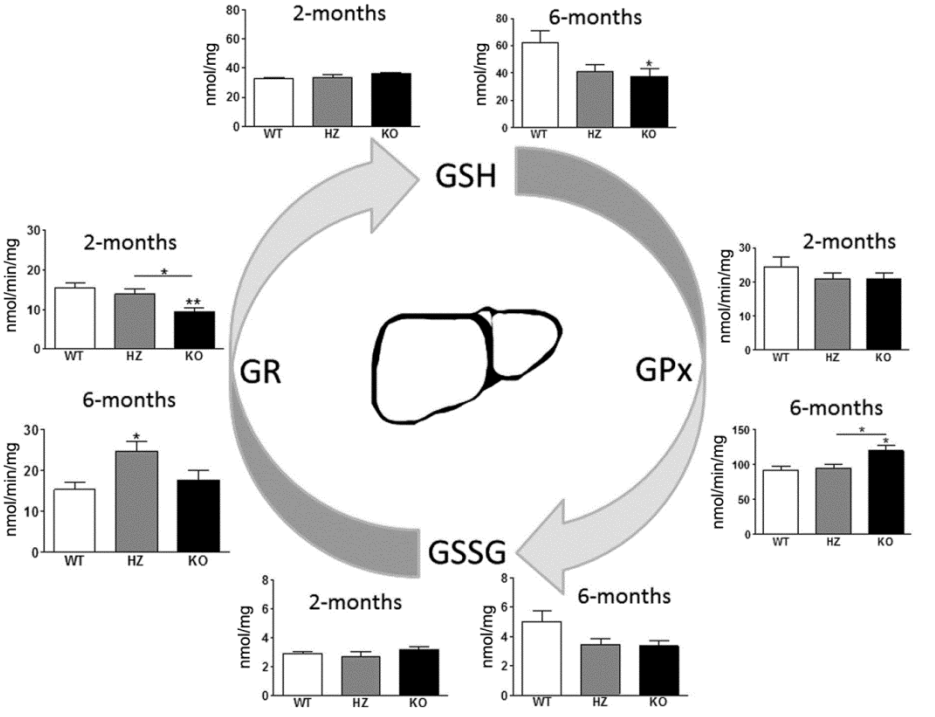


Figure 15. Schematic figure of glutathione system affected by knock-out of *Wfs1* in 2- and 6-month old mice in **liver** tissue. The x-axis corresponds to the concentration (nmol/mg protein) or the enzyme activity (nmol/min/mg protein). Asterisk with the line indicates significance between HZ and KO and asterisk without a line significance compared to WT. Values are mean ± SEM. * p < 0.05, ** p < 0.01; n=8. Abbreviations: GPx – glutathione peroxidase, GR – glutathione reductase, GSH – reduced glutathione, GSSG – oxidized glutathione, HZ – *Wfs1* heterozygous, KO – *Wfs1* knockout, WT – *Wfs1* wild-type. (Porosk et al. 2017)

5.4. The administration of UPF peptides and N-acetyl-L-cysteine

Firstly, to address the oxidative stress status after the administration of NAC, UPF1 or UPF17, the glutathione concentration in kidney, heart and liver was measured. Later, the activity of glutathione peroxidase and reductase was analyzed in the liver tissue as a main pool of glutathione in human body.

Kidneys. The administration of UFP17 increased the tGSH level by 1.8-fold ($F_{3,16}=12.79$, $p<0.001$) in 2-month old WT mice (Figure 16A). The concentration of GSSG was below the detection limit. Furthermore UPF peptides increased the tGSH concentration in WT and HZ, but did not have an effect in KO mice. In 6-month old mice, studied peptides did not alleviate tGSH concentration in kidney tissue (Figure 16B). Our previous results show reduced tGSH concentration in *Wfs1*-deficient KO mice (Porosk et al. 2017) and administration of antioxidative peptides seem not to improve tGSH level. (Unpublished data)

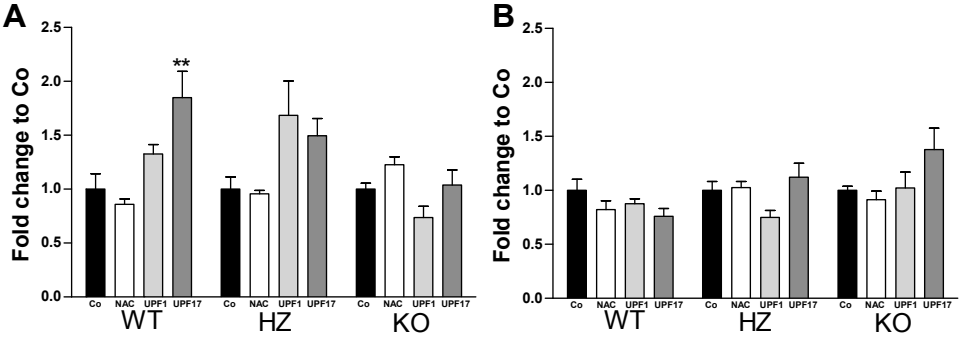


Figure 16. The concentration of total glutathione (tGSH) in **kidneys** of *Wfs1* wild-type (WT), heterozygous (HZ) and knockout (KO) 2- and 6-months old mice (A and B, respectively, n=8). Values are mean ± SEM. ** $p<0.01$. The relative concentration of tGSH of Co is 1.0 and NAC, UPF1 or UPF17 are normalized to Co (0.9% NaCl). (Unpublished data)

Heart. In the heart tissue of 2-month old mice, the reduced glutathione concentration was decreased in KO mice (Porosk et al. 2017). After the administration of UPF17 and UPF1, the GSH concentration was increased in WT and HZ, respectively (Figure 17A). The most informative, GSSG/GSH ratio, was decreased after the administration of UPF peptides in WT, but not significantly (Figure 17D). The oxidized glutathione level in HZ and KO was increased after the influence of NAC or UPF17, respectively (Figure 17B). This was also reflected to the GSSG/GSH ratio. (Unpublished data)

In 6-month old mice, only GSSG concentration was increased after the administration of every studied peptide and this also reflected in GSSG/GSH ratio (Figure 18)). Also in WT mice, UPF17 increased the concentration of GSH and the concentration of GSSG was slightly decreased after the administration of UPF peptides. As a result the GSSG/GSH ratio was decreased in WT mice, but not statistically significant. (Unpublished data)

