AMERICAN UNIVERSITY OF BEIRUT

RENIN-ANGIOTENSIN-SYSTEM IMBALANCE CONTRIBUTES TO CARDIAC AUTONOMIC AND RENAL DYSFUNCTION IN EARLY METABOLIC CHALLENGE

by

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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science in Pharmacology and Therapeutics
to the Department of Pharmacology and Toxicology
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
December 2020

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AMERICAN UNIVERSITY OF BEIRUT

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ACKNOWLEDGMENTS

Thanks to Almighty *ALLAH* for giving me the ability and strength to learn,understand and complete this thesis.

It is a genuine pleasure to express my sense of thanks to all those who made this work possible.

First and foremost, I am fully indebted to my advisor and supervisor, Dr. Ahmed El Yazbi who told me once, that a man should only be honored of nothing but his own achievements, not because of a place, a person or a thing, however after he becoming my advisor, I realized he was not totally right this time, for I was really lucky,honored and blessed to be his student, his guidance, patience and encouragement pushed me farther than I thought I could go, with out him the aims of this thesis would hardly have been accomplished.

I am also very thankful to my co-advisor Dr.Ramzi Sabra whose valuable suggestions and constructive criticism have contributed much to this work.

My appreciations go to Dr. Fouad Zouein and Dr. Sahar Koubar for accepting to be on board of the jury.

My gratitude is also extended to all members of Dr. El Yazbi's laboratory: Mrs Nahed Mougharbel, Nour, Hanin, Rana,and Dr. Safaa Hammoud for their help in experiments and computer work.

I would specially like to thank my friends Rana Abdallah, Nada Soubra and Batoul Al Harakeh for supporting me endlessly during this journey.

Lastly, I am deeply thankful to my wonderful mom for her unconditional care and support that helped me survive all the stress I encountered and not letting me give up.

AN ABSTRACT OF THE THESIS OF

Rana Mohamad Ghazi for Master of Science
Major: Pharmacology and Therapeutics

Title: Renin-Angiotensin-System Imbalance Contributes to Cardiac Autonomic and Renal Dysfunction in Early Metabolic Challenge

Cardiac and Reno vascular complications remain the major cause of morbidity and mortality associated with type 2 diabetes mellitus (T2DM). A growing body of evidence suggest that these complications can commence as early as the pre-diabetic stage before the appearance of frank hyperglycemia, this implicated the presence of mechanisms apart from those related to serum glucose control as a root cause of such pathology. Literature describes that poor cardiovascular (CV) outcomes linked to obesity can result from the interaction between adipose tissue (AT) and the Renin-Angiotensin-Aldosterone System (RAAS). Indeed, Angiotensin II (Ang II) the main effector of RAAS is known to be a resultant of and a contributor to AT inflammation and CV anomalies. Emerging data revealed that perivascular (PVAT) and perirenal (PRAT) adipose tissue inflammation play a role in the development of cardiac autonomic neuropathy (CAN) and kidney diseases respectively during early stages of metabolic derangement i.e. pre-diabetes (PD), however, whether there is increased production or sensitivity to Ang II at the levels of both PVAT and PRAT during the pre-diabetic stage has not been forthcoming. To investigate for that, the mildly increased caloric intake (HC)-fed rat model developed in our laboratory was used. This model shows all salient features of PD in the absence of hyperglycemia and hypertension. Control and HC Sprague-Dawley rats were fed their corresponding diets for 12 weeks, a solution containing either a slow-pressor dose of Ang II (0.8mg/kg/day) or a vehicle was subcutaneously infused during the last 2 weeks of feeding and two additional groups (fed either diet) received a three-week treatment of a non-hypotensive dose (10mg/kg) of an Angiotensin converting enzyme inhibitor,captopril. Both Ang II –treated groups and HC-fed rats showed a deterioration in the cardiac autonomic control affecting the parasympathetic activity as compared to their control counter parts .This parasympathetic CAN was further worsened in the HC-Ang II treated rats and treatment with captopril significantly improved the CAN phenotype in the HC group which might be indicative of an endogenous Ang II component contributing to the phenotype. Isolated perfused kidneys of HC-fed rats showed a normal reno-vascular relaxation that was not mediated by nitric oxide and prostaglandins as in controls,however the usage of captopril attenuated the response and preserved the vasodilatory endothelial mediators in these PD rats,on the other side, those effects were much more exacerbated in the perfused kidneys of HC-Ang II rats which showed a complete loss of all vasodilatory endothelial mediators and that explained the hypertensive phenotype observed in this group as well it further highlights the possibility of HC-diet associated increase in production of or sensitivity to AngII .The present results suggest that local PVAT and PRAT RAAS might be activated during early metabolic derangement and a sub-depressor dose of captopril might have a protective effect against these metabolic-induced pathologies.

CONTENTS

ILLUSTRATIONS

Figure Page

CHAPTER I
INTRODUCTION

A. Diabetes Mellitus :

1. Definition, Classification, and Etiology

Diabetes mellitus is a heterogeneous chronic metabolic disease characterized by hyperglycemia that is mainly attributed to impairment in insulin secretion, action, or both[1]. Despite the existence of several forms of the disease, it is broadly classified into two main categories: Type 1 and Type 2. Type 1 diabetes, previously known as juvenile-onset or insulin-dependent diabetes mellitus, is the less common type accounting for 5-10% of the cases and results from autoimmune destruction of the B cells of the islets of Langerhans in the pancreas leading to absolute insulin deficiency [1, 2]. This disease, which has a genetic predisposition, could be serologically detected via a range of autoantibody markers related to islet cells, insulin, GAD65, tyrosine phosphatase IA-2, and IA-2B. Type 2 (T2DM), previously known as adult-onset or non-insulin dependent diabetes mellitus, is the much more prevalent form encompassing 90-95% of the diabetic cases and is characterized by insulin resistance accompanied by a progressive decrease in the compensatory ability of the beta-cells for insulin secretion [1, 2]. This multifactorial disease has a less defined pathophysiology (sometimes considered idiopathic), nevertheless, genetic factors affecting insulin sensitivity and secretion as well as environmental factors like obesity, stress, aging, and decreased physical activity have shown to be important contributors to the pathogenesis of the disease [3].

2. *A Global and Economic Burden*

Diabetes mellitus (DM) has become a worldwide health epidemic. The global estimated prevalence for the disease is 463 million people in 2019 and this number is expected to increase by 51% to reach 700 million cases by the year 2045 [4]. The Middle East and North African (MENA) region is classified as one of highest areas in terms of the epidemiological distribution of the disease (55 million by 2019) where 1 in 8 people is diabetic and 1 in 2 deaths due to diabetes were in people under the age of 60 according to the International Diabetes Federation IDF 2019 [4] [5]. A study done by Hirbli *et al*. revealed that Lebanon has a relatively high prevalence with 339 among 3000 individuals are diabetic [6]. However, these statistical data do not precisely reflect the real number of cases as many go undiagnosed due to the asymptomatic nature of this disease [5]. It is worth mentioning that T2DM is much more prevalent in developing than in developed countries (69 versus 20%). This increased prevalence can be mainly attributed to dietary transitions towards western lifestyle *i.e.* a shift towards diets rich in refined sugars and saturated fat accompanied by reduced physical activity, all of which contributed to overweight and obesity, the well-known environmental predisposing factors to T2DM [1]. The World Health Organization (WHO) has declared diabetes as the seventh leading cause of death in 2016. In fact, It is estimated that 1.6 million deaths were attributed to DM during the same year[7]. Furthermore, it is believed that diabetic complications mainly cardiovascular ones (coronary heart disease, myocardial infarction, stroke, hypertension, heart failure) stand behind the high morbidity and mortality rates in the diabetic population [8]. On the economic side, the high incidence rate of this non-communicable disease has greatly impacted the global financial burden. In a study done by Bommer and his colleagues (2015)- in which epidemiological and economic data for 18 countries were analyzed- the evaluated global costs of diabetes were 1.31 trillion US $ which represents

around 1.8% of the global domestic product (GDP) [9]. The influence of this economic load comprises both the society (healthcare system) and the patient. It is divided into two main components: the direct costs that involve all the treatment expenses related to the disease like medications, hospitalization and other arising diabetic complications (micro- and macrovascular) and the indirect costs which encompass all activities that lead to reduced productivity and that include increased absenteeism, presenteeism (working while sick), morbidity and premature death that generally reduces the number of working years from death to retirement age. Typically the health care costs of a diabetic patient are 2.3 times higher than other individuals not suffering from the disease [10].

3. Prediabetes: a midway stage

Before the onset of overt diabetes, where the glycemic levels go beyond the defined cut-off values for diagnosis set by the different health organizations (WHO, American Diabetes Association (ADA), IDF), a metabolic stage referred to as Prediabetes (PD) arises [11]. This precedent stage (*i.e.* PD) is defined as an intermediate between normal and diabetic states [12]. Despite some of the disparities in terms of defining PD among the different organizations, the two major parameters used by all of them are: the impaired fasting glucose (IFG) reflected by the fasting plasma glucose (FPG) level and impaired glucose tolerance (IGT) determined via a 2-hour oral glucose tolerance test (OGTT) after the ingestion of 75g of anhydrous glucose solution, an additional diagnostic criteria HbA1C (glycosylated hemoglobin) is solely added by the ADA[11]. That is why PD is often referred to as IFG or IGT [12].

So generally speaking, there are two phases of metabolic dysfunction, one is hyperinsulinemic with normal blood glucose levels and is known as PD and an opposite one *i.e.* low insulin levels (hypo-insulinemia) and high blood glucose levels (hyperglycemia) and is recognized as T2DM [13].

Currently, the ADA(2020) has stated the ranges of four different markers as a differentiating tool for diagnosing the status of glucose metabolism (*i.e.* prediabetes versus diabetes). As for diabetes one or more of the following criterion should be met to confirm a diagnosis : (1) FPG ≥ 126 mg/dL (7.0 mmol/L), (2) a 2-hour plasma glucose level ≥ 200mg /dL (11.1mmol/L) during OGTT,(3) an HbA1C ≥ 6.5% (48mmol /mol) done based on a laboratory method standardized to Diabetes Control and Complication Trial (DCCT) assay,(4) random plasma glucose level ≥ 200 mg/dL (11.1mmol /L)[14]. On the other side PD screening is defined by the following: (1) IFG *i.e.* FPG between 100 and 125 mg/ dL, 5.6-6.9 mmol /L) and/or (2) a 2-hour plasma glucose level ranging between 140 and 199mg/ dL,7.8-11 mmol /L during OGTT, (3) HbA1C ranging between 5.7-6.4% (39-47 mmol /L) [14].Despite the credibility of the criteria used to diagnose this metabolic stage, PD has a lower reproducibility (50%) compared to diabetes (70%)[15]. It is considered a substantial risk factor for the development of T2DM [12]. According to the IDF, the number of prediabetic individuals is on the rise and is expected to reach 398 million by 2030[16]. Both impaired beta-cell function and insulin resistance (IR) are the main hallmarks seen in the early stages of PD. Moreover, IR and intermittent hyperglycemia could occur as early as 13 years before the emergence of overt diabetes according to the Whitehall study[17] . Briefly, during the early prediabetic stages, the blood glucose level often remains close to normal and that is highly attributed to the compensatory rise in insulin secretion via the pancreatic beta-cells which undergo an increase in both size and number[18]. Yet, as soon as the disease progresses the beta-

cells fall short in terms of their compensatory ability, IR aggravates, and relatively sustained hyperglycemia manifests. Lastly, the beta-cells are exacerbated and frank hyperglycemia arises, at this time T2DM officially kicks in [2].

Furthermore, being one of the well-known contributors to T2DM, the impact of obesity on the beta-cell function has been examined as well, it is believed that chronic overconsumption of calories leads to ultimate utilization of the adipose tissue (AT) in terms of storage capacity, as a consequence, the excess fat will tend to accumulate in non-adipose intraabdominal organs like the pancreas, skeletal muscles, liver, kidneys, etc. The outcome of that is the build-up of toxic metabolites as a result of lipid incorporation into non-oxidative metabolic pathways that result in beta-cell lipotoxicity a phenotype that is strongly correlated with the development of T2DM[12]. Herein, it is worth mentioning that beta-cell mass is subjected to inter-individual variabilities in terms of size and response to the various stressors and thus its compensatory ability *i.e.* for the length ot time these cells able to produce insulin in the face of progressive increase in IR[1]. Besides being one of the high-risk predictors of T2DM, PD itself represents a high probability factor for the development of microvascular and macrovascular complications [19]. As most of the elements of the metabolic syndrome including IR, increased serum triglycerides, decreased levels of HDL cholesterol, elevated blood pressure and body mass index BMI are present in this continuum phase (PD), it is, therefore, more likely for macrovascular problems to originate at this time point in the form of endothelial impairment affecting the large blood vessels, atherosclerosis and hardening of the arteries collectively contributing to the high risk of vascular and heart diseases during this stage [20]. Now concerning the microvascular deterioration, several studies including the one done by Sorensen *et al* 2016 (the Maastricht Study) have demonstrated its incidence at this metabolic stage [20], where it comprises the famous Triade seen with T2DM namely

retinopathy (in around 8% of prediabetic subjects as confirmed by the Diabetes Prevention Program (DPP) and the Gutenberg Study), nephropathy (an approximate of 15.5% individuals suffering from PD have shown increased levels of albumin during urine analysis (microalbuminuria)) and finally, neuropathy in particular peripheral (11-25% of the cases) and autonomic neuropathy revealed as diminished Heart Rate Variability (HRV) and higher incidence of erectile dysfunction[19].

