Investigation of the effects of prediabetes and non - obese type 2 diabetes mellitus on the heart in experimental models

Summary of the Ph.D. Thesis

Andrea Sója

Supervisor: Tamás Csont MD Ph.D.

Doctoral School of Multidisciplinary Medical Sciences

Department of Biochemistry

Albert Szent-Györgyi Medical School

University of Szeged

Szeged

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Publications related to the thesis

I. Sárközy M, Szűcs G, Fekete V, Pipicz M, Éder K, Gáspár R, **Sója A**, Pipis J, Ferdinandy P, Csonka C, Csont T.
Transcriptomic alterations in the heart of non-obese type 2 diabetic Goto-Kakizaki rats
Cardiovascular Diabetology (2016) 15:110
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II. Szűcs G[1], **Sója A[1]**, Péter M[1], Sárközy M, Bruszel B, Siska A, Földesi I, Szabó Z, Janáky T, Vígh L, Balogh G, Csont T.
Prediabetes Induced by Fructose-Enriched Diet Influences Cardiac Lipidome and Proteome and Leads to Deterioration of Cardiac Function prior to the Development of Excessive Oxidative Stress and Cell Damage
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1. INTRODUCTION

Unfortunately, nowadays a significant proportion of people consume too much, especially carbohydrate-rich food and drinks, and/or spend too little time exercising. These and many other factors can result in obesity and diabetes mellitus (DM). DM is a group of metabolic diseases with one common manifestation, i.e. hyperglycemia. The vast majority of cases of DM falls into two categories: type one- and type two DM (T1DM and T2DM). In the background of T1DM there is an absolute deficiency of insulin secretion. These patients are usually young and thin. As for the much more prevalent T2DM, the causes are resistance to insulin action and consequently an insufficient insulin secretory response. People struggling with this type of DM are primarily adults and obese. In addition to the two main types of DM, the significance of prediabetes also should be emphasized, since this hyperglycemic, pathological state preceding overt diabetes may strongly be associated with an elevated risk of heart diseases.

T2DM is the most common form of diabetes - it accounts for 90-95% of all DM cases and one of the most important complications of the disease is heart failure. Although researchers published a lot of data about DM and its effects on the cardiovascular system, there are still several uncertain and lacking information that would serve the better understanding of the pathomechanism in the background of the disease. Thus, further research on diabetes and its complications have great relevance. In the following paragraphs of the introduction, I will briefly demonstrate prediabetes and a less known but significant form of T2DM, i.e. non-obese T2DM, focusing on their connection with heart diseases. Besides, preclinical models that used for investigating these pathological states will also be discussed in this part.

1.1. Characterization of prediabetes: epidemiology, pathomechanism, cardiac effects

Prediabetes is a metabolic state when fasting plasma glucose (FPG) level is between 5.6 and 7 mmol/L, or blood glucose value measured at the second hour of an oral glucose tolerance test (OGTT) is between 7.8 and 11.1 mmol/L or hemoglobin A1c (HbA1c) level is between 5.7 and 6.5%. Prediabetes affects approximately 35% of the population and unfortunately may remain symptomless for many years. It should be stressed that even the above mentioned slightly elevated but nondiabetic levels of hyperglycemia and impaired glucose tolerance can have a cause-effect relationship to cardiovascular diseases (CVD).

Although only a limited amount of data is available about the effect of prediabetes on the heart, we know that the main macrovascular complications are coronary artery disease (CAD) and heart failure with preserved ejection fraction, characterized by diastolic dysfunction. Although the exact molecular mechanisms in the background of diastolic dysfunction in

prediabetes and DM are not clarified, some processes can be supposed, such as cardiac mitochondrial disorders, cardiac lipid accumulation and decreased Sarcoplasmic/Endoplasmic reticulum calcium ATP-ase 2a (SERCA2a) activity.

It was reported that the proper lipid composition in the heart is strongly correlated with cardiac function and greatly relies on proper cardiolipin (CL) content and species profile. CL is a crucial phospholipid (PL) of the mitochondria that takes part in essential processes like respiration and energy conversion. As the heart is full of mitochondria and CL accounts for approximately 15% of all membrane lipids, alterations in CL content and/or species profile can cause mitochondrial dysfunction which can lead to cardiac diseases including prediabetes and diabetes. Alteration of the lipid composition can be reached by the induction of de novo lipogenesis (DNL), as it has the capacity to modify the circulating nonesterified fatty acid (FA) profile. High-fructose diet is often used to induce prediabetes in rats, and since the metabolism of fructose leads to enhanced DNL in the liver, this model could be suitable to investigate the possible cardiac effects of the modified lipid pool in prediabetic state.

1.2. Characterization of DM: types, epidemiology, pathomechanism, cardiac effects

DM is a heterogenous chronic metabolic disorder characterized by hyperglycemia resulting from impaired insulin secretion, insulin resistance, or both. The disease can be described by FPG level higher than 7 mmol/L, or blood glucose value measured at the second hour of an OGTT higher than 11.1 mmol/L or HbA1c level more than 6.5%. It was reported by the International Diabetes Federation (IDF) that in 2019, circa 463 million adults were suffering from DM and this huge number was estimated to reach 700 million by 2045.

DM is classified by etiology and pathology as T1DM, T2DM and gestational DM (GDM). GDM affects about 5% of pregnant women and in most cases is an early form of T2DM. T1DM accounts for 5% to 10% of people with diabetes and it is primarily a genetic disease of the immune system. The remainder, about 90% of people with diabetes suffers from T2DM. T2DM is increasing in prevalence worldwide, and it is strongly associated with obesity and insulin resistance.