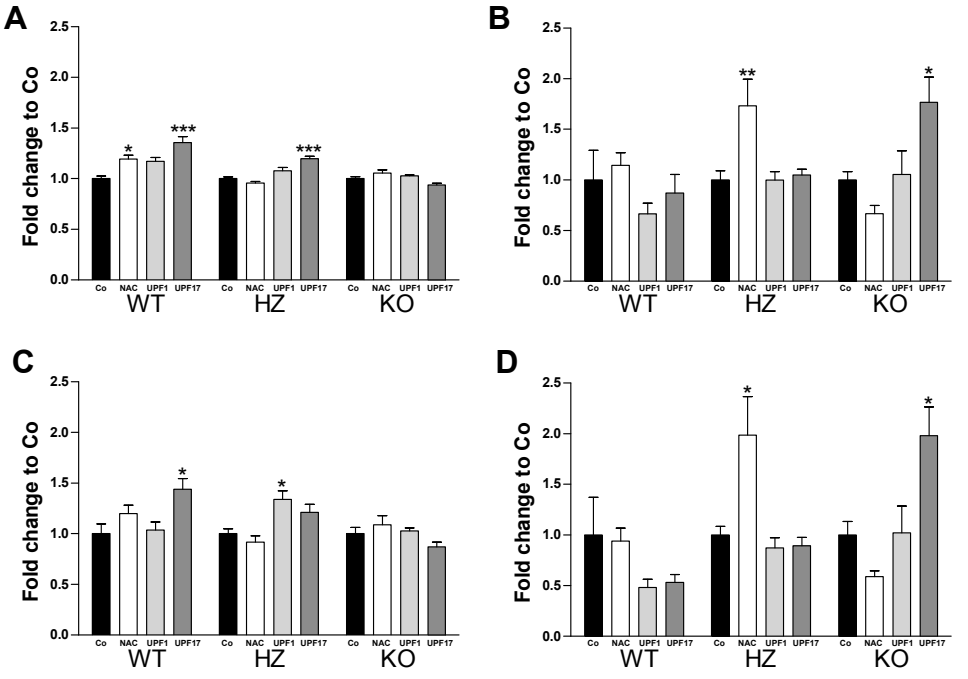


Figure 17. The concentration of total glutathione (tGSH) (A), oxidized glutathione (GSSG) (B), reduced glutathione (GSH) (C) and the glutathione redox ratio (GSSG/GSH) (D) in the **heart** of 2-months old *Wfs1* wild-type (WT), heterozygous (HZ) and knockout (KO) mice (n=8) after treatment with NAC, UPF1 or UPF17. Asterisk indicates significance to Co. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Values are mean ± SEM. (Unpublished data)

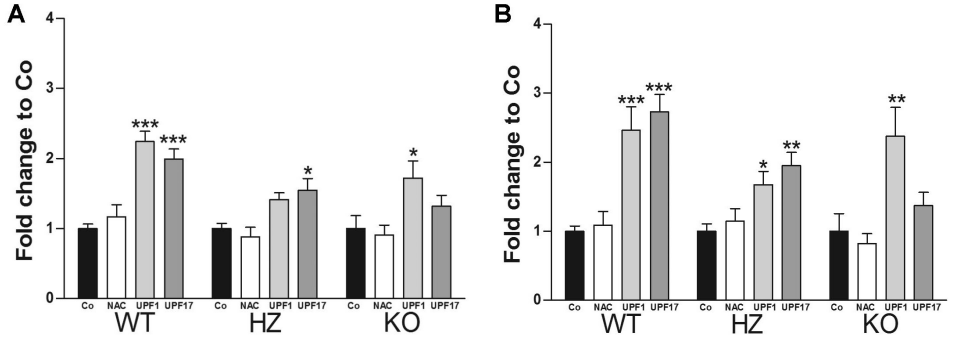


Figure 18. The concentration of oxidized glutathione (GSSG) (A) and the glutathione redox ratio (GSSG/GSH) (B) in the **heart** of 6-months old *Wfs1* wild-type (WT), heterozygous (HZ) and knockout (KO) mice (n=8) after treatment with NAC, UPF1 or UPF17. Asterisk indicates significance to Co. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Values are mean ± SEM. (Unpublished data)

Liver. The GSH and GSSG concentration and the activity of GR and GPx were measured in the liver tissue of *Wfs1*-deficient mice after the administration of saline, NAC, UPF1 or UPF17 (Figure 19). In 2-month old KO mice, the peptides reduced the concentration of GSH and GSSG, but the activity of enzymes did not change. In 6-month old mice, the UPF peptides slightly increased the concentration of GSH in WT mice, but did not alter the GSSG concentration and therefore the GSSG/GSH ratio was significantly reduced (Figure 20). (Unpublished data)

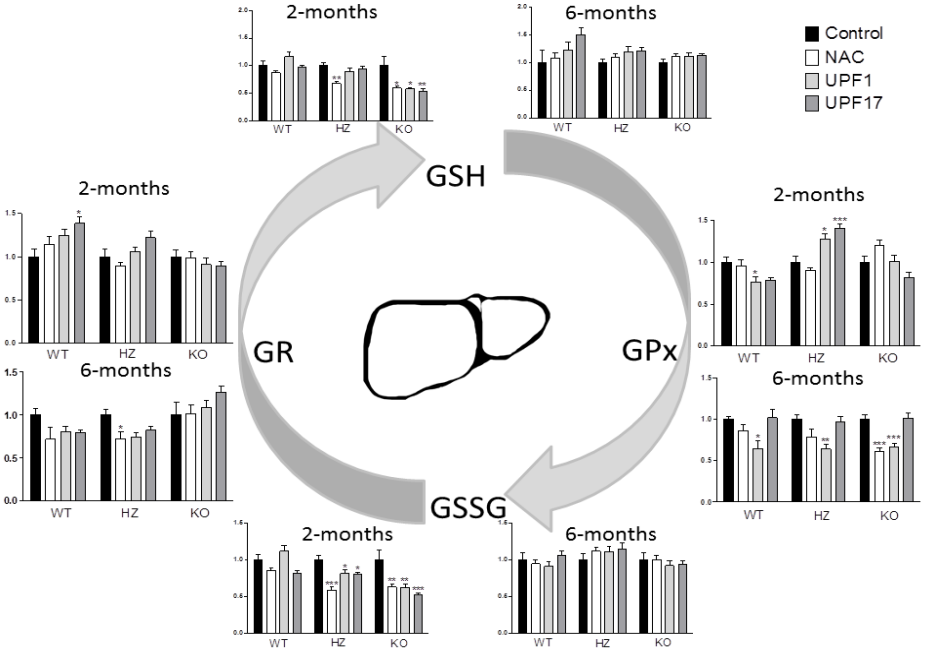


Figure 19. Schematic figure of glutathione system affected by the administration of NAC (white), UPF1 (light grey) and UPF17 (dark grey) in **liver** tissue of 2- and 6-month old *Wfs1* mice. The x-axis corresponds to the normalized concentration (nmol/mg protein) of GSH or GSSG or the enzyme activity (nmol/min/mg protein) against Control (0.9% NaCl). Values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n=8. Abbreviations: GPx – glutathione peroxidase, GR – glutathione reductase, GSH – reduced glutathione, GSSG – oxidized glutathione, HZ – *Wfs1* heterozygous, KO – *Wfs1* knockout, WT – *Wfs1* wild-type (Porosk et al. 2017)

