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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Development and characterization of a clinically relevant rat model of diabetic cardiomyopathy

Promotor :
Prof. dr. Virginie BITO

Copromotor :
Prof. dr. Quirine SWENNEN

Jirka Cops

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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2 Acknowledgements

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3 List of abbreviations

ACE	angiotensin converting enzyme
AGEs	advanced glycation end products
AKT2	RAC-beta serine/threonine protein kinase
ANP	atrial natriuretic peptide
ARB	angiotensin receptor blocker
AT_1	angiotensin II type 1 receptors
ATP	adenosine triphosphate
AWT	anterior wall thickness
BL	baseline
BNP	brain natriuretic peptide
CTGF	connective tissue growth factor
DAG	diacylglycerol
DCCT	diabetes conventional and complications trial
DCM	diabetic cardiomyopathy
DM	diabetes mellitus
DPP-4	dipeptidyl peptidase 4
EDV	end-diastolic volume
EF	ejection fraction
ESV	end-systolic volume
FA	fatty acid
FS	fractional shortening
GFR	glomerular filtration rate
GLP-1	glucagon-like peptide 1
GLUT	glucose transport protein
H&E stain	hematoxylin and eosin stain
HFD	high fat diet
HR	heart rate
IGF-1	insulin-like growth factor 1
I.p.	intraperitoneally
LV	left ventricle
LVEDD	left ventricular end-diastolic diameter
LVEDP	left ventricular end-diastolic pressure
LVESD	left ventricular end-systolic diameter
LVH	left ventricular hypertrophy
LVP	left ventricular pressure
MRI	magnetic resonance imaging
MTC stain	Masson's trichrome stain
NCX	sodium-calcium exchanger
NPD	normal pellet diet
NT-proBNP	n-terminal fragment pro brain natriuretic peptide
OGTT	oral glucose tolerance test

PKC	protein kinase C
PLN	phospholamban
PWT	posterior wall thickness
RAAS	renin-angiotensin-aldosterone system
ROS	reactive oxygen species
RyR	ryanodine receptor
SD	standard deviation
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
SR	sarcoplasmic reticulum
STZ	streptozotocin
SV	stroke volume
TGF-β	transforming growth factor beta
TD	transition diet
TnI	troponin I
UKPDS	UK prospective diabetes study
WHO	world health organisation
ZOL	Ziekenhuis Oost-Limburg

4 Summary

Introduction: Diabetic cardiomyopathy (DCM) is a devastating disease of the heart, which increases the risk of heart failure in individuals with diabetes mellitus (DM). DCM is characterized by structural and functional changes of the myocardium, which are attributable only to the presence of DM and not to confounding factors or diseases. To date, little is known about the pathophysiology of this disease and there is no specific treatment available. Therefore, a clinically relevant animal model, in which DCM is developed after induction of DM, is warranted. This study is based on the hypothesis that DCM can be induced in a rat model by using a high fat diet (HFD) and a single low dose streptozotocin (STZ) injection.

Materials and methods: In this study, three experimental groups were used: a first control group *(n=6)* consisting of male Sprague Dawley (SD) rats that were fed a normal pellet diet (NPD) and a second control group *(n=7)* which was fed a high fat diet (HFD, 60% fat, 20% protein and 20% carbohydrate (% total kcal)). Both control groups were injected i.p. with a vehicle citrate buffer. In the third experimental group *(n=11)*, DM was induced by combination of a HFD and a single low dose STZ injection (STZ, 35 mg/kg, i.p.). All animals were subjected to multiple *in vivo* measurements to investigate the occurrence of type 2 diabetes, whether rats have developed DCM and hence if the proposed animal model is a clinically relevant rat model for this pathology.

Results: One week after STZ injection, the oral glucose tolerance test (OGTT) showed significantly higher blood glucose levels in the STZ group, compared to the NPD and HFD group, indicating a slower glucose clearance in the STZ group. The last OGTT confirmed that the STZ group had significantly higher blood glucose levels two hours after glucose intake. In addition, the serum insulin response to this glucose challenge was significantly increased in the HFD group in the second and last OGTT, compared to the STZ group. Analysis of blood samples in the fed state demonstrated significantly higher plasma glucose and triglycerides levels in the STZ group in week 6. Plasma insulin levels in the STZ group were similar to the NPD group but were significantly increased in the HFD group in week 9 and 12. Conventional echocardiographic parameters changed significantly over time in all three groups although no significant differences between the experimental groups were observed. Both the HFD and STZ group showed a significantly increased left ventricular end-diastolic pressure (LVEDP) and a significantly increased time constant of left ventricular pressure decay during the isovolumic relaxation period (tau), compared to the NPD group. In addition, left ventricular pressure (LVP) increased significantly in the STZ group, compared to both the NPD and HFD group.

Conclusion: This study shows that the combination of a HFD (60% fat) and a low dose of STZ (35 mg/kg) is efficient to induce type 2 diabetes, a deteriorated left ventricular relaxation and an increased left ventricular pressure in a rat model. Moreover, rats which are fed only a HFD, will develop the insulin resistance syndrome. This nongenetic rat model has the additional advantage of being easy to induce and can be used in the future to unravel the molecular mechanisms of DCM.

5 Samenvatting

Introductie: Diabetische cardiomyopathie (DCM) is een ziekte die het hart aantast en die leidt tot een verhoogde kans op hartfalen bij patiënten met diabetes mellitus (DM). DCM wordt gekenmerkt door structurele en functionele veranderingen van het hartspierweefsel. Deze veranderingen zijn enkel toe te schrijven aan de aanwezigheid van DM en niet aan andere aandoeningen. Tot op heden is er weinig gekend betreffende de pathofysiologie van deze ziekte en er is geen specifieke behandeling beschikbaar. Daarom is er nood aan een klinisch relevant diermodel, waarin DCM wordt ontwikkeld na de inductie van DM. Deze studie is gebaseerd op de hypothese dat DCM kan worden geïnduceerd in een rat model door het gebruik van een hoog vet dieet en een eenmalige injectie met streptozotocine (STZ) in een lage dosis.

Methoden: In deze studie werden drie experimentele groepen gebruikt: een eerste controle groep *(n=6)* waarin mannelijke Sprague Dawley (SD) ratten een normaal pellet dieet (NPD) werden gevoerd. De tweede controle groep *(n=7)* kreeg een hoog vet dieet (HFD, 60% vet, 20% proteïne en 20% koolhydraten (% totaal kcal)). Beide controle groepen werden i.p. geïnjecteerd met een citraatbuffer. In de derde experimentele groep *(n=11)*, werd DM geïnduceerd met behulp van een hoog vet dieet en een eenmalige STZ injectie in een lage dosis (STZ, 35 mg/kg, i.p.). Alle dieren werden onderworpen aan verschillende *in vivo* experimenten om te onderzoeken of de ratten DM en DCM hebben ontwikkeld, en of dit model dus een klinisch relevant rat model voor DCM is.

Resultaten: Een week na de STZ injectie, toonde de orale glucose tolerantie test (OGTT) een significant hogere bloed glucose waarde in de STZ groep, vergeleken met de NPD en HFD groep. Dit geeft aan dat de STZ groep een tragere glucoseklaring vertoont. De laatste OGTT bevestigde dat de STZ groep significant hogere bloed glucose waarden vertoonde, twee uur na glucose inname. De serum insuline respons na een glucosetoediening was significant verhoogd in de HFD groep in zowel de tweede als laatste OGTT, vergeleken met de STZ groep. Bloedstaal analyse (van niet gevaste dieren) gaf aan dat de STZ groep significant hogere plasma glucose en triglyceride waarden vertoonden in week 6. Plasma insuline concentraties waren gelijkaardig in zowel de STZ groep als de NPD groep maar de HFD groep vertoonde significant verhoogde insuline concentraties in week 9 en 12. Conventionele echocardiografie parameters veranderden significant na verloop van tijd in alle drie de groepen maar er werden geen significante verschillen tussen de experimentele groepen waargenomen. Zowel de HFD als de STZ groep vertoonden significant verhoogde linker ventriculaire eind-diastolische druk (LVEDP) en een significant verhoogde tijdsconstante voor linker ventrikel drukverval tijdens de isovolumische relaxatieperiode (tau), vergeleken met de NDP groep. Verder was de linker ventriculaire druk (LVP) significant verhoogd in de STZ groep, vergeleken met de NPD en HFD groep.

Conclusie: Deze studie toont aan dat de combinatie van een hoog vet dieet (60% vet) en een lage dosis STZ (35 mg/kg) efficiënt is om type 2 diabetes, een verslechterde relaxatie van en een verhoogde druk in het linker ventrikel te induceren in een rat model. Het gebruik van enkel een hoog vet dieet in een rat model leidt tot de ontwikkeling van het insuline resistentie syndroom. Dit niet genetisch rat model heeft het voordeel dat het gemakkelijk te induceren is en dat het gebruikt kan worden in de toekomst voor het ontrafelen van de mechanismen die een rol spelen bij DCM.

6 Introduction

6.1 Diabetic cardiomyopathy

DCM is a heart disease in people with diabetes which may lead in time to heart failure. Rubler et al. were the first to describe this disease in 1972 based on the observations in four diabetic patients who presented with heart failure, without a history of hypertension, coronary artery disease (CAD), valvular or congenital heart disease. This clinical entity has been termed DCM and is defined as ventricular dysfunction that occurs independently of a recognized cause, such as previously mentioned [1]. Bell et al. (2003) defined DCM as 'a separate disease of the myocardium in patients with well-regulated DM' [2]. The natural history of DCM consists of an asymptomatic period, during which cellular abnormalities lead to diastolic dysfunction. Later on, this diastolic dysfunction may progress to left ventricular hypertrophy and even to systolic dysfunction [3]. The earliest signs of ventricular dysfunction are an impaired relaxation and a decreased compliance of the heart, together with a reduction in early diastolic filling and an increase in atrial filling [4]. In time, DCM may lead to clinical symptoms such as fatigue, shortness of breath, chest pain and peripheral and pulmonary edema [5].

Today, 180 million people worldwide have DM and this number will rise even more due to an increase in population, life expectancy, prevalence of obesity and a decrease in physical activity. The global prevalence of DM is expected to reach 300 million people by 2025 [4, 6]. As previously mentioned, DM can lead to development of DCM. Currently, the prevalence of DCM in diabetic patients is 12% worldwide [7]. Dandamudi et al. (2014) described a prevalence of DCM of 1.1% and a mortality of DCM of 31% in the global population by performing a population-based study [8]. The Framingham Heart study reported a twofold increase in heart failure in diabetic men and a five-fold increase in diabetic women, when compared with matched controls [9]. To conclude, diabetes is a strong and independent risk factor for developing heart failure.

DCM has been described to develop in three stages; an early, an advanced and a late stage. Although asymptomatic, the early stage displays, at the cellular level, changes such as hyperglycemia, insulin resistance, increased levels of free fatty acids, changes in calcium homeostasis and reduction of glucose transporters. In this stage, patients display diastolic dysfunction and heart hypertrophy. The next stage is the advanced stage and apoptosis and necrosis of cardiac myocytes will occur. In addition, an increase in angiotensin II and transforming growth factor beta (TGF-β) and a reduction of insulin growth factor-1 (IGF-1) is noticed. In the advanced stage, diastolic dysfunction is accompanied with fibrosis and an increase in left ventricular size, mass and wall thickness. In the late stage, microvascular changes, extensive myocardial fibrosis, cardiac autonomic neuropathy and a decreased ejection fraction are present. A significantly increased left ventricular mass, wall thickness and dilatation of the left ventricle (LV) can be observed using echocardiography. This late stage is characterized by severe diastolic and systolic dysfunction [10, 11].

To date, there is no single diagnostic test for DCM and therefore diagnosis can be challenging. To confirm the diagnosis of DCM, myocardial abnormalities have to be detected and other heart

diseases or contributory causes of cardiomyopathy have to be excluded. Different imaging techniques are available which make it possible to detect early cardiac features of DCM. Echocardiography is a reliable and non-invasive technique. This technique can detect significant abnormalities, such as left ventricular hypertrophy, diastolic and systolic dysfunction, before the onset of symptomatic heart failure. Early abnormalities are characterized by a normal ejection fraction with a reduced early diastolic filling, prolongation of isovolumetric relaxation and an increase in atrial filling. All of these characteristics confirm diastolic dysfunction. In addition, echocardiography allows a 'real-time' visualisation of the cardiac cycle. Furthermore, valvular anatomy and function can be assessed [12, 13]. A second diagnostic imaging technique is cardiac magnetic resonance imaging (MRI). Cardiac MRI can be used to asses diastolic function and according to Chuang et al. (2000) it is the 'gold standard' for measuring the left ventricular mass [12-14]. This technique can also be used for assesment left ventricular geometry and hypertrophy and for detection of wall motion abnormalities.