The pathophysiology of diabetic complications can be differentiated into three kinds: macrovascular, microvascular and neurologic. The common denominator of these complications is elevated blood glucose level (BGL). Neuropathy is estimated to affect 60% – 70% of people with diabetes depending on age, duration of diabetes, presence or absence of pain, and whether or not other causes of neuropathy are excluded. Microvascular disease affects capillaries all over the body; the eyes and the kidneys are the organs most obviously involved.

In case of the macrovascular complications mainly the large blood vessels of the heart, brain and legs are affected. The commonest manifestation of macrovascular disease is the atherosclerosis of the coronary arteries, which is the major cause of death in people with diabetes. It is common knowledge that besides atherosclerosis, diabetic patients also have an increased risk of developing diabetic cardiomyopathy (DCM).

T2DM patients are generally obese, however, around 20% of people suffering from the disease are non-obese in Europe and Asia. In the background of the non-obese T2DM there is a more pronounced reduction in insulin secretion and less severe insulin resistance when compared with the obese phenotype. Similarly to obese T2DM patients, non-obese ones also have an elevated risk of CVD and these cardiovascular complications include left ventricular (LV) hypertrophy, fibrosis and diastolic and/or systolic dysfunction. Nonetheless, the exact molecular mechanisms leading to these pathological alterations are not well-known. One possible way to expand our knowledge is the analysis of cardiac gene expression profile, since the up-or downregulation of certain genes can induce pathological processes and result in deteriorations in the heart. The analysis of the transcriptome is a frequently used method in different DM models, but as for non-obese T2DM, very little data is available.

1.3. Preclinical models of diabetes mellitus and prediabetes

Animal models play a crucial role in the research and characterization of disease pathophysiology. Basically, we can distinguish the following types of animal models: [i] genetic - or spontaneously ill - models, [ii] genetically modified models, [iii] diet-induced models, [iv] surgically created models and [v] chemically-induced models. Focusing on DM, the most common animal models are the chemically-induced and genetic models. In case of chemically-induced diabetes, streptozotocin (STZ) and alloxan are the main drugs to be applied. As for the genetic models of T2DM, Zucker Diabetic Fatty (ZDF) rats, Goto-Kakizaki (GK) rats and db/db mice are used most commonly.

For the investigation of the cardiac effects of non-obese T2DM, we used a genetic model animal, the GK rat, that is a widely known model animal of inherited T2DM. This special strain was developed by selective breeding of ordinary Wistar rats with the highest normal BGLs after applying OGTT. By week 4-5, GK rats can be characterized with a non-obese and slight hyperglycemic phenotype along with glucose intolerance and peripheral insulin resistance, which turns to a hyperglycemic insulin-deficient condition as they getting older.

GK rats show valuable characteristics that are more or less common and functionally present in human diabetic patients. However, hyperglycemia and glucose intolerance in these

animals are not connected with the development of obesity or hypertension. Another characteristic features of this non-obese rat strain are impaired insulin secretion in response to glucose, elevated glycosylated hemoglobin, altered heart and body weight, and various late complications including cardiovascular disorders. Since these cardiovascular disorders include hypertrophy, fibrosis and systolic and/or diastolic dysfunction, which can be found in the background of DCM, the development of DCM is very likely in GK rats. As in adult GK rats several cardiovascular complications has been shown to develop, and the pathogenesis of T2DM present in humans is well mimicked in this rodent, we can state that GK rat is an applicable model animal for the examination of the cardiac consequences of non-obese T2DM.

In addition to genetic and chemical animal models, high carbohydrate-diet-induced models are also used quite often to achieve experimental diabetic or prediabetic condition. As people nowadays consume more and more food containing a high amount of carbohydrates, applying chronic, diet-induced models seems to be an appropriate way to induce and examine the developed prediabetic or diabetic states. As a source of sugar in the chow and/or drinking water of the experimental animals, fructose, sucrose and glucose are most commonly used. In our study, we used fructose-enriched diet, since high-fructose diet is often applied to induce prediabetes in rats. As the metabolism of fructose gives rise to enhanced DNL in the liver, it might be a proper method to investigate the effects of the modified lipid pool in the heart, too.

2. AIMS

Our main goal was to investigate the cardiac effects of prediabetes and non-obese T2DM. For the prediabetic study, we used a chronic, high-fructose diet-induced rat model. Unlike in diabetes, not much is known about the molecular mechanisms causing diastolic dysfunction in prediabetes. It is common knowledge, however, that the metabolism of fructose leads to DNL in the liver. Thus, we were curious whether the altered lipidome has adverse effects on the heart, playing a role in cardiac dysfunction. Moreover, since increased apoptosis and oxidative stress often contribute to cardiac dysfunction, we also aimed to explore the effects of prediabetes on apoptosis and oxidative stress in the heart.

To investigate the cardiac effects of non-obese T2DM, GK rat, a well-known genetic model of the disease was chosen. The molecular mechanisms in the background of cardiac complications in non-obese T2DM are not known exactly. Though there are some literature data on the cardiovascular complications of non-obese T2DM, alterations in the heart of GK rats at the transcript level has never been studied before. So our goal was to examine the influence of non-obese T2 diabetes on cardiac alterations of the transcriptome in GK rats.