As the trajectory path from PD to T2DM is relatively long (10 to 13 years) and as most of the pathophysiologies seen with diabetes (IR, cardiovascular complications, etc.) are shared with PD[21], one can take advantage of this great opportunity by intervening both non-pharmacologically (lifestyle modifications) and pharmacologically during this period, in an aim to alter the natural path into an otherwise inevitable disorder[19]. In that aspect, several studies were done on different drugs, for instance, Metformin in the DPP study 2002, Pioglitazone in the ACT NOW study 2011, Liraglutide in the Satiety and Clinical Adiposity –Liraglutide Evidence SCALE study 2015, and others [22-24]. Many of them have shown promising results in either preventing or delaying the disease, however drug disutility (affect the patient compliance to the medicine provided), hepatoxicity, weight gain, and heart failure were among the most common limitations [21].

B. The Renin Angiotensin Aldosterone System

1. *General overview and basic components*:

The renin-angiotensin-aldosterone system (RAAS) is one of the complex humoral systems that is physiologically involved in the prolonged regulation of blood pressure, fluid,

and electrolyte balance through its coordinated control over the heart, the renals, the blood vessels, and the autonomic nervous system [25]. Disturbances of the function or activity of this system are involved in the development of cardiovascular diseases (CVDs) like hypertension, atherosclerosis, congestive heart failure, myocardial infarction, arrhythmia, aortic aneurysms, and nephropathy particularly in people with undergoing metabolic impairments like T2DM[26]. The classical activation of the systemic RAAS is initiated in response to different stimuli including decreased blood pressure, sympathetic overstimulation, low serum sodium chloride levels in the macula densa and reduced kidney perfusion pressure [27]. In this sense, renin, an aspartyl protease synthesized in the juxtaglomerular cells (JGC) of the renal afferent arterioles in the form of preprorenin and stored as prorenin and renin in vesicles inside these cells, is released by exocytosis after the proteolytic activation of prorenin via a set of enzymes like proconvertase I and cathepsin B [26]. Once in the circulation, the 44 KDa active enzyme (renin) cleaves its substrate angiotensinogen (AGN)- a glycoprotein that is mainly formed by the hepatic tissue and whose synthesis is triggered by various insults like inflammation, insulin and all types of steroidal hormones (estrogen, thyroxine, glucocorticoids, etc.)-into a decapeptide called angiotensin I (AngI) [26]. AngI is further processed via the angiotensin-converting enzyme (ACE) -a ubiquitous protein that is fundamentally present in the serum or bound to membranes (ectoenzyme) in particular to the endothelial cells of the blood vessels (mainly the pulmonary ones) and the epithelial cells' brush borders as in the kidney's proximal tubule cells- into the biologically active octapeptide called angiotensin II (AngII)[25, 27]. Moreover, ACE catalyzes the degradation of two powerful vasodilators namely bradykinin and Kallidin rendering them inactive[25]. Ang II is considered the main effector hormone of the RAAS system as it mediates most of its effects and that is principally through two G-Protein

Coupled receptors (GPCRs) namely the angiotensin 1 and angiotensin 2 receptors, AT1 and AT2 respectively[25]. Effects mediated through the AT1 receptors can result in three main outcomes, first a rapid increase in blood pressure through direct arterial vasoconstriction and enhanced central sympathetic outflow, second, a slow but prolonged pressor response through enhanced kidney salt reabsorption that is attained either directly by stimulating the Sodium/Hydrogen anti-porter in the proximal tubular cells or indirectly through the release of Aldosterone from the zona glomerulosa of the adrenal cortex. Aldosterone then binds its mineralocorticoid receptors which result in increased water and sodium reabsorption as well as enhanced potassium excretion at the levels of the distal and collecting tubules of the nephron. The net effect is elevated blood volume that leads to increased cardiac afterload as well as raised tension of the blood vessel walls and ultimately modulating the hemodynamic stability of the physiological system manifested as a rise in blood pressure[26, 28]. Third, cardiac and vascular dysplasia, in this aspect, Ang II will directly or indirectly (through aldosterone) promote phenotypical changes that affect the cardiovascular system mainly hypertrophy and remodeling through stimulating the release of growth factors (Fibroblast growth factor, Platelet-derived growth factor, transforming growth factor-beta, etc.) by triggering the expression of proto-oncogenes and increasing the formation of extracellular matrix by both the heart fibroblasts and the smooth muscle cells of the blood vessels[26]. On the other hand, the AT2 receptors are known to mediate mechanisms that oppose or counteract those facilitated by the AT1, despite the existence of crosstalk between both receptor types and the vastness of evidence suggesting the existence of heterodimerization between them[25]. These effects lead to antiproliferative, vasodilatory and natriuretic outcomes. It is worth noting that the AT2 receptors are distributed to a lesser extent compared to the ubiquitously abundant

AT1 and more studies are required to unravel all of its functional roles[27]. Apart from the previously described predominant systemic arm of RAAS whose effects are mainly transduced through the ACE, AngII, AT1 receptors axis [27], a large body of evidence proved the existence of an alternative pathway for Ang I processing that signals counteracting responses to the former and thereby providing cardiovascular protective outcomes that are attained through potentiating vasodilatory, anti-arrhythmic, anti-coagulant, anti-fibrotic and growth-suppressive effects [26, 27]. This novel arm mediates its actions through the ACE2, Ang 1-7, and Mas receptors axis [29].Moreover, our knowledge of the former endocrine RAAS has extended to include a local paracrine/autocrine arm as well, additional physiologically active peptides which are often degradation products of Ang II itself (Angiotensin III, Angiotensin IV, and Ang (1-7)), newly discovered receptors (AT4 receptors, Mas receptors) and different signaling pathways have been revealed as in Ang II production by distinct enzymes (no ACE enzyme required)[26]. As for distribution, the local systems are fundamentally present in the renal, cardiac, adipose, vascular, and adrenal tissues [25, 27]. In general for a local RAAS to exist the tissue must comprise all or at least some of the constitutional elements required for the *de novo* synthesis of angiotensin peptides i.e. mRNAs for AGN, ACE, renin, and others. Furthermore, the protein produced must be solely regulated via the tissue itself, and specific receptors for the formed product must exist either in the producing cells (autocrine) or nearby ones (paracrine)[25].

2. Adipose Tissue and Adipose tissue RAS: Role and Components

The concept that limits the role of the adipose tissue (AT) to energy storage and thermal isolation is no longer valid [30]. Actually, this previously-viewed passive tissue turned out to be one of the most metabolically active secretory organs with fundamental endocrine (systemic) and paracrine (local) effects [31]. The adipocytes are the major type of cells comprising the AT. These can exist in various phenotypes (White (WAT), Brown (BAT), and Beige AT) where each has its particular characteristics, morphology, and function. Moreover, the AT comprises non-adipocyte cells that include immune cells, preadipocytes, stem cells, and fibroblasts that are collectively referred to as stromal vascular fraction [32], in addition to nerve and connective tissue matrix [31]. Despite its complex composition, the AT acts as a single integrated entity that mediates its actions through releasing a wide variety of factors known as adipokines; a term that encompasses several adipose-derived proteins including adiponectin (role in enhancing insulin sensitivity and anti-inflammatory effects), leptin (associated with nutritional regulation via affecting appetite and energy consumption), fatty acids (involved in beta-oxidation), interleukin 6 (IL6) and Tumor Necrosis Factor-alpha (TNFα) (proinflammatory effects), resistin (impacts insulin resistance), monocyte chemoattractant protein -1 (MCP-1) ,plasminogen activator inhibitor-1(PAI-1), steroids, prostaglandins and others[30, 31]. Indeed, the AT expresses a number of receptors as well, through which it can respond to humoral and non-humoral ligands. Some examples include insulin, growth hormone, thyroid-stimulating hormone, AT1 and AT2 receptors, Glucagon-like peptide-1 (GLP-1), IL6, TNFα, and catecholamine receptors[31]. Adipose pools are relatively heterogeneous in terms of their location (Subcutaneous AT, Gluteo-Femoral AT, Ectopic AT), function, and composition [27, 33]. Storage and mobilization of fat at the level of AT are highly controlled by the sympathetic arm of the autonomic nervous system resulting in either lipolytic or anti-lipolytic effects that are mediated through beta or alpha 2 adrenergic receptors, respectively, and via insulin

as an anabolic hormone, that enhances lipid storage[34]. Adipocytic expansion often occurs through either hyperplasia that involves the induction of more preadipocytes to form mature cells or via hypertrophy that results in increased cellular volume and thus improved fat storage capacity[34]. Several insults including age,sex, weight, and caloric intake often affect the balance between the two mechanisms, whereby conditions leading to increased number of adipocytes maintain adequate AT functions and those resulting in adipocytic cellular enlargement are highly correlated with metabolic diseases like T2DM and obesity[34].

A mounting body of literature revealed the existence of mRNA of all RAS components and the expression of some at the protein level in the different adipose depots. These comprise the genetic material that encodes for renin and other enzymes like Cathepsin D responsible for Ang I formation and Cathepsin G involved in Ang II production[35]. Furthermore, as for receptor distribution, it has been shown that prorenin and renin receptors are mainly expressed on the cell membranes of the stromal vascular fraction in human AT[27], whereas the AT1, AT2, and Mas receptors are present on adipocytes and preadipocytes of both humans and rodents[36]. This local RAS system of AT accounts for 30% of circulating Ang II levels in rodents [37]. A study done by Cassis *et al* revealed that the amount of AGN mRNA in AT is 68% that of the hepatic tissue which further confirms the importance of this local system regarding its contribution in Ang II formation[38]. Locally, the binding of either renin or prorenin to their membrane receptors causes increased catalytic effect of the enzyme *i.e.* enhanced Ang II levels that often lead to stimulation of certain intracellular transduction pathways that lead to the formation of different modulatory factors and proteins[39]. Interestingly though, the AT RAS seems to operate in a solitary state independent of the systemic one as reflected in several studies wherein one of them, high levels of serum renin did not cause any modulations in the local generation of the enzyme[27]. Moreover,

the knock out of AT1 receptors in mice resulted in reduced expression of AGN mRNA in the liver cells but not in AT cells, additionally, AT2 deficient mice demonstrated an upregulation of AGN mRNA expression in AT[38]. With this in mind, one can assume that AT local RAS secretion has its separate regulatory pathways unrelated to those controlling the systemic form, however, the opposite is not true *i.e.* activation of the local arm of RAS impacts the systemic one [27]. A significant body of research relates the augmented Ang II synthesis in the AT to increased caloric intake that triggers AT inflammation and RAS stimulation, where at some point the excessive Ang II produced locally may escape the AT and enter the systemic circulation where it exerts multiple effects to be discussed later in details [27, 40, 41]. Another constituent of the local AT RAS is the monocarboxypeptidase ACE2[42]. This protease is mainly expressed in the form of mRNA and as an active protein attached to the adipose cell membrane[27]. Metallopeptidases such as ADAM 17 are needed to ensure the shedding of the enzyme and thus its activation. ADAM 17 is also involved in the liberation of the proinflammatory TNFα from the fat cells of obese people [42]. Having a higher affinity towards Ang II (400 fold times higher compared to Ang I), ACE2 catalyzes the octapeptide to form the vasodilatory heptapeptide Ang1-7 [27, 42]. By doing so, the level of the potent vasoconstrictor and fibrotic agent Ang II is reduced. Under normal conditions, Ang 1-7 is believed to be the counter-regulatory hormone that opposes the Ang II effects[43]. Actually, the aforementioned peptide (Ang 1-7) was able to improve the sensitivity of baroreflexes, ameliorate endothelial dysfunction, reduce heart and vascular remodeling and hypertrophy, reverse the vasoconstrictive impact of Ang II by modifying the vascular tone or via direct vasodilatory effects through the Mas receptors [33], which also mediate the decrease in the formation of NADPH oxidases and reactive oxygen species (ROS). Furthermore, it enhances adiponectin production that promotes insulin sensitivity [43]. In this aspect, based on Santos *et al*-Mas receptors deprived mice

developed insulin resistance accompanied with increased serum triglycerides and cholesterol, as well as down regulation in the insulin regulated glucose transporters in AT GLUT-4 [44].

It is worth noting that metabolic diseases involving hyperglycemia and/or insulin resistance (T2DM, Obesity) involve local upregulation of AT RAS where the resulting outcome is enhanced inflammatory processes and further exacerbation of insulin resistance and ultimately cardiovascular deteriorations[45].