Finally, biomarkers can be used in the diagnosis of DCM. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are cardiac hormones which are secreted in the atria and ventricles, in response to excessive stretching of cardiomyocytes, due to ventricular volume and pressure overload [15]. The functions of these hormonones are natriuresis, diuresis and vasodilatation. When myocardial stretch occurs, the prohormone proBNP is secreted and will be cleaved into active BNP and NT-proBNP. All of these fragments are detected in the circulation [16]. Therefore, these hormones could be used as a cardiac biomarker because they are sensitive and specific for heart failure. However, BNP may have a limited diagnostic use because it cannot reliably distinguish between systolic and diastolic heart failure [17, 18]. In addition, obesitas suppresses BNP release and hence causes lower levels of BNP [19] and obesitas is often present in patients with DCM. To conclude, the diagnosis of DCM currently relies on noninvasive imaging techniques. There is still no consensus concerning the precise imaging definition of DCM. However, when evidence of hypertrophy and diastolic dysfunction is observed, the diagnosis of DCM can be made.

6.2 Pathophysiology and pathogenesis of DCM

DCM is characterized by structural and functional cardiac changes such as diastolic and/or systolic dysfunction. These changes are caused by myocyte hypertrophy and myocardial fibrosis [7]. Moreover, hyperglycemia is present in diabetic patients and leads to an increase in cross-linking of collagen fibers, thereby contributing to a reduction in ventricular compliance [20-22]. Diastolic dysfunction, is present in 75% of diabetic patients and is considered to be the earliest and the most frequent fuctional change in DCM [23]. Diastolic disfunction occurs when the ventricle does not relax properly or when the ventricular wall is to stiff. Hence, the ventricle will not fill properly during diastole. Thus the definition of diastolic disfunction is an abnormal left ventricular relaxation pattern without clinical heart failure [13]. Systolic dysfunction may also be present in patients with DM, though its incidence appears to be lower than that of diastolic dysfunction [24]. Systolic dysfunction is defined as an impairement in the ability of the heart to eject blood and is characterized by a decreased ejection fraction. In patients with DCM, systolic dysfunction occurs rather late, often when signficant diastolic dysfunction has already developed. The mortality of these patients is 15-20% [13]. Both diastolic and systolic dysfunction can be evaluated with

echocardiography. In diabetic patients, diastolic and systolic dysfunction may be present in both the left and the right ventricle [25]. Finally, patients with DCM present with left ventricular hypertrophy (LVH). DM is an important contributer to LVH and myocardial stifness. LVH occurs as a result of hypertrophy of myocytes, perivascular and interstitial fibrosis and thickening of the basement membrane [26]. To date, there is still no clear concensus to define LVH. However, the 2003 European Society of Cardiology has determined a guideline. For men, LVH is said to be present when the left ventricular mass is > 125 g/m² and for women the treshold value is > 110 g/m² [13].

The next sections will give an overview of the most important mechanisms which play a role in the pathogenesis of DCM, which is multifactorial and not yet completely characterized. Several mechanisms act synergistically to induce DCM in patients.

6.2.1 Hyperglycemia and glucotoxicity

A first mechanism is hyperglycemia and subsequent glucotoxicity, due to insulin resistance caused by DM. Hyperglycemia leads to development of DCM by overproduction of reactive oxygen species (ROS) and advanced glycation end products (AGEs). ROS exert their harmful effects via cellular damage by oxidation and by interference in intracellular signaling pathways. ROS also cause DNA damage and induce apoptosis [27]. AGEs on the other hand, cause cross-linking of structural proteins, thereby altering their structure and function and leading to an increased myocardial stiffness. Moreover, AGEs provide a positive feedback loop on ROS production [27]. In addition, hyperglycemia and consequently also ROS and AGEs, alter the function of both the sarcolemmal calcium ATPase (SERCA) pump and the ryanodine receptor (RyR). This results in a decreased sarcoplasmatic reticulum (SR) calcium reuptake during diastole and an increased RyR-mediated SR calcium leak [28, 29]. Both mechanims lead in time to a decreased systolic and diastolic function, due to a disturbance in calcium homeostasis, as explained in section 5.2.4 [30]. Another mechanism that may lead to hyperglycemia, is a reduced insulin-stimulated glucose transport protein 4 (GLUT4) translocation to the plasma membrane of cardiac and skeletal cells [31]. This in turn leads to a reduced glucose uptake, a decreased glycolysis and to lipotoxicity [32], as explained in the next section.

6.2.2 Hyperlipidemia

Hyperlipidemia, concomitant with lipotoxicity, can also induce DCM. Hyperlipidemia occurs as a result of elevated circulating lipids and hyperinsulinemia. DM causes an enhanced hepatic lipid synthesis and an increased lipolysis in adipocytes. Moreover, insulin stimulates fatty acid (FA) transport into cardiomycytes [33]. This increased FA delivery will exceed the oxidative capacity of the cardiac cells and thereby leading to lipotoxicity due to β-oxidation of FA, which can impair calcium handeling as explained in section 6.2.4 [34]. Furthermore, high FA uptake stimulates ROS generation and leads to an increased oxygen demand, a decreased ATP synthesis and apoptosis [35].

6.2.3 *Hyperinsulinemia*

A next factor which plays a role in DCM is hyperinsulinemia due to insulin resistance. Increased levels of insulin promote cardiomyocyte hypertrophy by binding of insulin to both the insulin receptor and the insulin-like growth factor 1 (IGF-1) receptor. Insulin is able to bind to both of these receptors due structural similarities, although with a lower affinity for the IGF-1 receptor [36]. As mentioned before, in conditions of insulin resistance and subsequent hyperinsulinemia, cardiac substrate utilization shifts from using glucose to β-oxidation of FA. The diabetic heart is unable to switch back to glucose use and cardiac efficiency will be reduced [35].

6.2.4 *Disturbed calcium homeostasis*

Calcium is crucial for a good excitation-contraction coupling and normal cardiac function. Disturbances in calcium handling may lead to an altered cardiac function which may be the result of a decreased ability of the SR to remove calcium after contraction, due to reduced activitities of the sodium-calcium exchanger (NCX), the SERCA pump and the RyR. SR function is already abnormal at an insulin-resistant stage before manifestation of diabetes [37]. In a healthy heart, the NXC accounts for about 10-30% of diastolic calcium removal. However in the diabetic heart, the NCX activity is reduced due to hyperglycemia and insulin deficiency. According to Schaffer et al. (1997), these effects may be caused by changes in the phospholipid composition of the cell membrane as well as impaired translocation and/or activation of protein kinase C (PKC) in the diabetic heart [38]. In addition, a reduced SERCA activity causes an overload of cytosolic calcium concentration during diastole and an impaireds relaxation of the ventricle. A disturbed RyR function leads to dyssynchronous and diastolic calcium releases, also known as calcium sparks, possibly leading to ventricular arrhythmia [37]. To summarize, a disturbed calcium homeostasis leads to an impaired relaxation during diastole and in time to diastolic and even systolic dysfunction [39].

6.2.5 *Activation renin-angiotensin-aldosterone system*

As a compensatory mechanism in respons to cardiac impairment, the renin-angiotensin-aldosterone (RAAS) system will be activated. Activation or dysregulation of this system in patients with DM is achieved by binding of angiotensin II on angiotensin II type 1 (AT_1) receptors in cardiomyocytes. As a result, collagen metabolism is altered and cardiac fibroblast proliferation is stimulated wich causes an increase in interstitial and perivascular fibrosis [40]. Both mechanisms lead to cardiomyocyte hypertrophy and an increase in oxidative damage, apoptosis and necrosis [41].

6.2.6 *Mitochondrial dysfunction*

Hyperglycemia and increased β-oxidation will lead to the production of ROS and subsequent to mitochondrial damage. Myocardial contractility is disturbed due to a decreased ATP production in the heart [35]. DM also causes functional and structral changes in mitochondria [42].

6.2.7 *Activation of protein Kinase C*

Finally, protein kinase C (PKC) plays a role in the development of DCM. Hyperglycemia increases the synthesis of diacylglycerol (DAG) which activates PKC. Calcium handling in cardiomyocytes is disturbed by PKC, due to phosphorylation of proteins, such as RyR, troponin I and phospholamban (PLN), which are involved in the excitation-contraction coupling [43]. PKC activation results in reduction in blood flow, increased vascular permeability, extracellular matrix deposition and capillary basement membrane thickening [13].

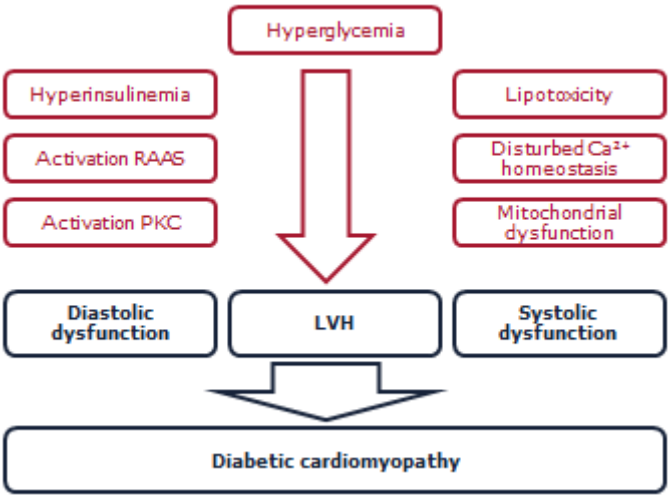

Figure 1: Overview of the most important mechanisms which play a role in the pathogenesis of DCM, adapted from Boudina et al. (2009) [44]. RAAS = renin-angiotensin-aldosterone system, PKC = protein kinase C, LVH = left ventricular hypertrophy.

To summarize, a variety of molecular mechanims play a role in the development of DCM. However, the pathophysiology of DCM remains to be fully elucidated.

6.3 *Risk factors*

Several risk factors for the development of DCM have been described [26]. A first risk factor is hyperglycemia which contributes to development of heart failure through various mechanisms, which are mentioned above. Therefore, a good glycemic control can lower the risk for development of DCM. The Diabetes Conventional and Complications Trial (DCCT) showed that patients with DM who are intensively treated, have a reduced incidence of DCM when compared with patients who are conventionally treated. The intensive therapy consisted of the administration of insulin three or more times per day and a daily adjustment of the insulin dose. The conventional treatment included one or two daily injections of insulin without daily adjustments of insulin dosage [45]. In the UK Prospective Diabetes Study (UKPDS), a risk reduction for myocardial infarction and death was described following intensive glucose-lowering therapy [46]. Insulin resistance and subsequent hyperinsulinemia is a next risk factor [47, 48]. Both factors lead to an imbalance in insulin and lead in time to development of obesity, hyperglycemia and hypertension [49]. A last possible risk factor is obesity. Patients with type 2 diabetes present often with central obesity which contributes to DCM due to its association with glucose intolerance and insulin resistance [26, 50].

6.4 Therapeutic options

The molecular pathways which play a role in DCM are still not clear. Therefore, no specific treatment for DCM exists. To date, therapeutic options for DCM consist of prevention of causative insults and medication which is used for general heart conditions. The goal is to ameliorate the cardiomyopathic state. Once DCM is established, prognosis is poor and management is challenging. Changing the lifestyle by eating healthy, losing weight, cessation of smoking and regular physical exercise, is a first cornerstone in the amelioration of DCM [26, 51]. In addition, improvement of glycemic control has been shown to be associated with a reduced risk for myocardial infarction, stroke and the likelihood to develop DCM [52]. Euglycemia can be achieved by several therapeutic drugs such as insulin, metformin, pioglitazones, sulfonylureas, glucagon-like peptide-1 mimetics (GLP-1), dipeptidyl peptidase-4 inhibitors (DPP-4) and amylin analogues [7]. However, optimal glycemic control will not be achieved by a single drug. Hence, a combination of drugs is applied in practice [51]. As mentioned before, poor glycemic control is also associated with increased levels of FA. Optimal glycemic control may be the best and most important strategy to prevent development of DCM. It has become clear that the sympathetic nervous system also plays a role in heart failure. Therefore, β-blockers are described for patients with DCM to prevent further cardiac remodeling, which results in a preserved LV function. In summary, β-blockers should be given to all diabetic patients with any evidence of heart failure [27, 53]. Next, due to a reduction of cardiovascular mortality in diabetic patients, ACE inhibitors or angiotensin receptor blocker (ARB) may be prescribed [12]. ACE inhibitors improve blood flow through the microcirculation and improve fibrosis in the myocardium by inhibition of angiotensin II. ARBs also reduce myocardial fibrosis [5]. Another class of drugs which may be used in the treatment of DCM are calcium channel blockers. They can reverse the disturbed calcium homeostasis and prevent myocardial changes. Calcium channel blockers are also antihypertensive drugs and they lower the heart rate and therefore, improve filling time [54]. Furthermore, Chen et al. (2009) reported that calcium channel blockers prevent the expression of a proapoptotic thioredoxin-interacting protein, thereby enhancing cardiomyocyte survival [55]. A last class of drugs is statins. Statins reduce circulating lipids levels and may be used for primary prevention in patients without established cardiovascular disease [56]. To conclude, no specific therapeutic strategies for treatment of DCM as such can be recommended and treatment is based on the general therapies used to treat heart failure.

