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The impaired change in plasma long-chain acylcarnitine level as a marker of insulin resistance

Diploma Thesis

Rīga Stradiņš University, Riga
Latvian Institute of Organic Synthesis
Laboratory of Pharmaceutical Pharmacology

Supervisor: Dr. Pharm. Elina Makarova

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ANNOUNCEMENT

I declare that this diploma thesis is my own, original, personal work. All literature and other resources, I used while processing, are listed in the literature list and are properly cited. The diploma thesis was not used to obtain different or the same title.

In …… date ……. …….

Petra Šišmová

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Inzulínová rezistence představuje jeden z faktorů, které by mohly vést k rozvoji diabetu mellitu 2. typu (T2DM). Zvýšené hladiny acylkarnitinů s dlouhým řetězcem (LC-acylkarnitiny) jsou spojeny s obezitou a T2DM a nedávné studie naznačují, že LC-acylkarnitiny mohou hrát roli ve vývoji inzulínové rezistence. Včasná diagnóza by mohla pomoci předcházet nástupu T2DM a vzniku souvisejících komplikací.

Cílem této studie bylo zjistit, zda-li by měření změn koncentrace acylkarnitinů během glukózo tolerančního testu (GTT) mohlo být použito jako nový marker inzulínové rezistence. Po intraperitoneálním podání glukózy C57bl/6N myším, krmených stravou s vysokým obsahem tuku (HFD), db/db myším a příslušným kontrolám, byly změřeny hladiny glukózy, inzulínu, glykovaného hemoglobinu, koncentrace volných mastných kyselin (FFAs) a acylkarnitinů, a to ve stavu nalačno a následně 1 a 2 hodiny po GTT. Využití glukózy bylo stanoveno měřením rychlosti příjmu 2-[1,2-^{3}H]-deoxy-D-glukózy ([^{3}H]-DOG) v tkáních citlivých na inzulín. Měření biochemických parametrů a příjmu [^{3}H]-DOG v tkáních ukázalo, že HFD myši představují model inzulínové rezistence v počátečním stádiu, zatímco db/db myši vyvíjejí diabetes 2. typu se závažným poškozením metabolismu glukózy. Pokles plazmatických koncentrací LC-acylkarnitinů během GTT u kontrolních myší byl vyšší ve srovnání s myšmi s inzulínovou rezistencí. Kromě toho, u db/db myší nedošlo během GTT k žádné změně plazmatických LC-acylkarnitinů. Redukce hladiny LC-acylkarnitinů by tedy mohla být považována za diagnostický marker inzulínové rezistence ve svalech.

Klíčová slova: diabetes 2. typu**,** inzulínová rezistence, acylkarnitiny

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Insulin resistance presents one of the factors that could lead to type 2 diabetes mellitus (T2DM). Increased levels of long-chain (LC)-acylcarnitines are associated with obesity and T2DM and recent studies suggest that LC-acylcarnitines could play a role in the development of insulin resistance. Early diagnosis of insulin resistance would help to prevent the onset of T2DM and the development of associated complications.

The purpose of this study was to determine whether the measurements of the changes in acylcarnitine concentrations during the glucose tolerance test (GTT) could be used as a novel marker of insulin resistance. After intraperitoneal administration of glucose in high fat diet (HFD)-fed C57bl/6N, db/db mice and respective controls the concentrations of glucose, insulin, glycated haemoglobin, free fatty acids (FFAs) and acylcarnitine levels in the fasted state and subsequently at 1 and 2 hours after GTT were measured. Utilization of glucose was determined by the measurement of the rate of 2-[1,2-^{3}H]-deoxy-D-glucose ([^{3}H]-DOG) uptake in insulin-sensitive tissues. Measurements of biochemical parameters and [^{3}H]-DOG uptake in tissues demonstrated that HFD mice represent a model of early stage insulin resistance, while db/db mice develop type 2 diabetes with severely impaired glucose metabolism. The decrease of plasma LC-acylcarnitine levels during the GTT in control mice was higher in comparison to mice with insulin resistance. Moreover, in db/db mice there was no change in plasma LC-acylcarnitines during GTT. Thus, the impaired change in LC-acylcarnitine concentration could be considered as a diagnostic marker of muscle-specific insulin resistance.

Keywords: type 2 diabetes, insulin resistance, acylcarnitines

CONTENTS

LIST OF ABBREVIATIONS

AA	*amino acid*
Acyl-CoA	*acyl-coenzyme A*
ACS	*acyl-CoA synthetase*
CPT	*carnitine palmitoyltransferase*
CACT	*carnitine/acylcarnitine translocase*
C57bl/6N	*mice strain, experimental model for diet-induced obesity and insulin resistance*
db/db	*mice strain, experimental model for obesity and type 2 diabetes*
db/L	*mice strain, control for db/db mice*
DM	*diabetes mellitus*
FFA	*free fatty acids*
GLUT	*glucose transporter*
GTT	*glucose tolerance test*
HbA1c	*glycated haemoglobin*
[^{3}H]-DOG	*2-[1,2-^{3}H]-deoxy-D-glucose*
HEGC	*hyperinsulinemic-euglycemic glucose clamp*
HFD	*high fat diet*
LC-FA	*long-chain fatty acids*
LC-acylcarnitines	*long-chain acylcarnitines*
Malonyl-CoA	*malonyl-coenzyme A*
MC-acylcarnitines	*medium-chain acylcarnitines*
SC-acylcarnitines	*short-chain acylcarnitines*
T2DM	*type 2 diabetes mellitus*
UPLC MS/MS	*ultra performance liquid chromatography – tandem mass spectrometer*

UNITS

AU	*arbitrary units*
µCi	*micro curie, a unit of radioactivity; 3.70 × 10^4 disintegrations per second*
Ci/mmol	*specific activity (SA); the amount of radiolabeled mass in a sample, often expressed as Ci/mmol*
cmp/g	*counts per minute (cpm); the output from the scintillation counter, the number of photons detected per unit time and standardized according to dilution of tissue homogenate - to get cpm/g of tissue*
mM	*mmol/L*
ng/ml	*nanogram per millilitre; the concentrations of standards of insulin's ELISA kit*
w/v in MilliQ water	*weight/volume; MilliQ - the name of water purification system*

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1 INTRODUCTION

Diabetes mellitus (DM) belongs to the group of the fastest growing metabolic diseases in the world. Insulin resistance characterized by impaired insulin action and its secretion plays an important role in the development of prediabetes and subsequently type 2 DM (T2DM). Increasing numbers of cardiovascular risks and other complications that are involved in diabetes, encourage searching for novel early detection methods of this disorder. Current diagnostic methods, which are used in clinical practice, mainly concern in measurements of disturbances in metabolism of the glucose and insulin (Melmed *et al.*, 2016).

One of the standard diagnostic methods of DM is the measurement of blood glucose in the fasted state, which reflects the acute state of the organism and is carried out after at least 8 hours of last meal (ADA - Diagnosis, 2012). Fasting blood glucose levels reflect the state of the organism, when the levels of blood glucose and insulin are the lowest, therefore do not display the ability of body to react on glucose intake. To establish the levels of chronic hyperglycaemia, another diagnostic marker is used - glycated haemoglobin (HbA1c). HbA1c describes the long-term fluctuation of blood glucose levels and does not reflect acute glucose changes, therefore it could be also useful to check the efficiency of diet in patients (Rybka, 2007).

Oral glucose tolerance test is frequently used method in diagnosis of DM. The increase of glucose is detected up to 2 hours after glucose administration and shows the exact concentrations of postprandial glucose and thus is considered as the most appropriate method to evaluate glycaemic changes. A disadvantage of this test is that it does not indicate the cause of disturbances in glucose tolerance (dysfunction of β-cells or insulin resistance).

The glucose metabolism disorder in patients with T2DM is related to decreased muscle insulin sensitivity. Hyperlipidaemia and stimulated fatty acid metabolism are suggested to be involved in the development of muscle insulin resistance (Martins *et al.*, 2012). Incomplete glucose metabolism to lactate in muscles, changes in liver gluconeogenesis and activity of adipocytes could mask the changes of glucose metabolism in mitochondria (Abdul-Ghani *et al.*, 2007).

The mechanisms of insulin resistance development are not completely clarified, but according to recent studies, dysregulation of fatty acid oxidation can play an important role in the development of insulin resistance (Martins *et al.*, 2012; Schooneman *et al.*, 2013;

van de Weijer *et al.*, 2013). Free fatty acids (FFA) are activated and converted to acylcarnitines to ensure transport from cytosol into the mitochondria for β-oxidation. Acylcarnitines are composed from free L-carnitine and acyl-coenzym A (acyl-CoA) substituents (Boden *et al.*, 2002). Increased plasma levels of acylcarnitines were found in experimental model of HFD-induced insulin resistance and also in patients with obesity and type 2 diabetes (Koves *et al*., 2008; Mihalik *et al*., 2010; Adams *et al*., 2009; Ramos-Roman *et al.*, 2012). The development of late complications of DM influences an oxidation of glucose and induces mitochondrial dysfunction, which could lead to damage of cardiac tissue and more likely to incidence of other severe cardiovascular diseases (Rolo *et al*., 2006). The synthesis of LC-acylcarnitines is limited by the increase of insulin levels during the fed state (Mihalik *et al.*, 2010). As the accumulation of plasma and muscle tissue LC-acylcarnitine levels in type 2 diabetes experimental model was detected only in fed state (Liepinsh *et al.,* 2016) and the amount of food intake could be highly variable, our experimental study was aimed to measurement of the changes in plasma LC-acylcarnitine concentrations during the fasted-to-postprandial state transition and to evaluate whether this measurement could be used as a marker of muscle-specific insulin resistance.

2 THEORETICAL PART

2.1 TYPE 2 DIABETES MELLITUS

2.1.1 Epidemiology

Type 2 diabetes mellitus (T2DM) also known as non-insulin dependent diabetes, belongs to the group of the most growing diseases in the world. This metabolic disorder represents 90 % of all cases of diabetes and the global prevalence has been increasing every year (Melmed *et al.*, 2016). According to International Diabetes Federation data, the severity and rapidly increasing incidence of this disease shows the fact, that till 2040, T2DM will affect 642 million people all over the world and will become one of the most frequent cause of death. In spite of an extensive development in prevention of T2DM, the number of patients with diabetes, including those who have not been diagnosed yet, is increasing constantly and is showing that global epidemic is still in progression (Melmed *et al.*, 2016). In 2016, The World Health Organisation has published a report on diabetes, summarizing basic information and data of this disease and calling for reduction of T2DM risk factors and for improvement of availability and a qualitative care of patients (WHO report, 2016).

2.1.2 Pathogenesis

The cause of T2DM is a complex of genetic, environmental and behavioural factors, involving eating habits associated with obesity and physical inactivity (Melmed *et al.*, 2016; Stumvoll *et al.,* 2005). Some of the risk factors as genetic predispositions, age or ethnicity are not possible to influence, but in the most of cases an important role plays insufficient prevention and patient's lifestyle. T2DM as a chronic disease characterized by insufficient tissue's insulin sensitivity, also called as insulin resistance, compensatory hyperinsulinemia or relative lack of insulin leads to the body's inability to react on this hormone (Table 1). This disorder is caused by disequilibrium between secretion and effect of insulin on glucose metabolism (Rybka, 2007). A key role in the development of impaired insulin secretion plays an inability of pancreas to produce enough insulin in early phase of insulin release, which gradually declines. Postprandially the amount of insulin increases to maintain the normal levels of blood glucose. Due to delayed secretion, the organism is not able to keep glycaemia in physiological range, resulting

to changes in values of postprandial glycaemia. If the pancreatic function is affected in the long term and cannot provide necessary amount of insulin, growing blood glucose level can lead to the development of hyperglycaemia (Rybka, 2007; Stumvoll *et al*., 2005).