3. Perivascular adipose tissue (PVAT) inflammation and RAS upregulation are potentially linked to early metabolic dysfunction.

a. PVAT: a distinctive adipose depot

PVAT is an adipose depot that surrounds blood vessels of all types (arteries, veins) with the exclusion of cerebral vessels [46, 47]. With a diameter that exceeds 100um, this type of AT is of peculiar importance, not only because of the protective feature it provides by serving as a cushion that preserves blood vessels and keeps them away from other organs in the vicinity [30]and for being a paracrine and endocrine organ- the case of other adipose pools- but also for its strategic location close to the vascular system and consequently the systemic circulation[33]. Besides, its structural features as being composed of both WAT and BAT makes it an organ with increased oxygen requirements, and thus increased vulnerability to any hypoxic- triggering factors [33]. In this context, several studies have demonstrated the existence of a cross-talk between PVAT and nearby structures like the vascular smooth muscle cells (VSMCs) and endothelial cells, and between PVAT and other distant organs (cardiac muscle, kidneys, liver, etc)[48] through a diversity of secreted adipocytokines that aid in the maintenance of normal homeostatic functions of the various

organs and vascular beds[33]. That is why pathologies targeting PVAT may result in vascular or organ dysfunctions [33]. Actually, it was not until 1991 when Soltis and his colleagues realized the impact of periaortic AT in modifying the vascular tone of underlying VSMCs located in the tunica media of the vessel [49]. At that time researchers found out that PVAT can modulate the vasoconstrictor effects of noradrenalin in blood vessels with intact AT [50]. The anti-contractile response observed was attributed to Adipocyte derived relaxing factors (ADRFs) released by this bioactive organ[51]. Although the nature of these ADRFs is still not well defined, yet, possible candidate molecules - to which the effect can be attributed – exist. Ang 1-7 is believed to be the most prominent effector followed by adiponectin, in addition to others including leptin, prostacyclin, methyl palmitate, *etc* …[51, 52]. The release of a particular mediator often depends on the vascular bed, the metabolic condition of PVAT, and the identity of the triggering agent [51, 52].

Almost all RAS elements are expressed in PVAT [53]. Under normal physiological conditions, and once needed, PVAT can generate Ang1-7 after the degradation of longer angiotensin peptides AngI and Ang II via the aid of ACE2 and /or other endopeptidases[26]. The produced heptapeptide diffuses across the blood vessel until it reaches the endothelial layer where it binds its specific Mas receptors, the consequence of which is eNOS activation and nitric oxide (NO) generation[49]. The endothelial NO will then mediate its vasodilatory effects by both enzymatic activations of soluble guanylyl cyclase and through direct stimulation of calcium-dependent potassium channels (Kca) that cause VSMCs hyperpolarization and thereby vasorelaxation [49].

b. PVAT inflammation and local RAS up-regulation

Metabolic diseases as obesity and diabetes have been associated with dysregulations in terms of adipokines secretion at the level of AT [49], which are simultaneously a resultant of and a contributor to inflammatory mechanisms as well as a promotor of cardiovascular events[33]. That is why obesity is regarded as a persistent low-grade inflammatory process [54]. Recent studies indicated that the initial symptoms of AT inflammation commence during the early phases of a metabolic disorder *i.e.* in the prediabetic stage and that PVAT is amongst the first fat pools to be affected [33]. Furthermore, the earliest signs of PVAT impairment have been detected with rat models of mild hypercaloric intake included adipocytic enlargement along with pro-inflammatory cytokines production, macrophage infiltration, mitochondrial deterioration, absence of PVAT's anti-contractile response in addition to decreased expression of the anti-inflammatory PPARϑ and increased tissue hypoxia[55]. Further, evidence has revealed the pathological overactivity of local AT RAS during episodes of diet-induced obesity[52]. As the RAS system is involved in cellular growth, so increased size of the AT in response to high caloric intake requires the presence of intact RAS components [27]. Masseira *et al* stated that AGN deficient HFD-fed mice did not undergo any increase in adipose tissue mass[37]. Hence, Ang II the main effector product of this pathway, and a growth factor is upregulated [27]. The later acts by inhibiting preadipocytes differentiation, enhancing adipocytes hypertrophy, and promoting lipogenesis [38, 56]. Here, it is worth noting that the usage of an ARB in a T2DM animal model (OLEFT rat) ameliorated the differentiation of fat cells, prevented the initiation of inflammatory signaling, and reduced its mediators (NF-kB, MCP-1, and PAI)[45]. Consequently, bigger, enlarged adipocytes result to accommodate the increased needs for lipid storage[27]. This occurs up until the adipose tissue expansion reaches a point in time where the tissue's limited vascularization capacity is unable to meet the

increased oxygen demands required by this bioactive organ[57]. Subsequently, the PVAT will be under the influence of a hypoxic milieu[33]. These conditions favor the recruitment of various immune cells and enhance the synthesis of several pro-inflammatory factors all of which contribute to PVAT inflammation[33]. Based on Zhang and his colleagues hypoxia-induced inflammation further potentiates the activation of the local AT RAAS through modulating the expression and activity of both ACE and ACE2; increasing the first and impeding the second[58], thereby favoring the arm dedicated to Ang II generation [51]. In turn, this vasoactive octapeptide causes the amplification of the undergoing inflammatory response as it enhances mitochondrial ROS formation (which was attenuated upon administration of AT1 receptor blocker in obese mice [38]), recruitment of macrophages, and activation of hypoxia-inducible factor-1 alpha (HIF-1α)[51]. By such means, a vicious inflammatory cycle arises. Generally speaking, an inflamed AT is a malfunctioning gland that fails its roles in secreting the right adipokines in adequate amounts to properly maintain metabolic and hemodynamic homeostatic effects[45]. Accordingly, pathological overproduction of Ang II strikes up at the expense of another counteracting adipokine Ang (1-7) affecting the balance between two opposing counter-regulatory axes *i.e.* the ACE/Ang II/AT1 versus ACE2/Ang (1-7)/Mas pathways[52].In the highlight of the preceeding, the question that arises is whether local RAS is activated at the level of PVAT during the prediabetic stage and whether it contributes to PVAT inflammation, this is something we are trying to answer in this research.

d. Local PVAT RAS overactivity could potentially lead to vascular dysfunction

Locally produced Ang II, rather than systemic one is considered to be the primary regulator of vascular inflammation [59]. AT1 receptors are the chief mediators of Ang II pro-inflammatory actions [59]. Metabolic incidents such as obesity or high caloric intake are associated with increased recruitment of inflammatory cells to PVAT[33, 60], however, as the physical and structural properties of the latter are not very well adapted to fit increased inflammatory cellular infiltration, the fat depot function will be compromised, this is mainly manifested as imbalances in terms of adipokines/chemokines secretions by this metabolically active organ[60]. Under such pathological circumstances, the local AT RAS becomes abnormally overactivated favoring the pathway contributing to Ang II formation, Ang II together with reactive oxygen species (ROS), TNFα, IL-6, resistin, and others trigger the activation of matrix metalloproteinases that catalyze the disintegration of VSMCs matrix, thus facilitating the migration of inflammatory mediators into them, which in part explains how dysfunctional PVAT results in vascular disease[60]. In this aspect, it is worth noting that T-cells are the major inducers of adipocytic Ang II[61]. Ang II is incorporated in a series of pleiotropic effects that go far beyond its well-known hemodynamic roles[45]. There is growing evidence that Ang II has a positive impact on each of the three stages of an inflammatory process[59].In this context, the locally released octapeptide enhances vascular permeability[62] by up-regulating the vascular endothelial growth factor (VEGF) synthesis and release[63]. Furthermore, Ang II promotes the expression of several adhesion molecules like P and L selectins, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule -1(VCAM-1) on both VSMCs and endothelial cells (ECs) and their specific ligands called integrins on certain leukocytes as well [63], thus contributing to extravasation of particular immune cells[63]. Rats infused with Ang II demonstrate elevated ICAM-1 and

VCAM-1 levels in the perivascular region of the cardiac muscle and the aorta respectively[59]. In the double transgenic rat model having both human renin and AGN genes, integrins such as αLB_2 and α_4B_1 are increasingly expressed at the surface of macrophages and monocytes[64] these play a role in increasing immune cell infiltration as their blockade causes a decrease in leukocyte permeation [59]. Additionally, an overactivated RAS upregulates the production of monocyte chemoattractant protein-1 (MCP-1) by endothelial cells of blood vessels, VSMCs, macrophages, and cardiomyocytes. This proinflammatory chemokine is significantly reduced after treatment with an ACEI or an ARB[59]. MCP-1 binds the C-C chemokine receptor 2 (CCR2) that plays a crucial role in mediating vascular inflammation and atherosclerosis triggered by Ang II up-regulation[63]. Moreover, both the precedent pathway (MCP-1/CCR2) and vascular inflammation were respectively attenuated in the monocytes and aortas of hypertensive rat models in response to treatment with an ARB[65]. In fact, mice lacking CCR2 receptors revealed the absence of leukocyte infiltration in blood vessel walls in the presence of Ang II[66]. Apart from this, Ang II stimulates the induction of endothelial COX-2 that is involved in the synthesis of vasoactive agents like prostaglandins and pro-oxidant species which results in endothelial functional deterioration[63]. Likewise, Ang II increases interleukin 8 (IL-8) that stimulates adhesion and migration of monocytes into the vascular wall [59]. Also, Ang II promotes the generation and secretion of IL-6, the later triggers AGN synthesis in the hepatocytes through a Janus kinase/Signal transducer and activator of transcription -3 (JAK/STAT-3) pathway[67]. The formed AGN further exacerbates the existing Ang II-induced vascular inflammation consequent to potential local AT RAS up-regulation, the outcome of which is an aggravation in the progression rate for the development of vascular dysfunctions and diseases[67].The inflammatory effects of Ang II are mostly

mediated via the transcription factor NF-kB and to a lesser extent by activator protein-1 (AP-1)[68]. As a matter of fact, Ang II is a pro-oxidant that triggers the activation of the enzyme NADPH oxidase (Nicotinamide adenine dinucleotide phosphate oxidase) which results in reactive oxygen species (ROS) formation[69]. Actually, superoxide generation via NADPH oxidase is doubled in Ang II –infused rats[67]. ROS, in turn, activates an NF-kB signaling cascade[12], consequently leading to activation of an IkB kinase inhibitor (IKK) -an upstream mediator in this transduction pathway- which in succession phosphorylates the inhibitor of NF-kB (IkB) resulting in its ubiquitination and subsequent separation from the NF-kB dimer[70], the latter is further translocated to the nucleus where it enhances the transcription of specific DNA consensus sequences encoding for certain inflammatory mediators including interleukins 1,6, and 8 (IL-1, IL-6, IL-8), TNFα, immunoglobulin superfamilies (ICAM-1, VCAM-1), MCP-1 and interferon 8[69].It is worth mentioning that the pro-inflammatory genes translated through Ang II up-regulation are often mediated via both AT1 (IL-6, AGN,MCP-1) and AT2 receptors (RANTES / CCL5 (regulated on activation, normal T expressed and secreted))[68]. Diseases such as atherosclerosis and kidney impairment are often associated with Ang II-induced NF-kB activation, and treatment with an ACEI often attenuates the resulting injury, in addition, rats infused with Ang II have shown enhanced NF-kB activity in both vascular and renal cells[68].Besides, the generated ROS deactivates the endothelial-derived vasodilator NO thus modifying the vascular tone[67], whereby, the administration of the antioxidant superoxide dismutase ameliorated the ROS-induced endothelial damage suggesting the direct influence of ROS as a contributor to endothelial impairment[67].It is noteworthy that ARB-treated rats show improved endothelial function in large vessels[67].The Framingham Offspring Study done on subjects with diet-induced obesity revealed that Ang II per se –in

particular the locally produced -is capable of causing arterial stiffness[52] as this vasoactive peptide happens to be a profibrotic agent as well. Ang II boosts the multiplication of VSMCs, augments collagen and extracellular matrix formation, and enhances the enrollment of inflammatory cells to the destined vessel[71]. Arterial stiffness was improved following the use of ACEIs or ARBs[52]. To further elucidate the Ang II-mediated inflammation, it is observed that the octapeptide via AT1 receptors can up-regulate the expression of Toll-like receptor-4 (TLR-4) in the VSMCs and the renal mesangial cells as revealed in both in vivo and in vitro studies [63, 72]. These receptors are involved in the production of ROS (through the interaction of the NADPH oxidase and Lipopolysaccharide), inflammation, and apoptotic cell death. Literature highlighted the impact of TLR-4 in mediating Ang II-triggered movement of adventitial fibroblasts in rat models, which were attenuated via TLR-4 blockers [63, 72], thereby perpetuating the existing inflammatory process and contributing to vascular structural modifications or remodeling[73]. Moreover,the Ang II infusion to TLR-4 -deficient mice exhibited remarkable reduction of vascular remodeling , ROS and NADPH oxidase levels[73]. Alternatively, Ang II negatively regulated the expression of the Peroxisome Proliferator-activated receptors (PPARs) that often mediate the propagation of anti-inflammatory pathways resulting in augmented Ang II –induced adipose inflammation[59]. It is believed that PPARs act via impeding the NF-kB transduction pathway, the sequelae of which is attenuated transcription of a diversity of pro-inflammatory factors including cytokines, ICAMs, VCAMs,chemokines etc, accompanied with reduced immune cells (macrophages, lymphocytes) infilteration[59].In this regard, chronic Ang II infusion in Sprague Dawley rats resulted in reduced PPAR activity and subsequently the anti-inflammatory effects accredited

to it. PPARϑ agonists (pioglitazone, docosahexaenoic acid) mitigated the Ang II-induced effects[59].