3. METHODS

3.1. Diet-induced prediabetes model

3.1.1. Experimental design

Male Wistar rats were kept under controlled temperature with 12/12 h light/dark cycles. Animals were divided into two groups and were fed with the following diets for 24 weeks: the control group was fed with a standard laboratory chow, while the fructose-fed group received a chow containing 60% w/w fructose. FBG was measured every 4 weeks, while at weeks 12, 16, 20 and 24 OGTTs were performed. At week 20 and 24, blood samples were taken to measure serum parameters such as insulin, triglyceride (TG), cholesterol, markers of heart and liver damage and oxidative stress. At the end of the feeding protocol, cardiac function was assessed by *in vivo* echocardiography and *ex vivo* isolated working heart perfusions. Following the perfusions, myocardial tissue was harvested for biochemical analysis.

3.1.2. Measurement of serum glucose levels and OGTT

Blood samples were collected from the saphenous vein and BGLs were measured using Accu-Chek blood glucose monitoring systems. In case of OGTT, after the measurement of baseline glucose concentrations, 1.5 g/kg body weight glucose was administered *per os* via gavage and BGLs were checked 30, 60 and 120 min later.

3.1.3. Measurement of serum and pancreatic insulin levels

Serum and pancreatic insulin levels were measured by enzyme-linked immunosorbent assay (ELISA). Insulin ELISA was carried out according to the instructions of the manufacturer from either sera or homogenized pancreatic tissue samples of fructose-fed and control rats.

3.1.4. Homeostatic model assessment for insulin resistance (HOMA-IR index)

HOMA-IR index was calculated by multiplying fasting serum insulin (μU/mL) with fasting serum glucose (mmol/L) then dividing by the constant 22.5.

3.1.5. Measurement of serum lipid levels

Serum total cholesterol (TC), TG, LDL and HDL (low-density and high-density lipoprotein) cholesterol levels were measured at week 24 in triplicate, using colorimetric assay kits applying enzymatic determinations adapted to 96-well plates.

3.1.6. Measurement of serum markers of liver and heart damage

Alanine- and aspartate aminotransferase (ALAT, ASAT), creatine-kinase (CK) and lactate dehydrogenase (LDH) enzyme activities were measured with UV assays. CK-MB enzyme activities were determined using an immunological UV assay.

3.1.7. Transthoracic echocardiography

Cardiac morphology and function were assessed by transthoracic echocardiography at week 24. Rats were anesthetized with sodium pentobarbital and two-dimensional, motion-mode (MM) and Doppler echocardiographic examinations were performed by the criteria of the American Society of Echocardiography with a Vivid IQ ultrasound system. Data of three consecutive heart cycles were analyzed by an experienced investigator in a blinded manner.

3.1.8. Working heart perfusion

Cardiac performance was assessed in isolated working rat hearts. Anesthetized rats were given heparin intravenously, then the hearts were isolated and the aorta was cannulated and initially perfused in Langendorff mode with Krebs-Henseleit buffer. Then, the perfusion system was switched to working mode according to Neely with recirculating buffer. Cardiac functional parameters including heart rate, coronary flow (CF), aortic flow, cardiac output, LV developed pressure (LVDP) and its first derivatives (dp/dt max and dp/dt min) and LV end-diastolic pressure (LVEDP) were measured.

3.1.9. mRNA expression profiling by qRT-PCR

Quantitative real-time - Polymerase chain reaction (qRT-PCR) was performed with gene-specific primers to monitor mRNA expression. To assess DNL, expression of sterol regulatory element-binding transcription factor 1 (*Srebf1*), stearoyl-CoA desaturase 1 (*Scd1*), fatty acid synthase (*Fasn*), acetyl-CoA carboxylase 1 (*Acaca*), carbohydrate-responsive element-binding protein (*Mlxipl*), elongation of very-long-chain fatty acids protein 6 (*Elovl6*), fatty acid desaturase 1 and 2 (*Fads1* and *Fads2*) were measured from liver samples. To assess cardiac hypertrophy, expression of myosin heavy chain α and β isoforms (*Myh6* and *Myh7*) was measured. RNA was isolated using Qiagen RNeasy Fibrous Tissue Mini Kit. Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) was used as control for normalization.

3.1.10. Lipidomics

Approximately 20 mg of the powdered left ventricle was directly extracted by adding methanol containing butylated hydroxytoluene and di20:0 phosphatidylcholine. After sonication, the mixture was shaken and centrifuged. The supernatant was transferred into a new Eppendorf tube and stored at −20 °C until mass spectrometry (MS) analysis. MS analyses were performed on an LTQ-Orbitrap Elite instrument.

3.1.11. Determination of oxidative stress: measurement of MDA and 3-NT levels

In order to determine the level of systemic and cardiac lipid peroxidation, serum malondialdehyde (MDA) and cardiac tissue MDA were measured by the following manner. Serum samples were mixed with 1.2 volumes of a stock solution of trichloroacetic acid,

thiobarbituric acid (TBA) and HCl, and heated for 30 min at 95 °C. After cooling and centrifugation, the supernatant was extracted in butanol and assayed spectrophotometrically.

For the measurement of 3-nitrotyrosine (3-NT), a double-antibody sandwich ELISA kit was used. Left ventricles were homogenized and centrifuged. 3-NT was measured according to the manufacturer's instructions and protocols, and optical densities were determined at 450 nm.