6.5 Animal model of diabetic cardiomyopathy

As mentioned above, DCM increases the risk of heart failure in individuals with diabetes. The pathophysiology of the disease is still not fully understood and there is no effective treatment available. Therefore, a greater understanding of how type 2 diabetes affects the heart is needed. Animal models of type 2 diabetes are currently the first line for investigating disease mechanisms and possible therapies. For relevance to the clinical situation, the proposed animal model must show the metabolic characteristics seen in patients and mimic the developmental process of the disease. Furthermore, the developed animal model must be easily available, practical and reproducible.

The existing animal models for the study of type 2 diabetes display a number of limitations. In some models, the pattern of disease initiation and development are different compared to the human clinical course. For example, some animal models develop type 1 diabetes, instead of type 2 diabetes, prior to development of DCM [57]. Genetic models also do not meet the criteria used in practice, because in these models the development of diabetes is predominantly genetically determined, in contrast with the human clinical situation [58, 59]. Another animal model is a model in which diabetes is induced by STZ. STZ is a naturally occurring chemical which is toxic for the Langerhans islet β-cells of the pancreas. As a consequence, insulin production will drop due to necrosis of β-cells. STZ causes DNA damage by DNA alkylation and is transported into β-cells by GLUT2. STZ will only harm the pancreas due to a high level of GLUT2 proteins [60]. However, in induced diabetic models, most of the studies report relatively high doses of STZ (> 50 mg/kg). These high doses destruct the insulin producing cells and this will result in insulin deficiency rather than insulin resistance. Thus, these models show symptoms and characteristics typical of human type 1 diabetes and therefore cannot be used in our study [61]. A last limitation, regarding cardiac research, is the extended periods which are needed to develop cardiac phenotypes. Mansor et al. (2013) mentioned that Zucker fatty rats showed cardiac metabolic dysfunction only after twelve months [62].

In the past, attempts have been made to develop a suitable animal model for type 2 diabetes by combination of a HFD and a STZ treatment in normal rats [62-64]. The HFD will induce peripheral insulin resistance and due to a relatively low STZ dose, β-cells will only be mildly impaired. This approach will lead eventually to insulin resistance and type 2 diabetes with a metabolic phenotype similar to patients. In the study of Reed et al. (2000), both control-fed and HFD-fed rats developed diabetes because a relative high dose of 50 mg/kg of STZ had been injected in rats [63]. Zhang et al. (2003), used a low dose of STZ of 15 mg/kg for inducing diabetes in HFD rats but it took more than four months to develop this animal model [64]. Finally, Srinivasan et al. (2005) have developed a rat model in which type 2 diabetes is developed in three weeks and which simulates the human syndrome, by using a HFD in combination with a relatively low dose of STZ of 35 mg/kg [65]. Thus, a STZ dose of 35 mg/kg seems to be the correct dose to induce type 2 diabetes in a rat model in a relatively short period of three weeks, as shown in table 1.

Table 1: Different doses of streptozotocin to induce type 2 diabetes

HFD + STZ (≥50 mg/kg) [63]	Insulin deficiency → type 1 diabetes
HFD + STZ (35 mg/kg) [64]	Insulin resistance → development of type 2 diabetes in three weeks
HFD + STZ (15 mg/kg) [65]	Insulin resistance → development of type 2 diabetes more than four months

Previous research shows that a dose of STZ of 35 mg/kg is the optimal dose to induce type 2 diabetes in rat model. HFD = high fat diet, STZ = streptozotocin.

The aim of this study is to develop a representative rat model of DM in which DCM can be developed. As previously mentioned, there is no appropriate animal model available for the study of the initiation and development of DCM. Therefore, we hypothesize that DCM can be induced in a rat model by using a HFD and low dose STZ injection (35 mg/kg). Our experimental approach is based on the animal models of Srinivasan et al. (2005) and Mansor et al. (2013). Their studies confirm that the combination of HFD-fed and low dose STZ-treated rat serves as an alternative model for type 2 diabetes simulating the human syndrome, which is also suitable for testing anti-

diabetic agents. Moreover, these rat models mimic the natural history and metabolic characteristics of the common type 2 diabetes in humans, in contrast to previous animal models. However, these papers only describe short term effects of the diet and the STZ injection on metabolism and development of type 2 diabetes. No follow-up in time was performed to study the effect of treatment on cardiac function and thus the development of DCM [62, 65].

As mentioned before, we want to develop a clinical relevant rat model of DCM. In our study, we will use the model described by Srinivasan et al. (2005) and Mansor et al. (2013) but we will perform a follow-up for a much longer time, in order to further confirm whether indeed, within this time frame, animals display features of DCM such as diastolic dysfunction, hypertrophy and fibrosis. It is not yet clear after how many weeks the rats develop DCM. Ouwens et al. mentioned that rats showed cardiac dysfunction after seven weeks of only high-fat feeding [66]. In the study of Mansor et al. (2013), HFD-fed low dose STZ injected rats were sacrificed after three weeks and they already showed cardiac alterations [62]. Therefore, our rats will be kept alive for minimal nine weeks after the administration of the STZ injection, so DCM can develop. The first objective is to induce DCM in rats by combination of a HFD and a STZ injection. A second objective is to investigate whether the proposed rat model is a clinically relevant model for this pathology. Multiple *in vivo* measurements are performed to investigate which animals have developed type 2 diabetes and subsequently DCM. After sacrificing the rats, *in vitro* evaluations will be performed on their tissue/cells to confirm the results that were discovered in the *in vivo* experiments. To summarize, the relevance of this study is the development of an easy to induce, reproducible, and suitable rat model of DCM, which may be used in the future for testing the effectiveness of new treatments or to unravel the molecular mechanisms of this pathology.

7 Materials and methods

7.1 Animals

Twenty-eight male age- and weight-matched Sprague Dawley rats (Charles River, France), weighing 125-150 g at the start of the experiment, were used in our study. The animals were housed in standard cages (two rats/cage) with a 12:12h light and dark cycle and a controlled room temperature of 22°C. Water was provided ad libitum for the duration of the study. Cage enrichment was provided in the form of playtunnels. All animals were fed a NPD (2018 Teklad global rodent diet, Harlan, Belgium), composed of 18% fat, 24% protein and 58% carbohydrate as a percentage of total kcal, for one week prior to the dietary manipulation. During the first two weeks of the study, the animals were handled daily to reduce handling-induced stress. All animal experiments were approved by the animal ethical committee of Hasselt University.

7.2 Timeline of the experimental work

Table 2 explains the timeline of the multiple *in vivo* measurements. When mentioning a particular week in the sections results or discussion, this refers to the experimental weeks in table 2.

Table 2: Overview of the in vivo measurements

Weeks	BL	1	2	3	4	5	6	7	8	9	10	11	12
1) NPD/citrate	NPD	NPD	NPD	NPD	NPD	NPD	NPD	NPD	NPD	NPD	NPD	NPD	NPD
2) HFD/citrate	NPD	TD	TD	HFD	HFD	HFD	HFD	HFD	HFD	HFD	HFD	HFD	HFD
3) HFD/STZ	NPD	TD	TD	HFD	HFD	HFD	HFD	HFD	HFD	HFD	HFD	HFD	HFD
Injection					x								
Handling	x	x											
Weighing	x	x	x	x	x	x	x	x	x	x	x	x	x
Blood sampling	x			x			x			x			x
OGTT	x					x							x
Urine collection	x						x						x
Echo	x												x
Hemo. meas.													x

This table gives an overview regarding the induction of type 2 diabetes and subsequent follow-up to study the development of DCM. The three study groups are shown with their respective diets and injection. The bold line at the beginning of week 4 indicates the time point when the animals were injected i.p. citrate buffer (1+2) or STZ (3). Furthermore, the in vivo measurements, with their respective time points, are shown (x). Hemodynamic measurement is an invasive procedure and was therefore only performed at the end of the study. Afterwards, the rats were sacrificed. BL = baseline, NPD = normal pellet diet, TD = transition diet, HFD = high fat diet, STZ = streptozotocin, OGTT = oral glucose tolerance test, echo = echocardiography, hemo. meas. = hemodynamic measurements.

7.3 Induction of type 2 diabetes

After one week of acclimatization, the rats were randomized into three groups: two control groups *(= NPD and HFD)* and a diabetes group *(= STZ)*. The first control group (n=8) was fed a NPD for the total duration of the study. The second control group (n=8) was fed an isocaloric HFD (Special Diets Services, UK) consisting of 60% fat, 20% protein and 20% carbohydrate as a percentage of total kcal. The diabetes group (n=12) was fed the same HFD and was injected with a single STZ dose. To facilitate the transition from a NPD to a HFD, the rats of the HFD and STZ group were fed

an isocaloric transition diet (TD, Special Diets Services, UK), composed of 35% fat, 20% protein and 45% carbohydrate as a percentage of total kcal. The rats were fed this TD for two weeks, before proceeding to the HFD. All rats were fed their respective diet ad libitum for the total duration of the study. After three weeks of dietary manipulation, the rats of the diabetes group were injected intraperitoneally (i.p.) with a low dose of STZ of 35 mg/kg (Sigma-Aldrich, Diegem, Belgium), freshly dissolved in an ice-cold 0.1M citrate buffer (pH 4.5; Sigma-Aldrich, Diegem, Belgium) [67, 68]. Both control groups were injected (i.p.) with the vehicle citrate buffer [65]. For the STZ/vehicle injection, the rats were anesthetized using isoflurane inhalation anesthesia (2%). The rats were followed-up in time for nine weeks and weighed weekly. Two rats died during the course of the study, one rat of the NPD group (n=7) and one rat of the HFD group (n=7).

7.4 Oral glucose tolerance test

Three oral glucose tolerance tests (OGTT) were performed: at baseline, one week after the STZ/vehicle injection (week 5) and at the end of the study (week 12), as shown in table 2. The rats were fasted overnight (for a minimum of sixteen hours) to achieve a baseline glucose level prior to the start of the OGTT. The next day, rats were administered a 100% glucose solution (2 g/kg body weight; Sigma-Aldrich, Diegem, Belgium) by oral gavage. A capillary blood sample (Capillary tubes for whole blood analyses with Analox, contains fluoride/heparin/nitrite, GMRD-054, Analox instruments, London, UK) was taken from the tip of the tail before (baseline) and at 15, 30, 60, 90 and 120 min after the glucose gavage. The capillary blood samples were mixed by turning of the capillary tubes during one minute before determining the blood glucose concentration using an Analox GM7 (Analox instruments, London, UK). Rats in the STZ group having a glucose level of ≥ 11.1 mmol/l (= 200 mg/dl), two hours after glucose gavage, were considered diabetic and therefore included in the study [69, 70]. Based on this threshold, one rat from the STZ group was excluded from the study (n=11).

In addition, an arterial blood sample (serum separator tubes, Multivette 600 Z-gel, Sarstedt, Germany) was taken from the tail artery for insulin analysis, before (baseline) and at 15 and 60 min after glucose feeding. Serum was separated by centrifugation (10000 g, 5 min) and samples were stored at -20°C until analysis. Insulin was measured using a rat ultrasensitive insulin ELISA (Sanbio, Uden, The Netherlands) according to the manufacturer's instructions. Rats were anesthetized using isoflurane inhalation anesthesia (2%), to perform the oral gavage, to obtain arterial blood samples and tipping of the tail.

7.5 Blood and urine collection

Arterial Blood samples were taken from the tail at baseline, in week 3, week 6, week 9 and at the end of the study (week 12), under isoflurane anesthesia (2%), for biochemical estimations (table 2). Plasma was separated by centrifugation (2000 rpm, 10 min) in plasma tubes (Multivette 600 Z, Sarstedt, Germany) and samples were stored at -20°C until analysis. Plasma samples were analyzed in the clinical laboratory of Ziekenhuis Oost-Limburg (ZOL) in Genk. Using an automated analyzer (Modular® P800-ISE900 System, Roche diagnostics, Mannheim, Germany), plasma samples were analyzed for concentrations of glucose, triglycerides, and total cholesterol.

For urine collection, rats were placed in metabolic cages for 24 hours at baseline, in week 6 and at the end of the study (week 12, table 2). Urine samples were analyzed for excretion of glucose, total protein, and creatinine and again these analyses were carried out in the clinical laboratory of ZOL in Genk (Modular® P800-ISE900 System, Roche Diagnostics; Mannheim, Germany), according to the manufacturer's instructions. Total protein was determined by a colorimetric biuret test and creatinine was determined according to the kinetic Jaffe method.

7.6 Echocardiography

Echocardiography was used to evaluate cardiac structure and function *in vivo* at baseline and at the end of the study (week 12, table 2), under 2% isoflurane anesthesia. Echocardiography was performed using the VIVID *i* ultrasound machine and a 10 MHz probe (GE Healthcare, Diegem, Belgium). A standard parasternal long-axis image and a short-axis image at midventricular level, were acquired using B-mode. LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), posterior and anterior wall thicknesses (PWT, AWT) were obtained from the parasternal short-axis view. LV end-diastolic volumes (EDV) and LV end-systolic volumes (ESV) were calculated as follows: $\pi * D_M^2 * B/6$. D_m indicates the systolic/diastolic diameter of the ventricle on midventricular short-axis view and B is the LV length on the parasternal long-axis image. Heart rate (HR) was determined using the M-mode. Stroke volume (SV) was defined as EDV minus ESV. Cardiac output was calculated as SV * HR. LV fractional shortening (FS) ([LVEDD-LVESD]/LVEDD * 100) and EF ([EDV-ESV]/EDV * 100) were calculated and expressed in %.