4. Ang II contribution to insulin resistance

Normally, insulin promotes its metabolic and cardiovascular effects through two major signaling pathways namely the phosphatidyl inositol-3-kinase (PI3K) and the Mitogen-activated protein kinase (MAPK)[74]. Briefly, the downstream events of the first transduction pathway include Akt (a serine/threonine kinase) phosphorylation that regulates the translocation of the glucose transporter GLUT-4 to the sarcolemma which enhances glucose uptake by insulin-sensitive tissues (skeletal muscles, AT, liver) and activates eNOS/NO signaling that result in vasodilatory, anti-inflammatory and anti-thrombotic effects. Moreover, it impedes VSMCs generation [45, 74].On the contrary, the MAPK pathway promotes the formation of the potent vasoconstrictor endothelin-1 (ET-1), enhances cellular growth and proliferation at the level of VSMCs rendering blood vessels more prone to atherosclerosis. Furthermore, it triggers the enlargement of the cardiomyocytes[45]. Upregulation of Ang II - consequent to metabolic dysregularition –impacts some common signaling pathways that happen to be co-regulated via insulin and as such, a crosstalk exists between the two [75]. Ang II halts the insulin/PI3K transduction pathway via enhancing phosphorylation of insulin receptor substrate 1 (IRS-1) at two serine residues Ser612 and Ser307 which result in detachment of the p58 subunit from the PI3K and /or the separation of the whole phosphorylating enzyme (PI3K) from its tyrosine kinase receptor (insulin receptor). The overall result is inhibition of all downstream effectors of this pathway, and consequently, all its favorable outcomes, which leads to reduced insulin sensitivity [45, 75]. It is important to highlight that the concentration of serum insulin favors one pathway over the other *i.e.*

PI3k/Akt versus MAPK [45]. Under physiologically normal metabolic conditions the fasting serum insulin concentration is within picomolar ranges (50-150pM), at this relatively low level, insulin preferentially triggers the activation of the PI3K pathway[45, 76]. Conversely, in states of metabolic impairment, as hyperinsulinemia commences, the fasting serum insulin becomes within nanomolar ranges which favors the opposing MAPK signaling, however, local RAS is concomitantly activated as well and the resultant Ang II produced together with elevated blood insulin promote MAPK signaling cascade[45]. In the vasculature, this cooperative over stimulation of the MAPK pathway is associated with deleterious events including endothelial impairment and arterial sclerosis[45].

5. Local perirenal adipose tissue (PRAT) RAAS activation and kidney dysfunction in early metabolic impairment

The PRAT is the fat tissue that surrounds or encapsulates the kidneys, until recently, this adipose tissue has been viewed as only mechanical support that protects the kidney [77], however emerging evidence revealed the existence of a substantial association between this visceral fat depot and the development of renal disorders[77]. The metabolic influence of PRAT can further exceed that of other fat tissues in particular during cases of obesity[77], it is also considered as an independent predictor of chronic kidney diseases (CKD) and subsequently cardiovascular (CVD) ones thus allowing PRAT to serve as a link between CKD and CVD. As to the tissue morphology and up until 11 months of age, the brown adipocytes (BAT) constitute the major type of fat cells in PRAT after which the tissue undergoes a progressive transition into WAT which is considered the major phenotype in adults, however during cold episodes especially in areas with chilly or icy weather like Siberia, the PRAT encounters a morphological conversion from WAT to BAT phenotype [77]. PRAT secretes a

variety of adipokines and pro-inflammatory factors that can reach the kidneys and modulate their function in a paracrine or an autocrine manner [77]. Furthermore, PRAT thickness – usually measured via ultra-sonographic techniques –was estimated to be higher in obese people with microalbuminuria compared to those without albuminuria, this marks PRAT as an independent determinant of renal damage[77]. The exact mechanism by which PRAT contributes to CKD is not fully elucidated, however, it is suggested that during a metabolic challenge (obesity) and as PRAT mass surrounding the kidney increases, the so formed AT mass creates an increased glomerular hydrostatic pressure that decreases the renal blood circulation, consequently leading to alterations in terms of renin release (increased activity of RAS system) and glomerular filtration[77, 78]. Greater PRAT thickness is often seen in patients with metabolic diseases and is accompanied by elevated insulin levels, dyslipidemia, and impaired fasting glucose, this observed effect is mainly linked to FFA-inflammatory cytokines-induced insulin signaling impairment. Excessive insulin levels contribute to salt retention, activate SNS, and affect the vascular morphology leading to kidney injury[77].

Studies reported that increased PRAT thickness is associated with both enhanced FFA release and albuminuria, the former enters the kidneys and results in renal lipotoxicity, moreover, the FFA through aggravating the oxidation of tetrahydrobiopterin favors the generation of superoxide via L-arginine over that of NO by impeding the endothelial NO synthase [79]. The diminished NO levels, in turn, result in the compensatory release of VEGF (vascular endothelial growth factor) from the podocytes which stimulate cellular proliferation and enhance the permeability of the endothelium, thereafter contributing to protein leakage[79]. One study (2020) attributed mild renal damage during early metabolic dysfunction (PD) - manifested by proteinuria and hyperfiltration -to PRAT inflammation,

moreover, the study reported deterioration in terms of renovascular function assessed by improper endothelial feedback accompanied by a relatively deficient NO and prostaglandin activity in addition to unregulated renovascular dilation that is possibly caused by an upregulated epoxyeicosatrienoic acid (EET) [80]. Whether local RAAS is activated at the level of PRAT during this early stage of metabolic impairment and whether it contributes to the PRAT inflammation and the pathogenesis involved is still an under-investigated issue.

C. Cardiac autonomic neuropathy (CAN): The beginning of diabetic cardiovascular complications

CAN is considered one of the common underdiagnosed diabetic complications affecting almost one fourth (25.3%) and one third (34.3%) of both type 1 and type 2 diabetic patients, respectively[81]. This type of diabetic neuropathies specifically impacts the autonomic nerve fibers that innervate the cardiac muscle and the blood vessels (i.e. the cardiovascular system)[82]. The Toronto Consensus Panel on Diabetic Neuropathy recommended an assessment for CAN to every diagnosed diabetic patient [83]. CAN is regarded as both an independent predictor and a prime cause standing behind the increased rate of cardiovascular (CV) derangements and consequently the morbidity and mortality associated with diabetes [84]. In this regard, type 2 diabetic patients with preexisting CAN who participated in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study were subjected to a mortality rate that is 1.55-2.14 times higher compared to their non-CAN counterparts [85].

The parasympathetic (PSNS) and sympathetic (SNS) are the two main divisions of the autonomic nervous system, while the first is involved in decreasing both the heart rate and blood pressure, the other is implicated in counteractive effects [86]. In this context, the

baroreceptors located in the aortic arch and the carotid artery play a key role in detecting variations in blood pressure via assessing the stretching degree of the blood vessel wall, the input is then relayed through an afferent nerve pathway to the specific integrative center in the medulla to be analyzed and the output is then transduced through an efferent arc to a specified effector organ (vasculature, heart)[87]. The cardiac autonomic function is often estimated based on the sensitivity and integrity of the aforementioned system [88].

1. Diagnosis :

The heart rate variability and baroreceptor sensitivity are the main methods used to assess the sensitivity of the baroreceptors and thus the functionality of this involuntary system, their reduction is often linked to CV pathologies including myocardial infarction(MI) and heart failure (HF)[89]

a. Baroreceptor sensitivity test (BRS):

As diabetic patients often suffer from the reduced function of their baroreceptors, BRS measurement could serve as an appropriate tool for CAN diagnosis[90]. In this regard, different approaches can be used.

i. The vasoactive drug method

This aims at evaluating the activity of the two components of the autonomic nervous system *i.e.* PSNS and SNS. For the first arm, we administer increasing doses of phenylephrine (PE) -an alpha-adrenergic agonist that does not directly affect the heart rate nor the CNS-that will cause elevation of the systolic blood pressure (SBP) which is measured for every corresponding dose of PE, at the same time changes in the beat-to-beat (R-R) interval happens in reflex-mediated feedback. The slope of the regression line depicting the changes in blood pressure

versus the R-R interval is calculated and its value is the judgemental factor in this test whereby steeper slopes are good functional indicators. Conversely, a vasodepressor like sodium nitroprusside (SNP) is used to assess the sympathetic arm [82, 91]

ii. Valsalva Maneuver

The procedure is performed by asking the patient to exhale against a closed airway or to blow continuously inside a balloon. The individual should be lying on his back where his ECG and beat-to-beat arterial pressure are recorded for five minutes for three successive times. Quantification of BRS is done during the phase of the sharp increase in BP using the linear regression plot of changes in ΔSBP versus that in R-R intervals[91, 92]

iii. Neck chamber method

In this technique, the carotid baroreceptors are either activated or inactivated via exerting a positive or negative pressure of known value to the neck. The slope of the regression line representing changes in R-R intervals versus the pressure applied on the neck [91].

iv. Spectral Analysis method

The BRS is analyzed based on interrelation between SBP and beat-to-beat intervals at distinct frequency domains whereby, the low- frequency bands in the R-R interval represents the sympathetic function and the high-frequency power is correlated with the parasympathetic activity [91].

b. Heart rate variability (HRV):

HRV is the measurement of inner beat-to-beat variations in heart rate. The variability in response is attributed to the competitive interaction between two opposing

tones: the sympathetic which increases the firing of the sinoatrial node and the parasympathetic which slows it down [93]. HRV permits detection of CAN at an earlier time point *i.e.* at the preclinical stage[83]. Actually, there are two sets of parameters that categorize HRV: Linear and non-Linear. As for the linear part, it consists of the time domains and the frequency domains of HRV [94]. The time-domain parameters are generally diagnostic or representatives of the parasympathetic function and these involve: the standard deviation of successive R to R intervals (SDNN) in addition to the root mean square of successive differences between beats (rmsSD). On the other hand, the frequency domain which is analyzed by the Fast Fourier transformation is composed of two pattern bands: high frequency (HF) which is measured by calculating the area under the heart rate spectral curve within the range of [0.15-0.4 Hz] is a representative of PSNS function, similarly, the SNS is measured at a lower frequency band [0.04-0.1 Hz] which is referred to as low-frequency domain (LF) and is indicative of the former function[84]. Other parameters include the total power (TP) and the LF/HF ratio which may also serve as indicators of the general tone. On the other side, non-linear parameters are analyzed from the Poincare plot of R-R intervals and these are estimates of the system's intricacy on transient and long terms [93].

2. Clinical manifestations of CAN

As hyperinsulinemia is one of the pathogenic factors leading to CAN and as the former is present during the prediabetic stage [95], CAN, in turn, is initiated during that phase before overt hyperglycemia is even present, however, the clinical symptoms only appear at progressed stages of the disease [96]. The early stages of the disease (subclinical) involve damage that affects the longest parasympathetic nerve fiber (vagus nerve) which is manifested by a

reduction in HRV. At this level, the sympathetic tone predominates and this augmentation is featured by symptoms like resting tachycardia [90-130 BPM] and exercise intolerance [96]. The sympathovagal imbalance alters the hemodynamic properties of the body including blood pressure (BP), cardiac output (CO), and heart rate [96]. Augmented sympathetic tone resumes up until advanced stages of the disease where sympathetic denervation ensues, the main feature of which is orthostatic hypotension manifested by a decrease in both SBP and diastolic blood pressure (DBP) by 20 mmHg and 10 mmHg, respectively. Indeed, this is accompanied by blunted baroreceptor sensitivity [97]. Furthermore, intraoperative instability is another clinical manifestation of the disease whereby the patient fails to adequately respond to the general anesthesia-induced hypotension and may require extra support [98]. CAN may also be involved or can exacerbate CVDs including coronary artery disease, hypertension, heart failure, arrhythmias and increase the rate of mortality and sudden cardiac death [83].

3. Pathogenesis of CAN

CAN involves a set of complicated pathways and molecular mechanisms that form the cornerstone through which ischemia followed by neuronal death results [83]. In this respect, the leading causes are hyperinsulinemia and hyperglycemia. Hyperinsulinemia disrupts the autonomic balance via augmenting the sympathetic activity [95], moreover, increased serum insulin levels in PD-as discussed earlier-is associated with AT inflammation followed by subsequent impairment in the adipocytokine balance. Evidence revealed a link between the latter and the parasympathetic-sympathetic tone and a further association between adipokines and the autonomic function[99]. Vinik and his colleagues were able to demonstrate an association between elevated serum IL-6 and deteriorated sympathovagal function (reduced SDNN) in both currently and previously diagnosed diabetic subjects[99]. Moreover, a positive

correlation between reduced high molecular weight adiponectin to leptin ratio (HMWA/L) and reduced LF/HF ratio (indicative of sympathetic overdrive)[99]. Another population study has shown that the onset of CAN occurs at an earlier time point with type 2 diabetic patients compared to those of type 1 [100], moreover, they observed that deteriorated efferent vagal tone is independently correlated to elevated pro-inflammatory cytokine IL-18, the latter is a risk factor of MI and coronary diseases [100]. This interrelation between inflammation and CAN applies to PD patients with impaired fasting glucose [101]. One study conducted in 2012 revealed that increased levels of TNFα and C-reactive protein (inflammatory biomarkers) are remarkably correlated with IR, serum insulin, fasting plasma glucose levels as well as the up-regulated heart rate observed in prediabetic subjects, additionally, Thiyagarajan *et al* confirmed a link between TNFα elevation and cardiovagal dysregulation [101]. On the other hand hyperglycemia via multiple pathways contributes to neuropathy or neuronal damage. Briefly increased serum glucose triggers the accumulation of advanced glycosylation end products (AGEs), increases oxidative stress, and ROS formation which all contribute to mitochondrial alterations accelerated stress at the endoplasmic reticulum and finally modifications of the membrane properties (permeability changes) and the endothelial function. The consequence of these dysregulations are translated by changes impacting transcription factors, gene expression and cellular activity subsequently leading to apoptosis followed by neuropathy [83, 102].To conclude, early screening of CAN is recommended for all pre- and diabetic individuals as a means to attain a better prognosis and prevent cardiovascular events[83].