3.1.12. Western blot

This technique was used to investigate changes of B-cell lymphoma 2 (BCL-2), Bcl-2-associated X (BAX), B-cell lymphoma-extra large (BCL-XL) and caspase-3 and 7 apoptotic proteins in the cardiac tissue, with actin or tubulin loading background. LV samples were homogenized and centrifuged. After quantification of protein concentrations of the supernatants, 25 μg protein was loaded. Then, Sodium Dodecyl Sulfate (SDS) - polyacrylamide gel electrophoresis was performed followed by the transfer of proteins onto a nitrocellulose membrane. Membranes were blocked and then incubated with primary antibodies overnight. Then, the membranes were incubated with secondary antibodies and the fluorescent signals were detected by Odyssey CLx.

3.1.13. Statistical analysis

Lipidomic data are presented as mean±SEM and statistical significance was accepted for $p<0.05$. Principal component analysis (PCA) was performed using MetaboAnalyst. All other parameters are presented as mean±SEM, and significance between groups was determined with two sample *t*-test or Mann-Whitney Rank Sum Test.

3.2. Non-obese genetic model of type 2 diabetes

3.2.1. Experimental design

Male GK rats and their age-matched male Wistar controls were obtained from Charles River Laboratories at the age of 6 weeks and were housed at 22 ± 2 °C with a 12:12-h light–dark cycle. The rats received standard rat chow and water ad libitum for 9 weeks after their arrival. Body weight, serum glucose, insulin, cholesterol levels and HOMA-IR were determined at 7, 11 and 15 weeks of age and OGTT was performed at week 15. At 15 weeks of age, rats were anaesthetized, hearts and pancreata were isolated, and then hearts were perfused according to Langendorff as described earlier in 3.1.8. After 5 min perfusion, ventricular tissue was frozen and stored at −80 °C until gene expression analysis.

3.2.2. Measurement of serum insulin levels

The method is the same as described earlier in 3.1.3. with some differences: at week 15 during OGTT, blood was collected at 0, 30 and 120 min for serum insulin level measurements.

3.2.3. RNA preparation and DNA microarray analysis

Total RNA was isolated from heart samples with Qiagen miRNeasy Mini Kit according to the manufacturer's protocol. On-column DNase digestion was carried out with the RNase-Free DNase Set. RNA concentration was measured by NanoDrop 1000 Spectrophotometer and RNA integrity was determined by an Agilent 2100 Bioanalyzer System. Samples with an RNA integrity number above 8.0 were used for further analysis.

Total RNA was labelled and amplified using the QuickAmp Labelling Kit. Labelled RNA was purified and hybridized to Agilent Whole Rat Genome 4 × 44 K array slides. After washing, array scanning and feature extraction was performed with default scenario by Agilent DNA Microarray Scanner and Feature Extraction Software 9.5.

3.2.4. mRNA expression profiling by qRT-PCR

In order to validate gene expression changes obtained by DNA microarray, qRT-PCR was performed with gene-specific primers. Total RNA was reverse transcribed and qRT-PCR was performed. Relative expression ratios were calculated as normalized ratios to rat glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), *Hprt* and ribosomal protein S18 (*Rps18*) housekeeping genes.

3.2.5. Gene Ontology (GO) analysis

It was performed using GO pathway analysis using DAVID bioinformatics system and database. The differentially expressed genes were submitted to DAVID to reveal significantly enriched biological functions/pathways.

3.2.6. Statistical analysis

Statistical analysis was performed by using Sigmaplot 12.0 for Windows. All values are presented as mean±SEM. Repeated measures Two-Way ANOVA was used to determine the effect of T2DM and age on FBG, serum insulin and cholesterol levels as well as glucose levels during OGTT. After ANOVA, all pairwise multiple comparison procedures with HolmŠídák post hoc tests were used as multiple range tests. Two-sample t test was used to determine the effect of T2DM on OGTT AUC, pancreatic insulin concentration, body weight, heart weight, heart weight/body weight ratio (HW/BW) and CF. $P<0.05$ was accepted as a statistically significant difference. Both in the microarray and qRT-PCR experiments, a two-sample t test was used and the p-value was determined to find significant gene expression changes. Gene expression ratios with p-value of <0.05 and log2 ratio of <−1.00 or log2 ratio of >1.00 (~2.0-fold) were considered as repression or overexpression in gene activity, respectively.

4. RESULTS

4.1. Diet-induced prediabetes model

4.1.1. Characterization of the animal model

In the present study, Wistar rats were fed with 60% fructose-containing chow for 24 weeks. FBG levels were slightly but significantly higher in fructose-fed rats at weeks 12, 16, 20 and 24, and OGTT AUC values were also significantly increased in this group at weeks 16, 20 and 24. HOMA-IR was significantly higher in the fructose-fed rats at week 20, although no significant difference was detected in serum insulin levels. Pancreatic insulin level was significantly higher in the fructose-fed group compared to controls.

Although body weight increased in both groups during the study, by the end of the 24-week feeding, the weight of the fructose-fed rats was significantly smaller. Weight gain during the study was decreased in fructose-fed rats. During the isolation of organs, we have observed macroscopical signs of fatty degeneration on the liver of fructose-fed animals. Despite these characteristic signs on the liver, neither serum lipid parameters, nor liver enzymes were increased in fructose-fed rats.

To further characterize metabolic changes in the liver of fructose-fed rats, qRT-PCR was performed. We examined different genes related to DNL and experienced significant increase in case of *Acaca* and *Elovl6*. Besides, a tendency of increase could be seen in *Fasn* expression.

