AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF HYPER-CALORIC INTAKE ON HEMODYNAMIC FUNCTIONS AND CARDIAC AUTONOMIC CONTROL: POTENTIAL MODULATION BY ANTI-DIABETIC DRUGS

by
OLA AHMAD AL-ASSI

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OLA AHMAD AL-ASSI

Approved by:

Dr. Ahmed El-Yazbi, Assistant Professor — Advisor
Department of Pharmacology and Toxicology

Dr. Fouad Zouein, Assistant Professor — Co-Advisor
Department of Pharmacology and Toxicology

Dr. Ramzi Sabra, Assistant Dean, Chairperson, Professor — Member of Committee
Department of Pharmacology and Toxicology

Dr. Assaad Eid, Associate Professor — Member of Committee
Department of Anatomy, Cell Biology and Physiological Sciences

Beirut, Lebanon
September 25, 2017

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AN ABSTRACT OF THE THESIS

Ola Al-Assi for Master of Science
Major: Pharmacology and Toxicology

Title: Effect of Hyper-Caloric Intake on Hemodynamic Functions and Cardiac Autonomic Control: Potential Modulation by Anti-Diabetic Drugs

Diabetes mellitus remains a public health challenge with considerable disease and economic consequences. Mortality and morbidity due to diabetes are related to the poor cardiovascular outcomes. A significant body of research describes the detrimental effect of hyperglycemia on vascular function and the importance of tight blood glucose control in delaying progression of diabetes micro-vascular complications. It is generally agreed that the impact of glycemic control on cardiovascular complications is influenced by the temporal framework in which an acceptable blood glucose level has been achieved, in what became known as the "Legacy effect" or the "Cardiovascular metabolic memory". On the other hand, recent clinical evidence paints a different picture, whereby treatment with certain anti-hyperglycemic drugs on top of the standard-of-care for diabetes and cardiovascular disease leads to cardiovascular risk reduction, in a manner that is not necessarily tightly linked to their effect on glycemic control.In parallel, recent studies reported cardiovascular abnormalities in animal models of high caloric intake before the development of frank hyperglycemia.

We recently developed a rat model of hyper-caloric feeding that was shown to develop vascular dysfunction without changes in blood pressure, serum glucose, or insulin levels. As such, we propose to use this model to characterize early hemodynamic changes occurring in the context of development of diabetes, study the underlying pathological mechanism, track the evolution of these disorders, and examine the effect of early treatment with some of the anti-hyperglycemic drugs with presumed cardiovascular benefit. Control and high calorie fed rat groups will be run side-by-side for 12 weeks. Body weight and caloric intake will be assessed regularly. Non-invasive blood pressure measurement and hemodynamic parameter assessment by echocardiography will be carried out at four-week intervals. Sub-groups of the high calorie fed rats will be given daily oral doses of metformin and pioglitazone for the last 2 weeks of the protocol. At the end of the 12-week period, rats will be instrumented with intra-vascular cannulas for invasive blood pressure measurement, baroreflex sensitivity assessment, and estimation of cardiac autonomic control through the calculation of heart rate variability. Finally, rats will be sacrificed and heart tissue will be collected for molecular studies.

We expect that high calorie fed rats will show a progressive deterioration of hemodynamic functions together with an impaired cardiac autonomic control. This is expected to be rescued in animals treated with metformin and pioglitazone. Whether the detrimental effect of hyper-caloric intake involves endoplasmic reticulum stress, impairment of autophagy and apoptosis of cardiac myocytes remains to be determined.

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CHAPTER I

INTRODUCTION

A. Diabetes

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia (increased blood glucose concentrations) caused by reduced secretion or action of insulin or both[1]. It is defined by abnormal fasting or postprandial glucose. Diabetes has two main classifications: type 1 and type 2. Type 1 is an autoimmune disease characterized by the destruction of pancreatic β cells with concomitant insulin deficiency and resistance. Type 2 encompasses insulin resistance and deficiency. This form accounts for 90-95% of diabetes[1]. Most patients having this type are obese or over weight[1]. The chronic hyperglycemia associated with diabetes is thought to be responsible for the long-term damage and dysfunction occurring in different body organs, mainly heart, blood vessels, nerves, eyes and kidneys[2, 3].

B. Diabetes incidence and prevalence

The change in human behavior, lifestyle and environment increased the prevalence and incidence of diabetes[4]. Overeating, smoking, excessive alcohol consumption and lack of exercise are adopted by people with no regard to their detrimental consequences. These behaviors are influenced by environmental stimuli which include: work load, stress, lack of time and motivation, rapid diet changes, multimedia and advertisement, among others[5]. The rapid dietary changes towards high saturated fat, high sugar, refined and low in fiber foods contributed to the increase

in diabetes and cardiovascular diseases [4]. According to the 2014 World Health Organization (WHO) Global Report on Diabetes, about 422 million people have diabetes worldwide. WHO estimated that approximately 1.5 million died of a cause related to diabetes in 2012. Diabetes is thought to progress to become the seventh leading cause of mortality by 2030[6].In the Middle East, high rates of diabetes have been observed[7]. Specifically in Lebanon, out of 3000 random patients, 339 have been previously diagnosed with diabetes[8], while out of 90individuals, 28 were not aware they have diabetes[9]. The prevalence of diabetes in greater Beirut in Lebanon is high and increasing and is accompanied by an increase in cardiovascular (CV) risk[9].

C. Economic and public health burden of diabetes

Diabetes is of great global concern because of its debilitating effects and great cost. According to the American Diabetes Association (ADA), the diagnosed diabetes costs have reached a total of $245 billion in 2012 and this burden is continuously increasing[10]. Multiple factors contribute to the increased impact of diabetes on public health. First, the incidence of developing diabetes increases with age and thus, an increased prevalence is expected with the demographic shift towards aging population. Second, the increased prevalence of obesity is a major factor driving the rise in the incidence of type 2 diabetes in young individuals. Additionally, genetic predisposition appears to underlie an increased susceptibility of diabetes[11].A significant proportion of newly diagnosed diabetic patients already present with microvascular complications[12]. Therefore, prior lifestyle should be further studied to assess whether it bears an influence as an early trigger of diabetic complications. In this context,

pathological stages early in diabetes development should not be undermined while addressing potential interventions to alleviate the detrimental sequelae of diabetes.

D. Stages of diabetes

Metabolic deterioration in diabetes occurs gradually in definable stages preceding the final decompensated phenotype. Each stage is defined by certain changes in metabolic parameters or/and beta cell function (table 1)[13]. However, the main stages are the pre-diabetic (undiagnostic) stage and the diabetic (diagnostic) stage[14].

Prediabetes is an intermediate metabolic state between having a normal glucose metabolism and diabetes[15]. This stage can last for years before developing diabetes[16]. Prediabetes is defined by a small increase in impaired fasting glucose, impaired glucose tolerance, and/or disturbed glycosylated hemoglobin levels (HbA1c) [15, 17]. Initially, a mild increase (89mg/dl) beyond the ideal level (80 mg/dl) of fasting glucose blood levels is seen. This unrecognizable increase is usually not considered abnormal (>100mg/dl) according to the WHO diagnostic criteria[18]. The chronic increase in blood glucose values, though limited, will cause an increase in insulin secretion. This compensatory mechanism is driven by the pancreatic beta cells, which undergo increased proliferation, mass and insulin secretion[13]. This process culminates into insulin resistance, which is characteristic of the pre-diabetes stage. Screening for prediabetes is not easy and the concordance between the tests is rarely achieved at this stage [15]. The American Diabetes Association (ADA) identifies prediabetes according to these values: fasting plasma glucose from 100 mg/dl to 125 mg/dl; HbA1c from 5.7% to 6.4%; and 2 hour values in the oral glucose tolerance test from 140 mg/dl to 199 mg/dl[19]. Patients with prediabetes appear to also have an increased risk for CVD[20].

This highlights the importance of recognizing these patients in order to allow intervention to lower the risk for developing diabetes and CV disease[15].

After the compensatory pre-diabetic stage, beta cells enter the stable adaptation phase. In this phase, glucose levels are mildly increased and beta cell function and differentiation are altered. The compensation for the increased glucose levels is lost and beta cells start to adapt for the continuous high glucose levels[13]. As glucose levels rise, pancreatic beta cell function changes and loss of glucose stimulated insulin secretion occurs. This loss is accompanied by phenotypic changes of the beta cells characterized by the appearance of new autoantigens making the cells less vulnerable to dysglycemia[21]. Accordingly, unstable early decompensation stage starts. It is considered as the first stage towards the progression of diabetes and glucose levels rapidly increase. The beta cells are no longer capable of maintaining the glucose levels within the normal range. The beta cell mass decrease, insulin secretion is not sufficient and insulin resistance is noted. At this point, the diabetes symptoms start to appear (weight loss, polyuria, polydipsia), yet, remain clinically unapparent. The glucose levels have an unstable range and no persistence of symptoms to allow easy diagnosis[13]. Consequently, the stable decompensation phase begins. Insulin levels at this stage are still enough for partial maintenance of glucose levels and to prevent ketoacidosis. Severe de-differentiation of beta cells and a 50 % reduction in beta cell mass along with slow beta cell apoptosis occurs in this stage. Finally, severe decompensation phase marks the last stage. Beta cell apoptosis and complete loss of mass and function are the hallmark of this phase. Patients in this phase depend on exogenous insulin to prevent ketoacidosis[13].

Table 1: Different stages of progression into diabetes [13]

Stage 1	Prediabetic, compensated stage	- Mild increase in glucose levels - Slight increase in insulin secretion - Mild increase in beta cell mass and proliferation
Stage 2	Stable adaptation	- Further mild increase in glucose levels - Altered beta cell function and differentiation - Phenotypic change in beta cells starts
Stage 3	Unstable early decompensation	- Rapid increase in glucose levels - Decrease in beta cell mass - Insufficient insulin secretion - Insulin resistance - Diabetes symptoms start
Stage 4	Stable decompensation	- Insulin levels can maintain glucose levels - No ketoacidosis - Severe de-differentiation of beta cells - 50 % reduction of beta cell mass - Slow beta cell apoptosis
Stage 5	Severe decompensation	- Complete loss of beta cell mass and function - Ketoacidosis - Insulin deficit diabetes

E. Cardiovascular diseases: a complication of diabetes

Cardiovascular diseases (CVDs) are a well-known complication of diabetes. About two-third of people with diabetes die because of CVDs [22]. Cardiovascular complications are considered the major and primary cause of morbidity and mortality in diabetes, where eight out of ten diabetic patients die because of cardiovascular diseases [23]. Diabetic patients have about 2 to 4 times higher cardiovascular mortality than non-

diabetic patients [24]. CVDs is a term that refers to any disorder in the heart or the blood vessels. It encompasses hypertension, arrhythmia, coronary artery disease (CAD), peripheral artery disease (PAD), cardiomyopathies, in addition to other disorders[11].CVD represents 31% of the global deaths[25] .Owing to the close correlation between diabetes and CVD, some Cardiovascular Medicine authorities consider diabetes as a cardiovascular disease[26].

For the longest time, diabetes was thought to be an independent risk factor of CVD. Framingham, Honolulu, and San Antonio Heart prospective studies along with many recent population studies in different countries linked the excess CVD risk in patients with diabetes to multiple interdependent factors [11, 24, 27]. The progression of CV disease and DM is a cumulative degenerative process. Thus controlling CVD lies in controlling diabetes [2].

F. Molecular pathway for developing CVD in response to hyperglycemia

Hyperglycemia activates different pathways leading to micro and macro-vascular damage. This will alter vascular function and structure causing long-term complications. Multiple complex and interconnected pathways activated by hyperglycemia alter the vascular structure through increasing the metabolic factors[28]. These pathways include: (1) increased synthesis of sorbitol, (2) increased synthesis of hexosamine, (3) glycosylation of proteins, (4) synthesis of “advanced glycosylation end” products (AGEs) and (5) oxidative stress[29]. A summary of these pathways is depicted in Figure 1. First, these pathways will alter the structure and extra cellular matrix composition of the microvasculature. AGEs and oxidative stress will commence these structural changes through activation of inflammation causing the increase in

collagen content of extracellular matrix[30] and decrease in capillary density[30]. The latter will cause a reduction and dysregulation in blood flow allowing increase in microvascular changes throughout the whole vasculature[29]. This continuous vascular damage will start to hit the large arteries causing endothelial dysfunction, atherosclerosis and macrovascular complications[31].

The microvascular structural changes are thought to promote the “metabolic memory”. In this context, early glycemic changes produce a long lasting effect on different organs (vessels, heart, retina, …etc.)[28]. At this point, early treatment canpotentially reverse the complications. Meanwhile, prolonged glycemic dysregulation leads to upstream macrovascular structural changes that will set a “point of no return” were structural fixation occurs and reversal of the vascular damage is no longer possible[31].

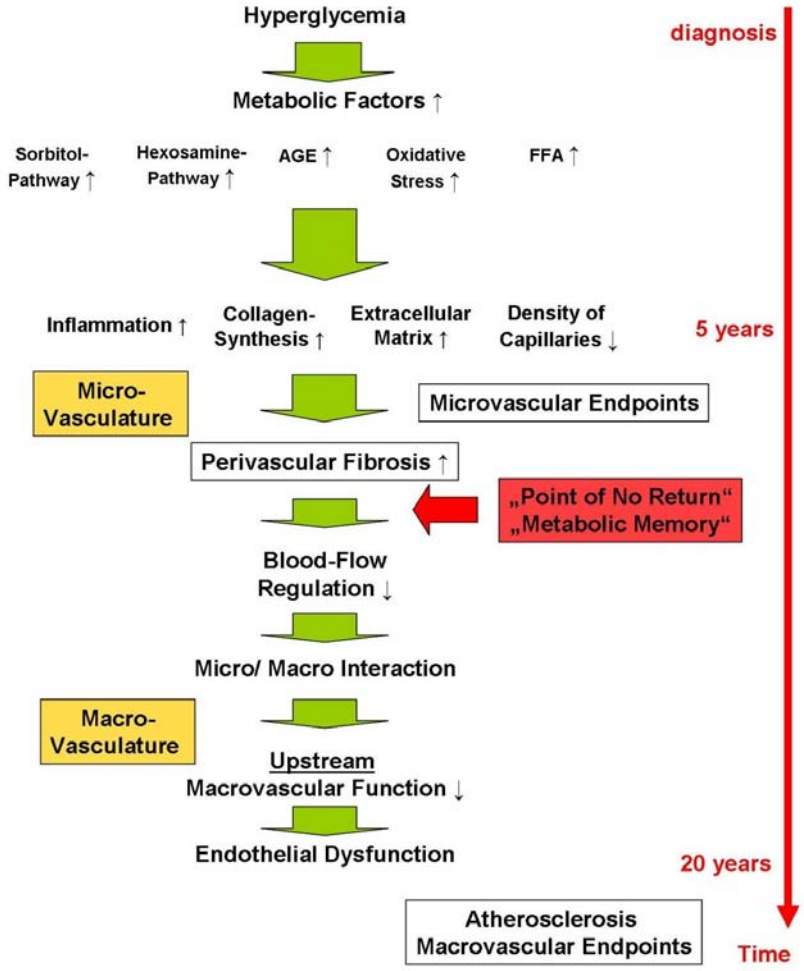


Figure 1: Molecular Pathways for developing complications in diabetes in response to hyperglycemia, adapted from [31]

AGE: advanced glycosylation end products, FFA: free fatty acid.