Hyperglycaemia is caused by a combination of a relative lack of insulin and insulin resistance as a result from decreased number of insulin receptors (Melmed *et al.*, 2016). The pathogenesis of T2DM has not been completely elucidated yet, but it is assumed that the transition from normal glucose tolerance to T2DM in genetically susceptible individuals is caused due to insulin resistance, dysregulation of hepatic glucose production, impaired glucose tolerance and progressive depletion of functional capacity of β-cells, that leads to inability of glucose utilization (Melmed *et al.*, 2016; Rybka, 2007). However, insulin resistance itself is not sufficient to cause manifestation of this metabolic disorder (Rybka, 2007). Chronically high levels of insulin limit its own effect, thus maintaining normal glucose concentrations, which means that patients could have insulin resistance, but without manifested DM (Piťhová, 2008).

Table 1: The main differences between type 1 and type 2 DM (Vlček *et al.*, 2010).

	Type 1 diabetes mellitus	**Type 2 diabetes mellitus**
Age	often diagnosed in childhood	usually diagnosed in adults (after age 30)
Nutrition	not associated with excess body weight	excess body weight (obesity)
Prevalence	≈ 10 %	≈ 90 %
Basis	autoimmune inflammation of the pancreatic islets	genetic predispositions, often as a part of metabolic syndrome
Defect	β-cells damage, minimal or no insulin secretion, absolute lack of insulin	decreased target tissue's insulin sensitivity (insulin resistance), relative insulin deficiency
Complications	at microvascular level (neuropathy, nephropathy)	at macrovascular level (atherosclerosis)

Among the other typical signs contributing to direct determination of diagnosis and creating a base of pathogenesis of T2DM, also disturbed glucose stimulated insulin secretion and an increased hepatic glucose production are involved (Melmed *et al.*, 2016).

According to clinical studies, T2DM is associated with many other abnormalities such as acceleration of lipolysis, deficiency of incretin hormone, increased tubular reabsorption in kidneys, excessive secretion of glucagon and an influence of central nervous system in metabolic regulation (Rybka, 2007). Currently, it is assumed that T2DM has polygenic character and is caused by a combination of insulin resistance, abnormal insulin secretion and other factors including genetics and lifestyle (Melmed *et al.*, 2016). Development of T2DM is also connected with accompanying metabolic complications as obesity, hypertension or dyslipidaemia thus increasing a risk of cardiovascular diseases (Melmed *et al.*, 2016). Symptoms of T2DM may be similar to type 1 DM, but they are not so expressed and T2DM may be diagnosed several years after onset, and in the most of cases the disease is only discovered due to developed complications.

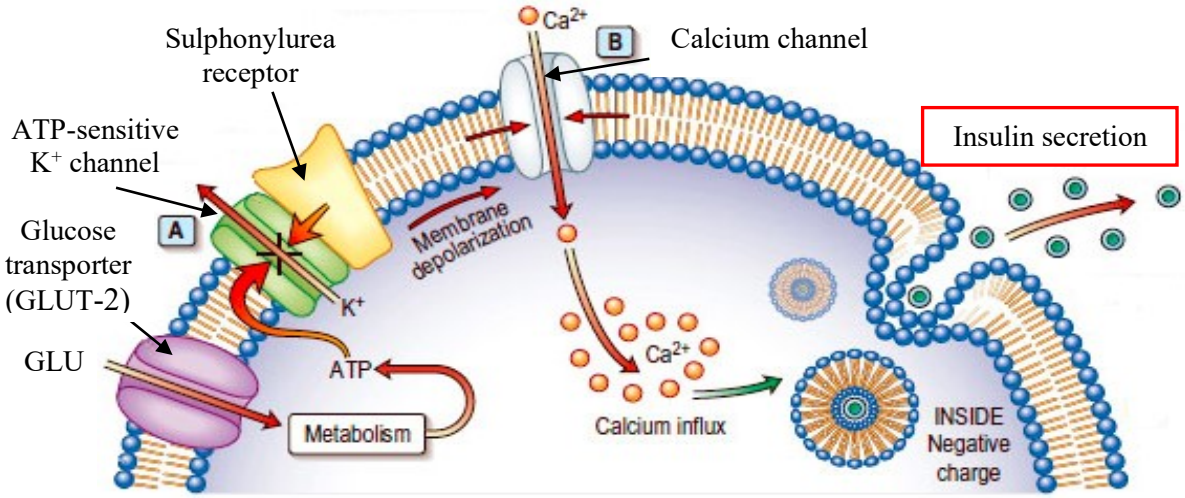
2.1.3 Insulin secretion

Insulin stimulates a signalling cascade, that starts by activation of insulin receptor from a subfamily of tyrosin-kinase receptor, including the insulin-like growth factor (IGF)-I receptor and the insulin-receptor related receptor (IRR). Signal transduction is mediated by binding of a specific ligand to cell surface of the receptor, composed of α and β subunits and after its activation, tyrosine phosphorylation and a conformational change of cellular substrates and receptor occurs, that is responsible for propagation of signalling (Melmed *et al.*, 2016).

Insulin secretion is influenced by multiple factors, but one of the most important is glucose and regulation of its metabolism. Activity of pancreatic β-cells, that are able to sense the changes in plasma glucose concentrations and to react on these changes by corresponding amount of insulin release, is necessary for normal metabolism. β-cells are clustered in islets, that are richly vascularised by a dense network of small blood vessels in order to provide 10-times more blood supply compared to the surrounding tissues. These capillaries contain small pores, called fenestrae that enhance permeability and thus allow a greater nutrient exchange and also rapid insulin diffusion into blood circulation (Cavaghan *et al.*, 2000). Glucose as a principal diet component and an essential source of energy is the primary impulse for insulin secretion. Intake of 75 g of glucose, which is absorbed in blood, will cause an increase in insulin plasma concentration from a basal level 20-30 pmol/L to 250-300 pmol/L during 30 minutes, while a similar amount of fat will increase the insulin concentration to 50-60 pmol/L (Fu *et al.,* 2013). Several

sensors of β-cells can measure circulating glucose and due to its concentration changes regulate insulin secretion. Among them, glucose-transporter 2 (GLUT2) is expressed in β-cells (Figure 1), also in the liver, in renal and intestinal absorptive cells, and GLUT4, that is primarily expressed in muscle and fat cells. Function of GLUT2 is not insulin-dependent and shows a low affinity to substrate, which leads to high glucose influx (Fu *et al.,* 2013). After entering β-cells glucose is phosphorylated by enzyme glucokinase and subtype hexokinase and only the rate of glucokinase activity is a limiting step in β-cell glucose metabolism (Fu *et al.,* 2013).

Figure 1: The mechanism of insulin secretion [online, cit. 2017-02-26] (http://www.medicinehack.com/2011/08/insulin-secretion-local-regulation.html)



Glucose is transported through the membrane by glucose transporter (GLUT). ATP, produced in glucose metabolism, will cause a closure of the adenosine triphosphate (ATP)-sensitive K+ channel (A), which leads to membrane depolarization. Subsequently, the opening of calcium channel occurs (B) and an increased intracellular calcium level activates protein kinase resulting in exocytosis of insulin granules.

2.1.4 Regulation of insulin secretion

Primarily insulin is secreted in response to glucose, if compared with other nutrients such as free fatty acids (FFA), amino acids (AA), various growth hormones (estrogen, melatonin, glucagon like peptide-1 (GLP-1)), that can increase glucose-induced insulin secretion, and thus regulates glucose utilization and storage (Fu *et al.,* 2013). If the body does not need more energy, insulin signals the liver and a surplus of glucose is stored as a glycogen. An inability to respond of insulin effect or lack of insulin can lead to the development of diabetes symptoms, which shows an importance of β-cells, connecting nutrient metabolism and endocrine system.

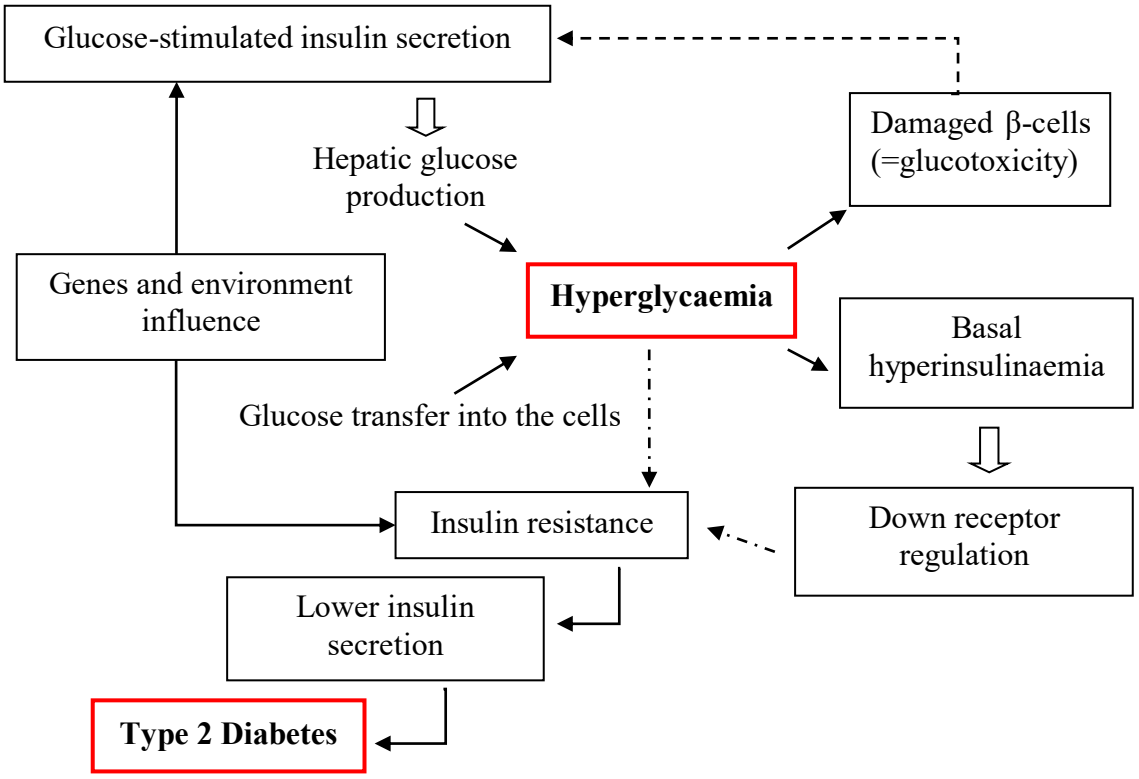
Beside glucose, insulin secretion is also affected by AA, various lipid metabolites and hormones. Although physiological concentrations of AA are not considerable insulin secretagogues, some of them can indirectly influence insulin secretion in β-cell (Fu *et al.,* 2013). After protein's catabolism, the majority of AA is metabolized to provide energy. Free AAs are released into the blood and increase glucagon secretion that results of increased blood glucose levels triggered insulin secretion.

FFAs do not only potentiate insulin secretion to compensate growing insulin need as a consequence of insulin resistance, but also stimulate glucose-stimulated insulin secretion. It is enabled by FFA receptor 1 (FFAR1, also known as GPR40 (the G-protein-coupled receptor 40)), located in β-cell and that could be involved in the regulation of insulin secretion. Adiponectin, leptin, GLP-1 and growth hormones belong among hormones, that can play a role in insulin secretion. Their effects are also mediated by specific receptors, which activate a cascade of metabolic events and in different range stimulate secretion of insulin (Cavaghan *et al.*, 2000; Fu *et al.,* 2013).