4.1.2. Heart function and morphology

Wall thicknesses and ventricular diameters were not changed significantly due to fructose feeding (except for anterior wall thickness). Though there was no difference in heart rate, ejection fraction and fractional shortening, the E/A ratio was significantly smaller in fructose-fed rats. Besides, LVEDP significantly increased, while cardiac output significantly decreased. Max and min dp/dt, LVDP, and aortic systolic and diastolic pressures were not changed between the groups.

Since the echocardiographic examination showed significant increase of anterior wall thickness in systole in the fructose-fed group, but the weight of the animals was significantly lower in this group - though the heart weight and HW/BW were not changed significantly -, we wanted to check the possible development of hypertrophy in the heart of the fructose-fed animals. In order to assess cardiac hypertrophy at the molecular level, mRNA expression of *Myh*6 and *Myh7* was measured. We have found that cardiac *Myh*6 mRNA level was increased in fructose-fed rats. However, *Myh6/Myh7* ratio did not differ significantly. Furthermore, we measured CK, CK-MB and LDH enzymes and found that none of them changed significantly in fructose-fed rats compared to controls.

4.1.3. Lipidomics

We have identified and quantified approximately 200 lipid molecular species encompassing 20 lipid classes. The clear separation of the sample sets into two nonoverlapping clusters indicates complex reshaping of the whole lipidome due to fructose feeding. Examining these alterations in more detail and comparing the molecular species patterns for the control and fructose groups revealed 100 statistically significant differences. One of the most noteworthy changes can be connected to the CL remodeling system. The level of matured CL showed significant decrease, while monolysocardiolipin (MLCL) level increased significantly, and consequently their ratio increased markedly in the membrane (MLCL/CL). Furthermore, at the molecular species level, we detected pronounced loss of the most abundant homosymmetric tetra18:2 species CL (72:8). This was the most prominent change not only in the context of membrane composition but also when considering absolute values. The loss in CL (72:8) was paralleled by elevations in practically all other asymmetric species independently on chain length and saturation for the fructose-fed animals. This altogether resulted in a dramatic drop of the CL "symmetry" factor calculated as the ratio of symmetric/asymmetric species. It is important to note here that we could not detect oxidized lipid species either in CL or in other oxidation-prone lipid classes. However, we could detect "asymmetry" defects already in the MLCL species profile.

Another important alteration of the lipidome was the general increase in lipid species with sum double bond (db)=1 in the fructose group. In parallel with the elevation of species with db=1, we detected significant depletion in species with db=2 in fructose-fed animals.

Furthermore, although the total cardiac TG content did not changed significantly, the prominent species profile change of the TG pool is worth mentioning. The robust relative increase in species containing saturated and monounsaturated FAs, in parallel with significant reductions in more unsaturated species, altogether led to the decrease of the double bond index (DBI), i.e., increase in saturation for cardiac TG.

The last interesting aspect of the complex lipidome remodeling was the reshaping of the sphingolipid (SL) pool, ceramide (Cer) and sphingomyelin (SM). We measured small but significant elevation in total cardiac Cer at membrane lipid compositional level. Besides, the total level of SM showed only an increasing tendency in the membrane, but its species compositions changed completely.

4.1.4. Oxidative stress

There was no significant increase in the levels of MDA in the serum or cardiac tissue, or in the cardiac level of 3-NT as compared to control values.

4.1.5. Apoptosis

Prediabetes did not affect the expression of proapoptotic caspase-7 and BAX, while the antiapoptotic BCL-2 was downregulated, thereby BAX/BCL-2 ratio increased significantly.

4.2. Non-obese genetic model of type 2 diabetes

4.2.1. Metabolic characterization of the GK rats

GK rats showed a significantly elevated FBG level at all time points as compared to controls. At week 11 and 15, BGL in GK rats was significantly lower as compared to week 7 blood glucose values. Serum insulin levels were significantly increased in GK rats at week 7 and week 11. However, there was no significant difference between serum insulin levels measured in GK and control rats at week 15. HOMA-IR was significantly increased at weeks 7 and 11 in GK rats when compared to controls. In contrast, increase of HOMA-IR did not reach the level of statistical significance at week 15 in GK animals. Serum cholesterol levels were significantly higher in GK rats as compared to control ones throughout the study duration.

Glucose levels during OGTT were markedly increased in GK rats in every time point of blood glucose measurements. AUC of BGLs during OGTT was significantly increased in GK rats. In addition, serum insulin levels in GK rats during OGTT were significantly lower 30 min after glucose loading and became markedly increased 120 min after glucose administration. Interestingly, pancreatic insulin levels were lower in GK rats compared to control ones, however, the values were not significantly different between the two groups.

4.2.2. Body weight, heart weight and CF

Body weight was significantly decreased, while heart weight and HW/BW were significantly increased at week 15 in GK rats. Interestingly, CF was significantly increased at week 15 in GK rats as compared to control hearts.

4.2.3. Cardiac gene expression profile and qRT-PCR in non-obese T2DM

Among the 41,012 genes surveyed, 507 genes whose expression was > ~2.0-fold up- or downregulated in hearts of GK rats relative to levels of control rats showed significant change in expression. According to our results, 204 genes showed upregulation and 303 genes showed downregulation in hearts of GK rats. Moreover, 138 genes showed more than threefold change of expression in hearts of GK rats. Among these 138 genes, 50 were significantly upregulated and 88 were significantly downregulated in GK rat hearts. The expression change of selected 28 genes was validated by qRT-PCR; 19 of these 28 genes have been confirmed by qRT-PCR.