G. Types of Cardiovascular complications of diabetes

As mentioned previously, the detrimental effect of diabetes impacts small and large vessels leading to micro- and macrovascular complications. Microvascular complications include retinopathy, nephropathy and neuropathy, while macrovascular complications include coronary artery disease (CAD), peripheral artery disease (PAD) and cerebrovascular disease. Retinopathy refers to the damage of the retina of the eye leading to blindness[32]. Nephropathy or chronic kidney disease encompasses damage of the kidney nephrons leading to their functional failure[33, 34]. Neuropathy involves the loss of peripheral nerve function and loss of sensation mainly in lower limbs, this may lead to leg amputations due to poor sensation of pain and diabetic foot infections[35, 36]. Macrovascular complications are driven by atherosclerosis[37].Hyperglycemia and hyperinsulinemia accelerate the development of atherosclerosis [38] thus these disorders are the main cause of mortality in diabetic patients[38].The association of these disorders with diabetes, mortality rate and morbidity in diabetic patients is discussed below.

1. CAD

The risk of developing a myocardial infarction in diabetic patients is high and myocardial ischemia is often asymptomatic in these patients[39, 40].Hyperglycemia has a main role in the early development of CAD by facilitating action of players in progression of atherosclerosis (monocytes and endothelial cells adhesion, proliferation and migration of vascular smooth muscle cells (VSMC), etc)[26].

2. PAD

Diabetes increases the risk of peripheral artery disease[41]. Diabetes aggravates the risk factors for developing PAD, including high blood pressure, abnormal cholesterol levels[42], obesity/overweight[43, 44] and history of previous cardiovascular disorders.

3. Stroke

Diabetes is a well-known cause of stroke especially strokes caused by infarctions or vascular diseases[39]. Diabetic patients have a 3-fold higher mortality risk from stroke compared to non-diabetics[45]. In diabetic patients, ischemic strokes are mainly caused by occlusion of small arteries penetrating the brain[46].Diabetic patients also have a high risk of carotid atherosclerosis andupon a carotid embolus these patients suffer from irreversible brain damage due to severe ischemia compared to non-diabetics[42].

4. Cardiomyopathy

Diabetic patients are at high risk of developing congestive heart failure[26]. The predisposing factors are: severe coronary atherosclerosis, prolonged hypertension, chronic hyperglycemia, microvascular diseases, autonomic neuropathy and glycosylation of myocardial proteins[47-49]. These factors are found to be aggravated by diabetes and controlling the above factors limits diabetic cardiomyopathy[26].

H. Cardiac autonomic neuropathy as an early trigger of diabetic CV complications

1. Definition and players involved

CAN development in diabetic patients remains unclear despite the significance of its negative impact on survival and quality of life[50]. Diabetes, being a metabolic disorder, causes enormous damage of peripheral and autonomic nerves. When damage of autonomic nervous system starts, the cardiovascular, genitourinary, gastrointestinal and neurovascular systems are disrupted[50]. CAN refers to the impairment of autonomic nerve fibers innervating the cardiovascular system (heart and blood vessels)[27]. These nerve fibers regulate heart rate, myocardial contractility, cardiac electrophysiology, and blood vessel constriction and dilatation[51] .This will lead to abnormal control of heart rate and vascular function. Hyperinsulinemia and hyperglycemia are the main pathogenic factors leading to diabetic cardiac autonomic neuropathy. Hyperinsulinemia , the main contributor in CAN, increases cardiac sympathetic activity leading to disruption of sympathovagal balance[52]. Meanwhile, hyperglycemia activates different pathways that contribute to the development of neuropathy like polyol pathways flux, advanced glycosylation end products, oxidative stress and protein kinase C[53]. The increased polyol flux regulated by aldose reductase activation is one of the main risk factors responsible for development of neuropathy. This pathway was found to be increased in peripheral nerve tissue of diabetic mice and high-fat diet fed rats and its inhibition improved diabetic neuropathy [54, 55]. As for advanced glycation end products (AGEs), they have been related to pathogenesis of diabetic neuropathy since they directly injure the nerve tissue's endoneurium causing axonal damage [56, 57]. In addition to these, oxidative stress and free radical production

induce mitochondrial changes and endoplasmic reticulum stress resulting in neuronal apoptosis and later neuropathy [58, 59]. In this context, the pathogenesis of diabetic complications is of great interest and novel pathways are mounting. Hyperglycemia has been shown to induce inflammation and increase inflammatory cytokines which have been detected in diabetic patients and are demonstrated to be connected with neuropathy through complex molecular networks [60, 61]. Different adipocytokines are being linked with neuropathy with particular attention to adiponectin and leptin that were found to affect neuronal function especially that of sensory nerves[62]. Regarding adiponectin, its concentration increases with obesity and weight loss and its function has been suggested to be linked to insulin sensitization and anti-atherogenic effects [63, 64]. Adiponectin was found to be a major marker for diabetic neuropathy[65]. As for leptin, its levels were higher in diabetic patients with parasympathetic neuropathy [66]. The exact role of these adipokines in neuropathy remains to be explored although some adipokines were shown to be helpful in predicting and treating different diabetic complications[67].

2. Cardiovascular manifestation of cardiac autonomic neuropathy

Recently, different studies predict CV risk in different diseases by cardiac autonomic neuropathy (CAN) [68]. CAN represents a main cause of mortality in diabetic patients, where it is associated with high risk of sudden death and cardiac arrhythmia [69, 70] although the exact mechanism remains obscure[71]. A speculation about CAN and sudden death revealed that the increased risk of QT prolongation in these patients can predispose them to ventricular arrhythmia leading to Torsades de Pointes, cardiac arrest and sudden death [71, 72]. Despite its recognizable negative

impact on diabetic patients, CAN remains one of the least understood, recognized and studied complication of diabetes [50]. The prevalence of diabetic CAN ranges from 12.2 to 22.1%, but it can vary between different populations and study designs [73]. Clinical manifestations that accompany CAN include: exercise intolerance, resting tachycardia, orthostatic hypotension, silent (painless) myocardial ischemia, orthostatic tachycardia and increased mortality risk [27]. These signs are very common in diabetes[74].

CAN in patients with type 2 diabetes might be a main contributor to increased mortalities from different CVDs. Recent studies demonstrate this relationship. Studies reported that cardiac autonomic neuropathy can be considered as an independent risk factor for stroke in subjects with type 2 diabetes[75].The DIAD study showed that cardiac autonomic neuropathy is an independent predictor of CHD and CV death in patients with type 2 diabetes[76]. Other studies reported that the recurrence of CVD in diabetic patients is related to the presence of CAN[77].

Cardiac autonomic neuropathy starts early in prediabetic stage and was found to be associated with metabolic syndrome where only slight glycemic changes are present, making it possible for early intervention when reversing the injury is still accessible[50, 51, 70].An early marker of cardiac autonomic neuropathy is impairment of parasympathetic tone and augmentation of sympathetic tone[50]. This imbalance in sympathetic/parasympathetic tone causes abnormalities in heart rate variability (HRV) and baroreflex sensitivity (BRS) making it more prone to arrhythmias[50]. Heart rate variability (HRV) and baroreceptor sensitivity (BRS) analysis are used to diagnose and evaluate CAN. Both HRV and BRS are reduced in CAN [78]. Reduced HRV has been shown to be directly linked to atherosclerosis, myocardial infarction and stroke[70].

3. Baroreceptor reflex sensitivity

a. Physiology of BRS

Baroreceptor reflex is a homeostatic mechanism to stabilize and maintain the arterial blood pressure. Baroreceptors are specialized pressure mechanoreceptors (stretch sensitive) located at the walls of the aortic arch and the carotid sinus [79]. Their function is to sense changes in blood pressure by responding to changes in arterial wall tension[80]. As a result, the cardiac and vascular components adjust their functions to maintain the arterial BP. These receptors are under the control of the sympathetic and parasympathetic neuronal reflexes of autonomic nervous system. They control blood pressure mainly by negative feedback mechanisms[81].

In response to increased blood pressure, the baroreceptors are stretched. The baroreceptor responds by increasing the firing to vasomotor center in the brain. Inhibition of sympathetic tone in the heart and vessels and activation of vagal tone occurs, leading to initiation of appropriate compensatory cardiovascular responses. The SA node firing, heart rate, cardiac contractility, stroke volume, venous return and peripheral vascular resistance decrease to counteract the increase in BP[82]. On the other hand, a sudden decrease in the blood pressure will decrease the firing of the impulses to the vasomotor center resulting in the activation of the sympathetic tone in the heart and the vessels and deactivation of the vagal tone. An increase in heart rate and cardiac contractility will increase the BP along with vasoconstriction[82].

b. Pathophysiologic role of BRS

In healthy individuals, the BRS can be influenced by many factors like: age, gender, baseline BP, obesity and body weight, physical activity, hormonal status and the

sleep cycle. Impairment of baroreceptor function has been linked to different diseases[83]. Baroreceptors appear to have a diminished function in diabetic patients and thus analysis of BRS can serve to diagnose CAN in diabetes [83]. A study that was done to analyze cardiovascular variability and baroreflex in type 2 diabetes mellitus (DM) patients without any manifestation of neuropathy found that vagal and cardiac baroreflex control were impaired in these patients[68]. Upon comparing the response to standing up in these patients, the sympathetic control to vessels and baroreflex response were preserved. All of these suggested that early signs of autonomic dysfunction in diabetic patients can be detected before signs of neuropathy become evident [68].

c. Methods for assessing BRS

Because of its strong prognostic value, various methods were developed for BRS assessment to study the functional integrity of the arterial baroreceptors[50, 84].

i. The vasoactive drug method

The alpha-adrenergic agonist phenylephrine is used for the assessment of BRS. First, an injection of phenylephrine is introduced which causes an increase in systolic blood pressure. Then, a change in the beat-to-beat interval (R-R interval) happens in a reflex mediated response[50, 79, 84]. A linear association between the increase in systolic blood pressure (SBP) and the consecutive increase in R-R interval exists. This association is expressed as the slope of the regression line representing the changes in SBP and R-R interval. The slope represents the BRS. BRS can be assessed using vasodilators like sodium nitroprusside and nitroglycerin[50, 84].

ii. Valsalva maneuver

It is performed by exhaling against closed airway or blowing continuously inside a balloon. Estimation of BRS is done by analyzing the linear regression of SBP and R-R interval changes during the phase of sharp BP increase[84, 85].

iii. Neck chamber method

Performed by applying a negative or positive pneumatic pressure on the neck region. Stimulation or deactivation of the carotid baroreceptors of this region will occur. Analysis of BRS can be performed by obtaining the slope of the regression of beat-to-beat interval vs the pressure applied [84].

iv. Spectral analysis method

Assessment of the BRS is dependent on the relationship between SBP and R-R intervals in different frequency domains. The high frequency power in the R-R interval reflects the parasympathetic function, while the low frequency indicates the sympathetic function[84, 86].

4. Heart rate variability

a. Overview

Cardiac autonomic function can be evaluated by analyzing the variability in heart beats. The interaction between the sympathetic and parasympathetic tones allows the variability in the cardiac beat-to-beat patterns over time[50].Using the time domain and frequency domain (power spectral density), the autonomic modulation of the heart can be quantified[87].The assessment of cardiovascular autonomic function and

dysfunction can be done using a simple and non-invasive technique called HRV analysis. The examination of HRV or R-R interval quantifies the variation in the successive QRS peaks. Different methods for analysis of HRV are present. They include frequency and time domain analysis [88].

Upon analyzing the HRV, detection of the R peaks is done. The distance between 2 consecutive R peaks is measured. Then, a selection of a stable ECG is evaluated. The results obtained are considered as a time series. The computation of first order statistical indices of variability can be done using it. However, the time domain does not allow continuous analysis of the successive heart beats[89]. The frequency domain analysis can be used to process the signal into a continuous wave. Analysis of periodic oscillations of heart rate at different frequencies and amplitudes is done using spectral analysis of HRV by fast Fourier transformation. Any noise or ectopic beats should be removed[90, 91]. Typically, HRV analysis is done on ECG signals. However, the arterial blood pressure can be used when heart beat intervals are not measurable. The systolic blood pressure wave could replace the R wave, where the SBP-SBP interval can replace the R-R intervals[92, 93].

i. Time domain analysis

Time domain analysis is dependent on the determination of successive R-R intervals. It quantifies HRV using mean or standard deviation indices[88]. The mean R-R interval and mean heart rate are considered as simple time domain variables. The standard deviation of normal-to-normal R-R interval, SDNN, represents the overall autonomic control over the entire recording. Removing ectopic beats and artifacts is required to accurately calculate SDNN. The standard deviation of the average R-R

intervals, SDANN, and the square root of the mean squared difference of successive R-R intervals, rMSSD, are other parameters calculated in time domain. The rMSSD represents an index for the variability in beat-to-beat caused by vagal activity[90].

ii. Frequency domain analysis

The change of the signal over time is represented by the time-domain while, the range of frequencies that the signal lies in is presented by the frequency domain. Fast Fourier Transform (FFT) transforms the time series heart rate data into frequency domain[94, 95]. FFT is based on a non-parametric algorithm and it computes the power-spectral density (PSD) of the HRV allowing the examination of the variability in the sinusoidal oscillations. PSD analysis represents how the power is distributed along certain frequencies. The area under the spectral curve represents the heart rate variability, HRV[90].

While analyzing the frequency-domain HRV variables, the heart rate frequency and their relative power is obtained in the beginning. The variability spectrum has peaks that reflect proportionate magnitude of heart rate variabilities occurring at different oscillation frequencies[94]. These peaks are referred as the spectral components of the HRV. The analysis of HRV in human outputs 3 main spectral components: very low frequency (VLF, ≤ 0.04 Hz), low frequency (0.04 - 0.15 Hz), and high frequency (0.15-0.4 Hz). However, different species have different frequency ranges. In rats the spectral components frequency ranges change a bit where the LF (0.25 - 0.75 Hz) and HF (0.75-3 Hz)[90].