D. The Mild hyper-caloric (MHC) animal model

The animal model adopted in this study is a modified form of the high-fat diet (HFD) animal model and the adjusted version is referred to as MHC fed animal model. Surwit and his

colleagues were the first to introduce the HFD model in 1988 in an attempt to study the pathophysiology of the metabolic syndrome and glucose intolerance. In fact and in contrast to previous models (*OB/OB* mouse, *db/db* mouse, *fa/fa* rat, *Gotokakizaki* rat, *OLFET* rat, etc..) the HFD model enabled them to track and elucidate the molecular mechanisms taking place during the early phases of the disease [103]. It is well known that increased fat intake (20 to 60% energy as fat) is associated with weight gain, hyperinsulinemia, and ultimately hyperglycemia[104]. Accelerated changes in dietary behaviors are highly related to the increased prevalence of T2DM. Accordingly, it is essential to provide the animal model with a dietary composition that mimics the western food enriched with saturated fats and refined sugars to simulate the ongoing changes[104, 105]. Fructose is one of the pivotal ingredients of a western diet found in a variety of beverages, prepared food, and syrup additives[104, 105]. However, based on a novel study the concomitant administration of high fat and high fructose for a long period (8 months) led to the emergence of a more robust phenotype of T2DM which maintained stable fasting and post-prandial hyperglycemia accompanied by a significant increase in IR[106]. The ADA endorsed a daily fat intake to range between 20-35% of energy to maintain adequate management of diabetes and its CV complications [106]. The MHC fed rat model is designed to consume around 38% of energy intake as fat which is slightly above the ADA recommendations, yet within the low range of HFD composition in the literature, inevitably reducing weight gain (minimizing the interference of the obesity factor) and providing a long window of time for the study of metabolic and cardiovascular impairments during this early phase of diabetes. The rat eventually gains all the prominent features of prediabetes including hyperinsulinemia, IR, dyslipidemia, elevated fat to lean ratio (Fat/lean) with no significant increase in body weight. With this model both stable hyperglycemia and

IGT were eliminated as those rats responded equally to their normal counterparts during the first twelve weeks of feeding, moreover, fasting hyperglycemia is initiated at week 16 which favors the development of the pathological machinery responsible for cardiovascular derangements, finally, after 24 weeks of feeding the MHC model shows overt hyperglycemia and is pronounced diabetic.

E. Modulation of CV deterioration and renal dysfunction via the ACEI, Captopril

During the past years, RAAS inhibition constituted a bedrock in pharmacotherapy for controlling hypertension and its cardiovascular and renal complications primarily in individuals with metabolic disorders [107]. Captopril is the first oral competitive inhibitor of the angiotensin-converting enzyme (ACEI) to be identified [108]. This potent sulfa-hydryl containing compound has 300000 times more affinity towards ACE than its natural substrate Angiotensin I [109]. Its hydrophilic nature prevents its distribution in the central nervous system and restricts its effects to the periphery [108]. Captopril like other ACEIs can suppress both the circulating and local Ang II production [110]. This RAAS inhibitor reduces the total peripheral resistance and subsequently the blood pressure predominantly via impeding Ang II formation and minimally by enhancing the accumulation of bradykinin a directly acting vasodilator inactivated by ACE to which the release of various vasoactive prostaglandins is attributed, the former effect further potentiates the hypotensive responses to captopril [108, 110]. Besides, Captopril is not associated with postural hypotension nor with reflex tachycardia because of the unchanged serum noradrenaline during the drug usage [108, 110]. It is mainly used in pathologies involving an overactivated RAAS system like hypertension, congestive heart failure (CHF), and chronic kidney diseases [111]. The anti-pressor effect of captopril is initially (at the beginning of the treatment) dependent on the serum renin levels,

however, this correlation is lost with continued drug therapy where the drug continues to exert a considerable decrease in BP despite the presence of normal or even low renin levels. Although the scientific basis or rationality behind that response is not well defined, yet some reports claim that even minute amounts of Ang II are enough to regulate BP and captopril can interfere with that or that its inhibitory effect on ACE opens the road for bradykinin to get into action [108]. Moreover, treatment with captopril increases the endogenous production of prostaglandins which enhances the depressor effects linked to the drug, the latter effects are abolished upon pretreatment with indomethacin (a cox inhibitor) [112]. Captopril has shown anti-inflammatory,anti-apoptotic, and anti-oxidative features. In a study where a model of oxidative stress is utilized, human coronary artery endothelial cells (HCAECs) treated with hydrogen peroxide (H_2O_2) in an aim to simulate some of the pathological modifications taking place in cardiovascular anomalies. Captopril was able to reverse the H_2O_2 –induced apoptosis via interfering with Akt phosphorylation in the Akt/mTOR signaling pathway that is usually activated by reactive oxygen species, which are fundamental players in the pathogenesis of diabetes and vascular dysfunction [113]. Another study reported that captopril was able to attenuate both PVAT inflammation and its consequent atherogenic effect (Ang II is a risk factor for atherogenesis through upregulating the adhesion of leukocytes to the vascular endothelium)[49]. Wire myography experiment conducted on rat mesenteric artery under established hypoxic conditions (in vitro) resulted in a deterioration in the anti-contractile function at the level of PVAT, incubating the vessel with captopril avoided the previous loss and maintained the PVAT function[51]. Apart from this, as mentioned earlier that RAS activation interferes with the insulin-mediated PI3K/Akt pathway and thus contributes to decreased insulin sensitivity at the level of different tissues. In this regard, it is worth noting

that IR besides its pre-mentioned outcomes can impact cardiac function via increasing fatty acid oxidation in the myocardium and enhancing myocardial-oxygen consumption[114]. Captopril was able to improve insulin sensitivity in a study done on *ob/ob* mice given a 4-week daily dose of the drug (4mg/kg) and the results involved amelioration of myocardial IR manifested by reduced oxygen consumption and enhanced cardiac function, in addition to improved fatty acid oxidation and decreased AMP-activated protein kinase (AMPK) [114]. In the obese Zucker rat an animal model with hyperinsulinemia, dyslipidemia, and glucose intolerance, captopril improved peripheral insulin sensitivity and reduced serum free fatty acid levels[115]. Furthermore, a large number of clinical trials like HOPE, CHARM, VALUE, and others have shown an approximate reduction of 22% in the development of T2DM in hypertensive patients treated with an ACEI[45, 114]. Additionally, in non-diabetic subjects with cardiovascular diseases or risk factors, captopril ameliorated glucose intolerance and enhanced insulin sensitivity[45]. It is established that this RAS inhibitor demonstrates renoprotective effects in both diabetic and non-diabetic patients where captopril mitigates kidney damage and suppresses inflammation by deactivating the NF-kB pathway[111]. Captopril decreases the vascular resistance in the renals thus improving the kidney blood flow[108]. At a dose of 37.5mg tid, this ACEI reduces proteinuria without affecting blood creatinine values via decreasing the intrarenal pressure in particular in diabetic nephropathy[108]. In a study performed by Saleh *et al* on aged, obese *Zsf1* rat model that shares many features of the cardiometabolic syndrome associated with heart failure with preserved ejection fraction (cardiac hypertrophy, chronic kidney disease), Captopril ameliorated left ventricular diastolic function and improved kidney hemodynamics manifested by reduced proteinuria,enhanced GFR and renal histopathology, moreover, the

drug retarded the progression of cardiac histopathologies,ischemic degenerative effects and necrosis [107].

F. **Rationale, Hypothesis, and Aims of the study**

A wide range of anti-diabetic drugs combined with the continuously optimized diabetic standard of care practices were able to successfully manage the glycemic levels in diabetic patients. However, these measures *per se* were not enough to halt or reverse the devastating consequences of the syndrome including cardiovascular and renal outcomes, which were even present in recently diagnosed diabetic patients. This implicates the presence of mechanisms other than hyperglycemia that could be involved in the pathogenesis of these complications. In a previous study we showed that HC diet was associated with CAN affecting the cardiac parasympathetic activity and renal function secondary to PVAT and PRAT inflammation respectively. Growing evidence revealed that diet induced obesity in both humans and rodents was accompanied with enhanced local production of Ang II in the adipose tissue, furthermore, the so-formed octapeptide was found to be involved in AT inflammation and remodeling and contributed to local insulin resistance. Moreover, Ang II has also been reported to play a role in vascular and renal dysfunction in metabolic diseases. Being said that, and as little emphasis was given to alterations in or the status of Ang II levels during the course of early metabolic disorders, we have carried out a new series of experiments that involved the usage of an exogenous slow pressor dose of Ang II in different metabolic conditions in an aim to assess their corresponding responses and to check whether a certain metabolic state can attenuate the sensitivity to and/or production of an endogenous Ang II peptide. Hence, we hypothesize that

increased Ang II signaling or sensitivity resulting from PVAT inflammation in prediabetes contributes to early cardiovascular and renal dysfunction.

As such, our specific aims are to:

1. Assess the sensitivity of hemodynamic, cardiac autonomic, and renal functions of prediabetic rats showing signs of PVAT inflammation to a slow-pressor dose of Ang II compared to control rats.
2. Examine the impact of a non-hypotensive dose of captopril on cardiovascular and renal functional alterations in prediabetic rats.

CHAPTER II

MATERIALS AND METHODS

A. Ethical Approval

All animal experiments were carried out in compliance with the study protocol number 16-10-386 approved by the AUB Institutional Animal Care and Use Committee (IACUC) in accordance 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; 150g) were allocated randomly to six different groups (n=8 each): (1) rats fed with normal chow diet for 12 weeks during which a mini osmotic pump (Alzet®MODEL 2ML2) containing normal saline (sham) was subcutaneously implanted in the animal mid scapular region at week 10 (**Control- Normal Saline ,NC-NS ,3Kcal/g**),(2) rats fed with normal chow diet for 12 weeks with implantation of mini osmotic pumps that continuously deliver angiotensin II (0.8mg/kg/day) at a rate of 5µl/hour for 14 days starting week 10 (**Control-Angiotensin II, NC-Ang II**),(3) rats fed with mild hyper caloric diet for 12 weeks during which subcutaneous implantation of mini osmotic pumps containing normal saline were inserted at week 10 (**HC-NS**, 4.035kcal/g),(4) rats fed with mild hyper caloric diet for 12 weeks during which an angiotensin II-containing osmotic pump is subcutaneously inserted at week 10, the later releases the drug at a rate of 0.8mg/kg/day (**HC-Ang II**), (5) rats fed with normal control diet and treated with captopril (10mg/kg) on daily basis starting week 9 (**NC-Cap**),(6) rats fed with mild hyper caloric diet and treated

with captopril (10mg/kg) on daily basis starting week 9 (**HC-Cap**). 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 and calorie intake was calculated daily based on the amount of food consumed. The treatment was freshly prepared on daily basis by crushing 25mg – captopril containing tablets, suspending them in water, and incorporating the required volume of suspended drug in a 5g wet-chow pellet of the issued diet (NC or MHC), then the drug-containing food pellet was administered to different groups starting week 9 once daily.

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: 54% from carbohydrates, 32% from proteins, and 14% from fat (of which 0.9% by weight saturated fat). The MHC diet was prepared in house and was composed of food-grade fructose (20% by weight, Santiveri Foods, Spain) and hydrogenated vegetable oil Mazola®,15% by weight, BFSA). Major electrolytes and vitamins were supplemented to match the concentration in ENVIGO diet and as suggested by the American Institute of Nutrition (Reeves, Nielson, and Fahey, 1993).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%). Diets were fed for 12 weeks period.

D. Osmotic Pump Implantation

An osmotic pump infusion (Ang II or normal saline) was implanted under the skin (subcutaneous) of the animal into an incision made dorsal to the scapular region using the Alzet® osmotic pump Model 2ML2 that delivered the solution at a rate of 5µl/hour for 14

days. The minipumps were incubated overnight at 37 degrees Celsius a day before insertion. On the next day, rats were given an antibiotic IP injection (gentamicin (40mg/1ml) at a dose of 1mg/100g body weight) 2 hours prior to the implant, the rats were then anesthetized using 70% of 1.034 mg/ml/kg ketamine/xylazine in 1:4 v/v mixture (AUB-MC Pharmacy). Once the animal was sedated the incision was made and the pump was inserted into place, the animal was then sutured, placed under warm conditions, and observed until it recovered from anesthesia before being transferred back to its cage.

E. Regular Gross Examination, Blood Tests, Sacrifice, and Organ Harvest

Non-invasive blood pressure measurement (CODA) and echocardiography were performed at week 10 before treatment and week 12 before sacrifice, random glucose measurement was done at baseline, 4, 10, and, 12 weeks. At the end of week 12, the rats were anesthetized by 100mg/ml thiopental (AUB-MC pharmacy), and the hemodynamic study was performed as described below. In the end, the rats were decapitated and blood was collected. The head was placed in ice and the brain stem was isolated. The thoracic cavity was exposed and the heart was flushed and isolated. The atria were removed and the ventricles were weighed and horizontally cut into three sections (apex,mid-section, base). The mid-section was placed in formaldehyde for further histological analysis. Other tissues isolated were stored at -80°C. The tibia bone length was measured for normalization purposes.