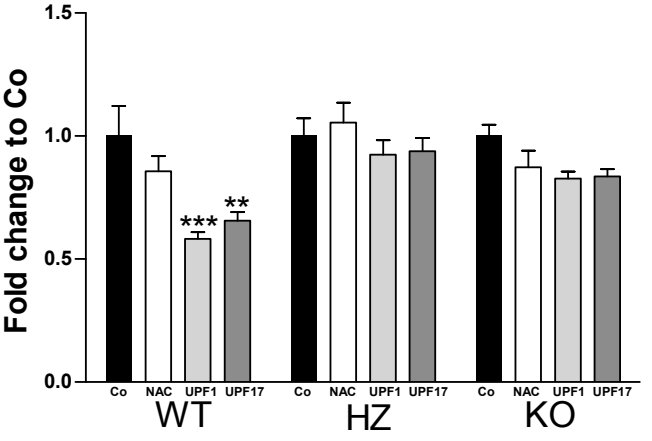


Figure 20. The glutathione redox ratio (GSSG/GSH) in the **liver** of 6-months old *Wfs1* wild-type (WT), heterozygous (HZ) and knockout (KO) mice (n=8) after treatment with NAC, UPF1 or UPF17. The relative GSSG/GSH ratio of Co is 1.0 and NAC, UPF1 or UPF17 are normalized to Co (0.9% NaCl). ** $p<0.01$; *** $p<0.001$. Values are mean ± SEM. (Unpublished data)

5.5. Hypothermia induces the stress response in different cell lines

There is little evidence provided about the therapeutic mechanisms of hypothermia, which is beneficial in several medical procedures. In paper III, we examined the effect of hypothermia on cellular stress pathways that have been related to ischemia reperfusion injury. One of the studied parameter was the measurement of intracellular reduced and oxidized glutathione level in HeLa, wild-type and Nrf2 KO mouse embryonic fibroblasts.

The concentration of total glutathione was significantly higher in wild-type fibroblasts after 24h of hypothermia when compared to normothermia (Figure 21). The opposite pattern was seen in Nrf2 KO cells, where tGSH was lower in hypothermic conditions. The concentration of oxidized glutathione was lower in WT cells in hypothermia conditions.

Hypothermic treatment of HeLa cells lead to an increase in tGSH (Figure 22). There was no effect on the concentration of GSSG.

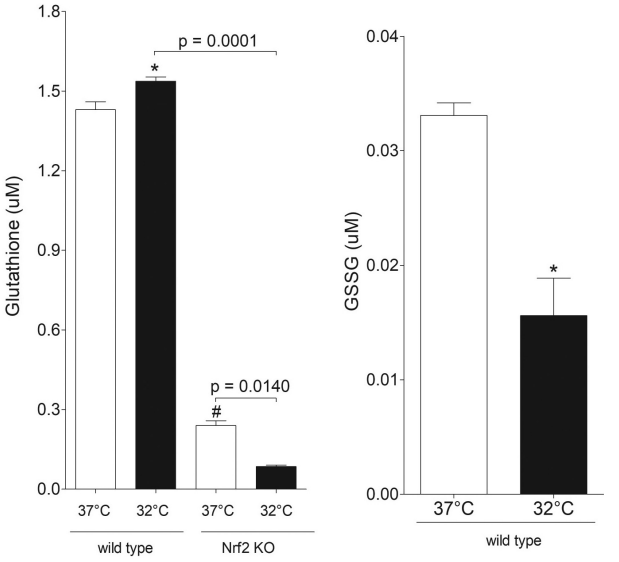


Figure 21. Effect of hypothermia on total cellular glutathione and glutathione disulfide (GSSG) levels in wild-type and Nrf2-deficient mouse embryonic fibroblasts. Mean values (+SEM) are shown. Statistical analysis was performed with Student's *t*-test. * $p<0.05$, # $p<0.0001$. (Eskla et al. 2018)

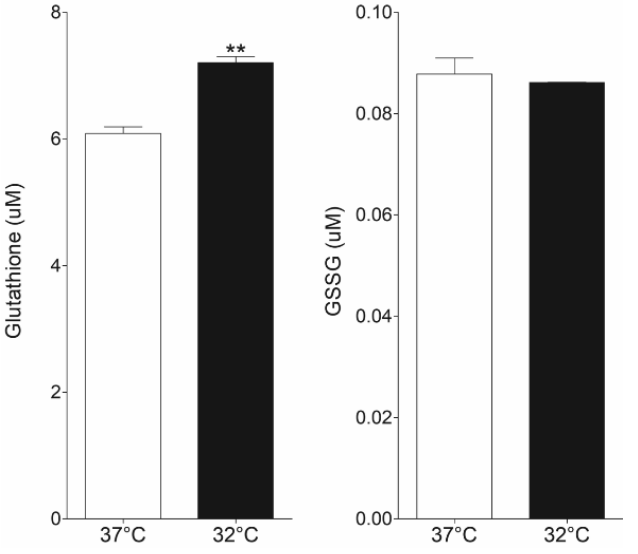


Figure 22. Effect of hypothermia (24 h) on total cellular glutathione and glutathione disulphide (GSSG) levels in HeLa cells. Mean values (+SEM) are shown. Statistical analysis was performed with Student´s *t*-test. ** $p<0.01$. (Eskla et al. 2018)

6. DISCUSSION

The pathology of Wolfram syndrome 1 is tightly related to endoplasmic reticulum stress, which mediates the upregulation of oxidative stress. The therapeutic mechanism of hypothermia lack widely accepted explanations, but it is known that it somehow leads to decreased status of oxidative stress. Therefore the molecular metabolic studies were carried out to describe the stress status in both models. Glutathione is the most abundant low-molecular-weight antioxidant peptide found in all tissues and functions to mitigate the harmful effects of oxidative compounds and the glutathione system is one of the main indicators of oxidative stress.

In the present study we firstly described the overall metabolism, including energetics and certain antioxidants of *Wfs1*-deficient mouse and continued with more specific characterization of the glutathione system of the mouse model.

Subsequently, we evaluated the effect of the administration of antioxidative peptides to reduce the stress in *Wfs1*-deficient mouse. And finally conducted a study to gather a knowledge about the hypothermia-induced stress response onto the different cell lines.

6.1. Untargeted metabolomics

Statistical analysis on metabolic profiles revealed that at 2 months of age there are relatively few statistically significantly different signals between WT and HZ while for both the number is high if compared with KO. At 6 month of age KO and WT have the highest number of different signals. Thus PCA and ANOVA both imply that KO and WT are different at both ages and HZ profile changes from WT-like profile towards KO-like profile over time. Due to the gene-dose effect HZ animals are expected to have intact wolframin, although at lower quantities than in WT mice (Punapart et al. 2014). Thus, the shift from WT-like profile towards KO profile is expected. The 2-month old KO mice have not yet fully developed the clinical WS or diabetes symptoms (Luuk et al. 2009). Our results show that, at the metabolome level, they already have significant disturbances in all investigated tissues.

Statistically significant changes were found in all tissues and the results in Table 1 and Figure 7 may initially seem controversial. However, in PCA the amplitude of signals determines the profile shape, thus the signals with higher absolute values are likely to get higher factor loadings. This is not the case for ANOVA and putting both analyses together we can conclude that liver and pancreas have more statistically significant changes among the high intensity, profile determining signals, and most of the statistical changes in blood and urine are of minor components.