Measurement of spectral components (VLF, LF, HF) can be made in absolute values of power (ms^2). HRV spectral analysis has cleared the modulatory effects of

neural feedbacks on the sinus node. HF mirrors the efferent vagal cardiac control. An injection of atropine completely removed the HF power implicating that HF is under control of PNS only[88]. The HF can be considered as a quantitative assessment of respiratory rate[89]. The variation in heart rate is thought to occur from peripheral reflux mechanisms (baroreceptors, chemoreceptors and atrial volume receptors), and partially from central origin[88]. Depending on the anatomy of nervous system and the slow removal of adrenaline, the time response of sympathetic system is longer than the vagal responses, and slower for variability in HF in HRV[96]. To emphasize this idea vagatomy, and blocking parasympathetic nervous system removed the HF variability. Controlled respiration and cold stimulation of the face have increased the variability in HF. Thus, the vagal control contributes in control of HRV under resting conditions[94].

LF is found to reflect sympathetic cardiac control. LF provides compensatory heart oscillations responsible for BP variability mediated by baroreceptors reflex mechanism. LF was also found to be related to Renin-Angiotensin System and thermoregulation. It is highly correlated to the thoracic preganglionic sympathetic outflow[90]. Upon bilateral removal of the stellate ganglion (collection of sympathetic nerves supplying face and arms), LF variability diminishes in dogs. In situations provoking the activation of the sympathetic system (hypotension, standing, moderate exercise, a 90-degree tilt), the LF appeared to increase[94].

b. Pathophysiologic role of HRV

Spectral analysis of HRV is used to understand the autonomic dysfunction and imbalance that happens in certain conditions and diseases. Strong correlation is present between HRV and the prognosis of different diseases. In hypertensive and pre-

hypertensive patients, the autonomic imbalance is due to increased sympathetic activity and reduced vagal tone and this is confirmed by LF/HF increase[89, 90]. In comparison with normotensive patients, hypertensive patients have higher LF and smaller HF suggesting a tilt in HRV. The chronic use of β-adrenergic blockers in HTN normalized the HRV values[97]. Patients with chronic heart failure have autonomic dysfunction and this can be quantified by a reduction in SDNN. SDNN appears to be a predictor of death and high risk of mortality in CHF [98]. In patients with a recent myocardial infarction, HRV is reduced thus reflecting the autonomic dysfunction that occurred post MI[99]. A depressed HRV was noted in sudden death and an increased ratio between low and high frequency ratio was found preceding ventricular tachycardia [97].

Diabetic patients have a decreased respiratory sinus arrhythmia and altered spectral analysis of HRV proposing a reduction in vagal activity[78].Spectral analysis of HRV is an appropriate method to assess the degree of diabetic autonomic neuropathy and detect autonomic abnormalities. LF increases while HF decreases in DAN and this method can be used to detect neuropathy in early stages of diabetes [100, 101]. In a 10 year prospective study done to investigate link between autonomic dysfunction and diabetes, an increased LF and decreased HF (ANS dysfunction) was noted to be connected with the development of diabetes in normal adults [102]. Diabetic patients having an increased LF had an increase in carotid intima artery diameter[103]. This reduction in LF increases the progression of atherosclerosis in these patients thus a link between atherosclerosis, decreased HRV and diabetes was found[83, 103].

Frontoni *et al.*[104] performed a study on off-springs of diabetic patients to test for early autonomic dysfunctions. They considered the off-springs as study subjects who have inherited insulin resistance (prediabetes). The insulin resistant off-spring

appeared to have abnormal blood pressure profile, sympathetic dominance characterized by increased LF:HF ratio and a decreased HF power[104]. Their findings suggested that insulin resistance can be considered as an early pathophysiologic change linked to early autonomic dysfunction [104].

I. Reducing cardiovascular complications in diabetes

Early on, it was believed that the duration of diabetes is the main factor that determines the extent of the complications [105]. However, newer studies showed that the high risk of CVD will start in the prediabetic stage before signs of the disease become evident [15]. Advancing the knowledge of interventions to reverse and limit CVDs in diabetic patients is a major concern of recent studies. Dietary and exercise interventions are recently being studied in reducing CV risk in prediabetic and insulin resistant patients[106]. It has been found that limiting fat and sugar consumption along with exercise is accounted to be beneficial for CVD[107]. Reducing CV risk can be established by minimizing the saturated fat intake and weight loss [108]. Many studies show that abnormal glucose levels are associated with increased risk of CVDs, MI, strokes and mortality[22]. Other studies found that insulin resistance accelerates CVDs, chronic kidney disease, obesity and dyslipidemia[19, 22].

1. Controlling HbA1c

Glycemic control is a well-known factor for reducing the cardiovascular complications in diabetic patients. The main parameter that predicts the likelihood of CVD is glycated hemoglobin (HbA1c)[109]. It has been debated for a long time whether glycemic control is accompanied by a reduction in CV risk or not. HbA1c

helped to provide a more comprehensive and graded risk of CV complications that might occur in diabetic patients. The American Diabetes Association (ADA) sets the target of HbA1c to be <7% but prefers it to be <6% (close to normal)[109]. An observational registry-based study on people with Type 2 DM showed that patients on a tight control of HbA1c and controlled blood pressure (130/80mmHg) have a considerable decrease of CVD, MI, coronary heart disease (CHD) and strokes after a 6-year follow-up when compared to other patients with uncontrolled HbA1c and blood pressure [110]. Another similar observational study also found that patients with HbA1c 6-6.9% have a decreased risk of CHD (20%) and CVD (16%) when compared to patients with HbA1c 7-7.9%. In these observational studies, the link between HbA1c and CV risk in people with Type 2 DM was demonstrated. These prospective clinical trials have assessed this association [22].These values are based on different epidemiological studies that found that each 1% increase in HbA1c is associated with a 15-18% increase in CV risk in diabetic patients. In support of these results, the UKPDS, ACCORD and ADVANCE prospective studies have investigated the effect of intensive glycemic control vs. standard glycemic control. HbA1c was found to be the strongest predictor of CVD risk[22, 111-113].Therefore, diabetic patients should have a controlled proper HbA1c to achieve the best CV outcomes.

2. Protection in prediabetes

Recent studies have shown that deteriorations in CV function in not exclusively limited to uncontrolled HbA1c. Many studies found that high CV risk is present in prediabetic adolescents[114]. A cross-sectional study performed to assess the prevalence of CV comorbidities in pre-diabetic and type 2 diabetic patients showed that

in both groups the comorbidities are statistically the same [115]. Several studies done on prediabetic patients for testing the risk of recurrent CV events and MI showed an increased risk in these patients [15]. Based on these studies and others, interventions to control CV risk in prediabetes, whether done pharmacologically or non-pharmacologically, might prove of significant value[15, 20, 116].

3. Potential role of antidiabetic agents

Current practice interventions towards reducing cardiovascular risk in diabetic patients are linked to optimization of HbA1c[117]. Until recently, the available evidence did not address whether there was a protective value for selection among individual antidiabetic drugs[118]. On the contrary, some reports described an increased risk for negative macrovascular outcomes associated with certain agents[119].The increase in myocardial infarction and heart failure risk was first suggested to be caused by the thiazolidinedione, rosiglitazone [120]. The Food and Drug administration (FDA) designed the TIDE study to compare the cardiovascular risk of thiazolidinediones (rosiglitazone and pioglitazone). Unfortunately, the study was terminated and no conclusive cardiovascular outcome was deduced. Later, the FDA increased the requirement for trials linking cardiovascular outcomes and different antidiabetic agents[117].Evaluation of Cardiovascular Outcome Results trials started afterwards to assess long term cardiovascular outcomes of different drugs. By the end of 2015, the first promising results were reported by the EMPA-REG OUTCOME trial, which assessed the cardiovascular morbidity and mortality outcome in diabetic patients receiving empagliflozin on top of the usual care [121]. As well, results from the LEADER trial examining CV risk in diabetic patients treated with liraglutide were

reported shortly afterwards [122]. Both drugs decreased the macrovascular outcomes and CV morbidity without significant additional improvement in glycemic control. These results indicate a potential preferential effect for certain drugs in affecting diabetic CV complications that is not necessarily related to their effect on glycemic control. Significantly, this idea was reinforced by other findings in 2015 and 2016 where antidiabetic drugs (metformin[106, 123] and pioglitazone[124]) were found to reduce cardiovascular risk in prediabetic patients who did not have blood glucose levels high enough for a diagnosis of diabetes.

J. Modulation of CVD by metformin and pioglitazone

Several antidiabetic drugs were seen to exert different protective roles. Many agents are studied for their cardio-protective role whether alone or combined. The main focus of these studies included vascular reperfusion, modulation of blood pressure, vascular remodeling, myocardial injury, arrhythmia score and mortality rate but very little is known about their link to cardiac autonomic neuropathy. My study is mainly concerned with the early effects of metformin and pioglitazone since they showed promising results in clinical trials studying prediabetic patients. Metformin and pioglitazone were studied for their cardio and neuroprotective roles. Thorough clinical studies have been done to test their effect on modulating CAN as an early event predisposing prediabetic and diabetic patients to CV complications.

1. Metformin

a. Overview

Metformin belongs to the Biguanide family and forms the cornerstone for diabetes therapy. It is the first line prescribed anti-hyperglycemic agent. It controls hyperglycemia in diabetic patients through decreasing hepatic glucose production, increasing uptake and increasing intestinal glucose utilization. As well, metformin is considered as an insulin sensitizer. It does not directly affect pancreatic β cells insulin secretion but has a protective effect on β cells. Metformin also improves insulin-dependent peripheral glucose utilization and caused slight weight loss[125, 126]. Metformin can be used as monotherapy or in combination with other anti-diabetic agents or insulin. Unlike many antidiabetic agents, it doesn't cause hypoglycemia or weight gain[127].

Many pleotropic effects have been found for metformin. They include improving lipid metabolism, an angioprotective effect, beneficial effects on diabetic cardiomyopathy and improved vascular reactivity[126]. Metformin has shown its efficacy in the treatment of PCOS, diabetic nephropathy, gestational diabetes and ameliorated CV risk.

b. CV protective role

Many studies revealed that treatment with metformin reduces the mortality from CVD in diabetic patients [117]. According to the American Diabetes Association, metformin reduces mortality in diabetic patients having heart failure[128, 129]. In the Diabetes Prevention Program, metformin significantly reduces the myocardial infarction events and this effect persisted for a 10 year follow up[106, 130]. Metformin

was also found to have better prognosis than other antidiabetic treatments with diabetic patients having chronic heart failure or acute coronary syndrome [131, 132]. Metformin also reduced ROS overproduction in endothelial cells thus reducing atherogenesis in diabetic patients [133, 134]. In addition to this, metformin activates endothelial NO synthetase and restores NO-dependent vasodilation in diabetic patient [135]. Metformin was also found to inhibit cardiac hypertrophy, fibrosis and cardiac myopathy in obese diabetic rats [127]. Metformin treatment in prediabetes provided positive effect in preventing of diabetes and CV risk reduction [133].

c. Diabetic autonomic modulatory effect

Regarding neuropathy, metformin was effective agent for attenuating the abnormal painful sensation in diabetic neuropathy [136]. Concerning the neuroprotective role, metformin prevents the loss of tactile function and protects the peripheral nerve endings in chemotherapy induced peripheral neuropathy [137]. An effective neuroprotective role for metformin was also noted against apoptosis of cortical neurons [138]. Metformin was found to have an anti-nociceptive effect in post-surgical pain through AMPK activation [139]. It is found to reduce neuronal apoptosis induced by status epilepticus [140] and shorten the duration of epileptic tonic-clonic seizures [141]. Metformin mediates the hippocampal neurogenesis in adults [142] and protects ischemia damages on hippocampus [143]. All the potential neuroprotective roles of metformin need further clarification since the ability to permeate the blood brain barrier has not been studied and needs to be investigated [138].

Little information was found to link the potential role of metformin on modulation CV risk through adjusting CAN (HRV and BRS). In a study performed by

Manzella et al, metformin improved the cardiac sympathovagal balance in obese type 2 diabetic patients by a direct effect on cardiac autonomic neuropathy. Metformin modulated HRV parameters, it increased R-R interval, total power and HF and reduced LF and LF/HF ratio suggesting a potential role for metformin on CAN [144]. In another study, 4 months of metformin treatment and diet in type 2 diabetic obese patients increased the sympathovagal balance and increased HRV parameters [83].

d. Molecular mechanism

On the molecular level, the exact mechanism of action remains obscure. The most studied mechanism is the activation of AMPK kinase. Metformin reduces cellular respiration by inhibiting mitochondrial oxidative phosphorylation. This inhibition drops the cellular energy charge (ATP). The drop in the energetic state of the cell activates AMPK kinase that is responsible for cellular energy homeostasis[127, 145]. This is accounted for many of its effects. In the hepatocytes, the activation of AMPK reduces the activity of acetyl-coenzyme A carboxylase and the expression of sterol regulatory element binding protein-1. This will result in increased fatty acid oxidation, reduced VLDL synthesis and improved hepatic insulin sensitivity[145]. AMPK activation is vital for metformin induced inhibition of hepatic glucose production, gluconeogenesis. In the periphery (skeletal muscles), metformin stimulates the glucose uptake in an AMPK-dependent mechanism[126]. Metformin produces these actions when it is taken in the therapeutic doses of 500, 750, 1000, 1500, 2000 g/day[127].

2. Pioglitazone

a. Overview and molecular mechanism

Pioglitazone is a thiazolidinedione that has an antidiabetic action[146]. It is a synthetic ligand of peroxisome proliferation (PPAR) nuclear receptors. PPARs are ligand activated transcription factors of the nuclear hormone receptors. They have a lipid and glucose homeostatic role[147]. Pioglitazone alters the transcription of genes responsible for carbohydrate and lipid metabolism leading to a change in the amounts of protein synthesis. Glycemic control in patients with type 2 diabetes is improved by pioglitazone due to improvements in insulin sensitivity and abnormalities in lipid metabolism. The activation of PPARγ 1 &2 will increase glucose transporter -1 and -4, enhance insulin signaling and reduce remodeling of adipose tissue. All of these increase glucose uptake and utilization in the periphery, decrease hepatic gluconeogenesis, decrease triglycerides thus reducing insulin resistance. Pioglitazone stimulates the differentiation of adipocytes leading to the generation of small insulin sensitive adipocytes[146, 148].

b. CV protective role

In a prospective study that was done on pioglitazone, it appeared to have an anti-inflammatory and anti-atherogenic effect which is independent of blood glucose control and dependent on PPAR γ activation. It also alters fat distribution and reduces LDL thus delays the progression of atherosclerosis and reduces overall CV risk[149]. The IRIS trial found that patients with insulin resistance receiving pioglitazone had a reduced risk of stroke or myocardial infarction[150]. In acute MI events in diabetic rats, pioglitazone reduces the infarct size, improves ECG findings, down regulates apoptosis

of cardiac tissue and improves the histopathological picture of the heart[151]. The endothelial function is also improved by pioglitazone, which also attenuates the progression of carotid intima media thickness [152]. As well, pioglitazone appears to have blood pressure lowering effect in hypertensive obese animal models[153].