4.2.4. Gene ontology analysis

Out of the 507 genes significantly altered by non-obese T2DM, 277 genes with known function were submitted to GO analysis and 115 were clustered into different categories. The

rest of the 507 genes were either unknown expressed sequence tags or unrecognized by the GO analysis database. The 115 analyzed genes were classified into five main categories such as (1) biological regulation, (2) metabolic process, (3) immune system process, (4) biological adhesion and (5) rhythmic process.

5. DISCUSSION

5.1. Diet-induced prediabetes model

We used a chronic, high fructose-fed rat model in order to induce prediabetes and to investigate the effects of this pathological condition on the heart. As previously mentioned, FBG levels were slightly but significantly higher in fructose-fed rats, and from week 16 the OGTT AUC values were significantly increased as well. Thus, we could verify the development of prediabetic condition with impaired glucose tolerance. In the fructose-fed group, the significantly increased HOMA-IR and pancreatic insulin levels, and the unchanged serum insulin levels demonstrate the emergence of a mild insulin resistance.

In the fructose-fed group, the significantly smaller body weight by the end of the 24-week feeding and accordingly, the decreased weight gain and the macroscopical signs of fatty degeneration on the liver can be connected and interpreted as follows. Our findings may indicate that the cause of fatty degeneration in the liver was DNL initiated by fructose feeding. Fructose may activate DNL due to its fast conversion to pyruvate bypassing the regulatory step of glycolysis**.** High-fructose diets can affect the metabolism of skeletal muscle through metabolic stress. For example, it has been shown that excessive fructose consumption increases the production of methylglyoxal in the liver, which leads to oxidative stress in the muscle. Activated DNL can result in endoplasmic reticulum stress and production of hepatokines that are known to negatively impact muscle energy metabolism and insulin sensitivity. These results might explain the experienced body weight gain decrease in the fructose-fed rats. In spite of the macroscopic signs of fatty degeneration in the liver, the unaltered serum lipid parameters and liver enzymes suggest an early stage of hepatic consequences in the fructose-fed animals.

For the further characterization of metabolic alterations in the liver of fructose-fed rats, qRT-PCR was performed. The expression of *Acaca*, that is responsible for the rate-limiting step of FA synthesis, was increased. Besides, the expression of *Fasn*, which catalyses the remaining steps of palmitic acid synthesis, showed a tendency of increase. These findings are consistent with previous results and indicate increased DNL in fructose-fed rats. *Elovl6* catalyzes the first and rate-limiting reaction of long-chain FA elongation cycle. It has been reported that *Elovl6* plays an important role also in nonalcoholic fatty liver disease (NAFLD) and steatohepatitis.

To follow-up the alterations caused by prediabetes, our study was continued by the examination of the heart. Echocardiographic data showed significantly increased anterior wall thickness in systole and significantly smaller E/A ratio. These findings might suggest a very early sign of a mild hypertrophy and diastolic dysfunction due to chronic high-fructose diet. However, the heart weight and HW/BW were not changed significantly. Therefore, in order to assess cardiac hypertrophy at the molecular level, we measured the mRNA expression of *Myh6* and *Myh7*. We found elevated cardiac *Myh6* mRNA level along with unchanged *Myh6/Myh7* ratio, that indicates the lack of hypertrophy according to the literature. As for the working heart perfusion, we observed that LVEDP significantly increased, while cardiac output significantly decreased in fructose-fed rats, indicating the appearance of a mild diastolic dysfunction in the prediabetic animals. It is widely known that LV hypertrophy is more common in diabetic patients and that 40-75% of people suffering from T1DM or T2DM have diastolic dysfunction. However, here we demonstrate that at this early stage of prediabetes cardiac hypertrophy has not developed yet, but the impairment of diastolic function occurs much earlier than the development of overt diabetes. Furthermore, the clinical laboratory markers of myocardial injury did not change significantly in fructose-fed rats compared to controls, indicating the appearance of cardiac dysfunction prior to severe structural/cellular damage.

Since presumably there was DNL in the liver of the fructose-fed rats, we were curious whether DNL was present in the heart as well. For this purpose, lipidomic analysis was performed. One of the most notable changes can be connected to the CL remodeling system. It is acknowledged that under normal circumstances, the levels of lysophospholipids (LPL) are kept low and CL remodeling requires only trace amounts of MLCL. Therefore, the significantly decreased level of matured CL in parallel with the significantly increased level of MLCL clearly report about an abnormal remodeling process in the fructose-fed group. Another remarkable alteration occured at the molecular species level is the pronounced loss of the most abundant homosymmetric tetra18:2 species CL (72:8). The decrease in CL (72:8) was accompanied by an increase in the other asymmetric species and these alterations altogether led to a marked drop of the CL „symmetry" factor in the prediabetic group.

Another notable feature of the lipidome alterations in the fructose-fed group was the general rise in lipid species with sum db=1, while PL species with db=2 showed significant depletion. The latter lipids mostly contained a saturated FA in *sn1* and a linoleoyl (18:2) group in *sn2* position of the glycerol backbone; these can function as potential acyl donors for the formation of tetra 18:2 CL for the transacylation reaction catalysed by tafazzin enzyme. As for

PL molecular species with highly unsaturated acyl chains, several significant alterations were seen. It was described in a Barth syndrome mouse model that the loss of tafazzin activity also caused complex changes of polyunsaturated PL species. Therefore, we can suggest that the imbalance in polyunsaturated PL species modifies the biophysical and signaling properties of the cardiac membrane.