7.7 Hemodynamic measurements

At the end of the study, invasive blood pressure measurements were performed under isoflurane anesthesia (1.5-2% volume supplemented by oxygen) in all animals. Briefly, a 2F high-fidelity pressure catheter (Millar Instruments, AD instruments, Spechbach, Germany), calibrated relative to atmospheric pressure before introduction, was advanced into the LV via the right carotid artery. After stabilization of the animals, LV pressure (LVP) and its peak time derivatives (dP/dt_{max}) and dP/dt_{min}) were recorded for at least ten min. Calculation of left ventricular end-diastolic pressure (LVEDP) and the time constant of LV pressure decay during the isovolumic relaxation period (tau) was performed with LabChart6 software (Millar Instruments, AD instruments, Spechbach, Germany). Abdominal circumference was assessed on the largest zone of the rat abdomen, with a flexible measuring tape. After pressure measurements, animals were euthanized with an overdose of pentobarbital (60 mg/kg) in order to collect tissues.

7.8 Organ weights

At the end of the experiment, hearts were excised and perfused with Tyrode's solution potassium chloride to stop the heart in diastole, weighed, sectioned into transverse slices at midventricular levels before fixation in 4% paraformaldehyde. Kidneys, liver and pancreas were also excised, kidneys and liver were weighed and all three organs were sectioned into transverse slices and fixed in 4% paraformaldehyde for 24 hours. Afterwards, one transverse slice from these organs was embedded in paraffin for hematoxyline & eosin staining (H&E stain) and/or Masson's trichrome

staining (MTC stain). One transverse slice was processed for cryosectioning and stored at -80°C until further analysis. A few transverse slices were frozen and crushed in liquid nitrogen and stored at -80°C for later analysis. Visceral fat mass was assessed by excising and weighing both the perirenal and epididymal adipose tissues. Finally, tibia was excised and tibia length was measured.

7.9 Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism5 software. Significance was determined by two-way ANOVA (after normality check of the residuals), except for the physical parameters, of which significance was determined using one-way ANOVA followed by Tukey's Multiple Comparison Test or by Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, when not normally distributed. Significance of the hemodynamic measurements parameters was determined by one-way ANOVA (after normality check) followed by Tukey's Multiple Comparison Test. A value of $P < 0.05$ was considered statistically significant.

8 Results

8.1 Body weight and physical parameters

In order to evaluate possible effects of the different diets on bodyweight between the treatment groups, bodyweight was recorded every week during the experimental period and is shown in figure 2. One rat from the NPD group was excluded from the experiment, after statistical analyses proved that this rat was an outlier for bodyweight as well as for the OGTT measurements. There was no significant difference in body weight between groups during the course of the study. The average total body weight gain was 385.4 ± 40.0 g, 460.9 ± 80.8 g and 373.4 ± 55.3 g for the NPD, HFD and STZ group respectively.

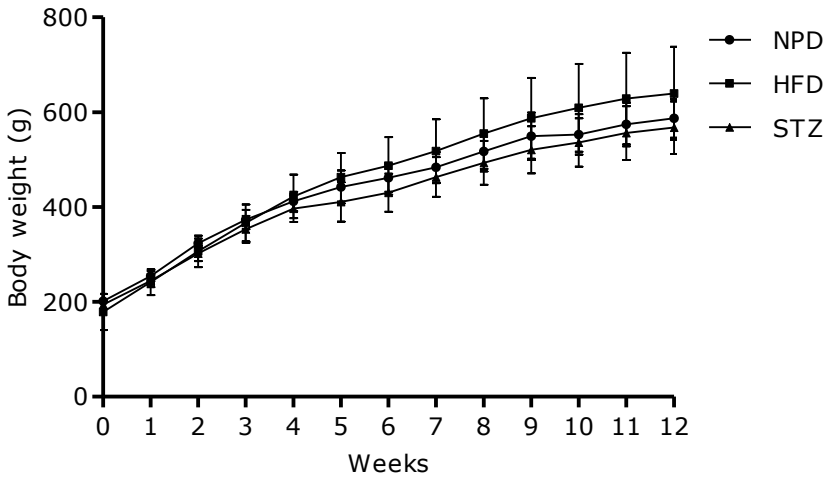

Figure 2: Body weight (g) at weekly intervals of rats consuming a normal pellet diet (NPD n=6), a high fat diet (HFD n=7) or a HFD in combination with a single low dose streptozotocin injection of 35 mg/kg (STZ n=11). Data are expressed as mean ± SD. NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin.

Heart and kidneys were weighed to evaluate possible differences between groups. Abdominal circumference, epididymal fat pad and perirenal fat pad were also weighed to determine whether the HFD and STZ groups were obese. Changes in physical parameters are shown in table 3. Organ and fat weight were normalized to tibia length. Perirenal fat/tibia length ratio was significantly increased in the HFD group, compared to the NPD group ($P < 0.05$). In addition, the STZ group tended to display a higher perirenal fat/tibia length ratio, compared to the NPD group. Epididymal fat/tibia length ratio and kidney weight/tibia length ratio tended to be higher in both the HFD and STZ group, compared to the NPD group. Heart weight/tibia length ratio and abdominal circumference/tibia length ratio were not influenced by the different treatments.

Table 3: Physical parameters from control rats consuming a NPD or a HFD and from diabetic rats induced by a combination of a HFD and a STZ injection.

	NPD (n=6)	HFD (n=7)	STZ (n=11)
Tibia length (mm)	46.8 ± 1.0	47.7 ± 1.0	46.6 ± 1.6
Heart weight (mg)	1610.0 ± 240.5	1717.1 ± 328.4	1616.4 ± 218.5
Heart weight/TL ratio (mg/mm)	34.5 ± 5.9	36.0 ± 6.7	24.7 ± 4.3
Kidney weight (mg)	3266.7 ± 301.5	3930.0 ± 460.3 ~~	3899.1 ± 360.5 **
Kidney weight/TL ratio (mg/mm)	68.9 ± 6.2	82.3 ± 9.3	83.9 ± 8.6
Abdominal circumference (mm)	220.8 ± 6.7	235.7 ± 20.3	224.5 ± 10.8
Abdom. circum./TL ratio (mm/mm)	4.7 ± 0.2	4.9 ± 0.4	4.8 ± 0.2
Epididymal fat pad (mg)	4361.7 ± 1830.8	7755.7 ± 3716.8 ~	5383.6 ± 1480.9
Epididymal fat pad/TL ratio (mg/mm)	92.6 ± 38.3	162.9 ± 77.7	115.4 ± 31.1
Perirenal fat pad (mg)	2341.7 ± 444.1	4787.1 ± 2242.1 ~~	3210.0 ± 794.4
Perirenal fat pad/TL ratio (mg/mm)	50.1 ± 10.1	100.2 ± 46.4 ~	68.8 ± 16.4

*NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin, TL = tibia length abdom. circum. = abdominal circumference. Data are expressed as mean ± SD. * = NPD vs STZ; ~ = NPD vs HFD. ~ = P < 0.05; **/~~ = P < 0.01.*

8.2 Effect of HFD and STZ on development of type 2 diabetes

An OGTT was performed in the beginning of the animal experiment to determine the baseline glucose response in fasted rats subjected to an oral glucose challenge. The baseline serum glucose concentrations of the rats at different time points are represented in figure 3A. In addition, arterial blood samples were obtained from the different groups, at baseline and after 15 and 60 min after glucose intake, and baseline insulin concentrations were determined (figure 3B).

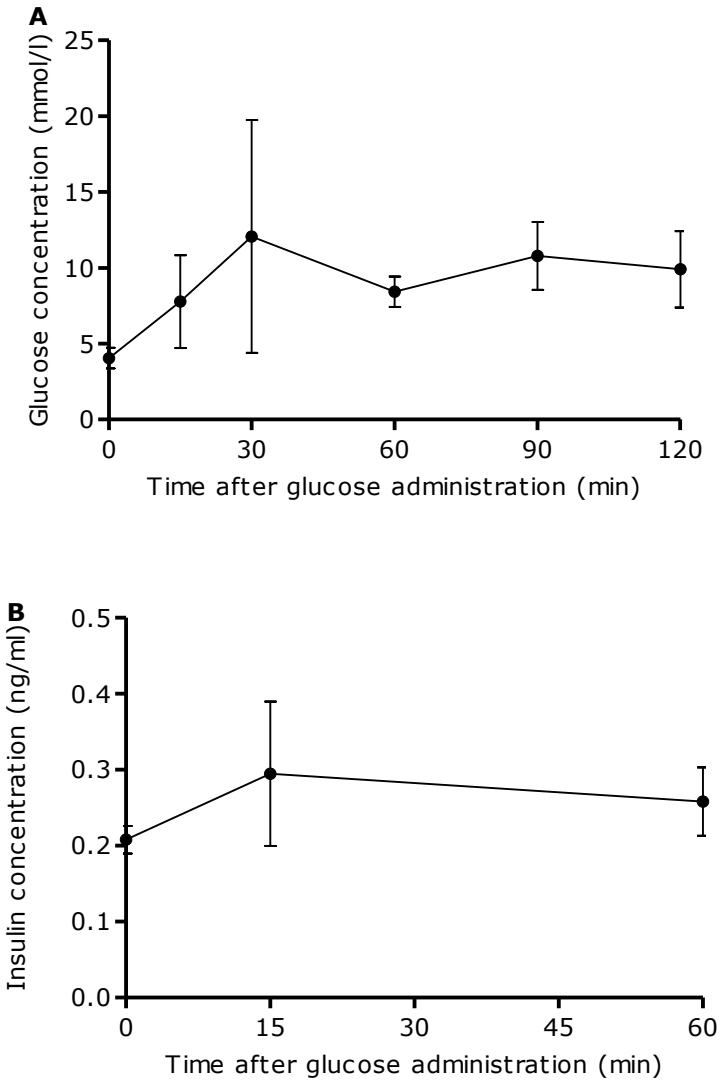

Figure 3: Baseline serum glucose (A) and insulin levels (B) measured after administration of an oral glucose challenge in fasted rats consuming a normal pellet diet (NPD). Data are expressed as mean ± SD (n = 6).

In week 5 (table 2), one week after STZ injection, a second OGTT was performed to determine which rats had developed diabetes. The results of the second OGTT are shown in figure 4. Rats of the STZ group had significantly higher serum glucose levels at 30 and 120 min after glucose administration, compared to the NPD group ($P < 0.05$ and $P < 0.01$; figure 4A). In addition, glucose concentrations were significantly increased in the STZ group at 30 ($P < 0.05$), 60 ($P < 0.05$), 90 ($P < 0.01$) and 120 min ($P < 0.01$), compared to the HFD group. No significant difference between the NPD and HFD group was observed at any time point. Two hours after glucose intake, the STZ group had a glucose level of 16.5 mmol/l, compared to 11.8 mmol/l in the NPD group and 11.5 mmol/l in the HFD group. Changes in serum insulin concentrations in the different experimental groups during the second OGTT, are shown in figure 4B. The HFD group had significantly higher serum insulin levels at 15 min after glucose administration, compared to the STZ group ($P < 0.01$). No significant differences were observed between the HFD and NPD group or between the NPD and STZ group.