In prediabetes, oxidative stress is suggested to be related to hyperinsulinemia or insulin resistance. Myocardial collagen accumulation, tissue damage and cardiac fibrosis occur as a response to increased reactive oxygen species. This explains the deteriorations in cardiac hemodynamics and diastolic dysfunction. The direct anti-oxidant of pioglitazone is related to its role on insulin resistance[154]. The decrease in oxidative stress reduces TGF-β, collagen accumulation, and prevents proliferation of vascular smooth muscle cells. All of these improve distensibility of aortic wall, reduce its stiffness, reduce further morphological changes and improve left ventricular diastolic function in prediabetic rat hearts[154, 155].

c. Diabetic autonomic modulatory effect

Besides the antioxidant effect, the anti-inflammatory effect of pioglitazone accounted for its neuroprotective and cardioprotective effect[156]. Pioglitazone's neuroprotective effect is both PPAR dependent and independent[154]. PPAR activation alleviates inflammation accompanied with acute and chronic neurological injuries. In acute neurological conditions like stroke, spinal cord injury and traumatic brain injury massive inflammation is responsible for the detrimental role. Pioglitazone induces the significant neuroprotective role by prevention of inflammatory cytokines and chemokines expression and promoting anti-oxidant mechanisms in the area injured[156]. In Parkinson disease, pioglitazone protected the dopaminergic neurons

and reduced Parkinson symptoms[157]. In multiple sclerosis, pioglitazone improved the neurological function[158]. Also, administration of pioglitazone in Amyotrophic lateral sclerosis (ALS) improved the neurological outcome and delayed the expression of ALS[156, 159].

In diabetic patients, pioglitazone ameliorates cardiac autonomic neuropathy and improved BRS thus reducing CV risk [83]. However, in a study done by Nerla *et al*., pioglitazone didn't show a significant effect on CAN in diabetic patients and no difference appeared in HRV analysis (only slight increase in LF/HF ratio in patients receiving pioglitazone)[160]. In another study done by Gianiorie *et al.*, pioglitazone improved the cardiac sympathovagal balance in diabetes, where it improved HRV in the orthostatic challenge[161]. Not enough information is present to tie the effect of pioglitazone on CAN and CV risk although in a study done on diabetic patients with recent MI, pioglitazone reduced sympathetic activity however they linked it to reduced insulin resistance [83].

Interestingly, pioglitazone was found to exert its protective role in low sub-therapeutic doses suggesting that its effect might not be mediated by controlling blood glucose or blood insulin levels[160].

K. Animal model used to study diabetes

In diabetes research, different animal models have been used to study diabetes and its comorbidities. The earliest model used to study the homeostatic role of pancreas in maintaining glucose levels was pancreactomised dogs. Lately due to legal and ethical restrictions, rodents are used to carry out most experiments. Diabetes can be induced by

toxin-mediated pancreatic damage, dietary interventions or by specific gene defects[162, 163]. Some of the rodent models of diabetes are summarized in Table 2.

Table 2: Different animal models of diabetes

Genetically induced spontaneous models	Experimentally induced nonspontaneous models
OB/OB mouse (monogenic model of obesity, leptin deficit) db/db mouse (monogenic model of obesity, leptin deficit) Zucker fa/fa rat (monogenic model of obesity, leptin resistant) GotoKakizaki GK rats (polygenic model) OLETF rat Diabetic torri rats NSY mouse KK mouse Israeli sand rat	Streptozotocin-treated rat High fat fed rats

All of the models mentioned in table 2 provided different presentations of diabetes and its complications. Ob/Ob, db/db and fa/fa models do not allow the study of diabetes in absence of interfering factors like obesity [164]. Also, they are monogenic genetic models of type 2 diabetes which make them a poor candidate for mimicking the human disease. The GK rats are polygenic models of type 2 diabetes thus, they are good candidates for studying the human disease[165]. However, due to long term inbreeding (>20 years) , these rats ended up having continuous stable levels of glucose intolerance

[166]and impaired insulin secretion following glucose uptake[167], which made them inappropriate for studying early diabetes progression. Furthermore, GK rats are born with a reduced mass of beta cells and impaired insulin secretion [166], hence no proper prediabetic study can be done. OLETF rats inherit diabetes mellitus though multiple diabetogenic recessive genes responsible for its induction. These rats show mild obesity, hyperglycemia and pancreatic beta cell structural changes[168]. Streptozotocin-diabetic rats are toxin mediated models of diabetes mellitus. Stable signs of diabetes are provided by this model (hyperglycemia, stable body weight, and increased food and water intake) but, no proper presentation of chronic diabetic complications as humans is provided by this model[169]. Diabetic torri rats are non-obese diabetic rat model. These rats have severe hyperglycemia and pancreatic changes to which result in severe diabetic complications[170]. On the other hand, the KK mouse inherits glucose intolerance and insulin resistance, they happen to be good models of obesity-associated diabetes[171]. NSY strain spontaneously develops diabetes mellitus in absence of obesity and severe hyperinsulinemia but in presence of marked impairment in glucose stimulated insulin secretion[172].

Our current interest in studying the potential early cardiovascular changes before any signs of metabolic derangement become manifest restricted our choice for an animal model. The model we are interested in is one that allows a reasonable window for study before and signs of insulin resistance, impaired insulin secretion and hyperglycemia appear without interference of other factors such as obesity and overt lipid derangement. This model should also facilitate early cardiovascular functional, structural and molecular study. We thus opted to use a mild hypercaloric intake rat model.

L. Mild hyper caloric model

The mild hypercaloric model (MHC), model adopted in this study, is a modified form of the high fat diet (HFD). The HFD fed animals are considered models for impaired glucose tolerance and metabolic syndrome. HFD model allows the discovery of molecular mechanisms underlying the early defects in diabetes unlike other animal models[173]. Surwit *et al*., were the first to introduce the model in 1988. This model was accompanied by insulin resistance and insufficient islets compensation[174]. It is a valid model to study the pathophysiology of type 2 diabetes and impaired glucose tolerance[173]. Increased fat intake (20-60 % energy as fat) increases body weight gain, causes hyperglycemia and a continuous increasing hyperinsulinemia[175]. In the present study, selection of a dietary composition simulating that taken up by humans consuming typical Western diet rich in refined sugars and saturated fat was essential. Fructose is particularly enriched in prepared foods, corn syrup additives, and carbonated beverages[176, 177]. The high fat, high fructose fed rat model is widely used in research involving vascular and metabolic dysfunction [178-185]. A recent comprehensive study[183] showed that, in contrast to HFD only, a combination of high fat and high fructose feeding on long term (8 months) led to the development of a more robust type 2 diabetes phenotype with maintenance of both fasting and post-prandial hyperglycemia (fasting hyperglycemia was not maintained in high fat only fed rats) and higher insulin resistance. Since, the American Diabetes Association recommends the ideal daily total fat intake to range between 20-35% of energy to manage diabetes and CV complications in adult diabetic patients [186], the mild hypercaloric model (38% energy as fat) was developed to be more reflective of the human dietary composition. It is slightly higher than the recommended

normal values of ADA and within the low range of HFD composition in the literature allowing minimization of weight gain, and prolongation of the time window prior to the development of overt markers of diabetes and/or prediabetes. The body weight gain was not significant in this model and was the same as normal fed rats, thus, the interference of obesity was removed. The stable hyperglycemia, impaired glucose tolerance and hyperinsulinemia were also removed and MHC rats responded to glucose intake the same way normal rats did after 12 weeks of the regimen. The MHC rat model develops stable fasting hyperglycemia at 16 weeks of feeding allowing proper and normal development of the debilitating mechanisms responsible for the complications. Details of the dietary composition and biometric and biochemical characterization of these rats will be provided in the methods and results sections.

CHAPTER II

AIMS OF THE STUDY

In diabetic patients, cardiac structural and functional deterioration were thought to commence after the development of diabetes. However, as mentioned earlier, recent evidence indicates that both could occur prior to signs of hyperglycemia becoming evident. Meanwhile, the potential mechanisms leading to CAN before diabetes develops have not been thoroughly studied and understood. Our work attempts to investigate the link between cardiac autonomic neuropathy and cardiovascular structural and functional deterioration prior to clinically detectable diabetes. Our specific aims are:

1. Examining whether CAN develops as an early complication of hypercaloric intake in the course of metabolic disease.
2. Examining the early structural and functional cardiac alterations in the course of development of diabetes.
3. Examining the role of metformin and pioglitazone in counteracting cardiac autonomic neuropathy associated with hypercaloric intake.
4. Addressing the association between increased calorie intake and inflammatory process leading to cardiac autonomic neuropathy.

CHAPTER III

MATERIALS AND METHODS

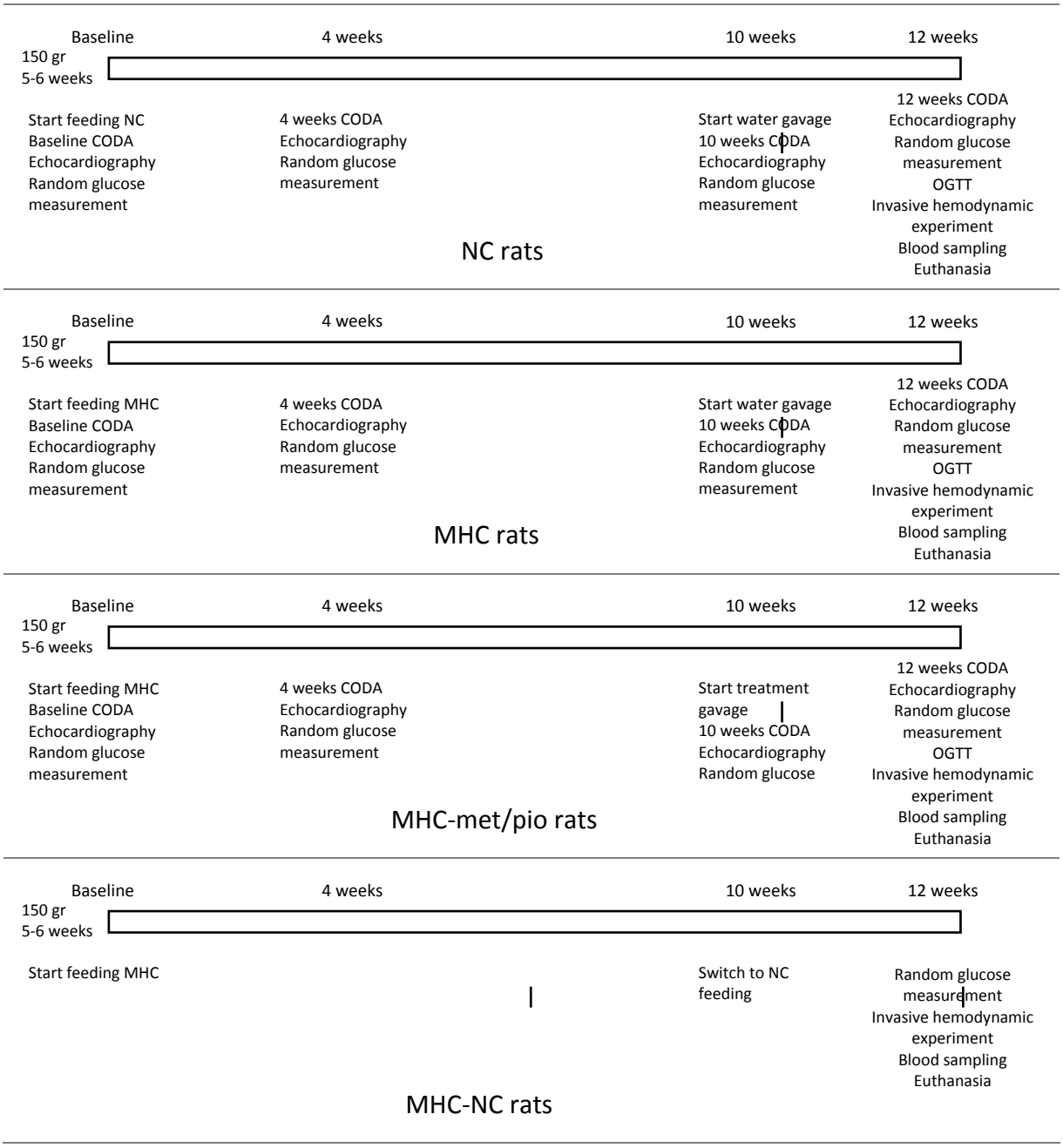
A. Ethical approval

All animal experiments were done according to study protocol number 16-10-386 approved by AUB Institutional Animal Care and Use Committee (IACUC) in compliance with the Guide for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research of the National Academy of Sciences, U.S.A.

B. Experimental design

Male Sprague-Dawley rats (5-6 weeks of age; 150 g) were divided into six groups randomly at the beginning of the study. Groups (each consisting of 8 rats) were divided as follow: (1) rats fed normal chow diet (Control, NC, 3Kcal/g), (2) rats fed with mild hyper-caloric diet (MHC, 4.035 Kcal/g), (3) rats fed with mild hyper caloric diet and treated with 100 mg/kg metformin at week 10 (MHC-met), (4) rats fed with mild hyper caloric diet and treated with 2.5 mg/kg pioglitazone at week 10 (MHC-pio), (5) rats fed MHC for 10 weeks then switched to normal chow for the remaining 2 weeks (MHC-NC) , (6) rats fed MHC *ad libitum* for 10 weeks then switched to pair feeding with MHC to match calorie intake of control rats on normal chow (MHC-LC). Other than rats in group 6, all rats had free access to food and water for the full 12-week duration. Rats were kept in a temperature- and humidity-controlled room, in a 12-hour light/dark cycle. Body weight was measured weekly along with the anus-to-nose length (ANL), and calorie intake was calculated daily based on the amount of food consumed. At week 10, oral gavage was performed for the different groups to administer the

treatment regimen. The control groups received water gavage for 2 weeks while the other two groups received freshly prepared suspensions of the antidiabetic agents. The experimental design timeline is depicted in Figure 2.



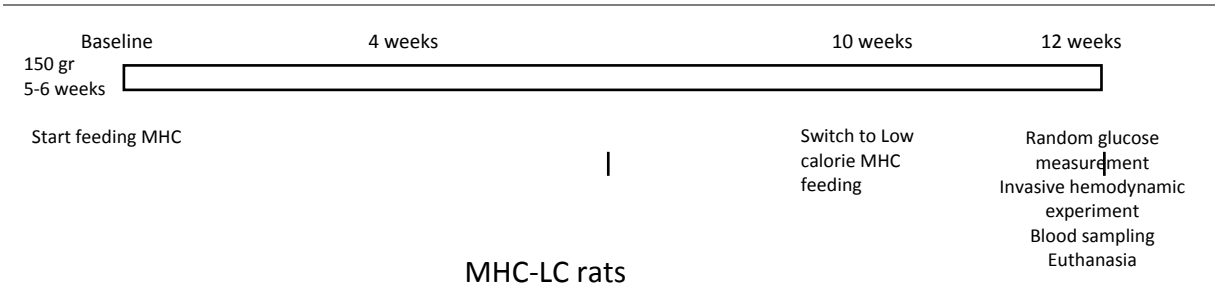


Figure 2: Experimental design timeline. NC: normal chow, MHC: mild hypercaloric, LC: low calorie, met: metformin, pio: pioglitazone, OGTT: oral glucose tolerance test

C. Food preparation and macronutrient composition

Normal chow diet (ENVIGO) was obtained from Teklad Rodent Diets (Madison, WI). This diet offers 3 Kcal/g distributed as follows: 32% from protein, 14% from fat (0.9% saturated fat by weight), and 54% from carbohydrates. The MHC diet was prepared in house through the addition of food grade fructose (20% by weight, Santiveri Foods, Spain) and hydrogenated vegetable oil (Mazola®, 15% by weight, BFSA). Major electrolytes were supplemented to match the concentration in ENVIGO diet. The final composition of the MHC diet by weight (calorie content) is: 18.06 % fat (38.68%,5% saturated fat by weight), 15.8% protein (15.66%), and 46.13% carbohydrates (45.73%). All diets were fed for 12 weeks.