Both PCA and ANOVA point to liver and pancreas as the organs with the most changed metabolism. Pancreas is indeed a tissue with high *Wfs1* expression (Inoue et al. 1998). Liver, however, is expected to have lower expression than

heart or kidney (Inoue et al. 1998). The high number of changes in liver may suggest its role in countering the systemic metabolic alterations. At younger age the liver keeps, maybe on its own expense, blood metabolome unchanged and therefore alterations in other tissues relatively low. Yet, surprisingly, liver has its metabolic profile nearly identical in all genotypes at older age, which contradicts a hypothesis for a permanent damage or metabolic reprogramming due to the observed early life events. Pancreas, on the other hand, degenerates over time.

6.2. Targeted metabolomics of *Wfs1*-deficient mice

6.2.1. Glucose utilization

With the development of diabetes in WS, the energy metabolism was expected to be severely affected, particularly in older animals. Indeed, at 6 month of age KO had the highest blood hexose levels (Figure 8). The lack of hyperglycemia at young age has been reported before (Ishihara 2004; Sedman et al. 2016), but interestingly despite normoglycemia the hexoses in urine were increased in KO animals. Hence, the kidneys leaked glucose, which may imply to decreased glucose reabsorption. In classical diabetic nephropathy the non-enzymatic glycosylation products from continuously high blood glucose are believed to cause glomerular basement membrane thickening leading to glomerular dysfunction. In humans the WS renal biopsy has been found compatible with diabetic nephropathy (Sumboonnanonda et al. 1997). With WS and hyperglycemia progression it may be that the initial tubular dysfunction is at later disease stages overshadowed by glomerular dysfunction explaining the contradiction between our results and the histological finding. Most importantly, the presence of early tubular dysfunction would give glycosuria a diagnostically different meaning than in diabetes, as in WS it would be an early marker while in diabetes it is a marker for late complication.

Lactate is the product of anaerobic glycolysis in most tissues and a source for gluconeogenesis in liver. The observed pattern where lactate level increases in insulin sensitive tissues of young KO animals and in older HZ mice (Figure 8) is in good accordance with the hypothesis that HZ genotype has the disease progression delayed. The heart of older HZ genotype may have its lactate in normal range due to decreased phosphohexose levels, which disallows further intensification of glycolysis and lactate rise. The insulin deficiency and intolerance might deny glucose use in older KO animals, and without glucose the lactate level normalizes again in KO genotype. In cardiac muscle the lactate increase was statistically not significant, but at the same time there was a significant increase of Ala levels. In muscles with high protein turnover and nitrogen excess, the pyruvate formed in glycolysis is converted to Ala via transamination. Increased lactate and Ala imply that glycolysis has intensified in KO mice heart.

6.2.2. Other energy sources

Lipids can be used for energy production directly in beta-oxidation. Additionally, lipids and amino acids can be used by the liver to produce ketone bodies, glucose or both in parallel. Acyl- and free carnitines reflect the availability of beta-oxidation substrates. Two to five carbon acyl residues depend on amino acid and ketone body utilization, but longer chains originate directly from fatty acids.

The levels of acylcarnitines suggest that availability of long chain acyl-carnitines in tissues has increased or remained at the WT level in *Wfs1*-deficiency, and the short-chain acyl and free carnitine imply that there is no stress on the carnitine shuttle. The initiation of insulin deficiency may lead to increased lipolysis, which increases availability of non-esterified fatty acids. In KO and WT comparison at young age pancreas and heart had increased triglyceride levels (Figure 9). At 6 months of age, a significant lipolysis had occurred in both tissues. At the same time liver had increased levels of di- and triglycerides or steroids. Increased lipolysis and decreased peripheral fat amount is further supported by the reportedly low leptin levels in *Wfs1*-deficient mice at 3 months of age (Noormets et al. 2014).

The branched chain amino acids (BCAAs) are also particularly valuable for covering energetic needs. Leu+Ile and Val were among of the most significant differences between the genotypes and also the few amino acids with a significant decrease in the liver of 2 month old KO mice. The striking difference between liver and extrahepatic tissues suggests that the use of BCAAs may be a more specific process than energy extraction. Interestingly, a very recent paper with branched-chain aminotransferase over-expression mouse model reported a liver specific decrease of BCAAs and development of glucose intolerance (Ananieva et al. 2017). It is unknown whether the mitochondrial branched-chain aminotransferase is somehow affected by WS or the observed effect on liver BCAAs can develop as a result of impaired glucose maintenance.

The few and small changes in the levels of ketone bodies leads us to conclude that the animals had no clinically significant ketosis. Only a minority of WS patients present with ketosis (Kinsley et al. 1995), thus this finding is in accordance with WS in humans.

6.2.3 Protein metabolism

As mentioned above in conjunction with glycolysis, Ala was significantly increased in the young KO heart (Figure 23). In liver, the place where Ala should be recycled to pyruvate and ammonia converted to urea, its level was not genotype dependent. From the urea cycle intermediates in the liver only citrulline did not show a tendency to decrease, which may be due to excessive ammonia and carbamoylphosphate, which keep the citrulline level up. Deficiency of oxaloacetate, possibly due to its use in gluconeogenesis limits available aspartate

for citrulline conversion to argininosuccinate. With urea also not significantly increased, accumulation of ammonia would remain the most plausible destination for excessive nitrogen. Unfortunately, free ammonia could not be measured reliably. From the measured amino acids only homocysteine and Orn showed no increase in the cardiac muscle of KO animals. Except for Orn, and the aromatic amino acids (Trp, Phe, Tyr and also His) young HZ mice had a similar increase of free amino acids in the heart. In a possibly related note, hippuric acid, one of the end products of Phe metabolism was increasingly excreted into urine in KO but not in HZ animals. Another tissue with global tendency of increased free amino acids was the pancreas. Here, however, was HZ not KO the main outstanding genotype. Two possible scenarios can explain the anomalous HZ pancreas. First is that the increase reflects a pathology, such as protein turnover increase due to improper folding in ER and HZ are developing it faster than KO. Tissue specific compensatory mechanisms for wolframin deficiency would be required for such effect. The second possibility is that both KO and HZ are affected, but due to different rates in KO a decrease in total protein, not an increase in free amino acids, is observed.