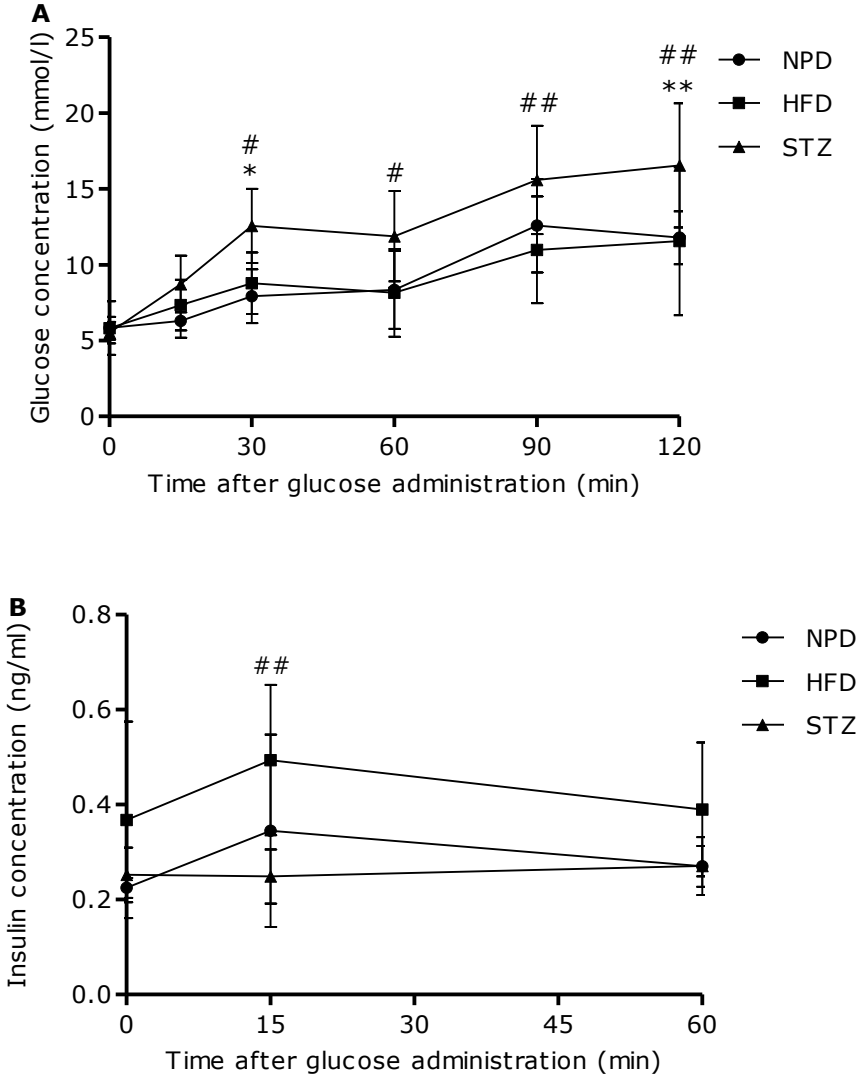

*Figure 4: OGTT performed in week 5. Changes in serum glucose (A) and insulin (B) concentrations following an oral glucose administration in fasted rats consuming a normal pellet diet (NPD —●— n=6), a high fat diet (HFD —■— n=7) or HFD in combination with a single low dose streptozotocin injection of 35 mg/kg (STZ —▲— n=11). Data are expressed as mean ± SD. * = NPD vs STZ; # = HFD vs STZ. */# P < 0.05; **/## P < 0.01. OGTT = oral glucose tolerance test, NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin.*

At the end of the study (week 12, table 2), a last OGTT was performed to check if the STZ group had diabetes and the results are shown in figure 5A. At 120 min, the STZ group displayed significantly higher glucose levels compared to the NPD and STZ group ($P < 0.01$). In addition, the glucose levels of the STZ group tended to be higher from 60 min after glucose intake onwards, compared to the other groups, although not significant. No significant difference between the NPD and HFD group was observed. After two hours of glucose intake, the STZ group had a glucose level of 24.7 mmol/l, compared to 17.0 mmol/l in the NPD group and 18.1 mmol/l in the HFD group. Figure 5B represents changes in serum insulin concentrations in the different treatment groups during the third OGTT. The HFD group had significantly higher serum insulin levels at baseline and after 15 and 60 min after glucose administration, compared to the STZ group ($P < 0.05$, $P < 0.001$ and $P < 0.05$ respectively). No significant differences were observed between the HFD and NPD group or between the NPD and STZ group.

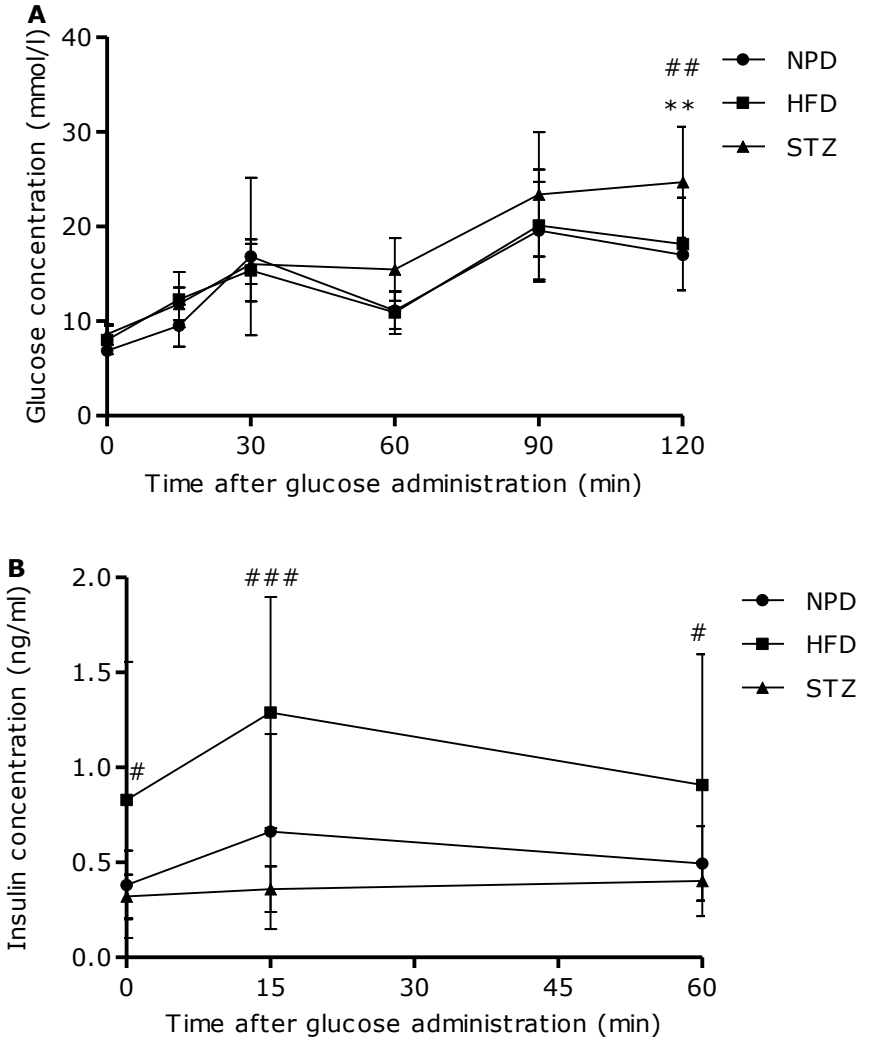

*Figure 5: OGTT performed in week 12. Changes in serum glucose (A) and insulin (B) concentrations following an oral glucose administration in fasted rats consuming a normal pellet diet (NPD ●— n=6), a high fat diet (HFD ■— n=7) or HFD in combination with a single low dose streptozotocin injection of 35 mg/kg (STZ ▲— n=11). Data are expressed as mean ± SD. * = NPD vs STZ; # = HFD vs STZ. # = P <0.05; **/## = P < 0.01; ### = P < 0.001. OGTT = oral glucose tolerance test, NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin.*

The increase in blood glucose concentration during the OGTT, expressed as (mmol/l)/min, was calculated by determining the slope of the line [$m = (y^1 - y^2)/(x^1 - x^2)$] for blood glucose measurements between 0 and 120 min. This slope was determined for all three groups from the OGTTs performed at baseline, in week 5 and in week 12 and is represented in figure 6. The STZ group showed a significant higher increase in blood glucose concentration per min in week 5, compared to the NPD and HFD group, ($P < 0.01$ and $P < 0.001$ respectively), and in week 12, compared to the NPD and HFD group ($P < 0.001$). No significant differences between the NPD and HFD group were observed.

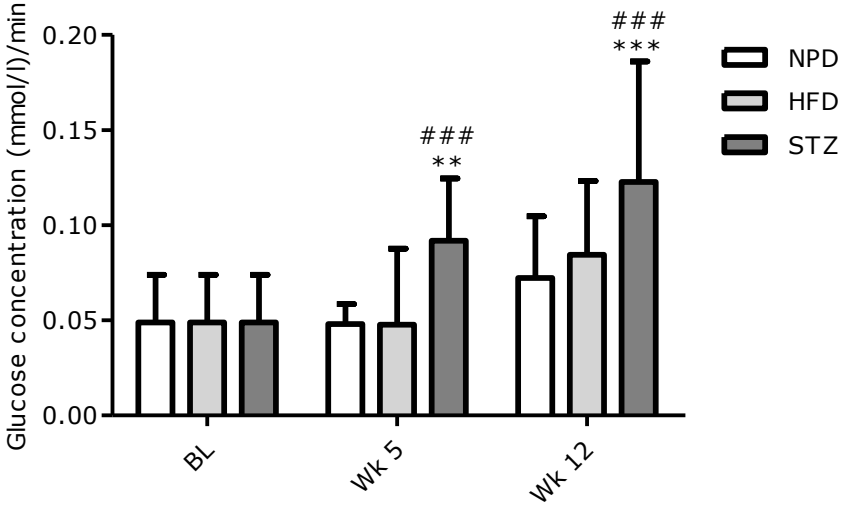

*Figure 6: Increase in serum glucose concentration per min [(mmol/l)/min,] following an oral glucose administration in fasted rats consuming a normal pellet diet (NPD —●— n=6), a high fat diet (HFD —■— n=7) or HFD in combination with a single low dose streptozotocin injection of 35 mg/kg (STZ —▲— n=11). This increase was calculated by determining the slope of the line for blood glucose measurements between 0 and 120 min from the OGTT performed at baseline (BL), in week 5 and in week 12. Data are expressed as mean ± SD. * = NPD vs STZ; # = HFD vs STZ. ** = P < 0.01; ***/### = P < 0.001. OGTT = oral glucose tolerance test, NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin.*

8.3 Plasma metabolites

Multiple plasma metabolites were measured to evaluate possible metabolic differences between the three experimental groups. Fed plasma glucose and insulin concentrations were determined to confirm the results from the OGTT. Fed plasma glucose levels from the three groups were obtained at baseline, in week 3, week 6, week 9 and in week 12. In week 6, which is two weeks after the STZ injection, the STZ group had significantly higher glucose levels compared to the NPD group ($P < 0.01$), as illustrated in figure 7A. Although, plasma glucose levels did not differ between groups in week 9 and 12, a trend of higher glucose levels in the STZ group was present. No significant difference between the NPD and HFD group was observed during the study period. Changes in insulin concentrations in the different groups during the experimental period are shown in figure 7B. The HFD group showed a trend towards higher insulin levels in week 6 and significantly increased plasma insulin concentrations in week 9, compared to the NPD and STZ group ($P < 0.05$ and $P < 0.001$ respectively), and in week 12 compared to the STZ group ($P < 0.01$). No significant differences between the NPD and STZ group were observed.

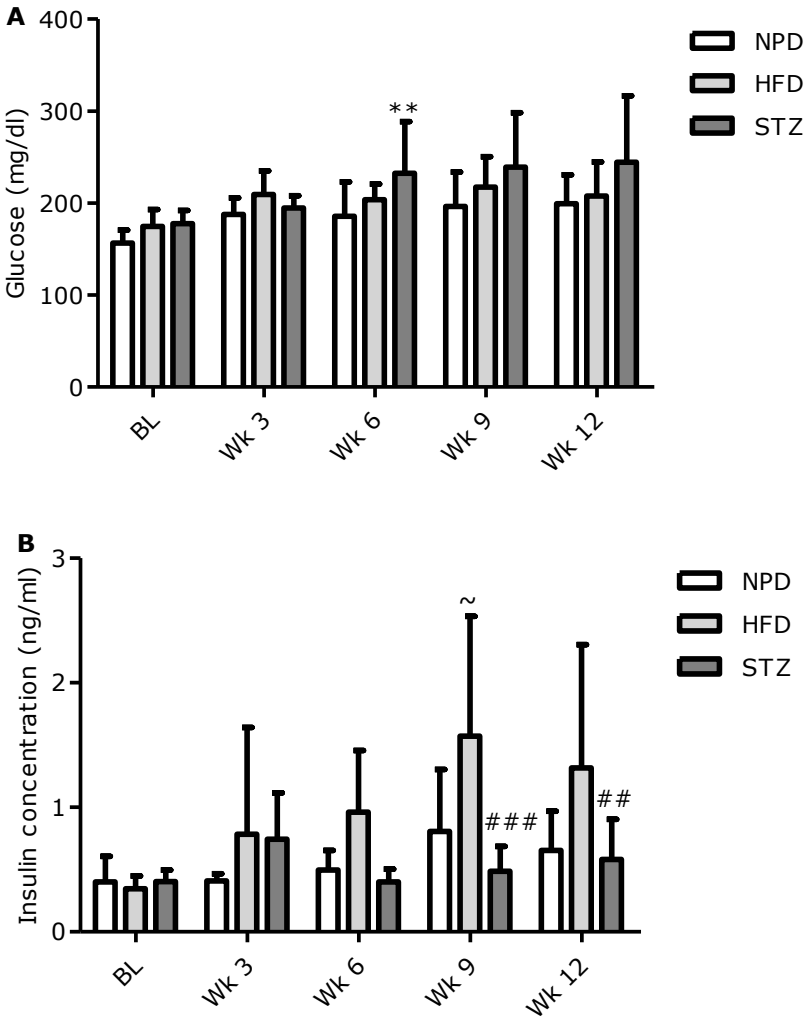

*Figure 7: Fed plasma glucose (A) and insulin (B) levels obtained at regular time points from the three experimental groups [NPD (n=6), HFD (n=7) and STZ (n=11)]. Data are expressed as mean ± SD. * = NPD vs STZ; ~ = NPD vs HFD; # = HFD vs STZ. ~ P = < 0.05; **/## = P < 0.01; ### = P < 0.001. BL = baseline, NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin.*

In order to investigate whether the HFD and STZ group are obese, plasma cholesterol and triglyceride levels were determined. As illustrated in figure 8A, the fed cholesterol levels did not differ between the three groups during the study. As shown in figure 8B, plasma triglyceride levels increased significantly in the STZ group in week 6 compared to the NPD group ($P < 0.05$) but the levels did not differ significantly between groups in week 12. Although not significant, triglycerides tended to be higher in the HFD and STZ group compared to the NPD group during the course of the study.