2.2 INSULIN RESISTANCE

The term insulin resistance presents damage of biological response to insulin. After exogenous insulin administration or postprandial endogenous secretion, insulin-stimulated glucose transport into insulin-sensitive peripheral tissues is facilitated. Relative lack of insulin or impaired insulin sensitivity is manifested as an inability to suppress gluconeogenesis in the liver, which leads to an increased hepatic glucose production, and its removal from the blood circulation reflects a level of glucose tolerance disorder (Piťhová, 2008). Insulin sensitivity is influenced by many factors as age, weight, body fat, use of medications and physical activity, and together with obesity belongs to the main pathogenetic factors of T2DM. Decreased tissue sensitivity to insulin action causes limited glucose utilization in peripheral tissues, postprandial hyperglycaemia and influences glucose production in liver, accountable for hyperglycaemia in the fasted state. In insulin-resistant individuals, glucose accumulation in blood can lead to chronic hyperglycaemia (Figure 2). Long-term high blood glucose levels cause serious health problems. Insulin resistance is manifested in adipose and muscle tissues and in the liver (Piťhová, 2008; Svačina *et al.*, 2003).

Figure 2: Development of insulin resistance and type 2 diabetes (Rybka, 2007).



Early prediabetic phase begins in young adulthood, manifesting as insulin resistance in peripheral tissues. Insulin levels are initially elevated in response to insulin resistance and slowly glucose desensitization develops. Subsequently, the insulin level gradually decreases and this leads to the onset of T2DM (Samuel *et al.*, 2012; Rybka, 2007).

The insensitivity of insulin is reflected in inability to suppress gluconeogenesis in the liver and to store excess glucose as glycogen that leads to increased glucose production in the liver. Peripheral insulin resistance in the muscles, adipose tissues and liver results in hyperglycaemia in the fasted state (Piťhová, 2008).

2.2.1 Skeletal muscle insulin resistance

The major site of glucose uptake in the postprandial state is skeletal muscle tissue. Insulin mediates glucose uptake into skeletal muscle cells, where it is either metabolised or stored as glycogen as a reservoir of energy. The synthesis of glycogen is regulated by effect of the enzyme glycogen synthase and approximately two-thirds of all glucose-6-phosphate is converted to glycogen (Defronzo *et al.,* 2009). Only after exceeding the liver capacity to synthesize glycogen, glucose is converted into triglycerides and is stored in adipose tissue. However, the high storage capacity of skeletal muscles determines that

insulin resistance will manifest earlier in the muscles than in adipose tissue and liver (Melmed *et al.*, 2016). The impairment of glucose disposal in relation to a defect in synthesis of glycogen is confirmed by the hyperinsulinemic-euglycemic glucose clamp (HEGC) (Melmed *et al.*, 2016; Guerrero-Romero *et al.*, 2010). This technique quantifies the insulin action as the rate of exogenous glucose infusion including residual glucose production in the liver in reaction to plasma insulin concentration, which is required to reduce glucose level. Approximately 80-90 % of glucose under hyperinsulinemic-euglycemic conditions is taken up by skeletal muscle, while adipose tissue uses less than 5 % of an infused glucose load, and thus this test reflects insulin sensitivity primarily in muscle tissue (Defronzo *et al.,* 2009).

2.2.2 The role of adipose tissue in insulin resistance

The relationship between insulin resistance and obesity has been widely studied. However, the exact role of fat deposits, visceral adipose tissue or subcutaneous adipose tissues in the development of insulin resistance is still discussed. In comparison to subcutaneous adiposity, the accumulation of visceral fat more contributes to the onset and an aggravation of insulin resistance at greater extent and thus increases liver gluconeogenesis, which leads to acceleration of hepatic glucose production glycaemia and to the risk of postprandial hyperglycaemia due to slower glucose metabolism in the muscles (Piťhová, 2008). Because of the lack or an insufficient action of insulin, a hormone-sensitive lipase is not inhibited and causes an increased FFA release from adipocytes. The sensitivity and secretion response of β-cells are dependent on glucose oscillation and an amount of FFAs. Fasting plasma concentrations of FFA in obese patients and patients suffer from T2DM are typically at the range from 600 to 800 μmol/L in comparison to 300-400 μmol/L in health subjects (Corcoran *et al*., 2007). Chronically increased FFA level causes hyperinsulinemia, manifesting in reduction of insulin secretion until its gradual extinction. Initial hyperinsulinemia is associated with a development of insulin resistance (Piťhová, 2008; Shanik *et al.*, 2008). Increased plasma FFAs levels can be caused by excessive lipid consumption or by increased release from visceral adipose tissues. If the storage capacity of adipocytes is depleted, the accumulation of lipids in other tissue leads to increased formation of toxic ceramides and other sphingolipids. These compounds are accumulated in the liver, muscles, pancreas and in arteries, and may contribute to the development of insulin resistance. Infiltration of FFAs and triglycerides

in β-cells leads to their failure, apoptosis and attenuation of secretion and synthesis of insulin (=lipotoxicity), what shows a key role of FFAs and lipid metabolism disorder in pathogenesis of T2DM. Plasma level of FFAs activates a cascade of inflammatory cytokines, a secretion of tumor necrosis factor and growth factors that maintain inflammation and a development of atherosclerosis (Piťhová, 2008; Polák *et al*., 2006). The aggravated metabolism influences the ability of endogenous hyperinsulinemia to compensate insulin resistance and induces a dysfunction of glucose tolerance. Increased weight and gradual obesity promotes inflammation of adipose tissue. Higher values of body mass index and increased amount of visceral fat increases levels of inflammatory markers (C-reactive protein, cytokines and adhesive molecules) (Piťhová, 2008). According to recent studies, the weight loss has considerably reduced a state of inflammation and inflammatory mediators produced in adipose tissue (Esposito *et al.*, 2003).

2.2.3 Insulin resistance and metabolic syndrome

Insulin resistance arises with many pathological states, but sometimes it evolves physiologically, for example during pregnancy or adolescence (Piťhová, 2008). In the most of cases, patients are affected by metabolic syndrome, also known as syndrome of insulin resistance. Metabolic syndrome presents a complex of clinical, biochemical and humoral abnormalities, caused by insufficient effect of insulin in glucose metabolism (Piťhová, 2008). A fat-rich diet creates one of the most important components of metabolic syndrome. Increased fatty acid flux to the liver and higher availability of FFA causes fat infiltration and organ damage, which leads to an accumulation of FFA and decline of β-oxidation. Dysfunction of β-cells, caused by elevated circulating FFA level, induces basal insulin release, but impairs glucose-stimulated insulin secretion (Boden *et al.*, 2002). Insulin helps to improve vascular permeability, but during insulin resistance and hyperinsulinemia the vasoconstriction is observed as a result of damaged endothelial function (Piťhová, 2008; Potenza *et al.*, 2009). Together with endothelial dysfunction due to insulin excess, an increased formation and lower regression of atherosclerotic plaque, higher proliferation of smooth muscle cells and collagen and a significant activity of low-density lipoprotein (LDL)-receptors support the onset and the acceleration of atherogenesis (Piťhová, 2008). Epidemiological studies show the risks of individual components of metabolic syndrome, but on the other hand treatment of associated diseases,

especially hypertension and dyslipidaemia, have reduced overall and cardiovascular mortality (Piťhová, 2008; Rybka, 2007).

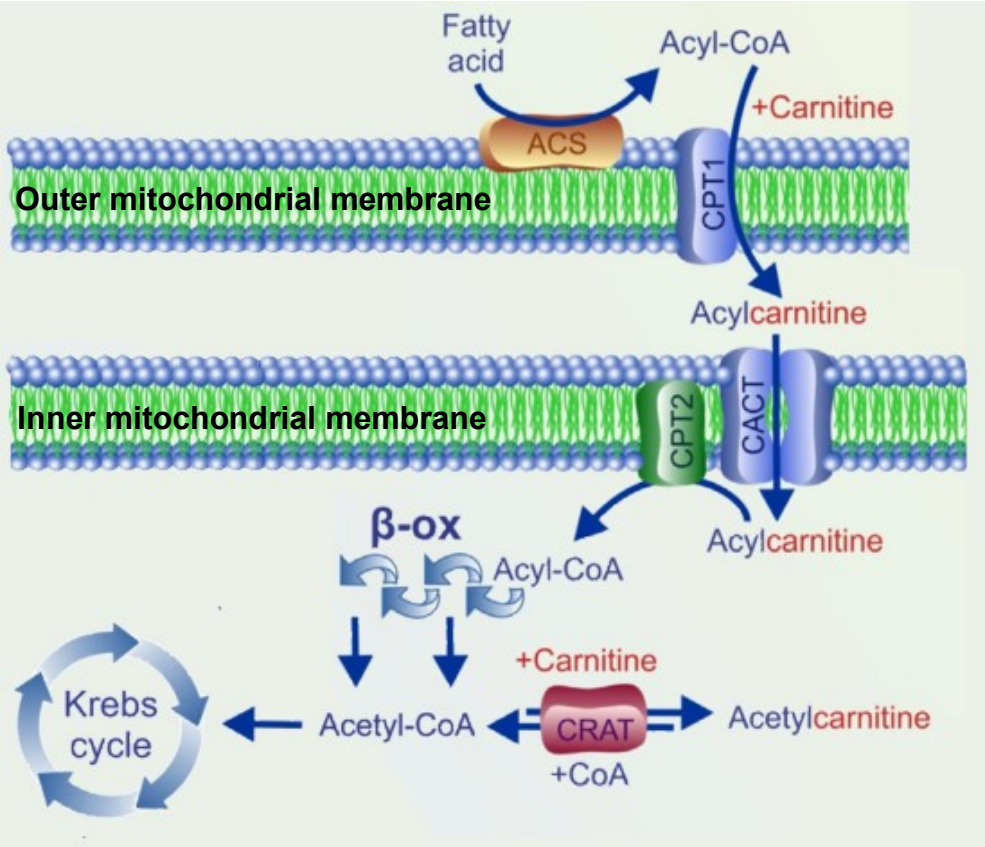
2.2.4 Fatty acid metabolism

In the human body the glucose and also considerable amounts of lipids are oxidized to ensure continuous energy supply. Stimulation of lipolysis and thus increased systemic availability of FFA provide energy during starvation and during increased physical activity. In patients with obesity and metabolic syndrome, elevated circulating concentrations of FFA are observed due to overnutrition and lipid accumulation. Early changes of FFA levels might present a predictive marker for the transition from impaired glucose tolerance to the development of T2DM associated with insulin resistance (Boden *et al.*, 2002).

The transport of activated long-chain fatty acids into the mitochondrion is mediated via the carnitine shuttle (Figure 3). The physiological importance in the mitochondrial metabolism of fatty acids lies in regulation of conversion of free CoA to acyl-CoA, which is necessary to FFA enter into the cell. In presence of the enzyme acyl-CoA synthetase (ACS), FFAs are activated by esterification to acyl-CoA, a starting metabolite for fatty acid β-oxidation and a major source of lipid biosynthesis. Thus, long-chain acyl-CoAs (LC acyl-CoAs) cross the outer mitochondrial membrane and after trans-esterification via carnitine palmitoyltransferase 1 (CPT1) are converted to respective acylcarnitines (Reuter *et al.,* 2012). Formed acylcarnitines are transported by carnitine-acylcarnitine translocase (CACT) from the inner membrane of mitochondria into the mitochondrial matrix, where β-oxidation takes place. In the last step, the acylcarnitines are reconverted back into free carnitine and LC acyl-CoA by palmitoyltransferase 2 (CPT2) and LC acyl-CoAs are subsequently oxidized (Schooneman *et al.*, 2013, Dambrova *et al.*, 2016).

FFAs or acyl-CoAs are not able to transport across the mitochondrial membrane alone, which shows the important role of L-carnitine and carnitine acyl-transferases (CPT1, CPT2) in metabolism of FFAs (Reuter *et al.,* 2012; Dambrova *et al.,* 2015).