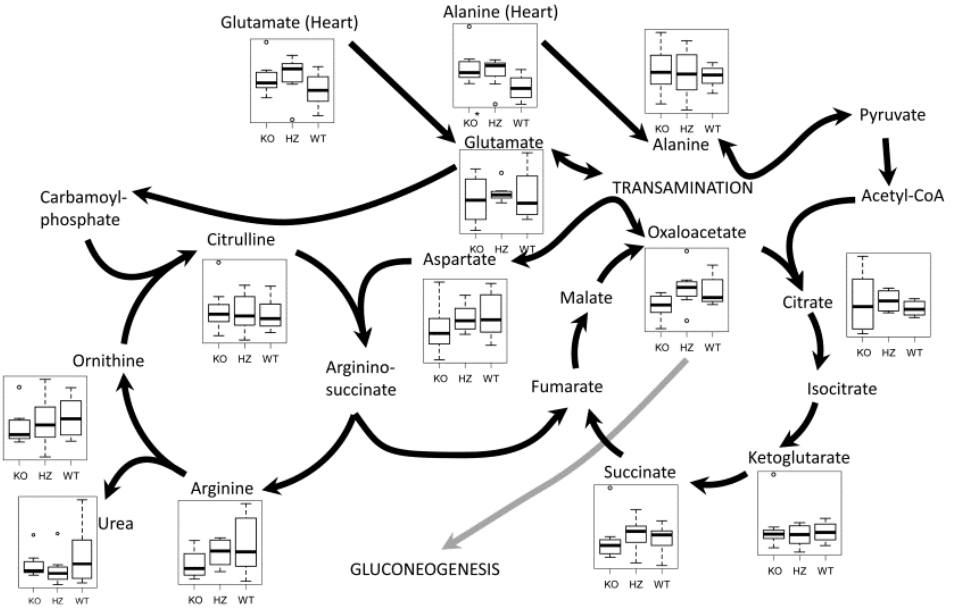


Figure 23. The citric acid cycle and nitrogen metabolism in *Wfs1*-deficient (KO – knock-out, HZ – heterozygous, WT – wild-type) mice. The nitrogen from peripheral tissues is transported to liver in form of alanine, glutamine and glutamate mainly. From glutamine/glutamate in liver ammonia is released and bound to carbamoylphosphate. Via transamination glutamate and alanine are closely related to the citric acid cycle and the latter is also related to the urea cycle where nitrogen is processed further to generate urea. (Porosk et al. 2017)

Significant changes in Pro and hydroxy-Pro levels were found. Pro itself has been shown to be required for ER stress tolerance in yeast (Liang et al. 2014). Therefore, higher incorporation of Pro into proteins and respective decrease in the pool of free Pro, may partly be a countermeasure to ER stress. Pro hydroxylation is a major post-translational modification for collagen. The process normally occurs in ER and is vital for collagen stability. Other proteins contribute minimally to hydroxy-Pro pool and it has therefore been suggested to reflect collagen amount or turnover (Neuman & Logan 1950). The mouse model used in this study is previously characterized by growth retardation despite increased growth hormone levels (Kõks et al. 2009). With ER stress being a molecular pathophysiology of WS the abnormal production of collagen and connective tissues may be the reason for limited growth and recompensatory increase in growth hormone. In relation to sports and recovery from traumas the role of growth hormone on protein synthesis has been studied and collagen been found to be a major product for growth hormone stimulation (Doessing et al. 2010). Excretion with urine is likely due to renal dysfunction (Selby et al. 1995).

6.3. Oxidative stress and glutathione system

6.3.1. Uric acid and cysteine metabolism

Purine and pyrimidine metabolism is most directly linked via nucleic acids and nucleotide pools. Varying responses in different tissues and involvement of both purines and pyrimidines may relate to different cellular proliferation in tissues. Kidney and liver having a higher rate of cell proliferation at young age causes nucleotide deficiency. The cardiomyocytes not undergoing proliferation, on the other hand, accumulate nucleosides and nucleobases.

Uric acid is a key intermediate in purine catabolism and it was non-significantly decreased in trunk blood and increased in urine at young age, suggesting a potential renal loss of uric acid in a similar way as noted for glucose. Decreased hypoxanthine and inosine indicates overproduction of uric acid not matching the purine catabolism. Both steps in hypoxanthine to uric acid conversion are performed by xanthine oxidase. This enzyme has in numerous studies linked to oxidative stress via superoxide generation, and may be relevant for diabetes development (Desco et al. 2002; Romagnoli et al. 2010). Evolutionarily uric and ascorbic acids have been suggested to have an overlapping role in antioxidative defence (Benzie 2000). The ascorbic acid level did not correlate with hypo- or hyperuricemia in *Wfs1*-deficiency undermining the potential uric acid and oxidative stress relationship hypothesis for WS.

The homocysteine, Cys and Met balance is also interesting because of thiol group redox sensitivity, their association with methylation reactions needed for nucleobase synthesis and homocysteine is also known to be an independent ER stress causing factor (Yu et al. 2013). KO mice had homocysteine decreased in the pancreas at young age, but other than that neither homocysteine nor Cys or

Met levels were disturbed by genotype. Therefore, homocysteine itself as ER stress factor, or Met and homocysteine involving methylation reaction are unlikely to have a significant impact to WS pathophysiology. In diabetes patients homocysteine levels have shown controversial values (Meigs et al. 2001; Smulders et al. 1999; Mazza et al. 2005), so the results are not contradicting diabetes pathogenesis either.

6.3.2. Glutathione system

Oxidative stress is strongly associated with ER stress. ER and oxidative stress reduce the GSH capacity and induce the synthesis of reduced GSH (Zeeshan et al. 2016; Harding et al. 2003). The present study examined the GSH system in *Wfs1*-deficient mice to characterize the extent of oxidative stress in several tissues under chronic ER stress. The results indicated that the GSH system was not identical in all tissues of *Wfs1*-deficient mice. It is particularly complex for the heterozygous mice, in which the tGSH levels may be up- or downregulated depending on tissue type and age.

One of the highest expression levels of *Wfs1* is found in heart tissue (Strom et al. 1998). In this research, *Wfs1* deficiency also exhibited the greatest effects on the GSH system in the heart. At 2-months old, when the disease has not yet fully manifested its clinical symptoms (Strom et al. 1998; Hofmann et al. 2003; Fonseca et al. 2005), the levels of both GSH and GSSG were slightly decreased in KO mice, but the GSSG/GSH ratio was increased, though not significantly. The administration of UPF peptides increased the concentration of GSH and decreased the GSSG/GSH in the heart tissue of younger WT and HZ mice. No positive effects on the glutathione system were found regarding to older littermates. This shows that the administration of UPF peptides reduce the GSSG/GSH only in the younger mice as the disease is not yet prolonged in 2-months old mice.

Enzyme levels favor the change in the ratio with a tendency of increased GPx activity and reduced GR activity. As the disease progresses, the changes become more significant. A previous GPx-1-KO study has suggested that GPx may have anti-ER stress effects (Geraghty et al. 2016), and therefore its upregulation may also be part of the UPR (Eletto et al. 2014). In addition to ER stress response, particularly at the older age (6 months), the GSH system may be altered by complications of systemic WS manifestations such as diabetes. In streptozotocin-induced diabetes, a decrease of GR activity in heart has been reported (Li et al. 2007).

The pancreas is another organ with high levels of *Wfs1* expression. Its exocrine and endocrine functions require active synthesis of proteins, and makes the pancreas particularly susceptible to the effects of *Wfs1* deficiency and UPR. Pancreatic β-cells have low levels of antioxidant enzyme expression and activity, including superoxide dismutases, catalase and GPx (Tiedge et al. 1997). By contrast, the catalytic subunit of γ-glutamylcysteine ligase, which is the rate-limiting enzyme for GSH biosynthesis, is highly expressed in pancreatic islet

cells (Tran et al. 2004). The inability to properly process insulin is a key event in WS pathophysiology and the development of diabetes is triggered by the deficiency of insulin (Shang et al. 2014). In the present study, whole pancreas tissue was examined, making the results more relevant for the larger exocrine function. With no identified significant changes in tGSH levels and with GSSG under the detection limit, oxidative stress is seemingly well controlled.