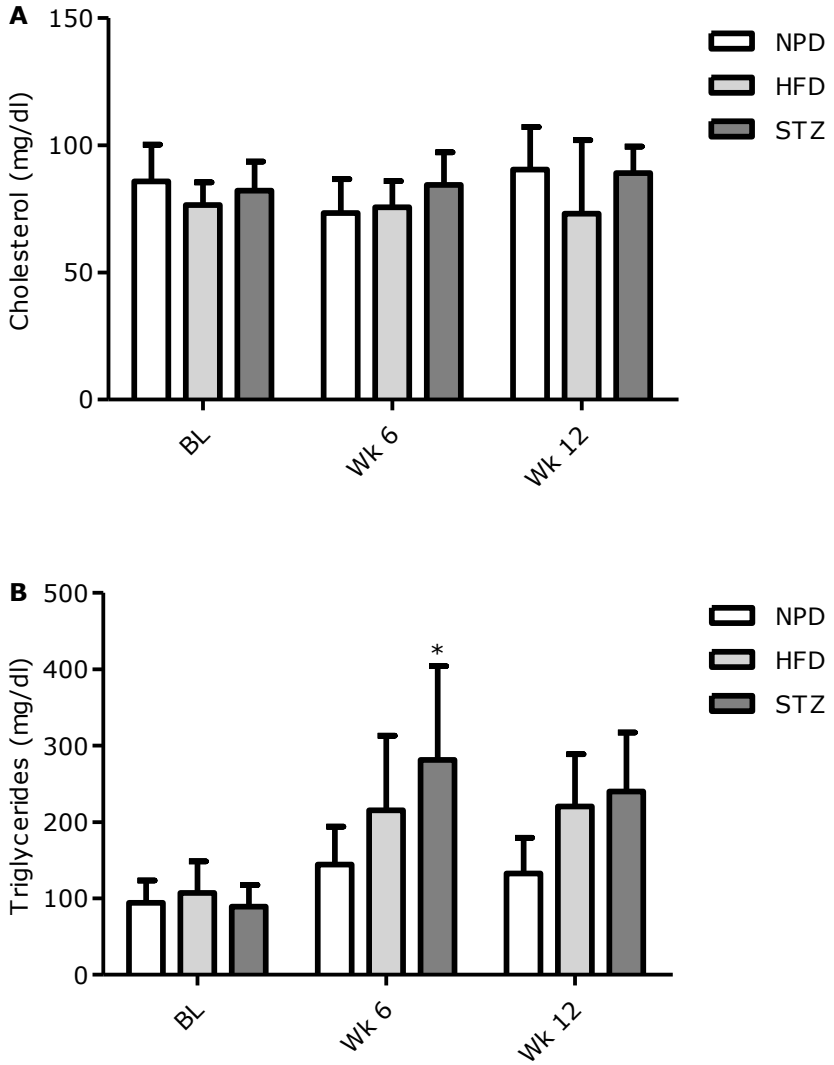

*Figure 8: Fed plasma cholesterol (A) and triglyceride levels (B) obtained at regular time points during the study period from the three experimental groups [NPD (n=6), HFD (n=7) and STZ (n=11)]. Data are expressed as mean ± SD. * = NPD vs STZ; P < 0.05. BL = baseline, NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin.*

8.4 Urine metabolites

Since diabetes can also lead to kidney damage and even to kidney failure, which is known as diabetic nephropathy, urine samples were obtained using metabolic cages and analyzed to evaluate possible differences between the three experimental groups. Figure 9A represents the urine creatinine concentration which is a useful measure for approximating the glomerular filtration rate (GFR). Urine creatinine concentration was significantly increased in the HFD group in week 6 and in both the HFD and STZ group in week 12, compared to the NPD group ($p < 0.01$). Total protein concentration, expressed as mg per g creatinine to correct for variations in urine concentration, was also investigated. As illustrated in figure 9B, no significant difference between groups was observed. However, a trend of higher total protein concentration in both the HFD and STZ group, compared to the NPD group, was present.

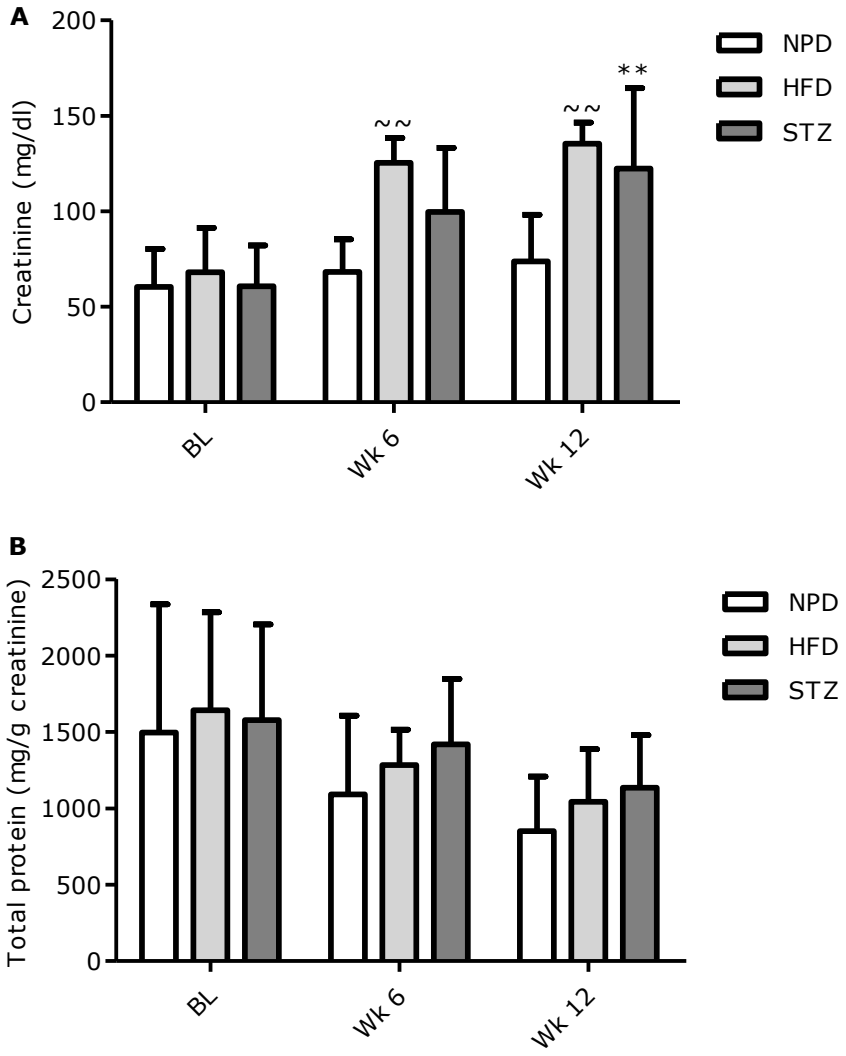

*Figure 9: Urine creatinine (A) and total protein (B) concentrations from the three experimental groups [NPD (n=5), HFD (n=6) and STZ (n=11)], obtained at baseline (BL), in week 6 and in week 12. Data are expressed as mean ± SD. * = NPD vs STZ; ~ = NPD vs HFD. **/~~ = P < 0.01. NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin.*

DM is characterized by polydipsia and hence water intake was assessed while the rats were housed on metabolic cages. The STZ group drank significantly more in week 12 compared to the NPD and HFD group ($P < 0.01$ and $P < 0.05$ respectively), as illustrated in figure 10. No significant differences between the NPD and HFD group were observed.

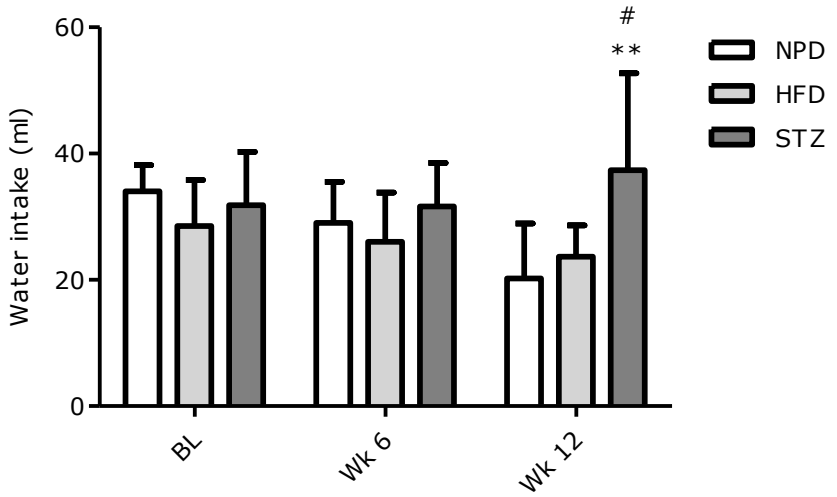

*Figure 10: Water intake per 24 hours in the three experimental groups [NPD (n=5), HFD (n=6) and STZ (n=11)], obtained at baseline (BL), in week 6 and in week 12. Data are expressed as mean ± SD. * = NPD vs STZ; # = HFD vs STZ. # P < 0.05; ** P < 0.01. NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin.*

Another symptom of DM is the presence of glucose in urine, which is known as glycosuria. Therefore, urine glucose levels were determined. Figure 11 represents the urine glucose concentration, expressed as mg glucose per g creatinine to correct for variations in urine concentration. No significant differences between groups were present.

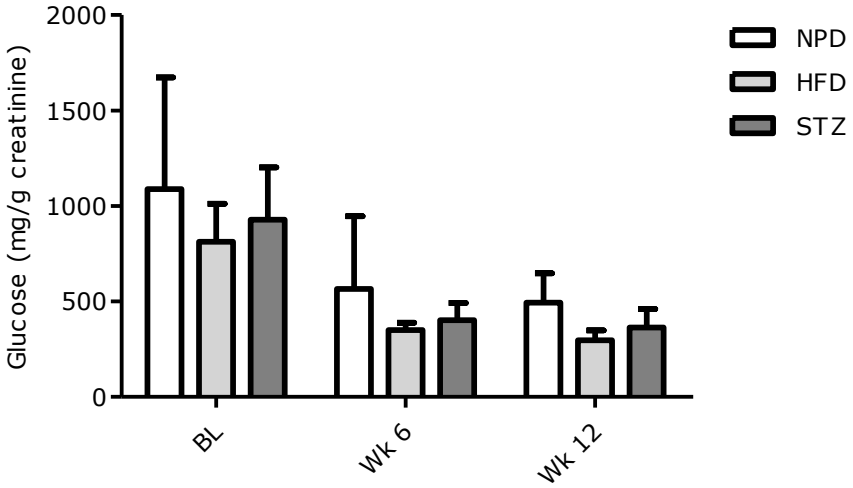

Figure 11: Urine glucose concentration (mg glucose/g creatinine) from the three experimental groups [NPD (n=5), HFD (n=6) and STZ (n=11)], obtained at baseline (BL), in week 6 and in week 12. Data are expressed as mean ± SD. NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin.

8.5 Echocardiographic parameters and hemodynamic measurements

As previously mentioned, DM can lead to development of DCM. Therefore, echocardiography was performed to evaluate the heart function of the different experimental groups. Conventional echocardiographic parameters obtained at the end of the study, are represented in table 4. No significant differences were observed between groups. Time had a significant effect on all parameters, except for EF. After 12 weeks, HR and FS decreased significantly ($P < 0.001$ and $P < 0.01$) and PWT, AWT, EDV, ESV and CO increased significantly in all three groups compared to baseline ($P < 0.001$ for all parameters except for AWT, $P < 0.01$, data not shown).

Table 4: Effect of NPD, HFD and the combination of a HFD and a STZ injection on echocardiographic parameters

	NPD (n=6)	HFD (n=7)	STZ (n=11)
HR (bpm)	367.0 ± 29.5	359.1 ± 27.6	376.7 ± 29.1
PWT (mm)	2.2 ± 0.4	2.0 ± 0.3	2.1 ± 0.4
AWT (mm)	2.4 ± 0.8	2.1 ± 0.4	2.5 ± 0.5
EDV (µl)	381.1 ± 34.7	454.1 ± 60.5	451.9 ± 78.1
ESV (µl)	90.9 ± 33.7	104.8 ± 52.9	99.7 ± 52.3
CO (ml/min)	105.8 ± 40.2	125.9 ± 22.7	132.2 ± 21.1
EF (%)	75.8 ± 6.7	77.3 ± 8.7	78.5 ± 8.7
FS (%)	24.6 ± 6.5	29.6 ± 9.0	31.4 ± 9.0

NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin, HR = heart rate, bpm = beats/min, PWT = posterior wall thickness, AWT = anterior wall thickness, EDV = end-diastolic volume, ESV = end-systolic volume, CO = cardiac output, EF = left ventricular ejection fraction, FS = fractional shortening. Data are expressed as mean ± SD.