Figure 3: The function of L-carnitine in the transport of FFA (Dambrova *et al.*, 2016 *Pharmacological research*)



After fatty acid activation to acyl-coenzyme A (Acyl-CoA) by long-chain acyl-coenzyme A synthetase (ACS), the acyl-CoA is transported through the outer mitochondrial membrane and converted to respective acylcarnitine via carnitine palmitoyltransferase-1 (CPT1). Formed acylcarnitine is transported into the mitochondria by carnitine/acylcarnitine translocase (CACT) and then palmitoyltransferase-2 (CPT2) converts the acylcarnitine back into acyl-CoA, which is oxidized to acetyl-coenzyme A (acetyl-CoA) in a process called β-oxidation. Acetyl-CoA may be further metabolized in the Krebs cycle or may be converted to acetylcarnitine by carnitine acetyltransferase (CrAT) (Dambrova *et al.,* 2016).

2.2.5 Lipid-induced insulin resistance

CPT1 is important regulator of fatty acid metabolism and postprandially its activity is inhibited by malonyl-CoA, the intermediate in fatty acid synthesis (Melmed *et al.*, 2016). In fasted state, adenosine monophosphate (AMP)-activated protein kinase inhibits acetyl-CoA carboxylase and malonyl-CoA levels decrease, thus allowing CPT1 activity and fatty acid oxidation (Schooneman *et al.*, 2013). By inhibition of CPT1, malonyl-CoA limits the transport of LC acyl-CoAs into mitochondria, which leads to the accumulation of long-chain fatty acids (LC-FA) in cytosol and to decline of FA β-oxidation (Koves *et al.*, 2008).

Lipid accumulation in insulin-responsive tissues influences insulin signalling pathway via various intermediates (diacylglycerols, ceramides, FFA, acyl-CoAs and LC- acylcarnitines) (Schooneman *et al.*, 2016; McCoin, 2015).

Recent studies suggest that relationship between insulin resistance and impairment of β-oxidation arises from mitochondrial oversupply of FFAs leading to increase of incomplete oxidation **(**Mihalik *et al.*, 2010; Adams *et al.,* 2009). In patients with T2DM decreased oxidative capacity in skeletal muscle, leading to lower conversion of FFAs to acyl-CoAs and a subsequent incomplete β-oxidation of fatty acids in mitochondria is observed (Aguer *et al.*, 2015).

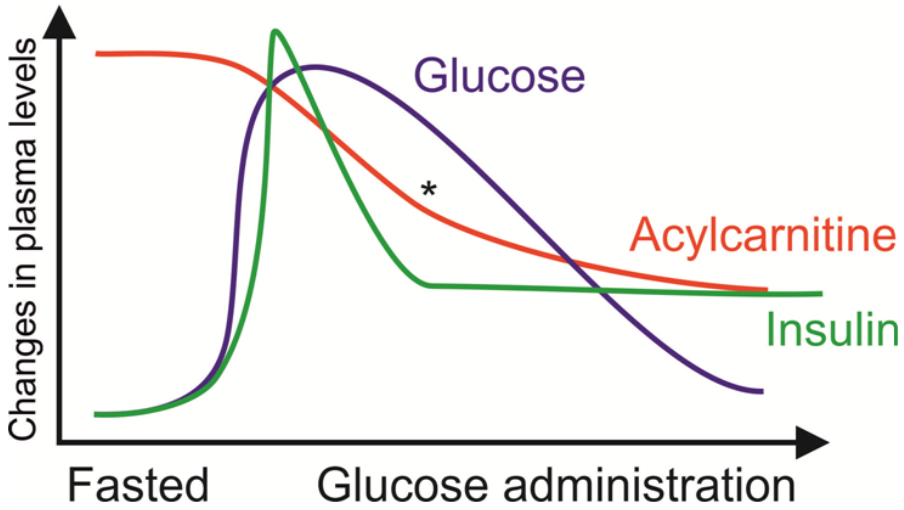
2.2.6 Long-chain acylcarnitines

Long-chain fatty acids are the main energy substrates in the heart and muscle tissues. Acylcarnitine accumulation during the fasted state is physiological, but in the case of myocardial infarction or other conditions related to metabolic disturbances the pathological increase is observed (Soeters *et al.*, 2009; Liepinsh *et al*., 2016). Physiological accumulation of LC-acylcarnitines in the fasted state regulates energy metabolism, but an excess of LC-acylcarnitines in the fed state could cause a progression of T2DM by impaired insulin signalling (Liepinsh *et al.*, submitted manuscript). Skeletal muscle is the major repository of free L-carnitine and therefore is considered as an essential contributor to the LC-acylcarnitine pool in plasma (Koves, 2008).

Traditionally acylcarnitines have been used as diagnostic biomarkers of inherited disorders of fatty acid oxidation (McCoin *et al.*, 2015), however, more recently profiling of blood acylcarnitines is used to identify a dysregulation in fatty acid oxidation associated with obesity and diabetes. Modestly increased plasma and tissue LC-acylcarnitine concentrations have been detected in obese patients with impaired glucose tolerance and T2DM **(**Mihalik *et al.,* 2010; Adams *et al.,* 2009). Increased levels of acylcarnitines were found also in an experimental model of HFD-induced insulin resistance (Koves *et al*., 2008). In recent study, the accumulation of LC-acylcarnitine levels in type 2 diabetes model was detected only in fed state as a result of insulin resistance or the inability of insulin to decrease the production of acylcarnitines (Liepinsh *et al.,* 2016). There are a few previous studies that describe the decrease of acylcarnitine levels in plasma after insulin infusion or glucose tolerance test and evaluate the relationship of acylcarnitine levels to insulin sensitivity (Zhao *et al.,* 2009; Mihalik *et al.*, 2010). However, the direct

association of changes of acylcarnitine concentrations with insulin sensitivity in muscles has not been displayed. After glucose administration, plasma glucose and insulin level increase; and acylcarnitine concentrations decrease significantly (Figure 4).

Figure 4: The changes of plasma glucose, insulin and acylcarnitine levels during transition from fasted to postprandial state after glucose administration. (This Figure was created without the reference)



The aim of this study was to measure the changes in plasma acylcarnitine concentrations during GTT and to evaluate whether this measurement could be used as a marker of muscle-specific insulin resistance.

2.3 DETECTION METHODS OF DIABETES AND INSULIN RESISTANCE

2.3.1 Hyperinsulinemic-euglycemic glucose clamp

Hyperinsulinemic-euglycemic glucose clamp (HEGC) is a method used for quantification of insulin sensitivity and it is based on measurement of direct insulin effect in tissues under steady-state conditions *in vivo*. The procedure involves an implementation of intra-venous insulin infusion, usually combined with glucose infusion, and the blood samples are collected during 3 hours. Insulin action is quantified as the rate of hepatic glucose production and exogenous glucose infusion needed to keep normal plasma glucose concentration in response to a fixed increment in the plasma insulin concentration. This technique maintains constant hyperglycaemia and adequate and sufficient insulin secretion is observed (Defronzo *et al.,* 2009). To characterize insulin resistance a series of indexes

such as the homeostasis model assessment (HOMA) or the quantitative insulin sensitivity check index (QUICKI) is used based on various combinations of fasting glycaemia and insulin levels (Singh *et al*., 2010; Guerrero-Romero *et al.*, 2010). Cross-sectional studies have indicated that detection of simple surrogate markers significantly correlates with the variability of insulin sensitivity measured by the HEGC (Lorenzo *et al*., 2015).

2.3.2 Glucose tolerance test

The oral glucose tolerance test (GTT) is widely used method to measure insulin release and level of insulin resistance. Increase of postprandial glycaemia reflects a reaction of organism to glucose administration and evaluates, if the body is able to keep glucose concentration in the normal range. The GTT is performed and glycaemia is measured in the fasted state after overnight fasting (usually 10 hours) glycaemia. In the following step, patient during 5-10 minutes should ingest a solution containing 75 g of glucose, which is dissolved in 250-300 mL of tea or water. After 60 and 120 minutes the blood samples are collected and glucose and insulin concentrations in plasma are measured. Another way of glycaemic response determination is the glycaemic curve, when blood is taken more often, typically at 30 minute intervals. The GTT provides information not only about plasma glucose levels, but also about the ability of pancreatic β-cells to secrete insulin and its sensitivity (Abdul-Ghani *et al.*, 2007).

The fasted glycaemia in healthy people is below the 5.6 mmol/L and 2 hours after ingestion of glucose solution is more than 7.8 mmol/L. In patients with diagnosed DM: glycaemia in the fasted state is above the 7.0 mmol/L, random glycaemia – above the 11.1 mmol/L and glycaemia after 2 hours – above the 11.1 mmol/L, together with characteristic clinical signs as thirst, polyuria or tiredness. In overweight patients repeated measurement is needed for confirmation of accurate diagnosis (Rybka, 2007). The absolute value of postprandial glycaemia is influenced by many factors as the severity of insulin resistance, the quality and quantity of secreted insulin response after food intake, and furthermore, an amount of sugar and glycaemic index of diet. Postprandial glycaemia belongs to the group of independent cardiovascular risk factors and knowledge of its level is necessary for right choice of therapy (Heine *et al.*, 2004). Together with HbA1c, postprandial glycaemia better reflects the overall glycaemic control in patients with diabetes, but simultaneously impaired glucose tolerance could also contribute to the development of primary and secondary complications of DM (Incani *et al.*, 2015).

Methods and surrogate markers describing β-cell function and an effectiveness of insulin action are summarized in Table 2. Among the commonly used detection methods the insulinogenic index (IGI), index derived from GTT, helps to estimate insulin level secretion after glucose administration and glucose/insulin ration (G/I) calculated from data in the fasted state, that is more sensitive and, thus, is used especially to measurement of insulin effect in tissues (Singh *et al*., 2010).

Table 2: Various methods of determination of insulin resistance (IR) (Singh *et al.*, 2010).

Hyperinsulinemic-euglycemic glucose clamp (HEGC)	Standard method for quantifying insulin sensitivity Direct measurement under steady-state conditions
Glucose tolerance test (GTT)	Detection of glucose intolerance
Fasting insulin	Detection of IR before clinical disease appears
Glucose/insulin ratio (G/I)	Highly sensitive and specific determination of IR
Insulinogenic index (IGI)	Index of β-cell function = δ I (0-30 min)/ δ G (0-30 min)
Homeostasis model assessment (HOMA)	Assessment of β-cell function and insulin sensitivity
Quantitative insulin sensitivity check index (QUICKI)	Mathematical transformation of fasting blood glucose and insulin sensitivity

2.3.3 Blood glucose and HbA1c

Fasting blood glucose is one of the main criteria of the diagnosis of DM and its value is determined by the rate of glucose production in the liver, which is primarily regulated by insulin. Measurement is carried out after at least 8 hours of last meal. Blood glucose levels are measured by glucose meter composed of a thin needle (a lancet) and a test strip which engages into a device to measure blood glucose. Optimal range in healthy subjects and well-controlled diabetes patients is under < 7 mmol/L. Increase of glycaemia levels can point to the acute state of the organism and its disability to utilize the glucose. Regular monitoring of fasted blood glucose levels informs patients about glucose concentration in blood to prevent possible risk of hyperglycaemia and also could help to control glucose level during a treatment of already diagnosed diabetes (Rybka, 2007).

The measurement of HbA1c shows the long-term fluctuation of blood glucose levels. HbA1c is a product of non-enzymatic glycation of haemoglobin, which is dependent on the average glycaemia during the previous 8 weeks and compared to the fasted state glycaemia is much more stable. HbA1c levels do not change during the day and after food ingestion and, therefore, HbA1c value does not have to be detected in the fasted state (Alqahtani *et al.*, 2013; Kilpatrick, 2008). Diagnostic criteria of HbA1c measurement are summarized in Table 3.

Table 3: Categories of glycated haemoglobin (HbA1c) values (Incani *et al.*, 2015; ADA - Standards, 2017).