Liver has lower *Wfs1* expression compared with heart or pancreas (Inoue et al. 1998). The liver serves a major role in the regulation of carbohydrate metabolism, such as maintaining the blood glucose level and homeostasis in general. An entire spectrum of liver diseases have been associated with type 2 diabetes, including abnormal liver enzymes, nonalcoholic fatty liver disease, cirrhosis, hepatocellular carcinoma and acute liver failure (Tolman et al. 2007). A decrease in GSH levels in the diabetic liver and remarkable increment of GSSG/GSH ratio have been reported previously (Furfaro et al. 2012). In the present study, 2-month-old *Wfs1*-KO mice at young age had GSH expression similar to WT expression levels. At 2 months of age hyperglycemia and diabetes has not yet manifested itself in mouse models (Ishihara et al. 2004; K Noormets et al. 2011), therefore changes from diabetic complications are not expected. At 6 months of age, however, the expected decrease was observed. A small GSH increase at young age could be expected in response to ER stress (Cullinan et al. 2003; Harding et al. 2003), which at that time is not overwhelming the compensatory mechanisms. It may be considered that the heart has either stronger stress owing to higher dependency on *Wfs1* or its compensatory mechanisms are weaker, which may lead to a tendency of GSH reduction even early on. The administration of antioxidative peptides in the liver increased the concentration of GSH in older mice and reduced the concentration of GSSG and GSH in younger littermates. This leads to the reduced GSSG/GSH and improved antioxidative status in both age groups.

Similar to the liver, kidneys express *Wfs1* at low levels. A commonly observed complication of WS and type 2 diabetes is diabetic nephropathy, which is a frequent cause of mortality in diabetic patients (Ibrahim & Hostetter 1997). It has been postulated that oxidative stress may be a key component in the development of nephropathy (Kashihara et al. 2010). Chronic exposure to high levels of glucose leads to a decrease in GPx activity in vascular endothelial and kidney cells (Urata et al. 1996; Catherwood et al. 2002). It has been demonstrated that exposure to oxidative stress inducers such as carbon tetrachloride increases GPx activity in rat kidneys (Szymonik-Lesiuk et al. 2003). Rats treated with ethanol exhibited increases in both GPx and GR activity in kidneys (Jurczuk et al. 2006). High glucose concentration has been reported to decrease γ-glutamylcysteine ligase expression and GSH levels in mesangial cell culture (Catherwood et al. 2002). Therefore increased GPx activity indicates an increased rate of GSH usage and the depletion of tGSH pool as seen in the present results. The administration of antioxidative peptides (NAC and UPF peptides) seem not to improve tGSH level.

In conclusion, the concentration of GSH was generally decreased in KO *Wfs1*-deficient mice. A slight, but not statistically significant increase was seen in liver at young age. In HZ mice, statistically significant or minimal increases of tGSH were observed in the kidneys and pancreatic tissue at older age. The upregulation of GSH in the liver of 2-month old KO mice is probably an attempt to control ER stress and depends on the expected expression of *Wfs1*.

The activity of the two main GSH redox enzymes, GPx and GR, also suggest that the early and late liver tissues are experiencing different situations. Early on, GPx activity remains unchanged in KO mice compared with 'healthy' WT, although GR activity is reduced. At 6 months old, GR activity in KO mice returns to similar levels as WT (or even surpasses the WT activity in HZ), but GPx activity was increased. The administration of studied antioxidants mainly reduced the activity of GR and GPx in older mice and inversely in the younger littermates.

The intraperitoneal administration of antioxidants seem to improve the glutathione status only in the liver and heart tissue of studied mice.

6.4. Hypothermia

The concentration of total glutathione was significantly higher in wild-type cells after 24h of hypothermia when compared to normothermia and the opposite pattern was seen in Nrf2 KO cells, where tGSH was lower in hypothermic conditions. This indicates that the activation of Nrf2 is required for the increase of GSH in hypothermic cells. The concentration of oxidized glutathione was lower in WT cells suggesting that hypothermia lowers the oxidative stress. GSSG was undetectable in Nrf2 KO cells probably due to low level of tGSH.

Hypothermic treatment of HeLa cells lead to the increase of tGSH. There was no effect on the concentration of GSSG, which once again suggests that there is no increase in oxidative stress.

In sum, we found evidence for increased levels of total glutathione and reduced oxidative stress after hypothermic pre-incubation.

6.5. The models of endoplasmic reticulum and oxidative stress

Hypothermia upregulated the expression of GCLc gene and the concentration of reduced glutathione both in MEFs and HeLa cells (Paper III) (Eskla et al. 2018)(Eskla et al. 2018) In addition, hypothermia temporarily activated Nrf2 transcription factor, which is a master downstream regulator of antioxidant response system. To add with, we have previously seen that the incubation of different cell lines with UPF peptides increases the expression of both catalytic (GCLc) and modifying (GCLm) subunit of GCL as well as the level of Nrf2 and

the concentration of GSH (unpublished data). These different oxidative stress models seem to be correlating and lead to the activation of Nrf2 transcription factor, up-regulation of Nrf2 target genes and GSH.

On the other point of view, hypothermia had negligible impact on the activity of ER-stress. No increase in the activity of endoplasmic reticulum stress response element reporter nor in the level of spliced isoform of X-box binding protein 1 (XBP1) was found in response to hypothermia (Paper III).

In conclusion, hypothermia seems to be more preferable model for studying oxidative stress as the pathology of Wolfram syndrome 1 corresponds to both upregulated ER and oxidative stress.

7. SUMMARY AND CONCLUSIONS

The metabolomic characterization of *Wfs1*-deficient mice revealed a broad spectrum of metabolic complications and affected glutathione redox status in the knock-out mice. Wolfram syndrome 1 is systemic and affects all organs (kidneys, liver, pancreas and heart) and bodily fluids (urine, blood) studied. The glutathione system in the heart, kidneys, liver and pancreatic tissues of *Wfs1*-deficient mice was characterized before and after the administration of antioxidants (N-acetyl-L-cysteine, UPF1 and UPF17), which improved the glutathione status in *Wfs1*-deficient mice. Additionally the glutathione redox status was analyzed under hypothermia conditions. Hypothermia reduced the oxidative stress status in different cell lines.

The results suggest the following conclusions:

1. Global profiling revealed that at 2 months of age the metabolism of WT and HZ mice is very similar, but the metabolism of KO mice is already affected by the disease. At 6 months of age the difference is even more striking.
   Targeted metabolomics approach revealed that at the whole organism level, the glucose use, gluconeogenesis and anaerobic glycolysis appear to be increased in the early stages of the disease, but later the energy demand is satisfied by intensified lipolysis. Furthermore, in the blood and liver tissue of KO mice, the progression of the WS proceeds from hypouricemia into hyperuricemia. Other low-molecular-weight antioxidants measured were inconclusive about oxidative stress in Wolfram syndrome.
   In conclusion, the metabolic profile of several tissues of *Wfs1*-deficient mouse model was thoroughly characterized by the metabolomics approach, which is essential for future research of WS.
2. The concentration of GSH was generally decreased in KO *Wfs1*-deficient mice, but a slight upregulation of GSH in the liver is probably an attempt to control ER stress. In the liver and heart the activity of glutathione peroxidase was increased and the activity of glutathione reductase was decreased in KO mice compared to WT littermates. In the kidneys KO mice, the activity of both enzymes increased. The results suggest that the glutathione system (GSH and related enzymes) has a distinct outcome in all studied tissues and confirms higher oxidative stress status in *Wfs1*-deficient mice.
3. The antioxidants had the highest effect improving the glutathione status in the liver and heart tissue of *Wfs1*-deficient mice. In the liver tissue, the studied antioxidants mainly reduced the activity of GR and GPx in older mice and inversely in the younger littermates. The UPF peptides could be potential antioxidants for reducing oxidative stress. (Unpublished data)
4. Hypothermia induced the highest level of total glutathione in wild-type mouse embryonic fibroblasts and HeLa cells, whereas the concentration of oxidized glutathione was decreased or remained unchanged, respectively. This suggests that there is no increase in oxidative stress level and the antioxidative defense system is upregulated.