F. 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 a specialized volume pressure recording (VPR) sensor to measure blood pressure changes that happen in a rat tail. Measurement was performed at weeks 10 and 12 on rats sedated via a 70% of 1.034 mg/ml/kg

ketamine/xylazine in 1:4 v/v 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 was excluded by the system. The blood pressure parameters obtained are: systolic, diastolic and mean blood pressure, heart rate, tail blood volume, and blood flow.

G. Echocardiography

Echocardiography is a technique used to assess 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 permits visualization of heart morphology. It is performed at weeks 8 (before drug use) and 12 (after drug use and before sacrifice). Rats sedated for the non-invasive blood pressure measurement underwent echocardiography before being placed on the CODA machine. Rats were placed on a panel and echocardiography was performed using Sonix TouchQ+ ultrasound (BK ultrasound, Peabody, MA). The parameters calculated were: left ventricular posterior wall diameter (LVPW), fractional shortening, and ejection fraction (EF).

H. Random Blood Glucose Measurement and HBA1c levels

Random blood glucose testing was measured at weeks 0, 4, 8, and 12. The lateral tail vein was pricked and enough quantity of blood was obtained. The measurement was done via Accu-check Performa glucometer (Roche Diagnostics, Basel, Switzerland). HBA1c levels were measured in blood samples obtained from the tail vein at the end of the feeding period using a bench-top Labona Check A1c analyzer (Greencross Medis, Cheonan-Si, South Korea) as per manufacturer instructions.

I. Invasive Measurement of Hemodynamic Parameters

The hemodynamic study was performed at week 12. Rats were anesthetized using 100mg/ml thiopental (AUB-MC Pharmacy). Tracheostomy was performed after which the right carotid artery was isolated, cannulated and connected to a Miller transducer to measure mean arterial pressure (MAP) and heart rate (HR). Then, the left jugular vein was isolated, cannulated and connected to a shunt for IV drug administration. Once the surgery was completed, the recording is allowed to stabilize for 30 minutes. Increasing doses of Phenylephrine (PE) were given (0.25, 0.5, 0.75, 1, and 2 µg) and the changes in MAP and HR were recorded. After giving the vasoconstrictor, the rat is allowed to rest for another 30 minutes after which ascending doses of Sodium Nitroprusside (SNP) were administered (0.75, 1, 2, 4, and 8 µg). Again, the changes in MAP and HR in response to SNP were recorded.

J. Baroreceptor Sensitivity

The vasoactive drug method was used to assess the BRS. This was executed using the modified Oxford method, where the relationship between cardiac autonomic control and mean arterial pressure during different vasoactive drug dose administration was calculated. The slope of the linear regression fit between ΔMAP and Δ HR was determined and used to validate the functionality of the baroreceptors. And so the steeper the slope, the better is the sensitivity and thus the functionality of the baroreceptors.

K. Nuclear Magnetic Resonance(NMR)

The body composition of the rat (lipids, proteins, water) was analyzed using the nuclear magnetic resonance machine (LF10 Minispec NF4433, Bruker, MA, USA). The animal was weighed and placed inside a cylindrical plastic box where it was made immobile

by squeezing it against the lower bottom of the cylinder. After which the cylinder containing the rat was inserted inside the NMR machine and the following parameters were estimated: fat mass, lean mass, fluid content, and the fat/lean ratio to detect different tissue densities. The body composition measurement was performed at a 4-week interval and values obtained from each rat were compared to a standardized, calibrated rat.

L. The rat isolated perfused kidney

The rat kidney was isolated and perfused based on the method described in previous studies [116, 117]. Briefly, before surgery rats were anesthetized with thiopental sodium (50mg/kg) given intraperitoneally, the abdomen was opened via a midline incision to expose the left kidney. The left renal artery was separated from its surrounding tissues and loose ties were made around both the renal artery and abdominal aorta proximal and distal to the renal artery .Once the aorta was ligated, the left renal artery was cannulated with a beveled 18-gauge needle coupled to a 5-ml syringe containing heparinized saline (100 U/ml) through a small incision made in the aorta. After which, the cannula was stabilized with ligatures, the kidney was flushed with heparinized saline and immediately dissected from its surrounding tissues. The kidney was then placed in a jacketed glass chamber that is kept at 37°C and constantly perfused with Krebs solution (in millimolars: NaCl, 120; KCl, 5; CaCl2, 2.5;MgSO4.7H2O, 1.2; KH2PO4, 1.2; NaHCO3, 25; and glucose,11) and gassed with 95% O2 and 5% CO2. The perfusion is done at a flow rate of 5ml/min adjusted by a peristaltic pump (Model P3; Pharmacia Fine Chemicals). The pump provided a pulsatile flow of the solution and the venous effluent was allowed to drain freely. Gould-Statham pressure transducer placed distal to the pump was involved in the continuous monitoring of the kidney perfusion pressure which were recorded on a LabChart-7 pro software (Power Lab 4/30, model ML866/P, AD Instruments,

Bella Vista, Australia).The experimental setup is allowed to stand up for 30 minutes to ensure the stabilization of the kidney perfusion pressure. To study the vasodilatory effects induced by carbachol (M3 agonist), the renal vascular tone was increased by continuous infusion of an alpha –adrenoceptor agonist phenylephrine (10 µM). The phenylephrine –preconstricted kidneys were infused for 20 min with or without L-NAME (nitric oxide synthase inhibitor, 100 µM), diclofenac (cyclooxygenase inhibitor, 7 µM) or both of them. Successive bolus doses of 0.1 ml carbachol (0.001, 0.01, 0.1, 1, 10 nmol) were injected after each infusion and the change in perfusion pressure was recorded.

M. Immunohistochemistry

The right kidneys and thoracic aortas placed in 10% formaldehyde solution were fixed in 100% ethanol, embedded in paraffin, cut transversely, and placed on slides. The staining was done simultaneously to all the slides for accurate immunohistochemistry comparison. Hematoxylin and Eosin (H&E) staining was used for the demonstration of nucleus and cytoplasmic inclusions, Mason Trichrome to detect renal and vascular fibrosis, Dihydroethidium (DHE) staining to detect the presence of reactive oxygen species within the tissue, and Periodic Acid-Schiff (PAS) staining used to examine morphological changes in various tissues taken from animals of different experimental conditions.

N. IBA-1 Immunostaining

For the detection of IBA-1, slides were first dewaxed by heating them in the oven at 55°C for 30 minutes, followed by progressive hydration in alcoholic solutions of increasing water content (xylol 2%, xylol 1%, ethanol 100%, ethanol 95%, ethanol 75%, water), the slides were placed in an antigen retrieval buffer (10mM sodium citrate buffer) and heated in a steamer for 15 minutes, upon cooling, the slides were washed and incubated in a blocking

buffer (10% normal goat serum, 1% bovine serum albumin, tris buffered saline TBS 1X) for 2 hours, after which the primary antibody (rabbit anti-IBA-1,1:100,abcam) was added and the slides were incubated overnight at 4°C. The next day, sections were washed with TBS-triton twice, followed by the addition of 0.3% H_2O_2 for 15 minutes to quench internal peroxidases and then the secondary antibody (horseradish peroxidase antibody HRP) in which they were incubated for 1 hour. The slides were then washed with TBS-triton twice and the sections were incubated with 35µl DAB solution for 10 minutes and then rinsed with water. Finally, the sections were counterstained with one drop of hematoxylin before examination under the microscope.

O. Statistical Analysis

Data were expressed as Mean ± SEM. All analysis was done using PRISM software. Comparisons between the different groups were performed using One Way ANOVA followed by Tukey's *post-hoc* test, as well as two-way ANOVA followed by Sidak's multiple comparison test (to compare different time points or concentrations among groups). P-value<0.05 was considered statistically significant.

P. Chemicals

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

CHAPTER III
RESULTS

I. Metabolic consequences of HC feeding.

Throughout the twelve-week period, HC-fed rats significantly consumed more calories compared to their control counterparts (Fig. 1A). Control and HC-fed rats treated with Ang II showed reduced calorie intake in the last two weeks of feeding coincident with Ang II treatment. Rats from all groups initially started with approximately the same weight (150 g) and continued to gain weight in the same manner except for HC-Ang II which had significant loss in terms of body weight during the last 2 weeks of feeding as compared to the HC-fed group (Fig.1B). An increased fat/ lean ratio was observed in HC-fed rats indicative of adipose expansion (Fig.1C). No changes in random blood glucose or HbA1c levels were observed among the different groups (Fig.1D&E).

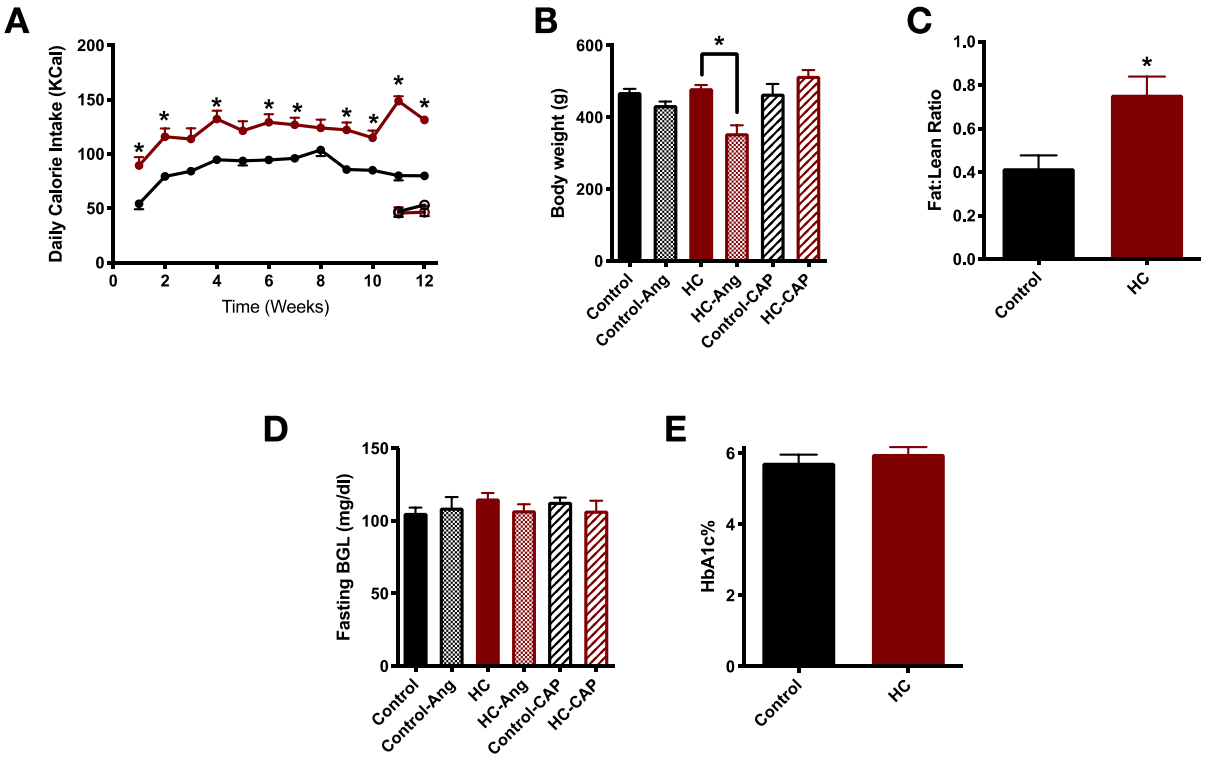

Figure 1. Daily caloric intake and metabolic parameters of rats in different treatment groups. A, Daily calorie intake in control versus HC-fed rats along the 12-week feeding duration (n=6). B, Body weight over 12-weeks of feeding for the different treatment groups (n=6). C, Body composition measured via NMR represented as fat/lean ratio for control versus HC rats (n=6). D, Random blood glucose levels for the different treatment groups (n=6). E, HbA1c levels at 12 weeks of control and HC feeding (n=6). Values are Mean ± S.E.M. Statistical significance was determined by two-way ANOVA followed by *post hoc* comparison for (A) or one-way ANOVA followed by *post hoc* test (B,D), and unpaired Student's t-test for C and E. * denote *P<0.05* versus corresponding values in control.

II. Non-invasive Blood Pressure Measurement and Echocardiography

Significant increases in systolic blood pressure were only observed in the HC- Ang II group compared to HC rats receiving normal saline (Fig. 2A). Echocardiographic parameters of heart function (Fig. 2B and 2C. ejection fraction, EF, and fractional shortening, FS) in the different groups remained comparable to that of the control.