D. Regular gross examination, blood tests, sacrifice, and organ harvest

Non-invasive blood pressure measurement (CODA), echocardiography and random glucose measurement were performed at baseline, 4, 10 and 12 weeks. At week 12, a day before sacrifice, oral glucose tolerance test (OGTT) was performed. The next day, the rats were anesthetized by 100 mg/kg/5ml phenobarbital and the hemodynamic study was performed as described below. At the end, the rats were decapitated and blood was collected. The head was placed in ice and different brain regions were

isolated. The thoracic cavity was exposed and the heart was flushed and isolated. The atria were removed and weighed, while the ventricles were horizontally cut into 3 sections (apex, mid-section, base) and weighed. The mid-section was placed in formaldehyde for further histological analysis. Other tissues isolated were stored at – 80 °C. The lungs were weighed wet and dry for estimation of water retention. The tibia bone length was measured for normalization purposes.

E. Non-invasive blood pressure measurement (CODA)

Rat blood pressure was measured non-invasively by tail cuff using CODA High Throughput Monitor (Kent Scientific, Torrington, CT). This method uses specialized volume pressure recording (VPR) sensor to measure blood pressure changes that happen in the rat tail[187]. Measurement was performed at weeks 0,4,10 and 12 on rats sedated using a 60% ketamine/xylazine mixture (AUB-MC Pharmacy). Rats were placed on a heated pad, tail cuff and VPR sensor were passed through the tail. 15 cycles were performed. Any irregular or unacceptable recording noted as false recording by the system were excluded. The blood pressure parameters obtained are: systolic, diastolic, and mean blood pressure, heart rate, tail blood volume, and blood flow.

F. Echocardiography

Echocardiography is a method for investigating cardiac morphological and functional changes. Parasternal long axis M-mode and B-mode were used. The M-mode allows monitoring of heart function while B-mode allows visualization of heart morphology[188]. It is performed at week 0,4,10 and 12. Rats were sedated using 60% ketamine/xylazine. Rats were placed on heating panel and echocardiography was done

using Sonix Touch Q+ ultrasound (BK ultrasound, Peabody, MA). The parameters calculated were: left ventricular diastole diameter (LVDd), left ventricular posterior wall diameter (LVpwd), left ventricle mass (LV mass), ejection volume (EV), and ejection fraction (EF).

G. Random Blood glucose measurement

Random glucose testing was performed at weeks 0, 4, 10, and 12. The lateral tail vein was pricked and an enough quantity of blood was obtained. The measurement was done using Accu-check Performa glucometer (Roche Diagnostics, Basel, Switzerland).

H. Oral glucose tolerance test

At the end of week 12, rats were fasted overnight. Rats were challenged using a 2g/kg, 20% glucose solution administered by oral gavage. They were gently restrained and blood glucose was measured at 0,15, 30, 60, and 120 minutes after glucose load. Blood was collected from a tail vein prick and glucose was measured using Accu-check Performa glucometer.

I. Measurement of Hemodynamic Parameters under anesthesia:

The hemodynamic study was performed at week 12. Rats were anesthetized using 100mg/kg phenobarbital (AUB-MC pharmacy). Tracheostomy was performed and right carotid artery was isolated, cannulated and connected to a Millar transducer to measure mean arterial pressure (MAP) and heart rate (HR). After this, the left jugular vein was isolated, cannulated and connected to a shunt to deliver drugs. After the

surgery was done, the recording was allowed to stabilize for 30 minutes. Increasing doses of Phenylephrine were administered (0.25,0.5,0.75, 1, and 2 μg) and the change in MAP and HR were recorded. The rat was allowed to rest for 30 minutes and increasing doses of Sodium Nitroprusside were administered (1, 2, 4, and 8 μg). The changes in MAP and HR in response to SNP were recorded.

J. Baroreceptor sensitivity

The vasoactive method was used to assess BRS. It was determined using modified Oxford method, where the relationship between cardiac autonomic control and mean arterial pressure during different vasoactive drug dose administration was calculated[189]. Slope of the linear regression fit between Δ HR and Δ MAP was used to validate the functionality of baroreceptors. The steeper the slope, the more functional the baroreceptors are.

K. Heart rate variability analysis

Using Lab Chart Pro 8 (AD Instruments Ltd, Dunedin, New Zealand), a derivative curve was obtained from the arterial pressure recording. Each peak in the resultant tracing was considered a systole. The R-R interval was represented by the distance between two consecutive peaks. Computations related to HRV and power spectral analysis were done by the HRV module of the software using the default settings for rat (pulse duration, 100-200 ms; LF, 0.25 - 0.75 Hz; and HF, 0.75-3 Hz). Time and frequency domain analyses were performed.

The time domain indices used were the average R-R interval of the accepted beats (ectopic beats were removed), SDNN, and rMSSD. In the frequency domain

analysis, only R-R intervals of acceptable normal heart beats were analyzed. The software performs power spectral analysis using autoregressive model and it automatically calculates the coefficients that define PSD.

L. Immunohistochemistry

The heart mid sections placed in 10% formalin were fixed in 100% ethanol, embedded in paraffin, cut transversely, and placed on clean slides. The staining was performed simultaneously for accurate immunohistochemistry comparison. For demonstration of nucleus and cytoplasmic inclusions Hematoxylin and Eosin staining is used, while for detection of cardiac fibrosis Masson trichrome was performed.

M. TUNEL analysis

Apoptotic myocytes were evaluated using Terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL) assay on paraffin embedded heart sections using a TUNEL assay kit (abcam, Bristol, UK). The assay was performed on four randomly selected tissue sections from each group. Sections were exposed to 100 µL Proteinase K solution for 20 minutes, then rinsed with TBS, followed by incubation with100 µL of 3% H202 for 5 minutes. Following this, the sections were rinsed with TBS and incubated with 100 µL TdT Equilibration buffer for 30 minutes. After this, incubation at 37°C for 90 minutes with 40 µL of TdT labeling reaction after covering the slide. The coverslip was removed and rinsed with TBS for 5 minutes. The cover slide was placed again, 100 µL of Stop buffer was added and incubated at room temperate for 5 minutes. Then, it was washed with TBS and incubation with 100 µL Blocking buffer at room temperature for 10 minutes was done. The Blocking buffer was

then carefully blotted and 100 µL of 1X Conjugate was immediately applied and allowed to incubate for 30 minutes. The slide was rinsed again with TBS and 100 µL of DAB solution was added and incubated for 15 minutes. Afterwards, the slide was rinsed with distilled water and 100 µL Methyl green were added for 3 minutes. In the end, dehydrate the slides by repeated immersion in 100% ethanol followed by xylene before adding a glass coverslip. Four fields/section were examined microscopically[190].

N. TGF-β immunostaining

For detection of TGF-β, the slides were deparaffinised followed by extensive washing in 70% ethanol and then placed in hydrogen peroxide/methanol to block endogenous peroxidase. Sections were incubated with the primary antibody (rabbit anti-TGFβ,1:100, abcam) then mixed with the blocking cocktail (2% normal goat serum, 0.1% Triton x-100, and 3% bovine serum albumin)and incubated overnight at 4 °C. The next day, sections were washed with TBS twice. Novolink polymer was added for 3 minutes, then washed with TBS twice. The sections were incubated with 35 µl DAB solution for 10 minutes and then rinsed with water. In the end, the sections were stained with one drop of hematoxylin before examination under the microscope [191].

O. Statistical analysis

Data were expressed as Mean ± SEM. All analysis was done using PRISM software. Comparisons between groups were done using One Way ANOVA followed by Dunnet *post-hoc* test. p value< 0.05 was considered statistically significant.

P. Chemicals

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Pharmaceutical grade metformin HCl, phenylephrine, and pioglitazone were obtained as kind gifts from regional pharmaceutical manufacturers (Merck, PharoPharma, and Hikma Pharmaceuticals).

CHAPTER IV

RESULTS

A. Calorie intake

On a daily basis, MHC group showed an increased energy intake of ~14 Kcal. ($P < 0.05$ on most days, Figure 3). Initially, calorie intake gradually increased with the progress of treatment duration. After three weeks of feeding, energy intake plateaued for both groups. For the remainder of experimental period, the MHC group consumed ~100 Kcal/24 hours, while the NC group intake continued at ~86 Kcal/24 hours.

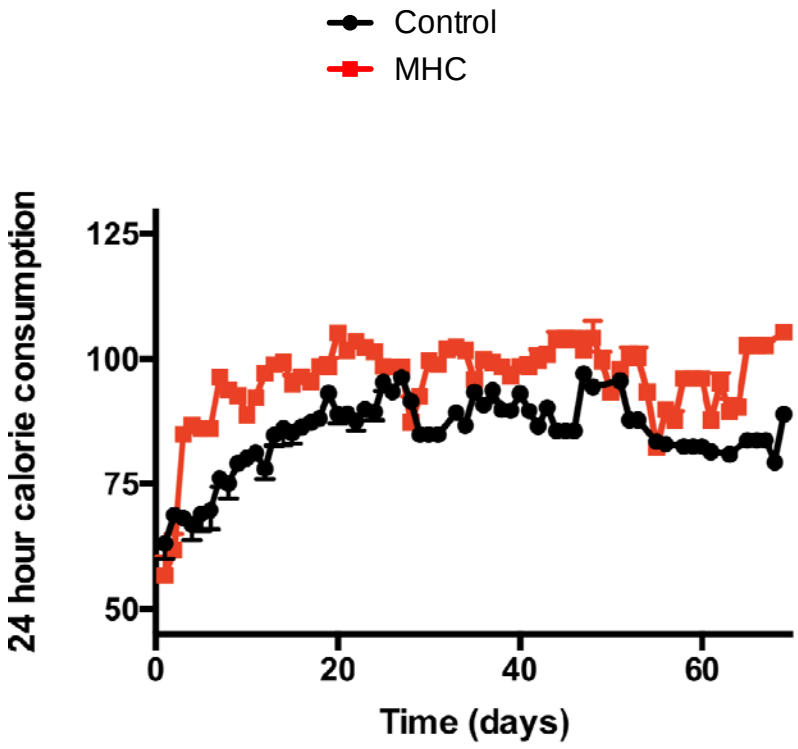


Figure 3: Calorie intake over 24-hour through 12 weeks. Values are Mean ± S.E.M. for 13 observations for control rats and 24 observations for MHC rats. Statistical significance was tested using ANOVA followed by Tukey Kramer *post hoc* multiple comparison. *P* was found to be < 0.05 for most treatment days.

B. Weight variation

Initially, rats from all groups started with approximately the same weight (172.7g ± 4.94). Rats from different groups continued to gain weight similarly throughout the experiment (Figure 4A). The BMI was calculated using ANL, and it showed no significant difference between different groups (Figure 4B). The weight change within each group in the last 2 weeks during the treatment was determined. Only rats receiving pioglitazone treatment showed significant increase in body weight when compared to NC and MHC. Meanwhile, rats that received metformin had no change in their weight during the treatment period when compared to NC and MHC (Figure 4C).

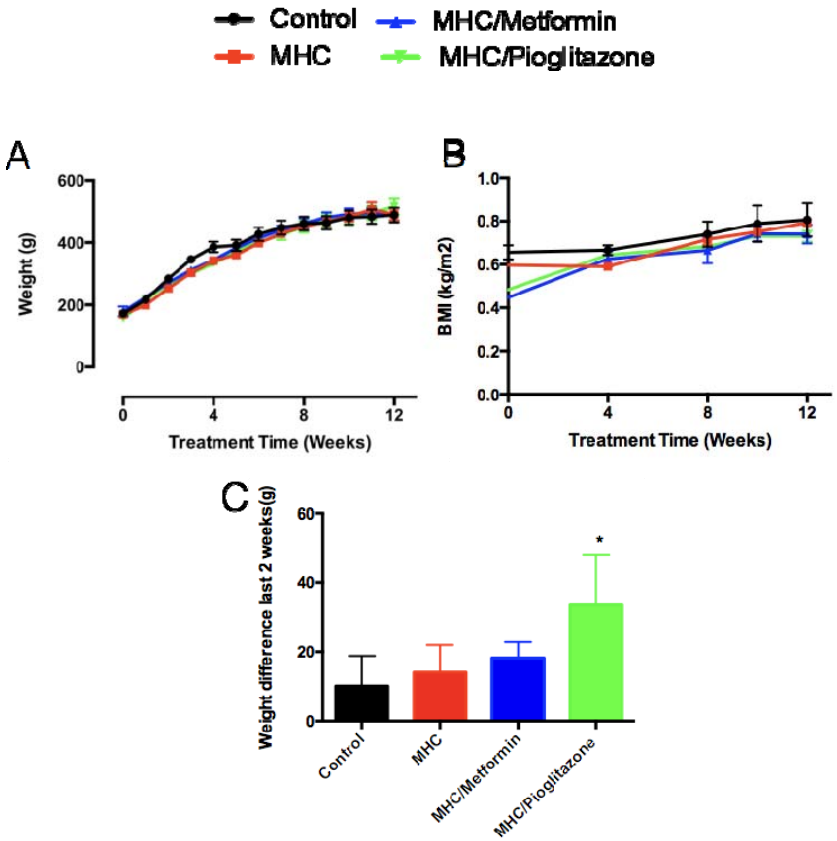


Figure 4: Changes in weight (panel A), BMI (panel B) over the 12 weeks treatment period, and weight variation in the last 2 weeks (panel C) of rats in different treatment groups. Values are Mean ± S.E.M. of eight rats. *denotes P<0.05 vs. control as estimated by ANOVA followed by Dunnett's post hoc comparison**.**

C. Random blood glucose and oral glucose tolerance test

MHC feeding did not induce any changes in random blood glucose levels through the first 10 weeks (Figure 5A). After treatment with metformin or pioglitazone no significant change in random blood glucose was noticed between the four groups in week 12 (Figure 5B). In the end of week 12, when treatment with metformin or pioglitazone ended, oral glucose tolerance test was performed. Comparing MHC and NC, no significance in OGTT was found between the two diet groups. Furthermore, neither metformin nor pioglitazone had a significant effect on OGTT (Figure 5C).