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SUMMARY IN ESTONIAN

Oksüdatiivse stressi roll Wolframi sündroom 1 ja hüpotermia korral

Sissejuhatus

Wolframi sündroom on haruldane autosomaalne retsessiivne haigus, mida iseloomustavad juveniilne diabeet (magediabeet, tüüp I suhkrudiabeet), nägemisnärvi kahjustus, kuulmishäired, progressiivne neurodegeneratsioon, endokriinsed kahjustused ja psühhiaatrilised probleemid. Wolframi sündroom on põhjustatud mõlemas alleelis esinevatest mutatsioonidest *WFS1* geenis, mille tõttu geeni produkt, wolframiin, ei oma enam tavapärast funktsiooni. Mutantse wolframiini puhul kuhjuvad voltumata valgud endoplasmaatilise retiikulumi luumenisse ning põhjustavad endoplasmaatilise retiikulumi stressi (ka oksüdatiivset stressi) ja sealse homoöstaasi häirumist ja apoptootilise raja käivitumist.

Metaboloomika on kiirelt arenev teadusharu, mis koos genoomika ja transkriptoomikaga võimaldab saada paremat ülevaadet organismis toimuvatest protsessidest. Metaboloomika keskendub organismi rakkudes, biovedelikes ja kudedes leiduvate madalmolekulaarsete ühendite ehk metaboliitide identifitseerimisele ja nende kontsentratsioonide kvantifitseerimisele. Antud metoodika abil on võimalik teada saada, milliste metaboliitide osakaal on muutunud võrreldes terve organismiga ja rakendada seda infot haiguste varajaste biomarkerite kindlakstegemiseks, haiguste diagnoosimiseks ning ravi kulu jälgimiseks. Uuritavate ühendite gruppi kuuluvad kõik madalmolekulaarsed struktuursed ehitusüksused, signalisatsioonielemendid ja metabolismi vaheühendid nagu nukleotiidid, aminohapped, suhkrud, vitamiinid, rasvhapped, lipiidid ning samuti ravimid ja nende metaboolsed derivaadid.

Eesmärgid

Antud töö peaeesmärgiks oli kirjeldada *Wfs1*-defektiga hiire metaboloomi ning oksüdatiivse stressi taset erinevates kudedes (maks, süda, neerud ja pankreas) ja biovedelikes (veri ja uriin) enne ja pärast antioksüdantide (N-atsetüül-L-tsüsteiin ja UPF peptiidid) manustamist. Lisaks analüüsida missugune on hüpotermia mõju glutatiooni süsteemile erinevates rakuliinides.

Täpsemad eesmärgid:

1. Kirjeldada *Wfs1*-defektiga hiire metaboolset profiili erinevates kudedest ja biovedelikest.
2. Kirjeldada terviklikku glutatiooni süsteemi arvestades ensüümide glutatiooni peroksüdaasi ja glutatiooni reduktaasi aktiivsuse muutust *Wfs1*-puudulikkusega hiirtel.
3. Analüüsida manustatud antioksüdatiivsete ühendite mõju glutatiooni süsteemile erinevates kudedes.
4. Kirjeldada kuidas hüpotermia mõjutab glutatiooni taset hiire embrüonaalsetes fibroblastides ja HeLa rakkudes.

Materjal ja metoodika

Uurimistöö läbiviimiseks kasutati isaseid *Wfs1*-puudulikkusega homosügootseid (KO), heterosügootseid (HZ) ja metsiktüüpi (WT) 2 ja 6 kuu vanuseid hiiri, kellele manustati 5 päeva intraperitoneaalselt antioksüdante 0,1 mg/kg (N-atsetüül-L-tsüsteiin ja UPF peptiidid). *Wfs1*-puudulikkusega hiir on hea mudel uurimaks Wolframi sündroomiga, endoplasmaatilise retiikulumi häiretega seotud patoloogiate ja ka üldiselt diabeeti, sest Wolframi sündroomi korral on diabeet põhjustatud ainult ühe geeni (*Wfs1*) defektist, mitte multifaktoriaalsetest põhjustest nagu enamasti.

Eksperimentaalses osas kasutati glutatiooni süsteemi uurimiseks laialdaselt kasutatud ja standardiseeritud spektrofotomeetrilisi test-komplekte. Enamik metaboloomilisi mõõtmisi viidi läbi mass-spektromeetriliselt, kasutades vedelik-kromatograaf tandem mass-spektromeetrit QTRAP 3200. Kogu eksperiment oli kooskõlas Euroopa Komisjoni direktiivi (86/609/EEC) ja Eesti loomkatse läbiviimise loakomisjoni loaga (nr. 36).

Tulemused ja arutelu

Wfs1-puudulikkusega hiire metaboolsel profileerimisel selgusid mitmed aspektid, mis viitasid kudedes esinevatele Wolframi sündroomi komplikatsioonidele, kaasa arvatud glutatiooni süsteemi muutustele. Glutatiooni süsteem oli muutunud ka hüpotermia tingimustes. Täpsemalt:

1. Wolframi sündroom on süsteemne haigus ning suunamata metaboloomilisel analüüsil selgus, et arvestades muutusi kõikides kudedes, erinevad nii 2 kui ka 6 kuu vanused KO hiired märgatavalt võrreldes HZ ja WT hiirtega. Suunatud analüüs näitas, et nooremad KO hiired kasutavad energia saamiseks eelkõige glükoosi, glükoneogeneesi ja anaeroobset glükolüüsi, kuid hilisemas vanuses kui haigus rohkem progresseerunud, eelistatult lipolüüsi. Lisaks esines noorematel KO hiirtel glükosuuria, mis tüüpiliselt diabeedi varajases staadiumis ei esine.
2. Redutseeritud glutatiooni kontsentratsioon on üldiselt KO hiirte kudedes madalam kui metsiktüüpi liigikaaslastel. Maksakoes täheldati mõningast GSH taseme tõusu noortel hiirtel, mis viitab GSH sünteesi intensiivistumisele stressitingimustes. Antioksüdantse ensüümi glutatiooni peroksüdaasi aktiivsus oli südames ja maksas KO hiirtel kõrgem ja glutatiooni reduktaasi aktiivsus madalam võrreldes WT hiirtega. Neerukoes oli mõlema ensüümi aktiivsus KO hiirtel kõrgem.
3. Antioksüdantite manustamine parandas eelkõige glutatiooni taset südames ja maksakoes ning suurendas vanematel ja vähendas noorematel hiirtel glutatiooni redoksüsteemi ensüümide aktiivsust.
4. Hüpotermia-indutseeritud rakkudes on kõrgem totaalse glutatiooni kontsentratsioon metsik-tüüpi hiire embrüonaalsetes fibroblastides ja HeLa rakkudes, kusjuures esimestes vähenes oksüdeeritud glutatiooni tase ja teises jäi see muutumatuks.