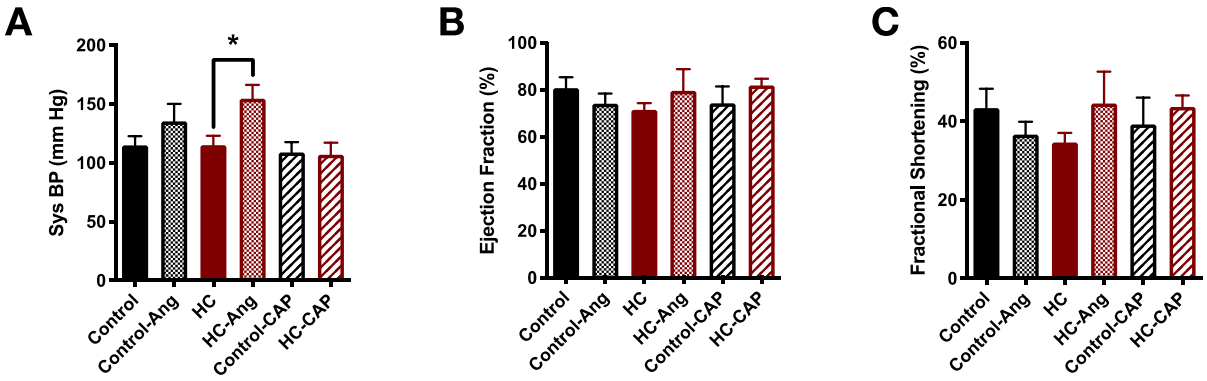

Figure 2. Non-invasive hemodynamic and echocardiographic parameters for the different treatment groups. A, Non-invasive systolic blood pressure; B, Ejection fraction (EF); and C, Fractional shortening (FS) at week 12 of feeding (n=6). Values are Mean ± S.E.M. Statistical comparison was done by one-way ANOVA followed by *post hoc* test. * denote *P<0.05* versus corresponding values in HC.

III. Baroreceptor Sensitivity (BRS) in control, high caloric, angiotensin -given and captopril -treated rats.

a. Cardiac (HR) and Vascular (MAP) Responses to vasopressor, phenylephrine (PE)

To assess the sensitivity of the parasympathetic arm of the baroreflex, the functionality of the baroreceptors was tested via the vasoactive drug method whereby

increasing doses of the vasoconstrictor PE separated by 5 minutes interval were given and the subsequent bradycardic responses were examined. An exaggerated vasopressor response to PE was noted in all Ang II-treated rats compared to other groups (Fig. 4A). Both Ang II-treated groups (given normal or high caloric diet) mounted blunted bradycardic responses to PE which was more evident with the group placed on HC feeding. Moreover, the decrease in HR (ΔHR) seen with increasing doses of PE was lower in HC rats than in controls, which was improved upon treatment with captopril (Fig.4B). All Ang II- treated groups along with the HC rats had a lower slope of linear regression of ΔHR versus ΔMAP as compared to controls (Fig.4C and 4D). Furthermore, this blunted BRS slope was more evident with Ang II-HC rats compared to HC only rats (Fig.4D). It is worth noting that treatment with captopril significantly improved the blunted BRS slope mounted by the HC rats and set it to a value comparable to normal controls (Fig.4D). Figure 3 depicts representative tracings of changes in MAP and HR in response to increasing doses of PE.

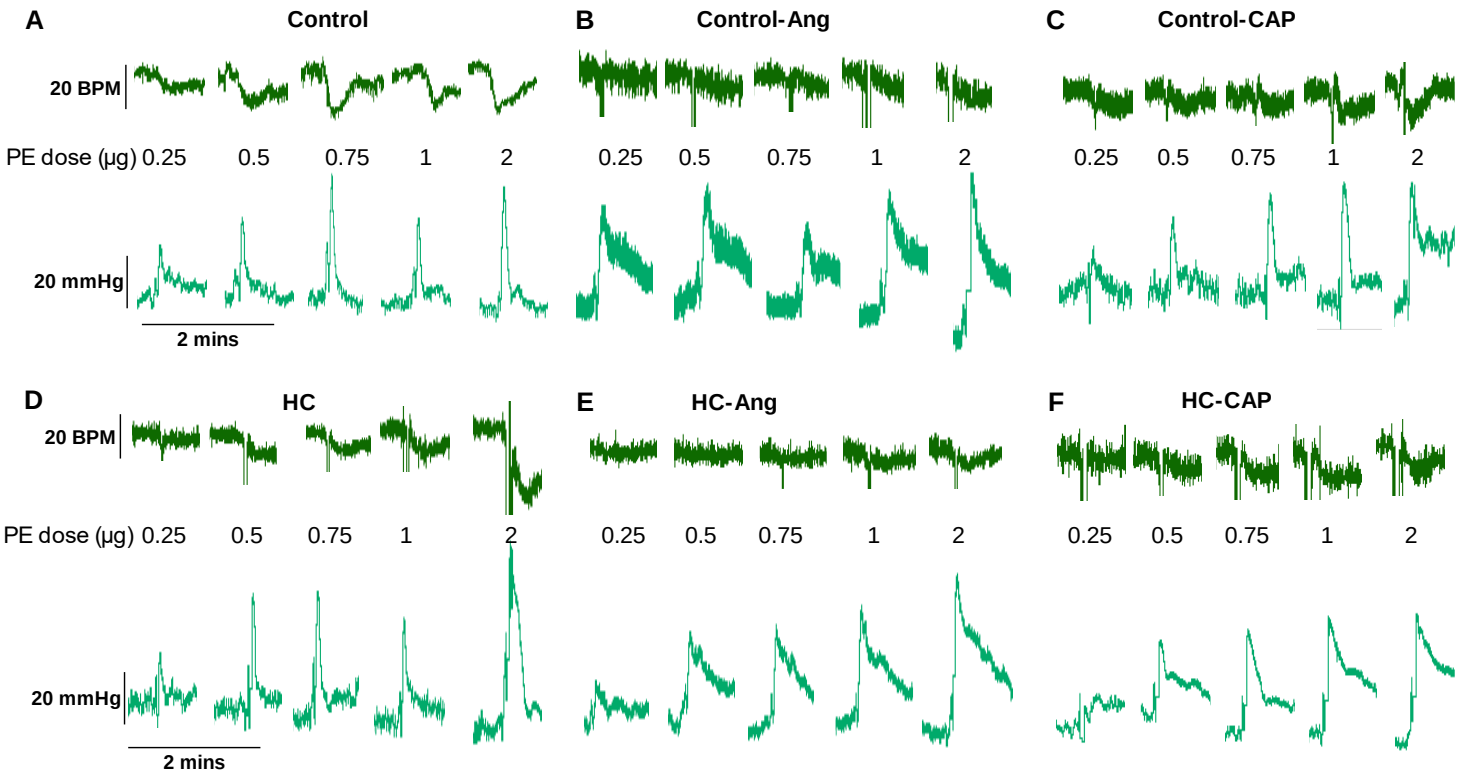

Figure 3. Representative tracings of pressor [mean arterial pressure (MAP)] and cardiac [heart rate (HR)] responses to increasing doses of phenylephrine (PE) in A, Normal control; B, Control-Ang II; C, Control-Captopril; D, HC; E, HC-Ang II; and F, HC-captopril rats. Vertical scale bars represent MAP (20 mmHg) and HR [20 beats/min (BPM)], and horizontal scale bars represent time (2 minutes).

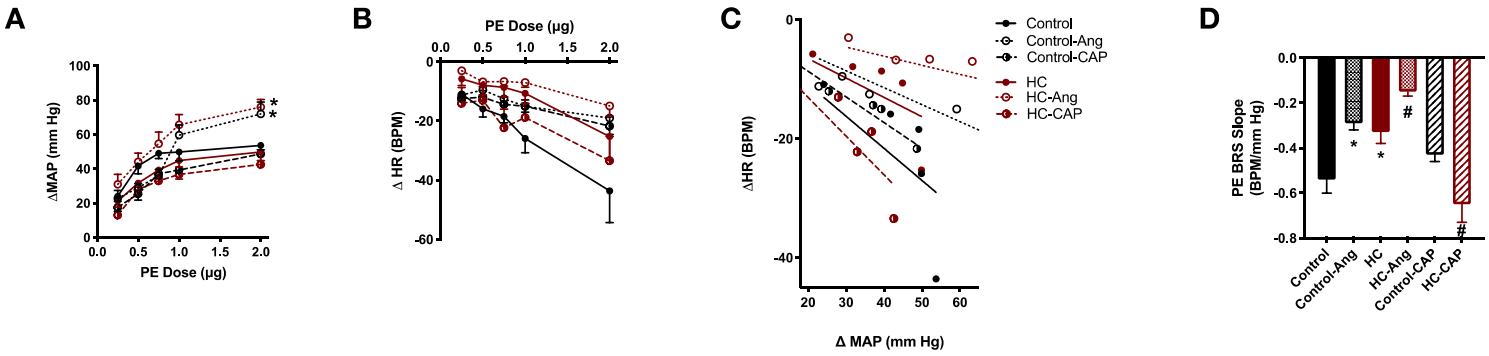

Figure 4. The effect of treatment with either slow-pressor doses of angiotensin II or non-hypotensive doses of captopril on the sensitivity of the parasympathetic arm of the baroreflex in both control and HC rats. A, Pressor effect of different doses of PE in different treatment groups. B, The reflex bradycardic response to different doses of PE. C, Best-fit regression lines for the correlation between changes in MAP in response to increasing PE doses and the reflex change in HR. D, Statistical comparison of the slopes of the best regression line of the ΔMAP versus ΔHR in response to different PE doses in different treatment groups. Values are Mean ± S.E.M. Statistical significance was determined by two-way ANOVA followed by Sidak's *post hoc* test (A, B, C) or one-way ANOVA followed by Tukey *post hoc* test (D). * and # denote *P<0.05* versus response at corresponding points in control and HC groups respectively.

b. Cardiac (HR) and Vascular (MAP) Responses to vasodilator, sodium nitroprusside (SNP)

The sensitivity of the sympathetic arm of the autonomic nervous system was assessed by measuring the tachycardic responses to increasing doses of SNP then the slope of the best fit regression line was determined. The steeper the slope, the better the functionality of the receptors were. Like in previously studied prediabetic rat models, all groups involved showed normal depressor responses to the various doses of SNP (Fig.6A). The change in heart rate (ΔHR) in response to SNP were not different among the groups

(Fig.6B). There were no differences in BRS in response to SNP (Fig.6C), which indicated normal reflex tachycardic responses and was further confirmed by the lack of statistical significance upon comparison of the different BRS slopes (Fig. 6D). Figure 5 depicts the representative tracing of changes in MAP and HR in response to increasing doses of SNP. The depressor responses induced by SNP remained unaltered in all treated groups.

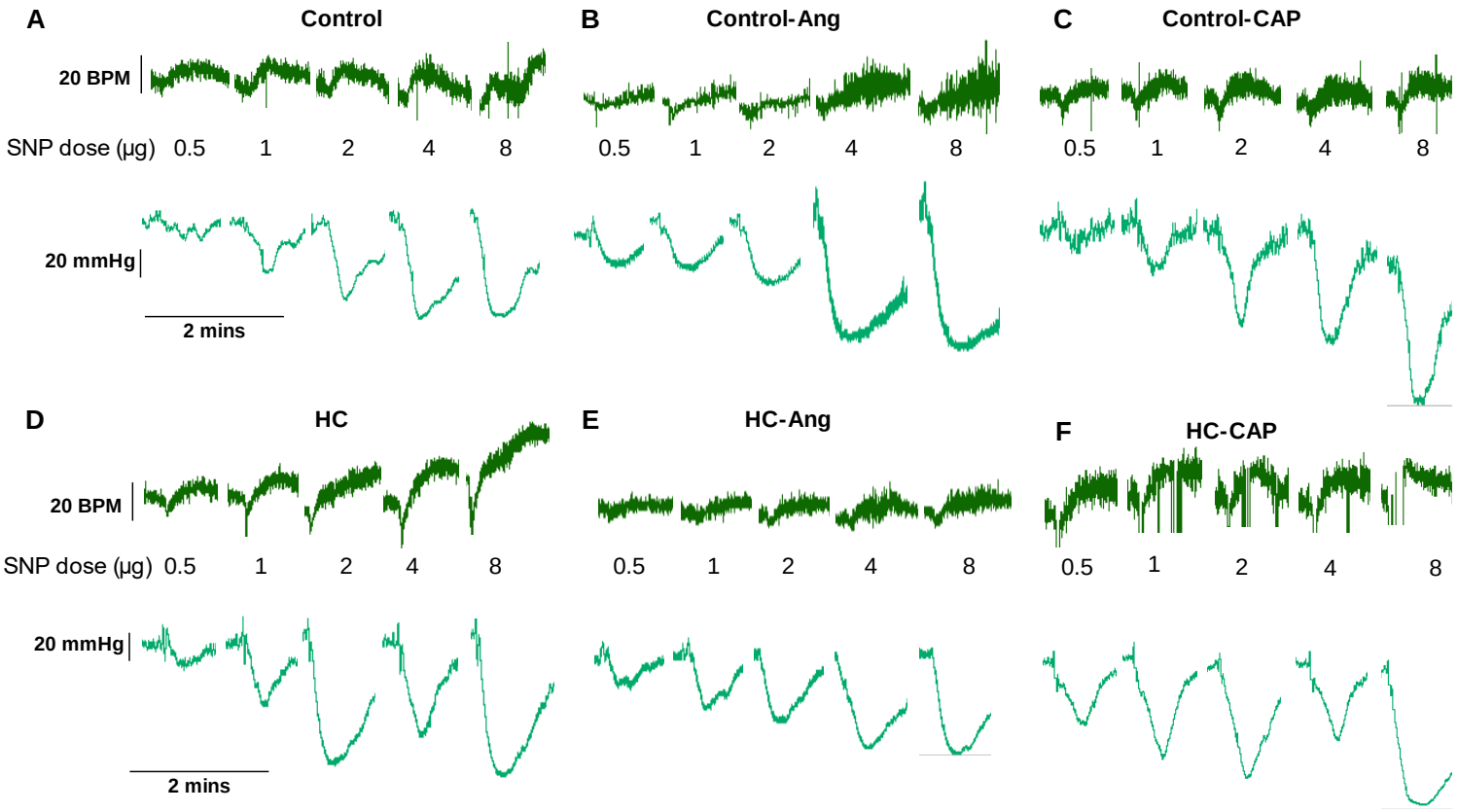

Figure 5. Representative tracings of depressor [mean arterial pressure (MAP)] and cardiac [heart rate (HR)] responses to increasing doses of sodium nitroprusside (SNP) in A, Normal control; B, Control-Ang II; C, Control-Captopril; D, HC; E, HC-Ang II and F, HC-captopril rats. Vertical scale bars represent MAP (20 mmHg) and HR [20 beats/min (BPM)], and horizontal scale bars represent time (2 minutes).