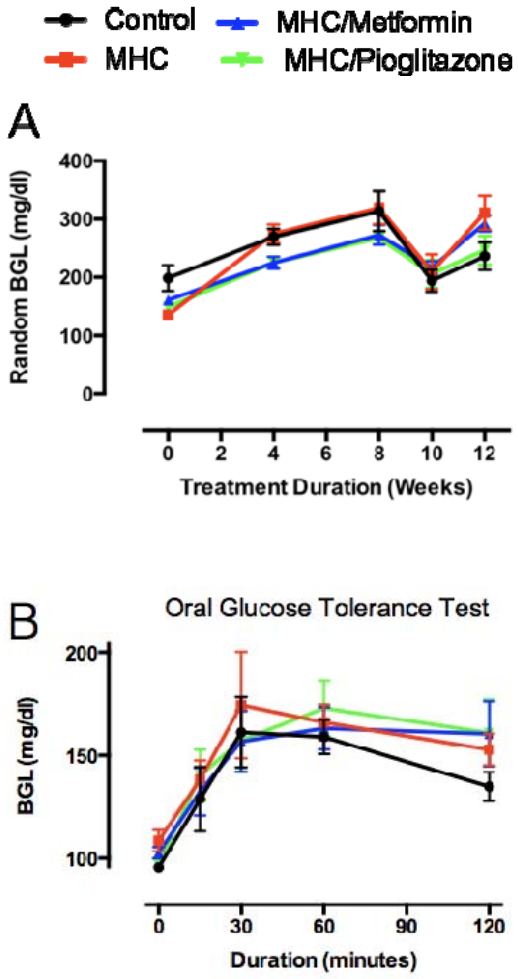


Figure 5: Random blood glucose measurement at weeks 0,8,10 and 12 (panel A) and oral glucose tolerance test at the end of week 12 (panel B) for rats from different treatment groups. Values are Mean ± S.E.M.

D. Non-invasive blood pressure measurement

Chronic feeding of MHC did not induce any changes in blood pressure throughout the experiment. Figure 6A shows the changes in SBP and Figure 6B shows the changes in MAP measured by tail cuff technique. No significant differences in SBP or MAP were found between the two diet groups (NC and MHC) from the beginning of the experiment and till week 12. In addition to this, rats receiving metformin or pioglitazone showed no significant difference in their SBP or MAP in the last two weeks. No significant difference was detected between Heart rate (HR) values among the four groups (Figure 6C).

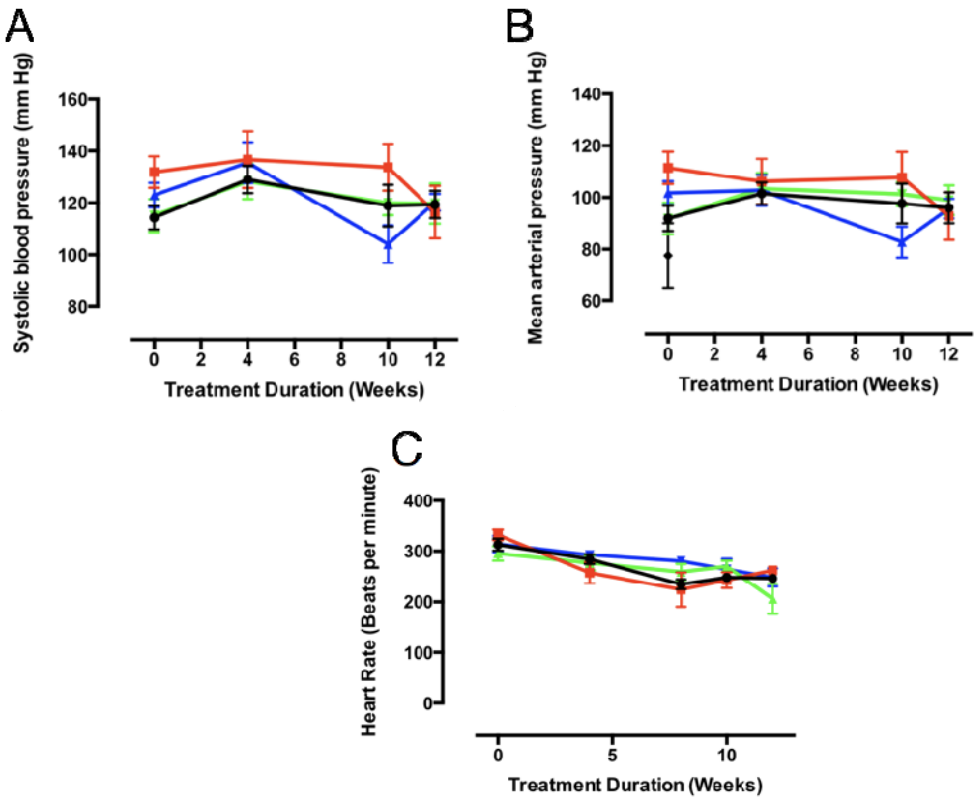


Figure 6: Non-invasive systolic blood (SBP, panel A), mean arterial pressure (MAP, panel B) and heart rate (HR, panel C) measurement at weeks 0,4,10 and 12 of treatment in different groups. Values are Mean ± S.E.M of eight observations.

E. Echocardiography

The echocardiography parameters calculated were left ventricular diastole diameter (LVDd), left ventricular posterior wall diameter (LVPWd), end diastolic volume (EDV), left ventricular mass (LV mass),and ejection fraction (EF). These parameters reflect the morphological (LVDd, LVPWd, and LV mass) and functional (EDV and EF) cardiac properties. Among the different groups, no significant changes in echocardiography parameters were noticed (Figure 7). However, these parameters showed an apparent rapid decrease in all groups in the early treatment phase. This is caused by the normalization of the parameters against the rat's body weight, where the rate of increase in the parameters measured was much less than the rate of increase in body weight. This becomes clear when these parameters are presented in absolute form (Figure 8).

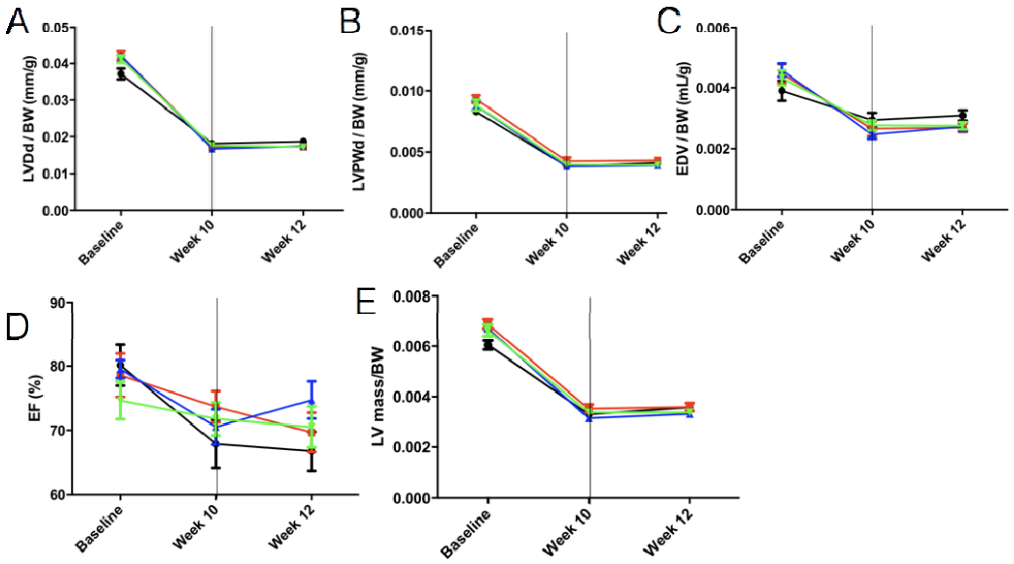


Figure 7: Normalized echocardiography parameters: left ventricular diastolic diameter (LVDd, panel A), left ventricular posterior wall diameter (LVPWd, panel B), end diastolic volume (EDV, panel C), ejection fraction (EF%, panel D) and left ventricular mass (LV mass, panel D) measured at weeks 0,10 and 12 for different treatment groups. Values are Mean ± S.E.M of eight observations.

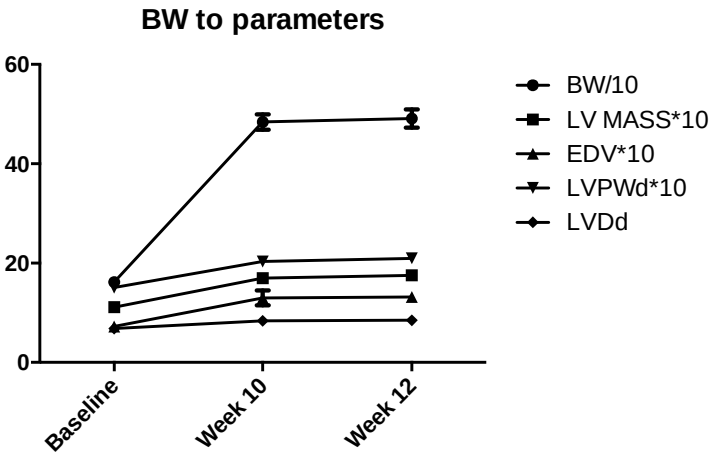


Figure 8: Absolute representation of echocardiography parameters and BMI variation. Values are Mean ± S.E.M of eight observations.

F. Water retention in lungs:

After sacrifice, lungs were collected and the wet weight was determined. Afterwards, lungs were allowed to dry for 24 hours in the oven and the dry weight was determined. The difference between wet weight and dry weight is considered as a marker for water retention. Upon comparison, no significant difference was present between NC and MHC. The treatment of MHC rats with metformin or pioglitazone showed no significant changes. No significant difference between the different diets (NC and MHC) was noticed (Figure 9).

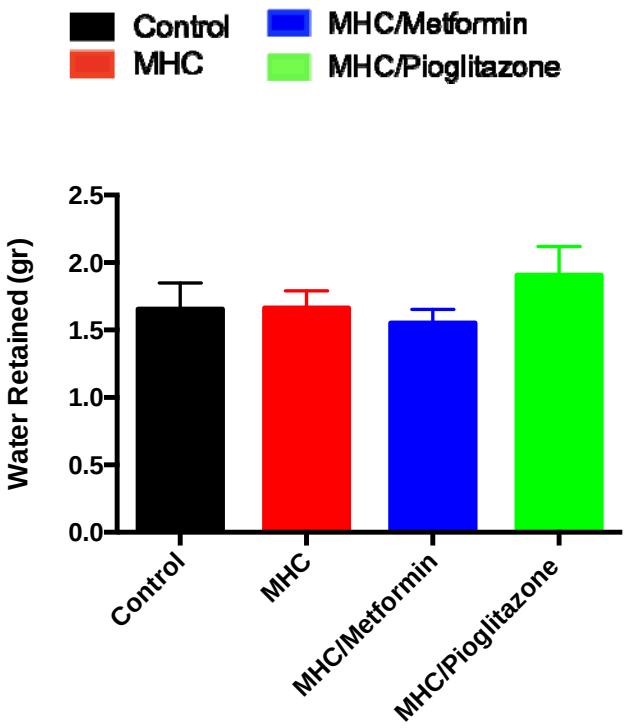


Figure 9: Water retention in rat lungs after sacrifice in the end of week 12 for different treatment groups. Values are Mean ± S.E.M of eight observations.

G. Invasive hemodynamic experiment

1. Invasive blood pressure measurement

Before performing the hemodynamic study and after the 30 minutes of stabilization, a stable five minutes recording was used to calculate the invasive SBP, DBP, and MAP after finishing the whole treatment period. As shown in Figure 10A, no significant difference exists in the invasive SBP between the six groups. In addition, no significant differences were detected among the six groups in DBP (Figure 10B) and MAP (Figure 10C).Concerning HR, similar to the previous parameters, no significant difference was found between the different groups (Figure 10D).

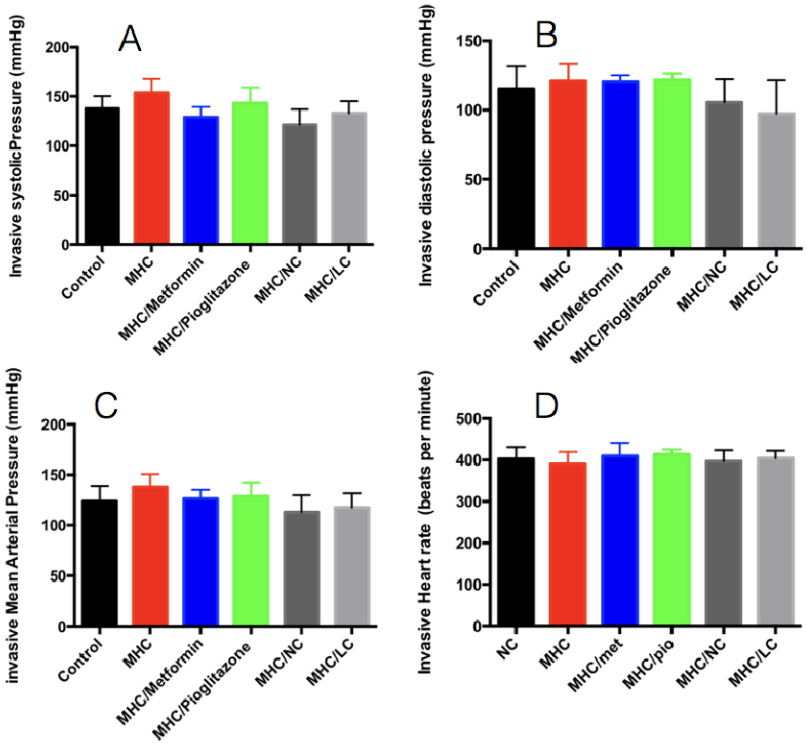


Figure 10: Invasive systolic blood pressure (SBP, panel A), diastolic blood pressure (DBP, panel B), mean arterial pressure (MAP, panel C) and heart rate (HE, panel D) measurement for different treatment groups after surgery. Values are Mean ± S.E.M of eight observations.

2. BRS

After the equilibration period, a series of PE and SNP doses were administered and the resulting change in MAP (ΔMAP) and HR (ΔHR) were recorded. An exaggerated vasopressor response to PE was noted in the MHC group (Figure 11A). A trend towards a reduction of the increased vasopressor response was evident only for rats treated with metformin, pioglitazone, or those switched to normal chow (MHC-NC); that was statistically significant at 0.75 µg PE. Significantly, rats switched to a lower daily calorie intake of the MHC diet (MHC-LC) did not show this reduction in

the vasopressor response. No significant differences were detected in Δ HR in response to different PE doses among the six treatment groups (Figure 11B).