Kokkuvõte

Antud uurimustöös kirjeldati põhjalikult mitme koe tasandil *Wfs1*-puudulikkusega hiirte metabolism Wolframi sündroomi tingimustes ning analüüsiti endoplasmaatilise retiikulumi ja oksüdatiivse stressi taset WS ja ka hüpotermia korral. Lisaks vaadati millist effekti omab antioksüdantide manustamine nende glutatiooni süsteemile ja kuidas hüpotermia mõjutab glutatiooni süsteemi mitmetes rakuliinides.

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PUBLICATIONS

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Formal education:

2013–	*PhD* in Medicine, Department of Biochemistry, Institute of Biomedicine and Translational Medicine, Faculty of medicine, University of Tartu, Estonia. The title of my thesis: "The role of oxidative stress in Wolfram syndrome 1 and hypothermia".
2011–2013	M*Sc* in Biomedicine, Department of Biochemistry, Institute of Biomedicine and Translational Medicine, Faculty of medicine, University of Tartu, Estonia. The title of my thesis: "Metabolic profiling of Wolfram syndrome 1 gene deficient mouse".
2007–2011	*BSc* in Gene technology, Department of Biochemistry, Institute of Biomedicine and Translational Medicine, Faculty of medicine, University of Tartu, Estonia. The title of my thesis: "Design, synthesis and properties of cell- and mitochondria-penetrating UPF peptides".
2004–2007	Hugo Treffner Gymnasium
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Professional experience:

2013–	Specialist of mass-spectrometry, Department of Biochemistry, Institute of Biomedicine and Translational Medicine, Faculty of medicine, University of Tartu.

Teaching and supervising experience:

2013–	Seminars of Biochemistry in the Faculty of Medicine
2011–	Supervisor of 7 undergraduate students in Pharmacy, Medicine and Gene Technology
2013–	Co-author of numerous e-courses in biochemistry

Scientific experience and projects:

PUT1416	Metabolomic biomarkers in a personalized medicine approach
PUT1169	Remote ischaemic preconditioning: from metabolomic phenotyping to clinical applications
ETF7856	Glutathione analogues library as a lead for predrug development and tool for mitochondrial medicine studies

SLOMR13012T	Development of efficient animal reproduction technologies for the sustainable cattle breeding
ETF9167	Mitochondria-penetrating peptides and mitochondrial diseases
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List of publications:

1. Ainelo, Andres; **Porosk, Rando**; Kilk, Kalle; Rosendahl, Sirli; Remme, Jaanus; Hõrak, Rita (2019). Pseudomonas putida responds to the toxin GraT by inducing ribosome biogenesis factors and repressing TCA cycle enzymes. *Toxins.*
2. Nõmm, Monika; **Porosk, Rando**; Pärn, Pille; Kilk, Kalle; Soomets, Ursel; Kõks, Sulev; Jaakma, Ülle (2018). In vitro culture and non-invasive metabolic profiling of single bovine embryos. *Reproduction, Fertility and Development.*
3. Eskla, Kattri-Liis; **Porosk, Rando**; Reimets, Riin; Visnapuu, Tanel; Vasar, Eero; Hundahl, Christian Ansgar; Luuk, Hendrik (2018). Hypothermia augments stress response in mammalian cells. *Free Radical Biology and Medicine.*
4. **Porosk, Rando**; Terasmaa, Anton; Mahlapuu, Riina; Soomets, Ursel; Kilk, Kalle (2017). Metabolomics of Wolfram syndrome 1 gene (*Wfs1*) deficient mice. *OMICS : a Journal of Integrative Biology.*
5. **Porosk, Rando**; Kilk, Kalle; Mahlapuu, Riina; Terasmaa, Anton; Soomets, Ursel (2017). Glutathione system in Wolfram syndrome 1-deficient mice. *Molecular Medicine Reports.*
6. Aug, Argo; Altraja, Siiri; Kilk, Kalle; **Porosk, Rando**; Soomets, Ursel; Altraja, Alan (2015). E-cigarette liquid affects metabolome of primary normal human bronchial epithelial cells. *PloS One.*
7. Meitern, Richard; Sild, Elin; Kilk, Kalle; **Porosk, Rando**; Hõrak, Peeter (2013). On the methodological limitations of detecting oxidative stress: effects of paraquat on measures of oxidative status in greenfinches. *Journal of Experimental Biology.*

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Õpetamine ja juhendamised:

2013 –	Biokeemia seminaride läbiviimine meditsiiniteaduste valdkonnas
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Teadussuunad ja projektid:

PUT1416	Metaboolsed biomarkerid personaliseeritud meditsiinis.
PUT1169	Kaugisheemiline eelkohastumus: metaboolsest fenotüpiseerimisest kliiniliste rakendusteni.
ETF7856	Glutatiooni analoogide sünteesitud „raamatukogu" kui ravimeellaste väljatöötamise lähtepunkt ning kui vahend mitokondriaalse meditsiini uuringuteks.

SLOMR13012T Loomade reproduktiivtehnoloogiate arendamine jätkusuutlikuks tõuaretustööks.

ETF9167 Mitokondrisse penetreeruvad peptiidid ja mitokondriaalhaigused.

IUT20-42 Metaboloomika ja arteriseina uuringute põhine fenotüüpimine (süsteemi bioloogia ja struktuuri- ning talitlusmarkerite kooskasutamine krooniliste haiguste uudsete diagnostiliste platvormide väljatöötamiseks).

Eesti Biokeemia Seltsi liige

Publikatsioonide loetelu:

1. Ainelo, Andres; **Porosk, Rando**; Kilk, Kalle; Rosendahl, Sirli; Remme, Jaanus; Hõrak, Rita (2019). Pseudomonas putida responds to the toxin GraT by inducing ribosome biogenesis factors and repressing TCA cycle enzymes. *Toxins*
2. Nõmm, Monika; **Porosk, Rando**; Pärn, Pille; Kilk, Kalle; Soomets, Ursel; Kõks, Sulev; Jaakma, Ülle (2018). In vitro culture and non-invasive metabolic profiling of single bovine embryos. *Reproduction, Fertility and Development.*
3. Eskla, Kattri-Liis; **Porosk, Rando**; Reimets, Riin; Visnapuu, Tanel; Vasar, Eero; Hundahl, Christian Ansgar; Luuk, Hendrik (2018). Hypothermia augments stress response in mammalian cells. *Free Radical Biology and Medicine.*
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7. Meitern, Richard; Sild, Elin; Kilk, Kalle; **Porosk, Rando**; Hõrak, Peeter (2013). On the methodological limitations of detecting oxidative stress: effects of paraquat on measures of oxidative status in greenfinches. *Journal of Experimental Biology.*

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

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