A summary of the invasive hemodynamics obtained at the end of the study, is shown in table 5. One rat from the STZ group was excluded from the hemodynamic measurements, after respiratory arrest during the pressure measurement (n=10). LVP increased significantly in the STZ group, compared to both the NPD and HFD group ($P < 0.001$ and $P < 0.01$ respectively). No significant difference was observed in LVP between the NPD and HFD group. Both the HFD and STZ group showed an increased LVEDP, compared to the NPD group ($P < 0.01$ and $P < 0.05$ respectively). Maximum and minimum value of the first derivate of LV pressure (dP/dt_{max} and dP/dt_{min}) did not differ significantly between groups. The time constant of LV pressure decay during isovolumic relaxation (tau), increased significantly in the HFD and STZ group, compared to the NPD group ($P < 0.01$ and $P < 0.05$ respectively).

Table 5: Hemodynamic characteristics of the different treatment groups

	NPD (n=6)	HFD (n=7)	STZ (n=10)
LVP (mmHg)	99.0 ± 7.1	105.4 ± 7.2 ##	118.4 ± 7.4 ***
LVEDP (mmHg)	8.4 ± 1.6	13.3 ± 3.9 ~~	14.3 ± 3.6 *
dP/dt_{max} (mmHg/s)	5296.0 ± 531.1	5909.9 ± 1274.9	5951.6 ± 910.3
dP/dt_{min} (mmHg/s)	-6116.3 ± 1085.3	-6005.7 ± 1529.7	-5900.1 ± 987.6
Tau (min)	0.008 ± 0.003	0.012 ± 0.002 ~~	0.012 ± 0.002 *

*NPD = normal pellet diet, HFD = high fat diet, STZ = streptozotocin, LVP = left ventricle pressure, LVEDP = left ventricular end-diastolic pressure, dP/dt_{max} = maximum value of the first derivate of LV pressure, dP/dt_{min} = minimum value of the first derivate of LV pressure, tau = time constant of LV pressure decay during the isovolumic relaxation period. Data are expressed as mean ± SD. * = NPD vs STZ; ~ = NPD vs HFD; # = HFD vs STZ. * P < 0.05, ##/~~ = P < 0.01; *** = P < 0.001.*

9 Discussion

9.1 Induction of type 2 diabetes

This study was initiated with the objective to develop an animal model for DCM, that would mimic the natural history seen in humans. Two rats died during the course of the study for unknown reasons, one rat from the NPD group and one rat from the HFD group. In addition, one rat from the NPD group was excluded after statistical analyses proved that this rat was an outlier for weight gain as well as for OGTT measurements, limiting the sample size of the NPD group to n=6 and the HFD group to n=7. Any difference between the NPD group and the HFD group is attributable to the effect of the HFD. In contrast, differences between the HFD group and the STZ group are the result of the STZ injection. When comparing the NPD group with the STZ group, any observed effect is a consequence of the combination of a HFD and a single low dose STZ injection.

9.1.1 Oral glucose tolerance tests

In order to diagnose diabetes in the different experimental groups, oral glucose tolerance tests were performed at baseline, 1 week after STZ injection (week 5) and at the end of the experiment (week 12). The baseline OGTT (figure 3A) shows the results of only six rats due to several problems. First, inappropriate capillaries were used and therefore the Analox GM7 was not able to accurately determine blood glucose concentrations. Second, the rats were anesthetized with isoflurane two consecutive days as the baseline echocardiography measurements were taken the day before the OGTT, resulting in the presence of viscous blood in the rats, which made it difficult to obtain arterial blood samples. These problems were solved before the start of the next OGTT.

A first clue to diagnose DM, can be found in the second OGTT which was performed one week after the STZ injection (figure 4A). Rats are considered diabetic when having a glucose level of ≥ 11.1 mmol/l (= 200 mg/dl), two hours after glucose intake upon fasting [69, 70]. Two hours after glucose administration, all three groups had a glucose level above 11.1 mmol/l [69, 70], except for one rat of the STZ group, thus limiting the sample size of the STZ group to n=11. The reason for this outlier is difficult to determine. Most likely, the STZ injection was not performed correctly. The STZ group displayed significantly higher glucose concentrations during the second OGTT from 30 min after glucose ingestion onwards, compared to the NPD and HFD group. Similarly, during the third OGTT, the STZ group showed higher glucose levels from 60 min after glucose administration onwards (although only significant at 120 min), compared to the NPD and the HFD group, thus indicating further increasing glucose levels. These high glucose values are similar to those previously described in literature [63, 71, 72], despite applying a lower dose of STZ (35 mg/kg in our study compared to 50 mg/kg, 45 mg/kg and 45 mg/kg respectively). Insulin response to the glucose challenge during the OGTTs was also investigated in all three groups. Serum insulin levels in the STZ group were similar to the NPD group in both the second and third OGTT (figure 4B and 5B), due to partial destruction of β-cells by the STZ injection. Similar low levels of insulin in the STZ-treated group were confirmed by previous studies [65, 72]. Considering the increased glucose levels in the STZ group compared to the other groups (figure 6), these rats seem to be insulin resistant and unable to maintain their glucose level in spite of normal insulin levels. The HFD group

displayed significantly higher insulin levels during both OGTTs, whereas glucose levels were similar to the NPD group, which indicates the presence of insulin resistance in this group while maintaining glucose levels in the normal range. This observation was supported by Kraegen et al. (1991) who showed significant insulin resistance in rats after just three weeks of high-fat feeding [73]. Ad libitum intake of a HFD causes a progressive accumulation of triglycerides in muscles. Due to this increased delivery of fatty acids and/or due to a reduced β-oxidation as a result of mitochondrial dysfunction, DAG and subsequently PKC is activated in muscle cells. PKC phosphorylates downstream activators, eventually resulting in a lowered RAC-beta serine/threonine protein kinase (AKT2) activity which in turn does not activate GLUT4 translocation to the cell membrane. Consequently, insulin-induced glucose uptake in muscles is reduced. In the liver, a lowered AKT2 activity leads to a reduced glycogen synthesis and to an increased gluconeogenesis [74]. Both mechanisms result in insulin resistance and a higher blood glucose level in time. In addition, the increase in blood glucose concentration during the OGTT between 0 and 120 min, expressed as (mmol/l)/min, was significantly increased in the STZ group in both week 5 and week 12, compared to the other groups (figure 6). For all of these reasons, the STZ group is considered diabetic.

In the second and third OGTT, all three groups showed blood glucose levels above 11.1 mmol/l two hours after glucose intake (figure 4A+5A). Moreover, all OGTTs show two peaks, the first peak appearing at 30 min and the second at 90 min. The first peak is the result of an increase in blood glucose due to the oral gavage. Nonetheless, the second peak should not be visible and the blood glucose concentration should have decreased by 90 min, as seen in literature [63, 71, 72]. The high serum glucose levels, as well as the second peak during the OGTTs may be explained by the use of isoflurane anesthesia. At 60 min, the rats are anesthetized again to obtain an arterial blood sample for insulin analysis. Isoflurane impairs insulin release, which causes in turn an increase in blood glucose concentration and subsequently hyperglycemia [75, 76]. Volatile anesthetics such as isoflurane, facilitate the opening of K_{ATP}-channels in pancreatic β-cells. Due to this opening, depolarization of the β-cell cannot occur and voltage dependent calcium channels are not activated. As a result calcium entry in the β-cell and subsequent insulin exocytosis is inhibited and blood glucose increases [77]. The actual glucose concentration is therefore probably lower. However, as isoflurane anesthesia was applied in a standardized way to all three groups, the relative difference between groups should be similar. This is a limitation for the study. However, as it is not possible to obtain arterial blood samples in a conscious rat, this problem could not be avoided.

In this study, an oral glucose bolus was administered to the rats to determine their glucose response. The main disadvantage of the OGTT is that the blood glucose concentration depends on the glucose passage through the stomach and on the glucose absorption by the small intestines. This can explain the absence of a pronounced peak and subsequent decrease in glucose values during the course of the OGTT. Therefore, performing an intravenous glucose tolerance test on rats by an infusion of glucose in the femoral jugular vein might be considered in the future, to accurately determine blood glucose concentrations.

9.1.2 Plasma glucose and insulin levels

A next clue to diagnose DM, can be found in the plasma glucose and insulin levels. The HFD-fed STZ-treated rats showed significantly increased levels of fed plasma glucose in week 6, two weeks after the STZ injection, confirming again that the STZ group has developed diabetes. However, fed plasma glucose failed to reach statistical significance in week 9 and 12 although a trend to increasing glucose levels in the STZ group was present (figure 7A). This may be explained by the fact that young rodents have the capacity to increase β-cell mass by spontaneous regeneration of pancreatic islets and hence attenuate diabetes [78, 79]. Likewise, increasing age may also play a role and cannot be excluded. The HFD and STZ group displayed a fed plasma glucose level of > 200 mg/dl (= 11.1 mmol/l) in week 6, in contrast to the NPD group. This effect persisted for the remainder of the study. The fasted serum glucose levels are similar in all three groups in week 5 (92.3 ± 15.3 mg/dl) and in week 12 (131.2 ± 20.2 mg/dl) (data not shown). In literature, rats are usually considered diabetic when fasting blood glucose ≥ 200 mg/dl [68, 80, 81], indicating that all three groups are not diabetic according to this guideline, in contrast to the results of the OGTT as explained before. To date, the World Health Organization (WHO) uses the presence of classic symptoms and an abnormal fasting plasma glucose test (>126 mg/dl or 7 mmol/l) or abnormal random plasma glucose (≥ 200 mg/dl or 11.1 mmol/l) to diagnose DM in patients. However, in many cases the fasting plasma glucose is still relatively normal and therefore diagnosis of DM will not be made yet. An OGTT is a better standard for diagnosing diabetes, prediabetes and gestational diabetes because this technique is more sensitive than the fasting plasma glucose test. Nonetheless, the OGTT is often not applied in practice because it is more expensive and less convenient [82]. Still, the results of our study showed that the OGTT is more reliable to diagnose DM than fasted or random plasma glucose levels and demonstrate the need to imply the OGTT in practice together with the fasting plasma glucose test.

The HFD and STZ group were already insulin resistant before the STZ injection due to the HFD, as shown in week 3 in figure 7B, displaying compensatory hyperinsulinemia (although not significant) to maintain a normal glucose balance. A HFD induces insulin resistance mainly through the glucose-fatty acid cycle [83]. Due to the excess fat intake, fatty acid availability increases which is preferred over glucose for β-oxidation. This blunts the insulin-mediated reduction of hepatic glucose output and reduces glucose uptake by skeletal muscle, which leads in turn to hyperglycemia and compensatory hyperinsulinemia, as explained before. Therefore, the slightest insult that could compromise pancreas function, such as exposure to STZ, may lead to hyperglycemia. When the NPD group would be injected with this low dose of STZ, they normally should not develop hyperglycemia because their normal defense homeostatic mechanisms would cope with this insult and maintain a normal glucose level [65]. After the STZ/vehicle injection, the STZ group displays a similar fed plasma insulin level compared to the NPD group, indicating only a relative insulin deficiency [65], due to partial destruction of the pancreas by STZ. The reduced insulin levels in the STZ group may also be explained by the induction and presence of late stage type 2 diabetes in the rats, instead of early stage type 2 diabetes. In patients, late stage type 2 diabetes is characterized by hyperglycemia and similar or lower levels of insulin compared with weight-matched controls, in contrast to early stage phase which is characterized by relatively high

levels of insulin [84]. The HFD group displayed significantly increased insulin levels at the end of the study, indicating the presence of the insulin resistance syndrome, characterized in patients by obesity, mild hyperglycemia, hypertriglyceridemia and compensatory hyperinsulinemia (figure 7B) [65]. Again, these observations regarding the glucose and insulin concentrations, are in agreement with previous studies [62, 65, 85]. Based on the increased fed plasma and fasted serum glucose levels and decreased plasma insulin levels after STZ injection, the diagnosis of DM can be made in the STZ group. The HFD group on the other hand, displays an impaired glucose tolerance and insulin resistance as indicated by the increased insulin levels.