BRS was determined by the linear regression of ΔMAP vs. ΔHR curve (Figure 11C). MHC blunted BRS as seen in the reduced BRS line slope and this was significantly different compared it to NC. The recovery of BRS was significant in metformin and pioglitazone treated rats where the regression lines were shifted towards a steeper slope mimicking that of NC. The introduction of a dietary change in MHC group allowed recovery of BRS only by MHC-NC but not by MHC-LC. Figure 11D represents the statistical comparison of the slopes assuring that recovery only happened in metformin, pioglitazone and MHC-NC groups where their slopes are statistically higher than the slope of MHC. Neither ΔMAP nor ΔHR in response to SNP appeared to show significant differences among the different groups (Figure 12A& 12B). As well, there were no differences in BRS in response to SNP (Figure 12C) as can be inferred by the lack of statistical significance upon comparison of the slopes (Figure 11D).

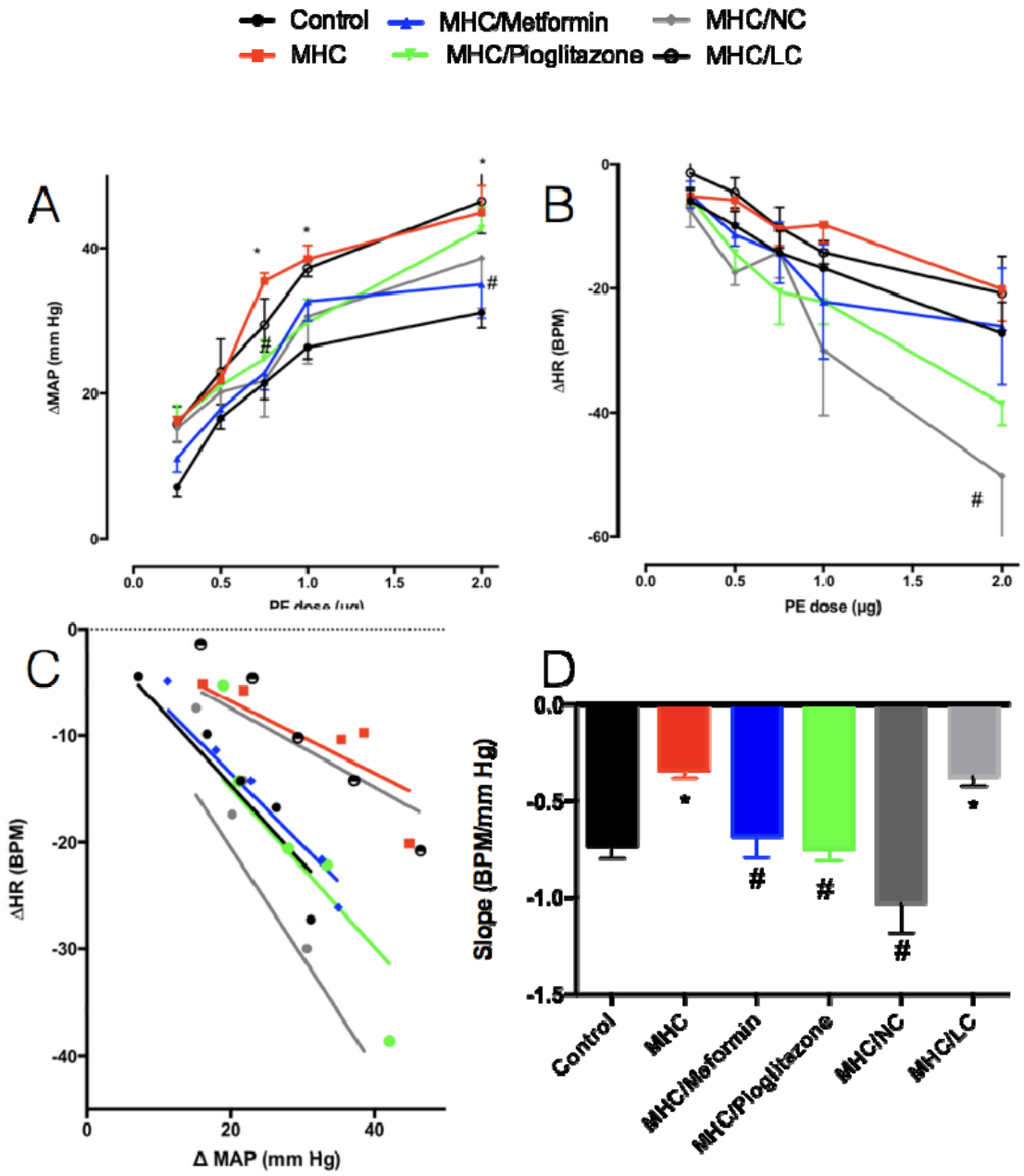


Figure 11: Changes in mean arterial pressure (MAP, panel A), heart rate (HR, panel B), linear regression of difference in MAP vs. difference in HR (BRS, panel C) and statistical comparison of the slopes in response to different phenylephrine (PE) doses in different treatment groups. Values are Mean ± S.E.M of eight observations. * and # denote $P<0.05$ vs. control and MHC groups as estimated by ANOVA followed by Dunnett and Tukey Kramer *post hoc* multiple comparisons, respectively.

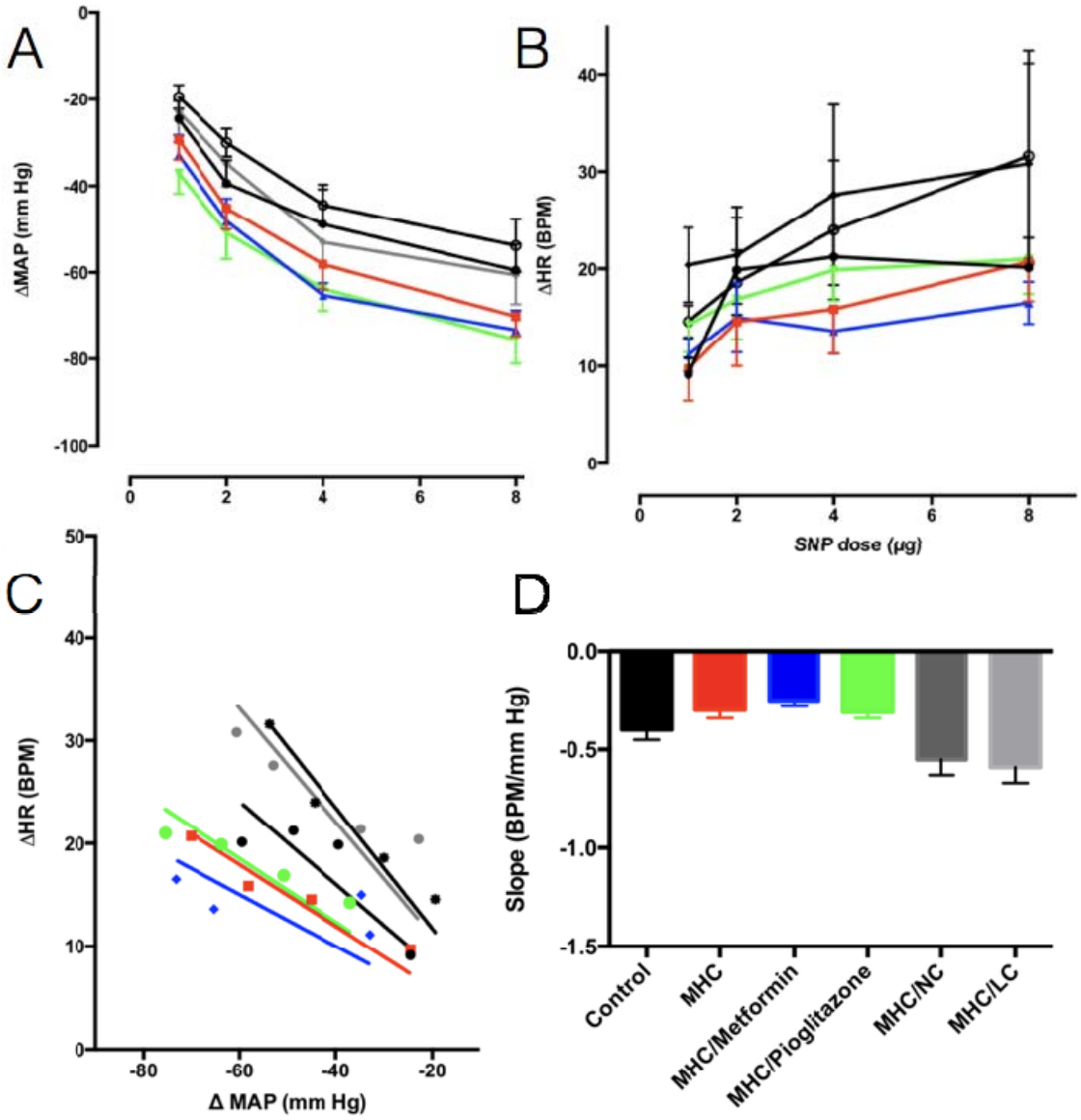


Figure 12: Changes in mean arterial pressure (MAP, panel A), heart rate (HR, panel B), linear regression of difference in MAP vs. difference in HR (BRS, panel C) and statistical comparison of the slopes in response to different sodium nitroprusside (SNP) doses in different treatment groups. Values are Mean ± S.E.M of eight observations.

3. HRV

In the end of the equilibration period, the derivative of a stable five minutes recording from arterial pressure was obtained and analyzed for determination of time domain and frequency domain parameters.

a. Time domain

Standard deviation of normal-to-normal R-R interval (SDNN) and the square root of the mean squared difference of successive R-R intervals (rMSSD) were obtained using the available software listed above. Figure 13A represents SDNN while Figure 13B represents rMSSD. Both time domain parameters were significantly reduced in MHC rats. Groups treated with metformin or pioglitazone greatly recovered both parameters. Switching the diet of MHC rat to normal chow (MHC-NC) restored the time domain parameters in a manner that mimics NC. Introducing a lower daily calorie intake of MHC (MHC-LC) did not restore the parameters and maintained them similar to MHC group.

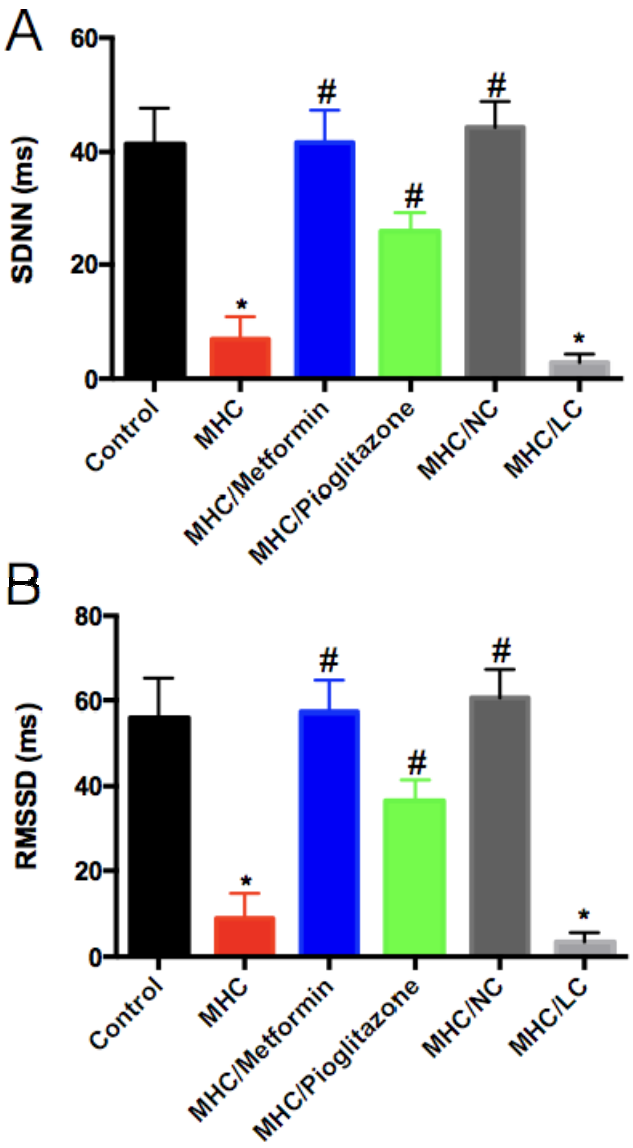


Figure 13: Changes in time domain measures of heart rate variability, SDNN (panel A) and rMSSD (panel B) in different treatment groups. Values are Mean ± S.E.M of eight observations. * and # denote $P<0.05$ vs. control and MHC groups as estimated by ANOVA followed by Dunnett and Tukey Kramer *post hoc* multiple comparisons, respectively.

b. Frequency domain

The PSD parameters (HF & LF) were extracted from the software. MHC group had an abolished power spectral density in LF and HF compared to NC group (Figure 14 A &B). Treatment with both metformin and pioglitazone restored both powers to a level not different from that of NC. The introduction of normal chow to MHC group (MHC-NC)allowed recovery of both powers. Lowering the daily calorie intake (MHC-LC) did not recover both powers.

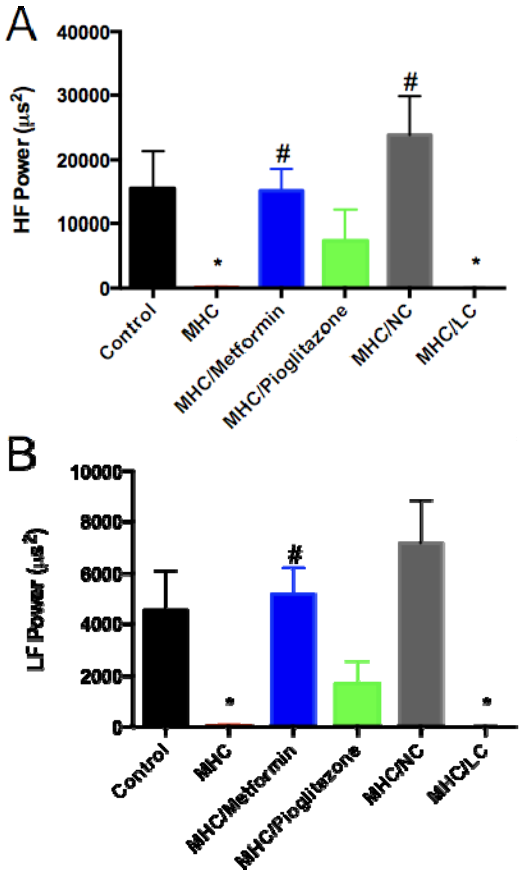


Figure 14: Changes in frequency domain measures of heart rate variability, high frequency (HF, panel A), and low frequency (LF, panel B) in different treatment groups. Values are Mean ± S.E.M of eight observations. * and # denote $P<0.05$ vs. control and MHC groups as estimated by ANOVA followed by Dunnett and Tukey Kramer *post hoc* multiple comparisons, respectively.

c. Average R-R interval

Neither dietary interventions nor drug treatment appeared to have a significant effect on average R-R interval. This was consistent with our observation of heart rate. Both in invasive and non-invasive recording, all treatment groups showed similar heart rate values (Figure 15).