Normal	≤ 38 mmol/mol	≤ 5.6 %
Prediabetes	39-46 mmol/mol	5.7-6.4 %
Diabetes	≥ 48 mmol/mol	≥ 6.5 %
Controlled diabetes	≤ 53 mmol/mol	≤ 7 %

It is assumed that effectiveness of HbA1c determination might be higher in obese patients in comparison to patients with normal weight, but direct association between amount of fat and HbA1c value has not been proved yet (Incani *et al.*, 2015).

2.4 DRAWBACKS OF CURRENT DIAGNOSTIC METHODS

In clinics, previously mentioned diagnostic methods are used to characterize disturbances in glucose tolerance and insulin sensitivity, but each of them has several drawbacks and limitations. Blood glucose testing as an integral part of diabetes treatment is the process, which helps to check the glucose control and reflects its changes. This useful method provides rapid information about actual glucose state in the body and is relatively cheap, but it is also associated with some disadvantages. The measurement reflects glycaemia at state when insulin concentration is the lowest and therefore it could not be linked to insulin action. Also in the early stages of insulin resistance the glucose levels in fasted state are not elevated and the levels of fasted state glucose are depending on the period length of fasting. To obtain precise and reproducible measurement, it is necessary to take into account all the possible factors that could affect blood glucose concentrations (Ginsberg *et al*., 2009).

The measurement of HbA1c describes long-term changes of glucose concentration, is one of the additional markers in diagnosis of diabetes and serves also as a proof to patient's compliance in the treatment. Normal blood glucose levels 2 hours after a meal indicate adequate function of β-cells, whereas increased glucose concentrations 2 hours after oral GTT show an impaired β-cells function. HbA1c is a poor indicator of postprandial glycaemia and captures only chronic hyperglycaemia without acute postprandial response. Interindividual variability determines different amount of deglycosylated enzymes that are responsible for haemoglobin degradation (Nathan *et al.,* 2008; Bonora *et al.*, 2011). Increased value of HbA1c represents increased glycation of proteins in the body, which shows a different biochemical abnormality and only secondary is associated with high blood glucose. Although, high HbA1c level is observed subsequently to an increase of blood glucose, there many data confirming do not exist how long the latency has lasted. The measurement of HbA1c has low sensitivity and in early stage of DM is not indicative (Bonora *et al.*, 2011).

In addition, in case of haemolysis the insulin can be degraded and cause error in the measurement of plasma concentrations of insulin. Hyperinsulinemic-euglycemic glucose clamp method is not appropriate to detect insulin resistance in mice because of necessity to administrate general anesthetics which induce marked insulin resistance (Windelov *et al.,* 2016). Mentioned current clinical tests do not characterize muscle insulin resistance and mitochondrial function. Novel method of muscle insulin resistance detection, used in combination with current methods, would improve and personalize early diagnosis and treatment of patients with diabetes and pre-diabetes.

3 EXPERIMENTAL PART

3.1 ANIMALS

Twenty male C57bl/6N mice (7 weeks old, Envigo, Netherlands), 10 db/db and 10 db/L mice (7 weeks old, Envigo, Netherlands) were housed in special boxes under standard conditions (21-23 °C, relative humidity 50 % ± 10 %, 12 hours shifted light-dark cycle) with unlimited access to food and water. The experiment was carried out in accordance with the guidelines of the European Community (2010/63/EU), local laws and policies and was approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia. The experiments were conducted under the supervision of FELASA C certified specialists.

Two weeks before the start of treatment mice were adapted to local conditions. C57bl/6N mice were divided into 2 groups. Control group received standard diet (R70 diet, Lantmännen, Sweden) and the other group received HFD (Western RD, Special Diets Services, UK) for 8 weeks (Figure 5).

Figure 5: Diet-induced obesity model in C57BL/6 mice (https://www.taconic.com/mouse-model/diet-induced-obese-dio-b6).

3.2 EQUIPMENT

To measure blood glucose a MediSense Optium (Abbott Diabetes Care, Maidenhead, UK) blood glucose meter and diagnostic strips were used. Determination of acylcarnitine levels was accomplished by the UPLC MS/MS method using liquid chromatography system Waters Acquity and masspectrometer Waters Quattro Micro or Waters Xevo TQ-S. This method is applied for quantitative determination of various biological molecules and their metabolites in different biological states.

3.3 METHODS

3.3.1 Glucose tolerance test and biochemical measurements

In the fasted state the action of insulin is limited, and fatty acid oxidation rate and related acylcarnitine levels are increased. The aim of this experiment was to detect the decrease of acylcarnitine content in the plasma after glucose administration in the model of insulin resistance and type 2 diabetes and to evaluate this change of LC-acylcarnitines as an indicator of insulin resistance. Before the performance of GTT, mice were fasted overnight and the glucose solution (0.5 g/kg of body weight) was administered intraperitoneally. The blood samples were obtained from the tail vein at 0 (fasting), after 1 and 2 hours to measure biochemical parameters and acylcarnitine profile. The fed state blood samples were collected 2 days before GTT. To obtain plasma, the samples were centrifuged at 3000 rpm for 10 min at 4 °C. Concentrations of plasma insulin were determined with an ELISA kit (Millipore, Billerica, USA), the HbA1c levels in blood was measured by an automated haemoglobin testing system (DCA Vantage Analyzer, Siemens, USA) in fasted state and plasma concentrations of FFAs were evaluated by using commercially available enzymatic assay kit from WAKO. This enzymatic method is based on colorimetric determination of non-esterified fatty acids in plasma. FFAs are converted to acyl-CoA in the presence of acyl-CoA synthetase. Subsequently, acyl-CoA is oxidized by acyl-CoA oxidase while simultaneously hydrogen peroxide (H_2O_2) is formed. In the presence of peroxidase, H_2O_2 creates blue-purple pigment by quantitative oxidation condensation with 3-methyl-N-ethyl-N (β-hydroxyethyl) aniline and 4-aminoantipyrine. The absorbance is measured at 550 nm and concentrations of FFA are calculated using standard curve.

3.3.2 Assay of glucose uptake

The uptake of [^{3}H]-DOG was performed to evaluate the insulin sensitivity of HFD-fed and db/db mice tissues. The amount of [^{3}H]-DOG in the insulin-sensitive tissues was determined 10 min after the subcutaneous injection of 1 μCi of 2-[1,2-^{3}H]-deoxy-D-glucose ([^{3}H]-DOG specific activity, 60 Ci/mmol). [^{3}H]-DOG solution in saline was injected 1 hour and 50 min after glucose administration in db/L and db/db mice, and 50 min after glucose administration in C57bl/6N mice. The mice were killed by decapitation and the samples from heart, muscle, liver and adipose tissue were obtained.

Heart, muscle and liver tissue were homogenized in water (1:5, w/v in MilliQ water) and adipose tissue in the 1% Igepal solution (1:3, w/v in MilliQ water + 1% Igepal). The content of [^{3}H]-DOG was measured by liquid scintillation method (Marques *et al.*, 2013; Temple, 2015).

3.3.3 Determination of acylcarnitines

The concentrations of short-chain (SC), medium-chain (MC) and long-chain (LC) acylcarnitines in the plasma were measured by the UPLC MS/MS method using an ultra performance liquid chromatography system Waters Acquity and mass spectrometer, against a seven-point standard curve of palmitoylcarnitine (Makrecka *et al.*, 2014; Liepinsh *et al.*, 2016). The range of standard curve was from 10 ng/mL to 1000 ng/mL.

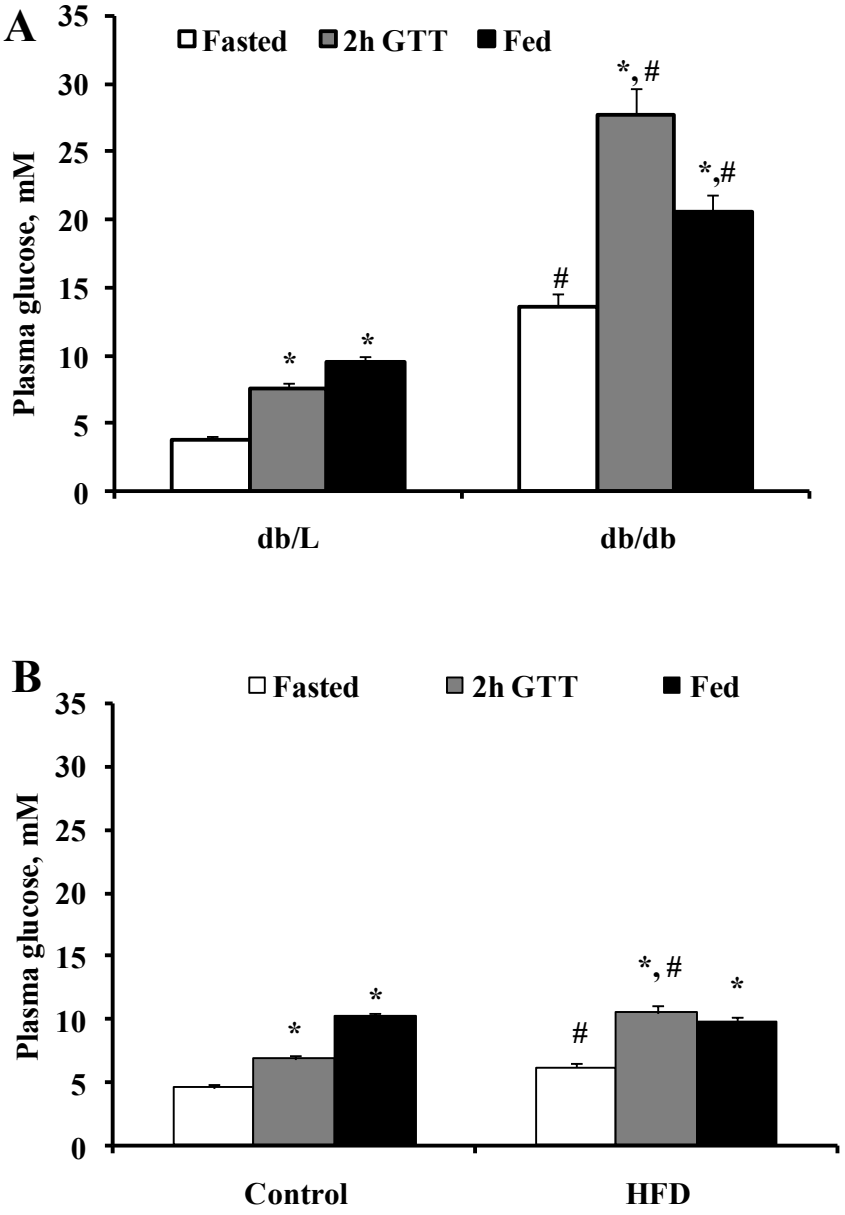
3.3.4 Statistical analysis

Values are represented as the average ± standard error mean(SEM) of 5-10 animals. Statistically significant differences compared to control group or fasted state were tested by Student's t-test or paired t-test, respectively. Repeated measurements one-way ANOVA with Dunnett's post-test was used to compare the differences from the baseline. The differences were considered significant when $p < 0.05$. The data were analyzed using GraphPad Prism 3.0 statistical software (GraphPad Inc., USA).

3.4 RESULTS

Fasting blood glucose is one of the main criteria of the diagnosis of DM and its value is reflected by the rate of glucose production in the liver, primarily regulated by insulin (Melmed *et al.,* 2016; Abdul-Ghani *et al.,* 2007). Our samples were determined in the fasted, fed state and 2 hours after GTT (Figure 6). The blood plasma glucose levels in db/db mice compared to db/L mice were 4 and 2-fold higher in the fasted and fed state, respectively (Figure 6 A). In db/db mice also a 4-fold increase in plasma glucose concentration 2 hours after GTT compared to db/L was observed. Glucose levels of db/L and db/db mice in the fed and postprandial states were significantly increased by 1.5 to 2.5-fold in comparison to fasted state (Figure 6 A). In mice fed with HFD we noticed only moderate increase of glucose levels: in the fasted state by 35 % and 2 hours after GTT by 53 % compared to respective control group (Figure 6 B). In the fed state of control and HFD-fed mice (after 8 weeks of diet) no significant differences in glucose concentrations were observed.