As mentioned earlier, the total cardiac TG content did not show significant alteration due to fructose feeding, however, there was a great species profile change at the TG pool, namely the increase in species containing saturated and monounsaturated FAs and decrease in more unsaturated species. As a consequence of these alterations, cardiac TG saturation increased, which together with the monoene rise and 18:2 reduction in membrane PLs might indicate the upregulation of DNL.

As the last notable aspect of the complex lipidome remodeling, we have to mention the reshaping of Cer and SM. Cer has a central role in SL metabolism as it functions as a mediator of the eukaryotic stress response. Its role is mainly associated with growth inhibition; the most investigated being its function as a proapoptotic molecule. In our work, a small but significant elevation occured in total cardiac Cer at membrane lipid compositional level due to mainly the elevation in very long chain Cer-24 species. It was showed that in confluent Michigan Cancer Foundation-7 (MCF-7) cells C-24 Cer species influenced cell cycle arrest, but not apoptosis. From these findings we assume that in our model the alterations in Cer led to pathologic cardiac function by changing the membrane biophysical properties rather than triggering apoptosis. SM is considered as the major structural mammalian SL which accumulates in microdomains. Its total level showed only an increasing tendency, while its species compositions changed entirely. These data suggest microdomain reorganization and thus the modulation of the membrane physical state and signaling properties as a result of chronic high-fructose diet.

As oxidative stress has been linked to the impairment of cardiac function, we continued our study by the measurement of this factor. We found no significant elevation in the levels of MDA and 3-NT as compared to the control values, and we could not detect any oxidized lipid species. It is important to emphasize that in our model, we could see many significant alterations in the cardiac lipidome before the development of any changes of oxidative stress markers.

The last probable aspect we examined which might be related to cardiac dysfunction is apoptosis. Regarding our western blot results, prediabetes had no effect on the expression of proapoptotic caspase-7 and BAX, while the antiapoptotic BCL-2 was downregulated. Our data suggest early dysregulation of antiapoptotic proteins in prediabetic state. However, these alterations are not remarkable enough to consider apoptosis as an essential factor in prediabetes.

5.2. Non-obese genetic model of type 2 diabetes

A further goal of this thesis was to investigate how non-obese T2DM influences cardiac gene expression pattern. The model we chose was the spontaneously diabetic GK rat, which is - as for the pathomechanism - considered to be very similar to the non-obese T2DM in humans. It was reported that the abnormal glucose regulation in the GK rat develops in association with imperfect insulin secretion and also with insulin resistance. BGLs were significantly higher in GK rats at each time of measurement and we observed significant difference in BGLs between the different time points. At week 11 and 15, the BGL of GK rats was significantly lower compared to the value measured at week 7. Furthermore, serum insulin level was significantly elevated at week 11 in GK animals compared to week 7 values. Serum insulin levels along with HOMA-IR were significantly increased at week 7 and 11 indicating rising insulin resistance and compensative hyperinsulinemia. At week 15, the pancreatic insulin level of GK animals was moderately decreased referring to the exhaustion of beta cells. It must be noted that these findings are in accordance with literature data demonstrating that beta cell mass along with insulin production continuously decreases from birth to adulthood in GK animals.

At week 15, body weight was significantly decreased in GK animals proving a non-obese phenotype of T2DM. Moreover, heart weight and HW/BW were significantly elevated implying the development of cardiac hypertrophy. As a further confirmation of increased cardiac size, we found that the CF was significantly higher in GK rats as compared to the values of the control animals. These findings proving the presence of cardiac hypertrophy indicate the development of DCM in GK rats.

Surprisingly, there are only a few studies where qPCR was performed to investigate the gene expression changes involved in the development of impaired cardiac morphology/function in GK rats. Therefore, this study can be considered the first to characterize overall alterations in the cardiac transcriptome in male GK rats. These significantly altered genes are classified here into such categories as metabolism, signal transduction, membrane and structural proteins, etc. Besides, several other genes with unknown function in the heart were also changed in response to diabetes. In the following part, some genes related to **DCM** showing significantly altered expression, selected from the previously classified specific clusters, will be discussed.

In our present study, we have shown altered expression of numerous genes being a factor in the structure formation of the myocardium and possibly connected with DCM, based on GO analysis. These genes include for instance downregulation of collagen, type V, alpha 3 (*Col5a3*) and myosin, light chain 7, regulatory (*Myl7*). Collagen V regulates collagen fibril geometry and strength especially in the pancreas and skeletal muscle. It was reported that skeletal muscle of

Col5a3$^{-/-}$ mice was defective in glucose uptake and mobilization of intracellular GLUT-4 glucose transporter to the plasma membrane in response to insulin, resulted in glucose intolerance, insulin resistance and hyperglycemia. In addition, decreased expression of the genes playing a part in contractility such as *Myl7* could cause sarcomeric dysfunction and DCM.

Another group of genes which showed altered expression due to T2DM function as receptors and ion channels. These genes are the following: e.g. downregulation of adrenoceptor alpha 1d (*Adra1d*) and sarcolipin (*Sln*). The downregulation of *Adra1d* has been shown earlier in connection with cardiac hypertrophy and in STZ-induced DM by our research group. *Sln* is a central regulator of SERCA. The abnormal function of *Sln* leads to elevated SERCA activity, resulting aberrant intracellular Ca^{2+} handling and atrial remodeling with dysfunction.