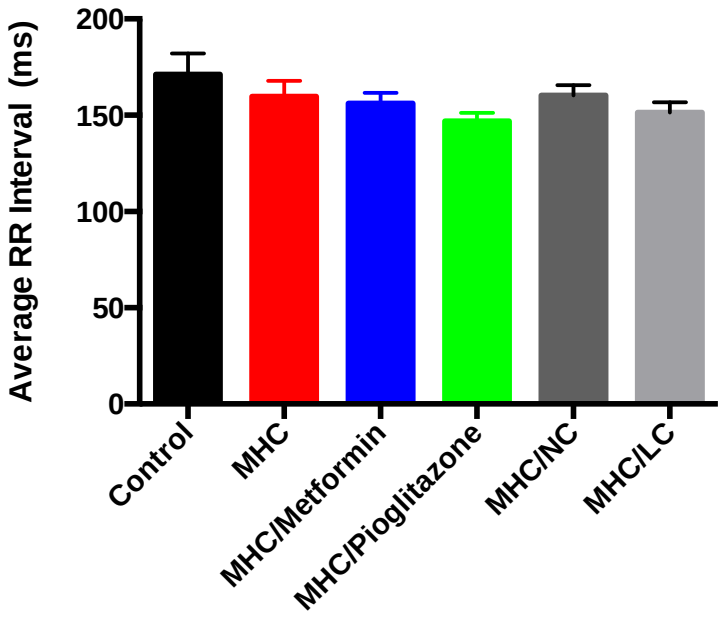


Figure 15: Changes in average R-R interval in different treatment groups. Values are Mean ± S.E.M of eight observations.

H. Histology, TUNEL and Immunostaining

Histopathological examination of the heart midsection using hematoxylin and eosin and trichrome staining showed normal myocyte arrangement and distribution. Also, no signs of tissue or cellular damage appeared in MHC fed rats. However, in agreement with previous data from our laboratory, TGF-β staining was increased in MHC fed rats and decreased with metformin or pioglitazone treatment. In addition, TUNEL analysis showed an increased staining in tissue sections from MHC fed rats, which was reduced by treatment with metformin or pioglitazone (Figure 16).

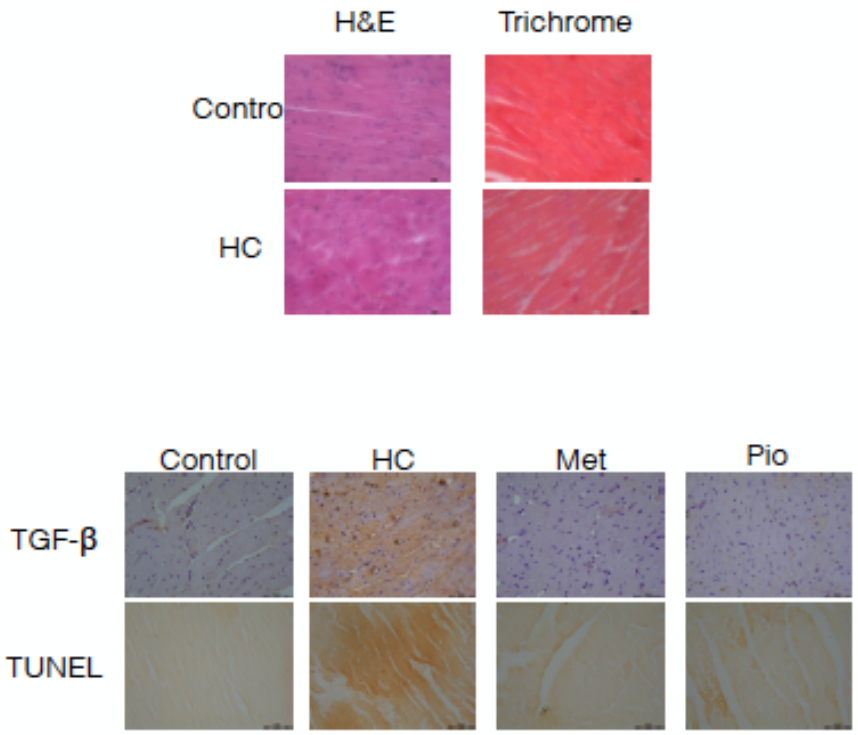


Figure 16: Representative micrographs of H&E, trichrome, immunostaining for TGF-β, and TUNEL stain for ventricular mid-section from different treatment groups.

CHAPTER V

DISCUSSION

The main purpose of this study was to determine whether cardiac structural and functional deterioration starts early in the course of metabolic alteration and examine the possibility of reversing these changes using antidiabetic agents. In order to prove this association, hypercaloric intake in rats was used as a model of mild metabolic challenge. This model receives 38% of energy intake as fat, which is slightly higher than the ADA daily total fat recommendations (20-35% energy as fat) but within range of HFD compositions (20-60% energy as fat) reported in the literature. Periodic non-invasive blood pressure, echocardiography, random blood glucose, OGTT, and invasive hemodynamic examinations were performed. No significant differences were noted among the NC and MHC groups in non-invasive systolic blood pressure, non-invasive MAP, HR, random blood glucose, echocardiography parameters, and OGTT. Up to twelve weeks of treatment and prior to invasive examination and sacrifice, neither structural nor functional cardiac changes were obvious. Further invasive investigations of cardiovascular function were performed to assess the functionality of cardiac homeostatic mechanisms and integrity of cardiac autonomic innervation. At this stage, multiple functional differences became apparent indicating a detrimental effect of high calorie intake and a potential corrective effect of metformin, pioglitazone, and dietary interventions involving reduction of saturated fat intake.

Significantly, when compared to NC group, the MHC group had an exaggerated vasopressor response to PE (Figure 10A) and blunted BRS as seen in the

reduced BRS line slope (Figure 10D), but no significant difference in the depressor response to SNP (Figure 11A)or in HR in response to PE or SNP (Figure 10B & 11B). The exaggerated vasopressor response is in line with previous findings from our laboratory showing an increased sensitivity to contractile agonists observed in isolated vessel preparations in these animals[192]. This BRS alteration implied that MHC fed rats had a reduction in vagal control but maintained integrity of sympathetic control. Alongside, significant reduction in HRV time domain parameters (SDNN and rMSSD, Figure 13A & 13B) and complete abolishment of HRV frequency domain parameters (HF and LF powers, Figure 14A & 14B) were present in MHC group. The reduction in SDNN signifies a reduction in overall cardiac autonomic control and this was confirmed by the concomitant reduction in HF power (parasympathetic) and LF power(sympathetic). In parallel, a reduction in rMSSD was seen in MHC group indicating an impairment of vagal control of variability in R-R intervals. These results are in agreement with previous findings in diabetic patients comparing time domain and frequency domain variables to normal adults. A significant reduction in both domains was noted among diabetics[193].In addition, a 10-year prospective study done on diabetic patients found that a consistent trend in PSD (increased LF and decreased HF) overwhelms the frequency domain analysis of these patients[102].As well, signs of sympathovagal imbalance characterized by a reduction in time domain and frequency domain parameters were present in prediabetic[194] and metabolic syndrome patients[195].On the other hand, no significant difference was seen in average R-R interval between the two groups (Figure 15) which is in line with a lack of change in the heart rate implying that the measured deteriorations were due to loss of autonomic cardiac balance and not due to direct cardiac structural changes affecting the inherent

cardiac automaticity. This is in agreement with the echocardiographic and histopathologic findings showing no alterations in cardiac macro/microscopic structure. The changes seen in TGF-β and TUNEL staining could signify an early molecular insult that might potentially lead to cardiac structural involvement over time. Hence, in our treatment model, early cardiac functional deteriorations are associated with CAN in absence of any signs of prediabetes. Previously, cardiac dysfunction in diabetic and insulin resistant patients was linked to early structural changes. The Framingham study, was one of the early reports that tightly linked cardiovascular diseases in diabetes mellitus to early cardiac structural changes characterized by increased LV wall thickness and mass[196]. Other studies like the Strong Heart Study similarly confirmed the association between diabetes mellitus, early echocardiographic changes and adverse cardiovascular outcomes[197]. To our knowledge, this is the first report indicating that early cardiac functional deterioration is linked to CAN despite lack of evidence for weight variation and insulin resistance.

Following the characterization of cardiovascular functional impact and CAN associated with hypercaloric intake, the question of the reversibility of these deteriorations became paramount. The presence of saturated fat in MHC food formula and its metabolic load rose a question about association of adipose inflammatory pathways in the observed deleterious effect. Indeed, unpublished results from our laboratory showed an increase in inflammatory cytokine expression in the peri-vascular adipose tissue[198]. Similar findings were reported for other adipose tissue reservoirs in rats receiving high fat diet. However, our model did not show a rise in systemic concentrations of adipose tissue-derived inflammatory mediators (unpublished data). The idea of reversing the observed deterioration using certain antidiabetic drugs with

potential anti-inflammatory action was tested using metformin and pioglitazone. The selection of metformin and pioglitazone was based on previous studies showing their cardioprotective effects. In the Diabetes Prevention Program, metformin significantly reduced the myocardial infarction events in prediabetic patients and this effect persisted for a 10 year follow up [106, 130]. Furthermore, the IRIS trial found that patients with insulin resistance (prediabetic) receiving pioglitazone had a reduced risk of stroke or myocardial infarction[150].Roles for antidiabetic drugs beyond glucose lowering effect are being addressed and the anti-inflammatory role is proposed for many protective effects[199]. In many trials, metformin was found to reduce CV risk, yet the mechanism remains obscure. Recent studies, suggest that metformin has a direct anti-inflammatory action independent of metabolic parameters improvements (hyperglycemia and insulin resistance)[200]. Through activating AMPK dependent and independent pathways metformin inhibits multiple inflammatory cytokines that are believed to be responsible for the potential diabetes mellitus independent cardioprotective[201] and neuroprotective anti-inflammatory activity[202]. Pioglitazone exerts an anti-inflammatory effect through binding to PPAR receptors that might account for its cardioprotective and neuroprotective effects[156]. Indeed, the anti-inflammatory effect of pioglitazone starts prior to its glucose regulating effect[203]by inhibiting inflammatory cytokines and chemokines thus reducing overall CV risk [204].To this end, two treatment groups were included in this study; metformin treated group (MHC-met) and pioglitazone treated group (MHC-pio). Both drugs were used in doses equivalent to the low end of those used in humans with the intent of having a minimal impact on blood glucose levels[160, 205].The lack of an effect on blood glucose level was confirmed by regular random blood glucose measurement and an oral glucose

tolerance test at the end of the treatment period. As well, potential undetected fluctuation in blood glucose levels were ruled out by the lack of difference in the serum concentration of advanced glycation end-products (unpublished data). As such, it could be deduced with reasonable confidence that the produced effect is due to the cardioprotective and anti-inflammatory role of these drugs.

As expected, no significance difference in non-invasive BP parameters, echocardiography, random blood glucose level nor OGTT were noted in metformin or pioglitazone treated rats. However, a trend towards a reduction of the increased vasopressor response to PE was evident for rats treated with metformin and pioglitazone. Again, this is in agreement with findings from *ex vivo* experiments on isolated vessel preparations [192].Both agents allowed recovery of BRS to values similar to those seen in rats receiving NC. Meanwhile, neither the depressor effect in response to SNP nor HR was affected by either dugs. In addition, time domain and frequency domain parameters recovered in rats treated by metformin or pioglitazone. Thus, metformin and pioglitazone implemented their cardioprotective roles by restoring cardiac autonomic control and reviving the vagal atony in a manner independent on blood glucose, blood pressure and reperfusion. Different experiments investigated metformin ability to improve cardiac sympathovagal balance in obese type 2 diabetic patients, where total power and HF were increased while LF/HF and LF were decreased [83, 144]. On the other hand, pioglitazone administration to diabetic patients ameliorated CAN [83] and improved cardiac sympathovagal balance and BRS[161].

In order to further address the association between adipose inflammation and CAN, two additional treatment groups were examined. The first was a group that was switched to normal chow for the last two weeks (MHC-NC), while the second was

switched to pair feeding with MHC to match calorie intake of control rats on normal chow (MHC-LC).The consumption of high fat diets was found to be accompanied by adipose tissue inflammation marked by increase in inflammatory adipokines, while energy restricted diets decreased expression of inflammatory markers [198]. Consistent with these results, another study found that excess dietary fat (HFD), and not excess calories, increases inflammatory signals in hypothalamus that were attenuated by low fat diet [206]. Among the adipokines, adiponectin and nesfatin have been found to exert metabolic and cardiovascular regulating function within the paraventricular nucleus (PVN) located in hypothalamus. PVN is an integrative autonomic center that is involved in regulating cardiovascular function[207]. The exact proposed cardio/neuroprotective underlying mechanism for metformin or pioglitazone through normalizing these adipokines reversing early CAN cannot be discounted for the direct effect of metformin or pioglitazone on neurons and their ability to penetrate blood brain barrier has been never tested before. Our study aimed to differentially assess the effect of high calorie intake vs. the nature of calorie source. The introduction of NC reduced the vasopressor response to PE and restored BRS. Whereas lowering the calorie intake while maintaining a high saturated fat content of the chow elicited the same increased vasopressor response to PE seen in MHC fed rats and did no restore BRS. No significant difference was noted among the depressor effect to SNP and the HR. The time domain and frequency domain parameters were only restored by switching to normal chow(NC) and not lowering the calorie intake (LC). Accordingly, it is our view that deterioration in cardiovascular control is caused by increased proportion of saturated fat in diet rather than increased calorie intake *per se.* Reducing the contribution of saturated fat to total daily calories reversed the deteriorations observed.

Therefore, inflammatory processes induced by high saturated fat intake commences CAN leading to cardiovascular deteriorations, in absence of any alarming signs, which can be restored by administration of low doses of metformin, pioglitazone or by healthy diet containing low amounts of saturated fat.

Future research would be required to elucidate a number of lingering questions and address the clinical utility of these findings. The potential effect of MHC feeding on the adipokine profile needs to be systematically addressed. Whether an alteration in the relative abundance of different adipokines is related to the discrepant neuronal function and whether that ties into the development of early CAN remains to be determined. The question remains whether drugs like metformin and pioglitazone, and potentially statins, would exert a normalizing effect on the disrupted adipokine profile. On the other hand, without direct testing of blood brain barrier penetration of metformin or pioglitazone a direct neuronal effect cannot be ruled out. Indeed, high fat intake was associated with endoplasmic reticulum stress in neuronal tissue [208], which is known to be alleviated by these drugs [209, 210]. And finally, gender differences in the cardiac and autonomic response to MHC feeding requires examination together with the potential detrimental effect on the off-spring.

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