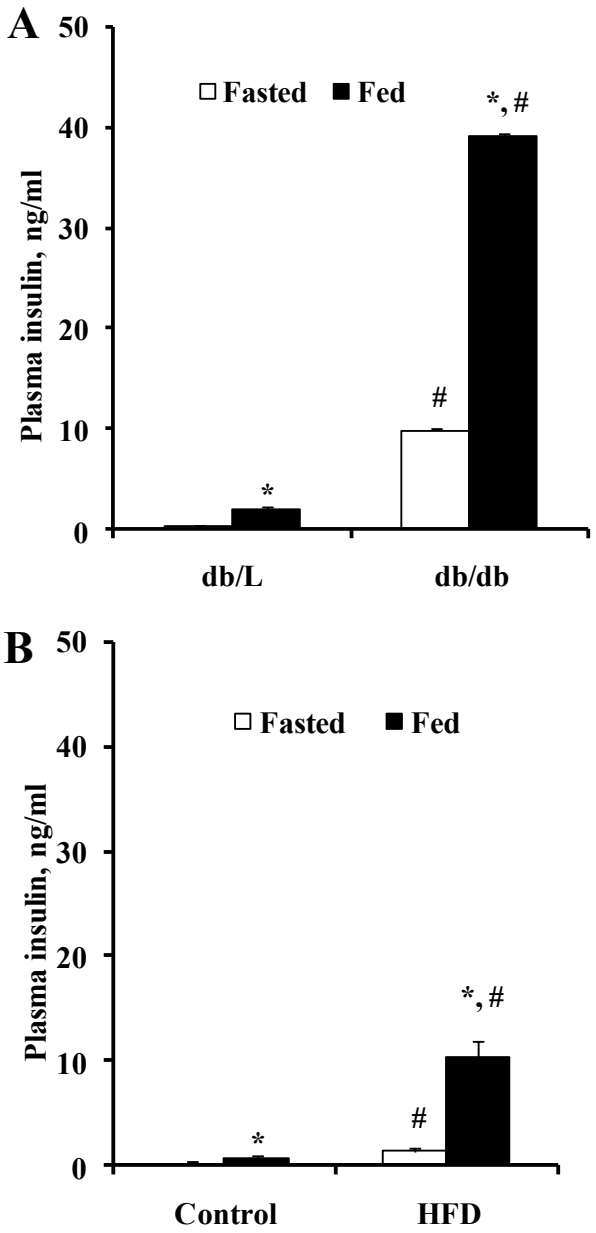
Figure 6: Glucose concentrations in the fasted, fed state and 2 hours after glucose tolerance test in control, HFD-fed *C57bl/N, db/L* and *db/db* mice



*Significantly different from fasted state (paired t-test, $p < 0.05$). #Significantly different from respective control group (Student's t-test, $p < 0.05$).

We also monitored the levels of insulin, whose secretion is related to blood glucose concentration. Long-lasting stimuli of ingestion-induced insulin secretion causes down regulation of insulin receptor, protecting the cells before the excessive decrease of glycaemia. During transition, back to the fasted state, occurs to opposite effect. In individuals with well-preserved insulin sensitivity, both reactions are in equilibrium (Piťhová, 2008). The development of hyperinsulinemia was determined in db/db and HFD-fed mice in the fasted and fed state and in respective control groups (Figure 7).

Figure 7: Insulin concentrations in the fasted, fed state and 2 hours after glucose tolerance test in control, HFD-fed *C57bl/N, db/L* and *db/db* mice.

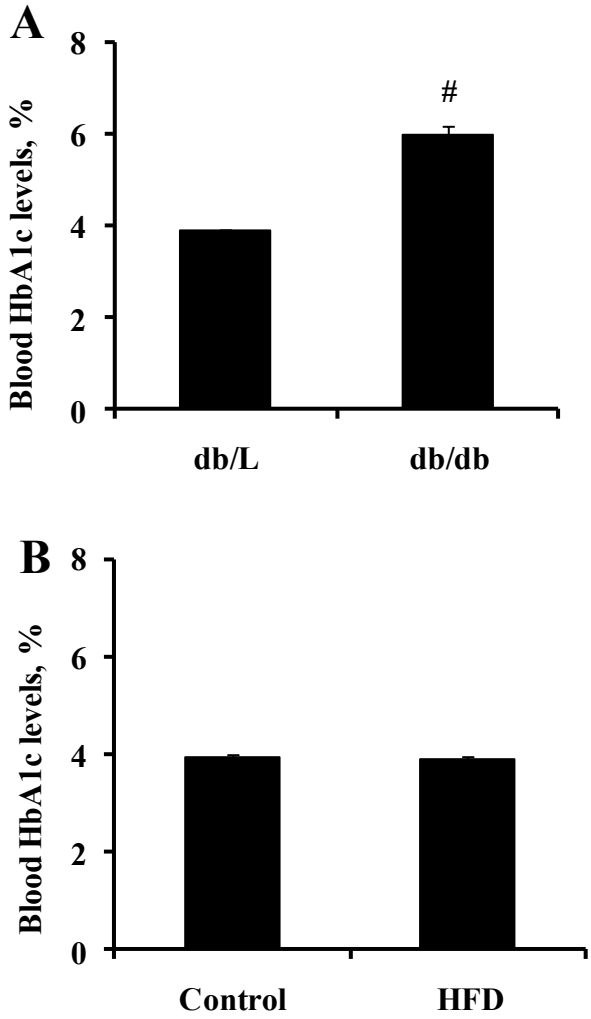


*Significantly different from fasted state (paired t-test, $p < 0.05$). #Significantly different from respective control group (Student's t-test, $p < 0.05$).

Insulin levels in db/db mice plasma were increased by approximately 20 to 36-fold compared to db/L mice in both (fasted and fed) states. In HFD-fed mice the insulin levels were about 6 times lower than in db/db mice (Figure 7 A and B). 8 weeks HFD induced a 6 and 13-fold higher insulin levels in the fasted and fed mice, respectively, compared to control group.

The measurement of HbA1c shows the long-term fluctuation of blood glucose levels. HbA1c concentrations do not change during the day and after food ingestion (Alqahtani *et al.,* 2013), which means, that HbA1c value did not have to be detected in the fasted state. In db/db mice, the HbA1c was increased by approximately 50 % (the range of prediabetes category is 5.7-6.4 %) in comparison to db/L (Figure 8 A). As shown in the Figure 8 B, the levels of HbA1c after 8 weeks of HFD remained unchanged.

Figure 8: Concentrations of HbA1c in control, HFD-fed *C57bl/N*, *db/L* and *db/db* mice.

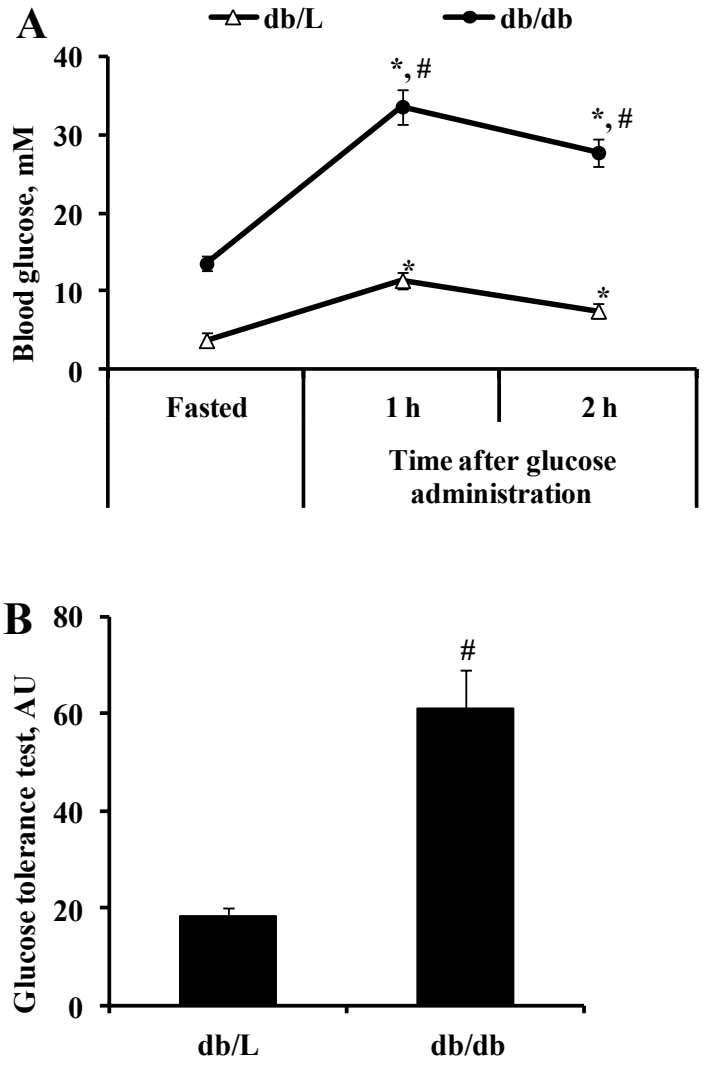


#Significantly different from respective control group (Student's t-test, $p < 0.05$).

The inability of organism to react on glucose load was manifested as glucose tolerance disorder. Limited glucose utilization in peripheral tissues due to decreased tissue sensitivity to insulin action can lead to the development of hyperglycaemia, which may fluently convert into a chronic state (Piťhová, 2008; Rybka, 2007). The measurement was accomplished by determination of blood glucose in the fasted state and 1 and 2 hours after

GTT. The glucose tolerance was considerably impaired in db/db mice and the area under the curve was 3-fold greater than in db/L mice (Figure 9 A and B), reflecting significant disturbance in glucose metabolism.

Figure 9: Glucose tolerance in *db/L* and *db/db* mice.

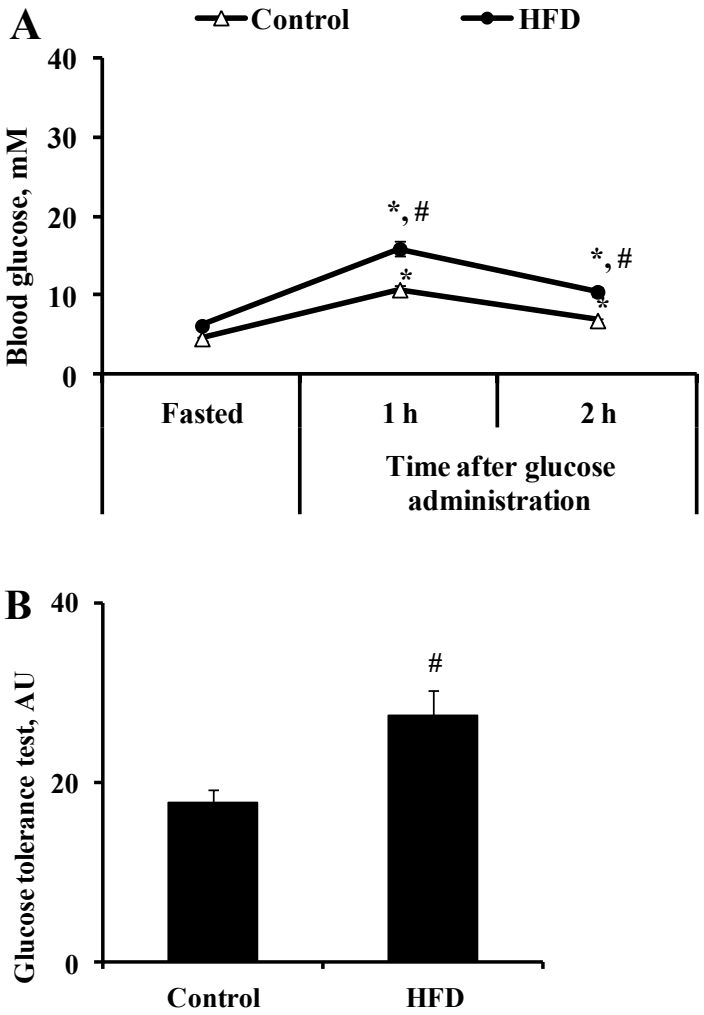


*Significantly different from fasted state (Repeated measures one-way ANOVA with Dunnett's post-test, $p < 0.05$). #Significantly different from respective control group (Student's t-test, $p < 0.05$).