A main cluster of significantly changed cardiac genes induced by non-obese T2DM was related to signal transduction, regulation of transcription and biological processes (for example downregulation of signal transducer and activator of transcription 3 (acute-phase response factor) (*Stat3*) and upregulation of Jun D proto-oncogene (*Jund*)). *Stat3* takes part in several physiological processes such as proliferation, apoptosis and cardiac survival particularly during myocardial ischemia/reperfusion injury, although its role is contradictory in these processes. Large number of studies have reported that cardiac *Stat3* expression was reduced in DM, potentially resulting cardiac dysfunction. Other literature data demonstrated that cardiac *Stat3* expression was increased in DM, inducing hypertrophy. In our present work, *Stat3* was downregulated as assassed by microarray, however, qRT-PCR did not confirm this. It is conceivable that the expression of *Stat3* depends on the duration of DM and the stage of DCM. *JunD* regulates genes involved in antioxidant defense mechanisms. Another major function of *JunD* is the modulation of insulin/insulin-like growth factor 1 signaling and longevity.

After discussing genes regarding DCM, there is another important group to mention in which we saw significantly altered expression in response to non-obese T2DM. These cardiac genes are related to immune and antimicrobial response (e.g. downregulation of complement component 3 (*C3*) and upregulation of chemokine (*C-X-C* motif) receptor 6 (*Cxcr6*). The altered expression of these genes is in line with the increased susceptibility to infections in DM.

Finally, there were several novel genes with altered expression in this study which have not been previously related to diabetic changes in the heart (for instance downregulation of mesothelin (*Msln*) and upregulation of kallikrein 1-related peptidase C3 (*Klk1c3*)). Some other altered genes were not categorized into specific functional groups or indicated as yet uncharacterized, predicted genes and fragments, which might have important roles in diabetes.

5.3. Conclusion

In summary, we can state that the designed comprehensive analysis of alterations in the heart caused by prediabetes and non-obese T2DM was performed successfully. In rats chronically fed a high-fructose diet we found an early stage of prediabetes that was associated with deteriorated cardiac function and the total reshaping of the myocardial lipidome - especially CL pool. It is very important to highlight that cardiac lipidomic alterations preceded the development of sizeable hypertrophy, apoptosis and oxidative stress in the heart.

In our other study, we have demonstrated for the first time that non-obese T2DM can be connected to a thorough alteration of the transcriptome in the heart of GK rats, represented by numerous up-and downregulated genes in connection with the disease. Nevertheless, future studies are necessary to investigate the precise role of these specific genes in the development of cardiac consequences (i.e. DCM) of non-obese T2DM.

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ABBREVIATIONS

3-NT: 3-nitrotyrosine
A: Atrial (flow velocity)
Acaca: Acetyl-CoA carboxylase 1
Adra1d: Adrenoceptor alpha 1d
ALAT: Alanine aminotransferase
ASAT: Aspartate aminotransferase
AUC: Area under the curve
BAX: Bcl-2-associated X
BCL-2: B-cell lymphoma 2
BGL: Blood glucose level
BCL-XL: B-cell lymphoma-extra large
C3: Complement component 3
Ccl12: Chemokine (C–C motif) ligand 12
Cer: Ceramide
CF: Coronary flow
CK: Creatine kinase
CL: Cardiolipin
Cma1: Chymase 1, mast cell
Col5a3: collagen, type V, alpha 3
CVD: Cardiovascular diseases
Cxcr6: Chemokine (*C-X-C* motif) receptor 6
db: double bond
DBI: Double bond index
DCM: Diabetic cardiomyopathy
DM: Diabetes Mellitus
DNL: de novo lipogenesis
E: Early (flow velocity)
ELISA: Enzyme-linked immunosorbent assay
Elovl6: Elongation of very-long-chain fatty acids protein 6
Epn3: Epsin 3
FA: Fatty acid
Fads1: Fatty acid desaturase 1
Fads2: Fatty acid desaturase 2
Fasn: Fatty acid synthase
FBG: Fasting blood glucose
FFAs: Free fatty acids
FPG: Fasting plasma glucose
Gapdh: Glyceraldehyde-3-phosphate dehydrogenase
GDM: Gestational diabetes mellitus
GK: Goto-Kakizaki
GO: Gene ontology
HDL: High-density lipoprotein
HOMA-IR: Homeostatic Model Assessment for Insulin Resistance
Hprt1: Hypoxanthine phosphoribosyltransferase 1
HW/BW: heart weight to body weight ratio
Jund: Jun D proto-oncogene
LDH: Lactate dehydrogenase
LDL: Low-density lipoprotein
LV: Left ventricular
LVDP: Left ventricular developed pressure
LVEDP: Left ventricular end-diastolic pressure
MDA: Malondialdehyde
MLCL: Monolysocardiolipin
MS: Mass spectrometry
Msln: Mesothelin
Myh6: Myosin heavy chain α isoform
Myh7: Myosin heavy chain β isoform
Myl7: myosin, light chain 7, regulatory
OGTT: Oral glucose tolerance test
PCA: Principal component analysis
PL: Phospholipid
qRT-PCR: Quantitative real-time - Polymerase chain reaction
Sln: Sarcolipin
Scd1: Stearoyl-CoA desaturase 1
SEM: Standard error of the mean
SERCA: Sarcoplasmic reticulum Ca^{2+}-ATPase
SL: Sphingolipid
SM: Sphingomyelin
Srebf1: Sterol regulatory element-binding transcription factor 1
Stat3: Signal transducer and activator of transcription 3
STZ*:* Streptozotocin
T1DM*:* Type 1 diabetes mellitus
T2DM: Type 2 diabetes mellitus
TBA: Thiobarbituric acid
TG: Triglyceride