9.1.3 Plasma triglyceride and cholesterol levels

Apart from glucose and insulin, the HFD-fed STZ-treated rats also showed abnormalities in lipid metabolism as evidenced by increased plasma triglyceride levels, which may lead to cardiovascular complications in time (figure 8B). These increased plasma triglyceride levels are the result of consuming a HFD, as the HFD group showed the same trend. However, plasma cholesterol failed to reach statistical significance (figure 8A). Khan et al. (2012) mentioned that earlier studies have shown wide variations in the lipid and cholesterol profile of STZ-treated rats. These variations can be attributed to differences in age, weight, gender as well as the dose and route of STZ exposure [86]. The use of a HFD also leads to a couple disadvantages. First, the HFD leads to lipemia in blood samples which present as 'milky' blood samples. Lipemia occurs when blood is drawn in the fed state. A high concentration of lipoprotein particles is present in the blood sample which causes turbidity of the sample and can lead to false positive (protein, glucose calcium) or false negative results (sodium, potassium) [87]. Therefore, combining fed and fasted blood samples is advisable. Second, the HFD may also lead to aggressive behavior as observed a few times in the HFD and STZ group. Hilakivi-Clarke et al. (1996) showed that male animals which were fed a HFD, exhibited an aggressive behavior and the latency to a first aggressive encounter was significantly lower in these animals. They concluded that dietary fat can increase aggressive behavior in male rats, possible by elevating circulating estradiol levels [88].

No significant difference in body weight between groups was observed (figure 2). This can be explained by the fact the rats were fed an isocaloric NPD or HFD which means each rat received equal amounts of calories. However, the source of calories is different. The HFD contains 60% fat as a percentage of total kcal, in contrast to the NPD which contains 18% fat as a percentage of total kcal. In order to have an idea of the effect of the treatments on body composition and the onset of obesity, two different fad pads were excised and weighed. Indeed, the rats fed a HFD displayed relative more perirenal and epididymal fat (table 3), indicating a higher fat level in the body. However, as body weights were not different, the animals were not overtly obese.

To summarize, the STZ group is considered to have diabetes and more specifically type 2 diabetes for the following reasons. First, the rats are fatter than the NPD rats as indicated by the relative higher visceral fat mass and the presence of hypertriglyceridemia. Second, the rats are hyperglycemic as indicated by the OGTT. Third, they are insulin resistant and after the STZ injection, they display a relative insulin deficiency [71, 84]. Fourth, the dose of STZ has also an impact of the phenotype of diabetes. A high dose of STZ will induce a robust β-cell depletion which

leads to clear features of type 1 diabetes such as insulin deficiency, hyperglycemia, polyuria, polydipsia and sudden and drastic weight loss [84]. In this study a single low dose of STZ was used and the rats continued to gain weight, they were insulin independent and water intake was significantly higher at the end of the experiment. And fifth, the order of pathological events, fatter rats with clinical signs of obesity followed by β-cell failure favors a mimicking of type 2 diabetes rather than type 1 diabetes [84]. For all of these reasons, one can conclude that the rats of the STZ group have indeed developed type 2 diabetes. In addition, the HFD group is insulin resistant as indicated by the increased insulin.

9.2 Changes in kidney function

DM may also induce diabetic nephropathy. Various urinary parameters, such as creatinine, total protein and glucose levels, were assessed to investigate kidney function. Urine creatinine concentration was significantly increased in both the HFD and STZ group in week 6 and week 12 (figure 9A). Jain et al. (2015) also demonstrated an increased urine creatinine level in their HFD-fed STZ-induced diabetic rats, using a similar experimental protocol [89]. In general, creatinine levels are considered to assess kidney function. Creatinine is a byproduct of muscle breakdown and is excreted (virtually completely) and unchanged by the kidneys, mainly by glomerular filtration. Therefore, creatinine levels can be used to calculate creatinine clearance, which is an accurate measurement for assessment of the GFR. When filtration in the kidney deteriorates, creatinine levels will rise. In this study, an increased urine creatinine level may be a first indicator of kidney damage and more specific diabetic nephropathy, due to the development of DM. Urine creatinine levels may also be increased due to highly concentrated urine. However, this is in contrast to the presence of polydipsia in the STZ group, as indicated in figure 10. Creatinine clearance was not calculated in this study because fasted plasma creatinine concentration is required for this calculation [90]. However, this parameter was not determined and this aspect requires further research.

Next, total protein was assessed and expressed as a protein/creatinine ratio to correct for differences in urinary concentration (figure 9B). This ratio is a measurement of proteinuria, indicating the presence of excess proteins in the urine and therefore a deteriorated filtration function. Total protein/creatinine ratio tended to be higher in both the HFD and STZ group compared to the NPD group. This observation is confirmed by the study Danda et al. (2005). They mentioned that hyperglycemia is a major factor in causing proteinuria. The degree of proteinuria correlates with the degree of hyperglycemia, since rats with type 1 diabetes presented with higher glucose levels and significantly higher protein excretion rates, compared to rats with type 2 diabetes [85]. Hyperglycemia leads to abnormal activation of PKC, which in turn stimulates the production of growth factors, such as TGF-β1 and connective tissue growth factor (CTGF). TGF-β1 can also be activated by AGEs, which are produced as a consequence of high glucose levels, as mentioned in section 6.2.1. These growth factors are responsible for increased deposition of extracellular membrane and increased permeability of glomerular basement membrane, followed by expansion of the mesangium which leads to progressive narrowing of the capillary lumens and thus to a decline in GFR [91, 92]. As a result of this, increased filtration of proteins will occur leading to proteinuria.

Urine glucose tended to be increased in the STZ group. However, when expressed as mg glucose per g creatinine to correct for variations in urine concentration, urine glucose levels tended to decrease in the HFD and STZ group, although not significant (figure 11). In normal circumstances, glucose is not present in the urine because the kidneys reabsorb the filtered glucose. When blood glucose levels rise in case of untreated DM, and exceed a particular threshold, the proximal tubule becomes overwhelmed and glucose is excreted in the urine. Frequent high blood glucose levels may lead in time to kidney and filter damage, as mentioned before.

In conclusion, the increase in urine creatinine concentration and the tendency for a higher total protein/creatinine ratio, indicate that mild kidney damage is present, probably as a consequence of the development of DM.

9.3 Evaluation of cardiac function

The ultimate goal of this study is to develop a rat model of DCM and to evaluate cardiac function in the different groups. As mentioned in the introduction, there is no consensus after how many weeks rats develop cardiac dysfunction. LV pressures were measured by cardiac catheterization (table 5). One rat of the STZ group was excluded due to respiratory arrest during the pressure measurements (n=10). The STZ group displayed a significantly increased LVP, compare to the HFD and NPD group. LVP is a measure of left ventricular end-systolic pressure. This higher end-systolic pressure might lead in a next phase to development of hypertrophy and in the final stage to dilation of the heart and consequently deterioration of the heart function. In addition, LVEDP as well as tau, which is a measurement for the time to relax, were increased in both the HFD and STZ group compared to the NPD group. These parameters indicate a deteriorated relaxation of the LV and hence a stiffer heart in both groups. The increased stiffness results from an increased cardiac fibrosis which is a common hallmark of DCM. Fibrosis is caused by both AGEs and ROS, in response to high glucose levels. Glucose enters the cell via GLUT1 and GLUT4. High glucose levels generate AGEs which can crosslink collagen. On the other hand, ROS are produced by the mitochondria in response to high glucose levels but are also produced by the pathway that produces AGEs. Both AGEs and ROS can activate particular cell signaling pathways such as PKC, which in turn yield enhanced collagen production and fibrosis and hence to a decreased compliance of the heart [93]. The changes in hemodynamic parameters are in agreement with previous experimental investigations [94-96] and indicate the early start of diastolic dysfunction due to a deteriorated relaxation of the LV.

With the use of echocardiography, conventional echo parameters were evaluated (table 4). All parameters significantly increased in all groups at the end of the study, except for HR and FS which were both significantly reduced and EF tended to decrease in time (data not shown). The rats were six weeks old at baseline echo and eighteen weeks old when the final echo was performed, and during this period the rats grew and gained weight. Both factors contribute to a larger heart in accordance with increased volumes, cardiac output and wall thicknesses and a decreased HR, EF and FS. FS is the fraction of any diastolic dimension that is lost in systole and thus another way of measuring left ventricle performance. FS and EF are similar in rat and human echocardiography [97] and the normal range of FS is 25-45%. Therefore, all three groups displayed a normal FS. No

significant differences in echo parameters were observed between groups at the end of the experiment. However, the HFD and STZ groups tended to display higher values for ESV, EDV, CO and FS, compared to the NPD group. The increased volumes may indicate the presence of dilation of the ventricles and development of hypertrophy [93]. Our observations for the different echocardiographic parameters are in line with the results described in previous studies, although they applied a different experimental protocol to induce DM and DCM [67, 94, 98]. Ti et al. (2011) developed a similar model as ours, however they applied a combination of a HFD (34.5% fat) and a dose of STZ of 27.5 mg/kg to induce DCM in rats. They showed a moderate decrease in E/A ratio in diabetic rats, six weeks after the onset of diabetes. In addition, after 16 weeks of diabetes, EF, FS and E/A ratio were further decreased in the diabetic group [94]. The E/A ratio is a marker of LV function and diastolic dysfunction and can be evaluated using Doppler echocardiography. The E represents the early filling velocity and the A represents the atrial kick or atrial filling velocity. In a normal heart, the E/A ratio > 1 and the reversal of the ratio (E < A) is accepted as a marker of diastolic dysfunction. However, due to a much faster heart beat in rats compared to patients, E and A peak are often fused which makes it difficult to accurately determine this ratio. Therefore, E/A ratio was not determined in the current study.

To summarize, both the HFD and STZ group displayed a deteriorated relaxation of the LV and the STZ group showed an additional increased LVP. Due to the order of pathological events, induction of type 2 diabetes before the development of cardiac abnormalities, we may presume that the deteriorated relaxation is possibly the result of the start of the development of DCM. However, *in vitro* experiments to investigate the presence of fibrosis or other molecular changes, have not yet been performed. In the future, BNP levels also could be determined as these levels are unaffected by high glucose levels, in contrast to ANP [99]. For all of these reasons, diagnosis of DCM cannot yet be made with great certainty. However, when it becomes apparent that the STZ group has indeed developed DCM, they are probably in between the early and advanced stage of DCM, as was explained in section 6.1 of the introduction.

9.4 Limitations

There are several limitations that should be recognized. First, this was a pilot study and the number of animals in each group is limited. As a result, the statistical power may not be strong enough to draw a definite conclusion about the development of DCM, due to a HFD in combination with a single low dose of STZ. Second, after nine weeks of diabetes, no significant difference in echo parameters between groups was observed. In the future, the rats have to be followed for a longer period of time to detect possible differences between groups. Third, echocardiography was only performed at baseline and at the end of the study. Therefore, we have no insight in changes in the echocardiographic parameters during the remainder of the study. Fourth, periodic plasma metabolites were determined in fed blood samples. Lipemia may lead to false positive or false negative results. Fifth, kidney function was deteriorated as indicated by the various urinary parameters. In the future, microalbumin levels should be determined as this is a more sensitive marker of kidney damage. Normally, microalbumin will be reabsorbed by the kidney but when the kidney is damaged by a high glucose concentration, the tubular function of the kidney will deteriorate and more microalbumin is excreted. In addition, creatinine clearance could be

determined as this is an accurate measurement for assessment of the GFR. Sixth, *in vitro* experiments were not yet performed and thus cardiac dysfunction may also originate due to other risk factors. And finally, increasing age has also an influence on all of these results, which cannot be excluded nor avoided.

10 Conclusion and future perspectives

In conclusion, this study showed that the combination of a HFD (60% fat) and a low dose of STZ (35 mg/kg) was efficient to induce type 2 diabetes and cardiac abnormalities in a rat model. The diagnosis of type 2 diabetes was confirmed by the results from the OGTTs, fed plasma glucose levels and fasted serum glucose levels, body weight, insulin levels and water intake. As mentioned before, the HFD causes peripheral insulin resistance and the STZ injection mildly destructs the pancreatic β-cells and leads to a transition from an insulin-resistant state to a state of type 2 diabetes. The pressure measurements indicated the presence of a deteriorated relaxation of the LV in both the HFD and STZ group and in addition a higher LVP in the STZ group. This nongenetic rat model has the advantage of being easy to induce, is less expensive than genetic models and can be modified for different severities of diabetes. In addition, this model has moderately elevated and constant blood glucose levels without the need for insulin treatment. Moreover, possible kidney damage can also be evaluated. In the future, the development of DCM in this rat model has to be confirmed *in vitro* by subjecting the hearts to a MTC and/or H&E staining to check for presence of fibrosis. In addition, changes in calcium homeostasis and changes in glucose transporters such as GLUT1 and GLUT4 could be investigated. The study could also be repeated with larger groups and could be carried out for a longer period of time so the diabetic rats are able to develop cardiac dysfunction with distinct features of DCM. As mentioned before, echocardiography should be performed at regular time intervals and strain, strain rate and BNP levels could additionally be determined. Finally, high blood glucose level can induce kidney damage. Therefore, it should be investigated if this rat model for DCM also could represent a clinically relevant rat model for diabetic nephropathy, by determining microalbumin, blood urea nitrogen levels and creatinine clearance rate.

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Development and characterization of a clinically relevant rat model of diabetic cardiomyopathy

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
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