In contrast, in HFD-induced insulin resistant mice, the glucose tolerance was moderately impaired in comparison to mice with normal diet, which indicates a slighter reduction of metabolic function. Recent studies demonstrate, that growing lipid accumulation, itself, is not so deleterious as an excess of lipid metabolism intermediates (diacylglycerols, ceramides and acyl-CoAs) linked to HFD (Cox-York *et al.,* 2014).

The area under the curve after GTT was increased by 44 % compared to control group (Figure 10 A and B).

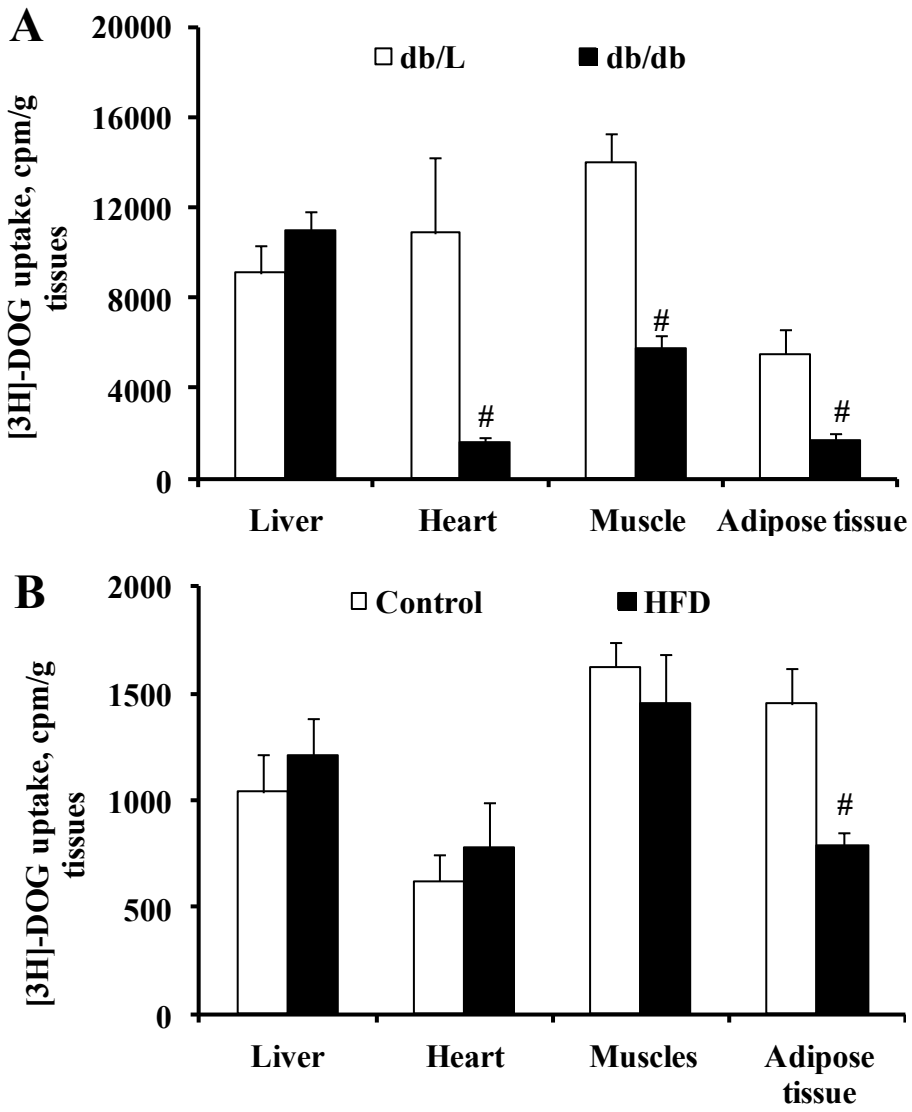
Figure 10: Glucose tolerance in control and HFD-fed *C57bl/N* mice.



*Significantly different from fasted state (Repeated measures one-way ANOVA with Dunnett's post-test, $p < 0.05$). #Significantly different from respective control group (Student's t-test, $p < 0.05$).

In addition to observing the glucose and insulin blood levels, we also measured the insulin sensitivity. Evaluation of the insulin action in db/db and HFD-fed mice tissues was performed by measurement of the uptake of [3H]-DOG. Tissues samples were isolated from liver, heart, muscle and adipose tissue. In the db/db mice, the amount of radio labelled glucose was decreased in heart, muscle and adipose tissue by 2 to 7-fold compared to db/L control mice (Figure 11 A), indicating diminished rate of insulin-stimulated glucose uptake in these organs.

Figure 11: The uptake of ^{3}H-DOG in liver, muscle, heart and adipose tissue in control, HFD-fed *C57bl/N* mice, *db/L* and *db/db* mice.



#Significantly different from respective control group (Student's t-test, $p < 0.05$).

The glucose uptake was not significantly changed in the liver, heart and muscle tissue of HFD-fed mice compared to control group, but in adipose tissue the glucose uptake was decreased by 1.8-fold (Figure 11 B). These results show that db/db mice as a model of type 2 diabetes developed pronounced hyperglycaemia, hyperinsulinemia and considerably impaired glucose tolerance, which resulted in diminished rates of glucose uptake in heart, muscle and adipose tissues. On the contrary, HFD-fed mice displayed early changes in insulin sensitivity – like hyperinsulinemia, moderate changes in glucose homeostasis and reduced glucose uptake only in adipose tissue.

During GTT also changes of FFA levels as a major component of lipids were determined (Table 4). In postprandial state in healthy individuals the insulin induces the

inhibition of lipolysis in adipose tissue that results in decreased plasma FFA availability. Obtained results demonstrate that 2 hours after GTT the amount of FFA was considerably decreased by 30-35 % in healthy animals (Table 4). In diabetic db/db mice and HFD-fed mice no significant changes in FFA levels during GTT were determined, thus indicating impaired insulin response in adipose tissue of mice with diabetes and insulin resistance.

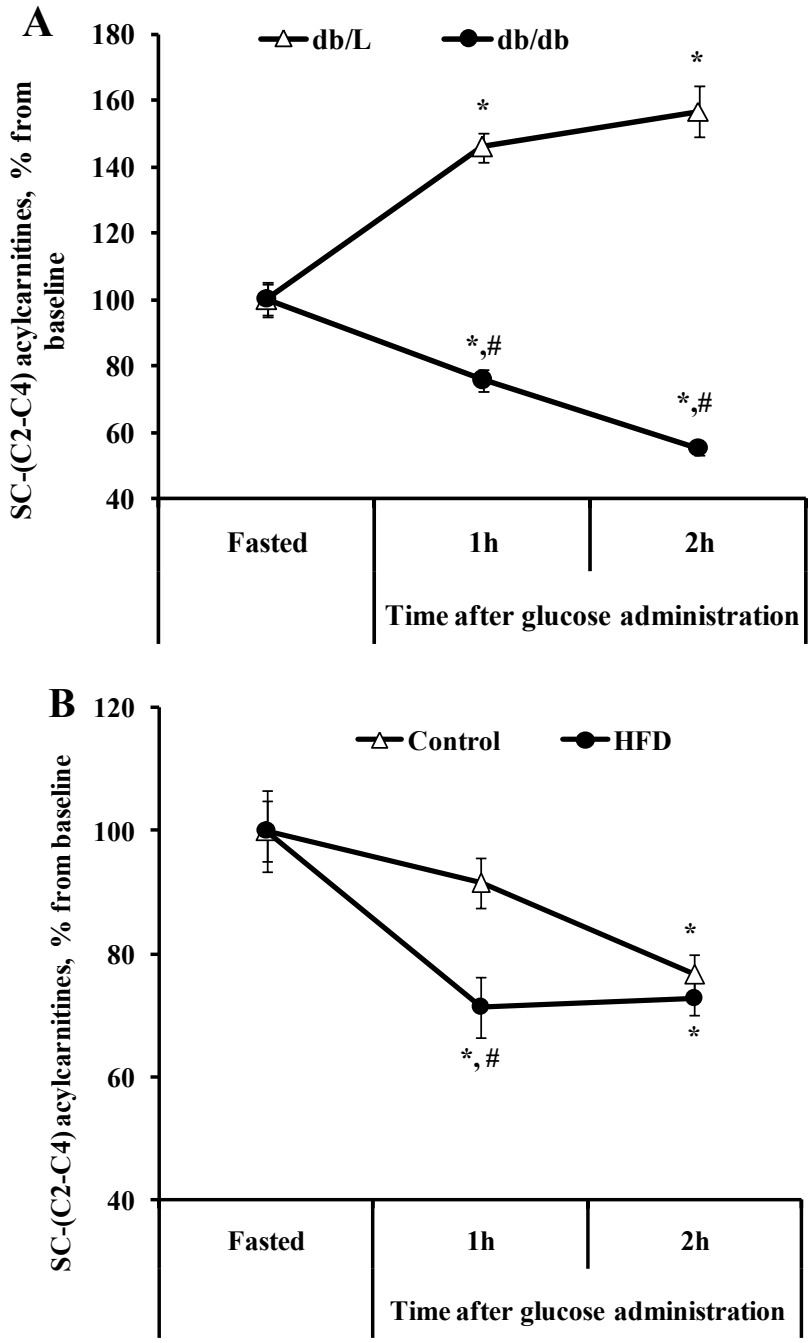
Table 4: Changes in plasma concentrations of FFA in control, HFD-fed *C57bl/N, db/L* and *db/db* mice during glucose tolerance test.

Changes in plasma concentration of FFA, %			
		Time after glucose administration	
	Fasted	**1 hour**	**2 hours**
Control	100 ± 9	58 ± 3*	65 ± 2*
HFD	100 ± 11	83 ± 6#	91 ± 4#
db/L	100 ± 6	61 ± 4*	72 ± 6*
db/db	100 ± 7	100 ± 10#	105 ± 13#

*Significantly different from fasted state (Repeated measures one-way ANOVA with Dunnett's post-test, $p < 0.05$). #Significantly different from respective control group (Student's t-test, $p < 0.05$).

The main purpose of our experimental study was determination of acylcarnitine levels change *in vivo*. Acylcarnitines as intermediates play an important role in FFA metabolism (Schooneman *et al.,* 2013; Mihalik *et al.,* 2010) and their higher/lower concentrations could indicate a possible metabolic disturbance. During our experiment, the changes of acylcarnitine plasmatic concentrations with different length of chain were observed. Figure 12 shows the changes in SC-acylcarnitine (C2-C4) plasma concentrations in transition from fasted to fed state in experimental models of moderate insulin resistance and type 2 diabetes. 2 hours after the glucose administration significant increase of SC-acylcarnitine levels by 57 % in db/L mice plasma was observed, while in the db/db mice plasma SC-acylcarnitine concentrations decreased by 45 % (Figure 12 A).

Figure 12: Changes in short-chain (SC)-acylcarnitine concentrations in plasma of control, HFD-fed *C57bl/N, db/L* and *db/db* mice during glucose tolerance test.

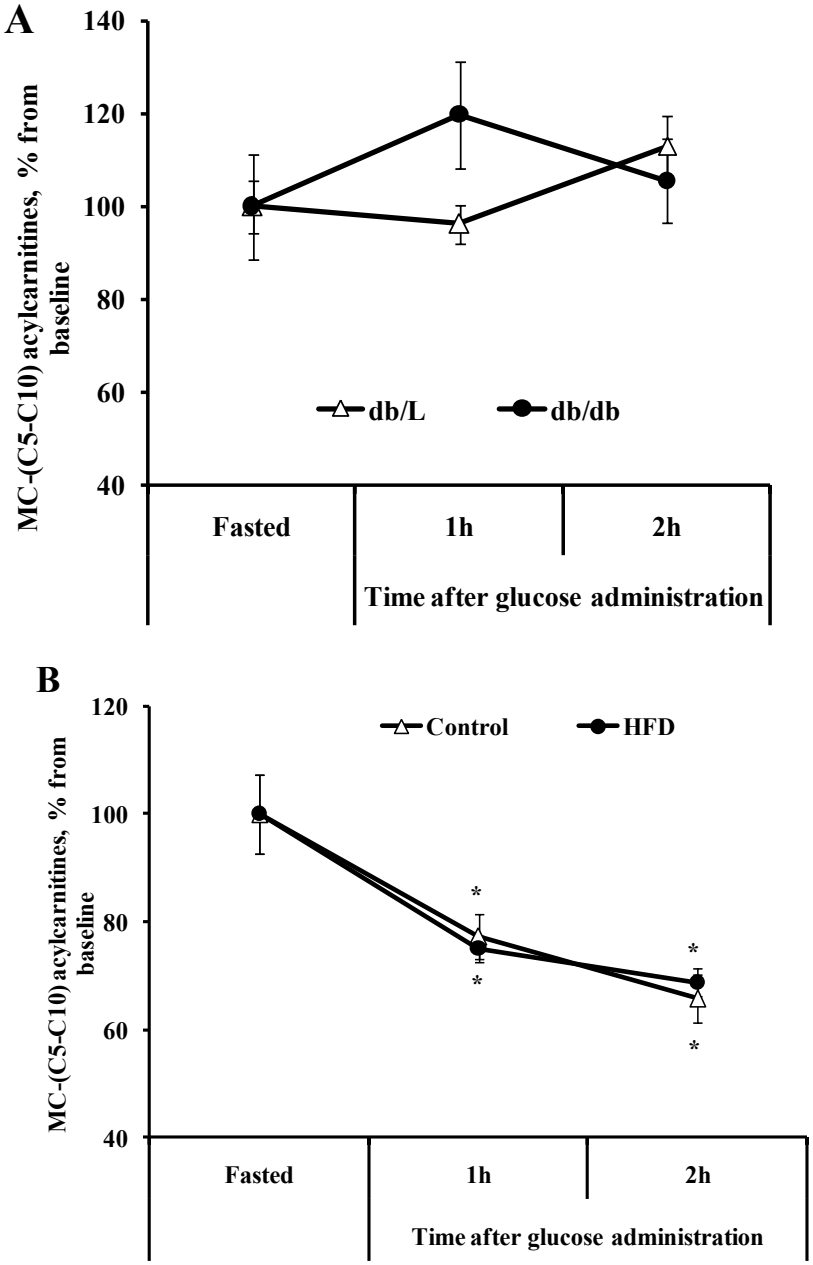


*Significantly different from fasted state (Repeated measures one-way ANOVA with Dunnett's post-test, $p < 0.05$). #Significantly different from respective control group (Student's t-test, $p < 0.05$).

The plasma levels of SC- and MC-acylcarnitine (C5-C10) were decreased equally in the chow and HFD-fed mice plasma by about 25 and 30 %, respectively (Figure 12 B and 13 B). Furthermore, there were no significant differences in circulating MC-acylcarnitine

concentrations after glucose load between the *db/db* and *db/L* mice (Figure 13 A), indicating that changes in plasma SC- and MC-acylcarnitine concentrations do not characterize the severity of insulin resistance.

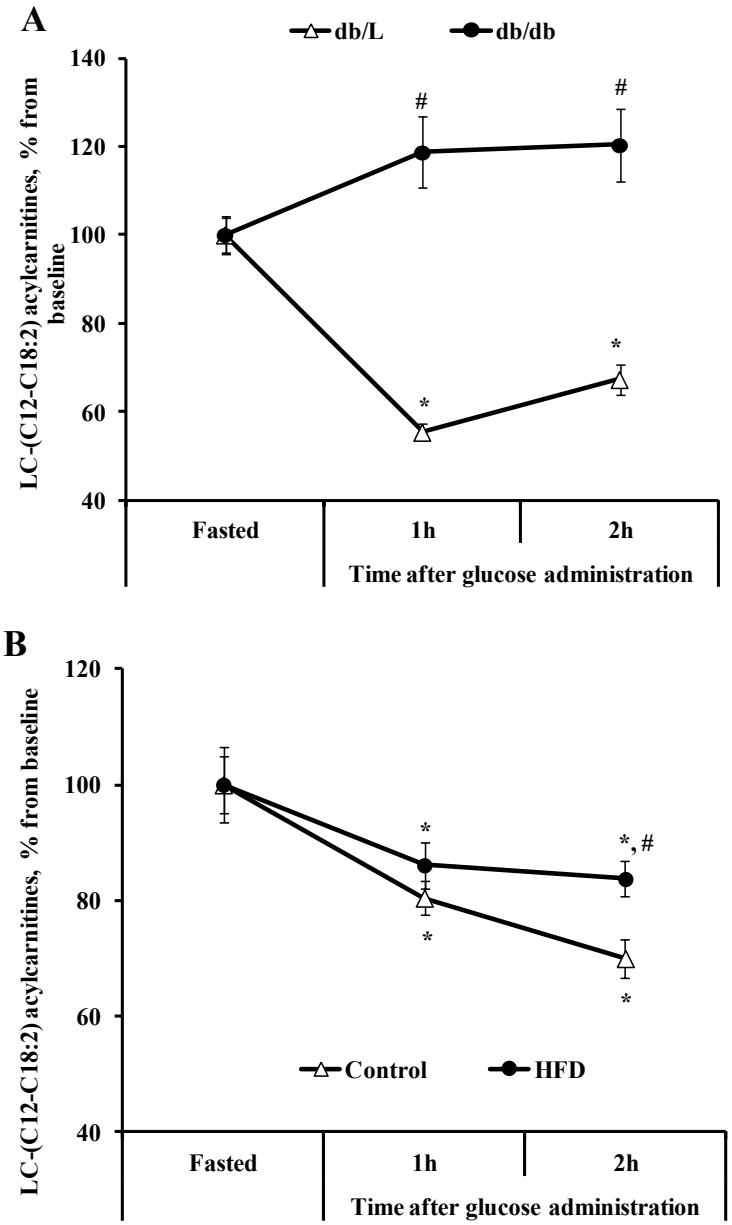
Figure 13: Changes in medium-chain (MC)-acylcarnitine concentrations in plasma of control, HFD-fed *C57bl/N, db/L* and *db/db* mice during glucose tolerance test.



*Significantly different from fasted state (Repeated measures one-way ANOVA with Dunnett's post-test, $p < 0.05$).

In healthy control *C57bl/N* and db/L mice, the concentrations of LC-acylcarnitines (C14-C18:2) in plasma decreased in response to insulin release after the glucose administration by 30-33 % (Figure 14 A and B).

Figure 14: Changes in long-chain (LC)-acylcarnitine concentrations in plasma of control, HFD-fed *C57bl/N, db/L* and *db/db* mice during glucose tolerance test.



*Significantly different from fasted state (Repeated measures one-way ANOVA with Dunnett's post-test, $p < 0.05$). #Significantly different from respective control group (Student's t-test, $p < 0.05$).

On the contrary, diabetic db/db mice did not respond to insulin stimuli and plasma LC-acylcarnitine concentration was not significantly decreased during the GTT (Figure 14 A) whereas in HFD-fed insulin resistant mice the administration of glucose decreased LC-acylcarnitine levels only by 14-16 % (Figure 14 B).

The obtained data indicate that the smaller changes in circulating LC-acylcarnitine concentrations during GTT are characteristic for insulin resistance state and impaired change in LC-acylcarnitine concentration could be used as a diagnostic marker of muscle-specific insulin resistance.

4 DISCUSSION

Our study was divided into 2 parts - sample preparation, where I was the most involved, and evaluation of the results. The samples were taken from db/db mice group and its respective control and HDF-mice group, respectively. Individual markers were measured via blood glucose meter, automated haemoglobin testing system and ELISA kit (insulin level) and enzymatic WAKO assay kit (FFA). The acylcarnitine levels were determinated by using of HLPC MS/MS. The obtained data were statistically processed in GraphPad Prism 3.0 statistical software and subsequently compared to other studies, dealing with the development of insulin resistance.

According to our experiment, the results show, that the unchanged response or smaller changes in circulating LC-acylcarnitine concentrations after glucose administration during GTT are characteristic for insulin resistance state and impaired change in LC-acylcarnitine concentration could be used as a diagnostic marker of muscle-specific insulin resistance. The extent of decrease in LC-acylcarnitine plasma levels during the fasted-to-postprandial state transition reflects the sensitivity of endogenous insulin.

The obtained data indicate that db/db mice developed the common features of severe type 2 diabetes like pronounced hyperglycaemia, hyperinsulinemia, considerably impaired glucose tolerance, and diminished rates of glucose uptake in insulin sensitive tissues. On the contrary, HFD-fed mice displayed early changes in insulin sensitivity. As the accumulation of plasma and muscle tissue LC-acylcarnitine levels in type 2 diabetes experimental model was detected only in fed state (Liepinsh *et al.,* 2016) and the amount of food intake could be highly variable, in our study the decrease in LC-acylcarnitine plasma levels after glucose administration was measured. The fact that acylcarnitine levels in fasted state are not indicative was supported by a recent study showing that improvement of insulin sensitivity resulted in an increase of fasted state acylcarnitine levels (Schooneman *et al.*, 2016), although the acylcarnitine levels in fed state were not detected.

SC- and MC-acylcarnitine levels were reduced equally in healthy and HFD-fed animals, but in mice with type 2 diabetes no association between those and the degree of insulin resistance was observed, confirming that changes in plasma SC- and MC-acylcarnitine concentrations do not reflect the severity of insulin resistance and could not be used as diagnostic markers of this disorder.

In our study significant decrease in LC-acylcarnitine plasma levels during the GTT was detected in healthy control animals, while this decrease was less pronounced in animals with moderate insulin resistance and no changes were observed in the model of type 2 diabetes. In insulin resistant subjects, the insulin produced after the glucose load is not able to limit the LC-acylcarnitine production in muscles adequately and its plasma concentration remains high, while in insulin-sensitive individuals a significant decrease of circulating LC-acylcarnitines by at least 30 % in postprandial state was observed. In line with these observations, in healthy volunteers the levels of plasma LC-acylcarnitine dropped by 60-70 % during the oral GTT (Zhao *et al.*, 2009). In addition, insulin infusion reduced all species of plasma acylcarnitines but in patients with T2DM the reduction of LC-acylcarnitines was less pronounced compared to healthy and obese individuals (Mihalik *et al*., 2010).

Also total FFA concentrations in plasma of healthy volunteers were considerably decreased during GTT by 90 % (Zhao *et al.*, 2009). Similarly, in our study the total plasma FFA levels in healthy control animals were decreased approximately by 30-40 %, but no significant changes were observed in experimental models of obesity and type 2 diabetes. The impaired changes of plasma FFA levels from fasted to fed state indicate the metabolic inflexibility and disturbed ability to switch to carbohydrate metabolism but rather reflect the insulin resistance of adipose tissue.

5 CONCLUSION

In conclusion, the obtained data provided an evidence that the smaller changes in circulating LC-acylcarnitine concentrations during GTT are characteristic for insulin resistance state and impaired change in LC-acylcarnitine concentration could be used as a diagnostic marker of muscle-specific insulin resistance. Further clinical studies are needed to confirm the usefulness of this method to detect early changes in insulin sensitivity.

6 LITERATURE

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