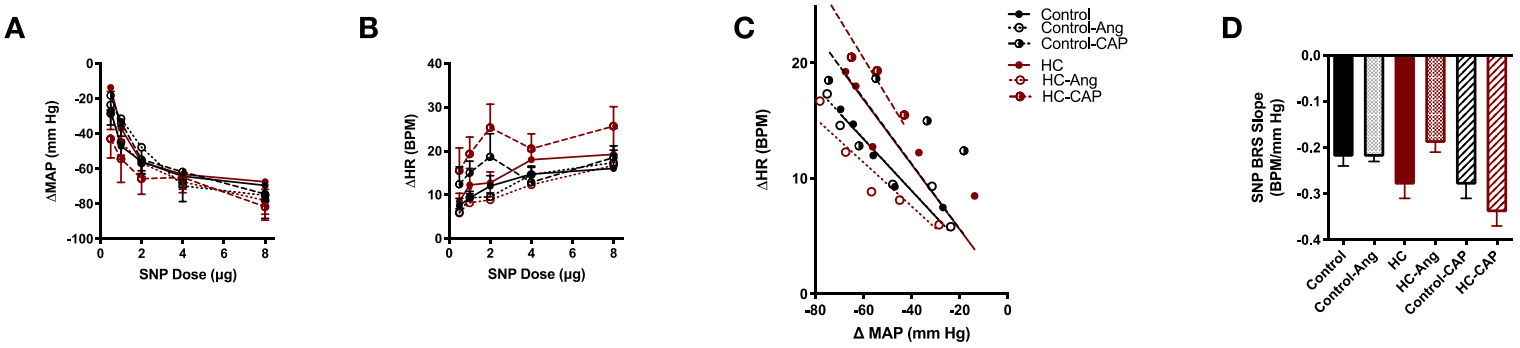

Figure 6. The effect of treatment with either slow-pressor doses of angiotensin II or non-hypotensive doses of captopril on the sensitivity of the sympathetic arm of the baroreflex in both control and HC rats. A, Depressor effect of different doses of SNP in different treatment groups. B, The reflex tachycardic responses to different doses of SNP. C, Best-fit regression lines for the correlation between changes in MAP in response to increasing SNP doses and the reflex change in HR. D, Statistical comparison of the slopes of the best regression line of the ΔMAP versus ΔHR in response to different SNP doses in different treatment groups. Values are Mean ± S.E.M. Statistical significance was determined by two-way ANOVA followed by Sidak's *post hoc* test (A, B, C) or one-way ANOVA followed by Tukey *post hoc* test (D). * and # denote *P<0.05* versus response at corresponding points in control and HC groups respectively.

IV. Isolated Rat Kidney Perfusion Test

To test for the integrity of the renovascular endothelial function, the isolated perfused kidney test was performed. Rats from different groups underwent surgical isolation of their right kidneys after which the change in perfusion pressure mounted by PE in response to increasing doses of CCh was assessed. Despite showing some endothelial-

dependent relaxation to CCh, the isolated perfused kidneys from HC-fed rats did not show a reduction in this response after treatment with blockers of endothelial mediated vasodilators *i.e*. prostaglandins and nitric oxide (Fig.7D). In NC-Ang II treated rats the vasodilatory response to CCh persisted. However, the addition of endothelial mediator blockers flipped the dose response curve into a constrictor response as compared to NC rats (Fig.7B). The HC-Ang II treated group showed a lack of vasodilatory response to increasing doses of CCh which was not affected further by the addition of the blockers (Fig.7E). Control-Cap treated rats (Fig.7C) showed similar responses to that of the control rats (Fig.7A). HC rats treated with captopril improved the endothelial function as seen by the reverted dose response curve to a phenotype similar to that of the control group (Fig.7F)

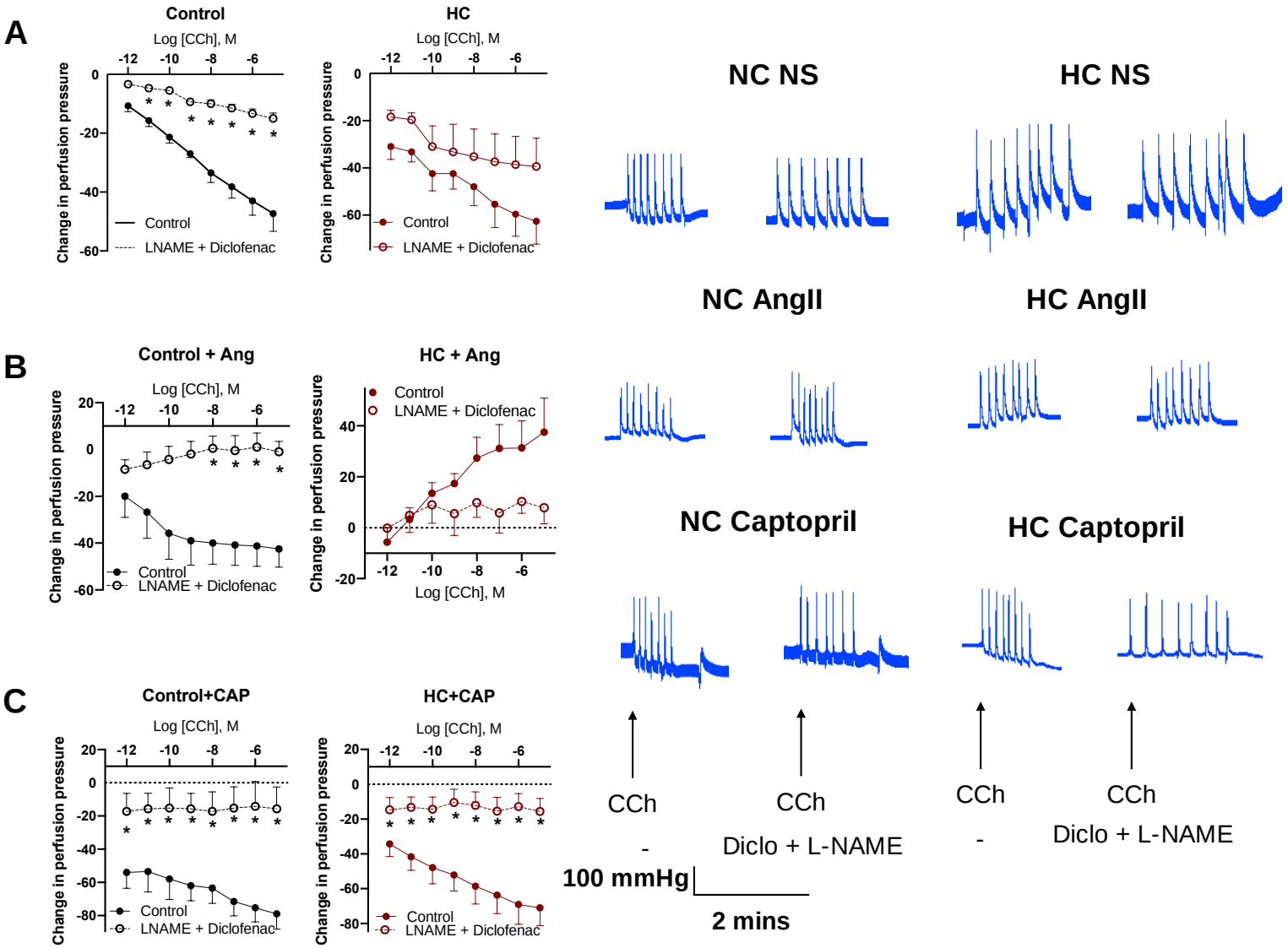

Figure 7. Changes in renal perfusion pressure as a function of increasing doses of the endothelium- dependent vasodilator carbachol (CCh) in isolated perfused rat kidneys preconstricted with PE (10μM) in the absence and presence of different inhibitors of prostaglandins [Diclofenac, cyclooxygenase inhibitor] and nitric oxide [LNAME, a non-specific endothelial nitric oxide synthase inhibitor (eNOS)]. A, Responses to CCh-induced vasodilation in isolated perfused kidneys of control rats in the absence and presence of LNAME/Diclofenac mixture. B, Responses to CCh-induced vasodilation in isolated perfused kidneys of NC-Ang II rats in the absence and presence of LNAME/Diclofenac mixture. C, Responses to CCh-induced vasodilation in isolated perfused kidneys of NC-Cap treated rats in the absence and presence of LNAME/Diclofenac mixture. D, Responses to CCh-induced

vasodilation in isolated perfused kidneys of HC-fed rats in the absence and presence of LNAME/Diclofenac mixture. E, Responses to CCh-induced vasodilation in isolated perfused kidneys of HC-Ang II rats in the absence and presence of LNAME/Diclofenac mixture. F, Responses to CCh-induced vasodilation in isolated perfused kidneys of HC-Cap treated rats in the absence and presence of LNAME/Diclofenac mixture. Values are Mean ± S.E.M. Statistical significance was determined by two-way ANOVA followed by Sidak's *post hoc* test. * denotes $P<0.05$ versus controls. To the right, the representative tracings of the isolated rat kidney perfusion test, it represents endothelial-dependent vasodilatory responses to increasing carbachol doses in the absecence and presence of LNAME +Diclofenac mixture the corresponding inhibitors of eNOs and COX enzymes. Vertical scale bars represent MAP (100 mmHg), and horizontal scale bars represent time (2 minutes)

V. Microscopic changes in Aortic tissues

Hematoxylin and Eosin (H and E) staining indicated no changes in the aortic medial thickness in the different groups as depicted by their corresponding micrographs and related quantification (Fig.8)

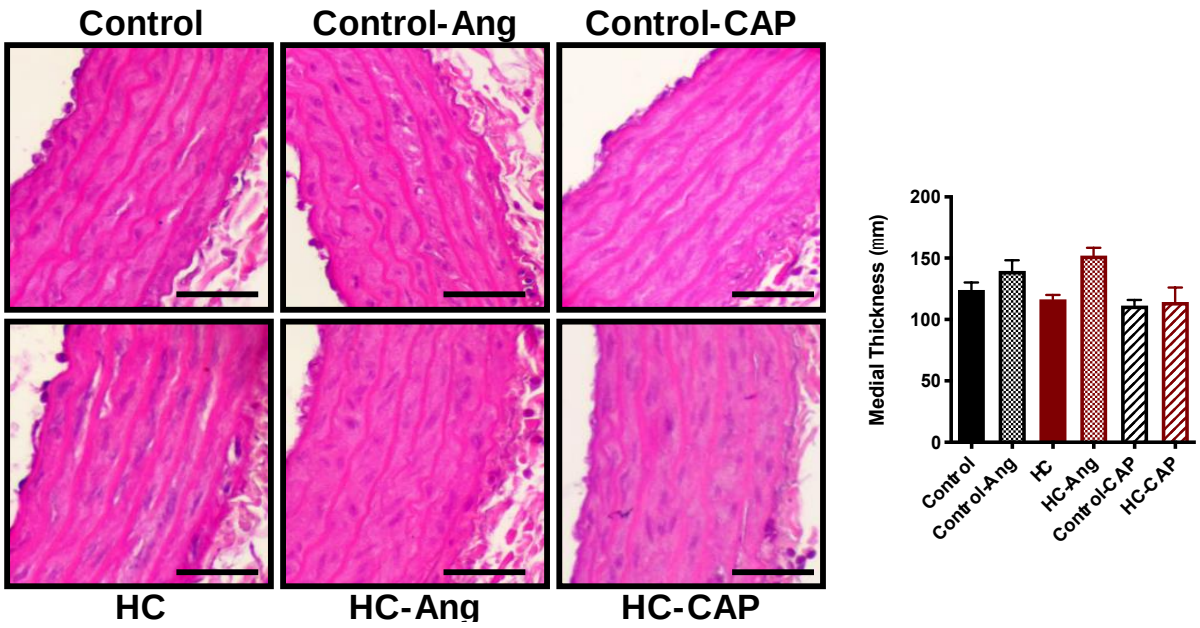

Figure 8. H&E stained micrographs of aortic sections together with quantification of medial thickness. Scale bars are 50 μm. Depicted data represent ± S.E.M of results obtained from 9 slides from 3 different rats per. Statistical significance was determined by one-way ANOVA followed by Tukey *post hoc* test.

VI. Inflammation in Brainstem

As compared to controls, both Ang II treated groups (control and HC) and HC rats showed elevated IBA1 levels in the brain stem. These levels indicative of microglial activation and consistent with increased inflammation were much more aggravated with the HC-Ang II group as compared to HC only fed rats. Moreover, captopril treatment ameliorated this inflammatory phenotype in HC-rats manifested by significantly reduced IBA1 levels (Fig.9)

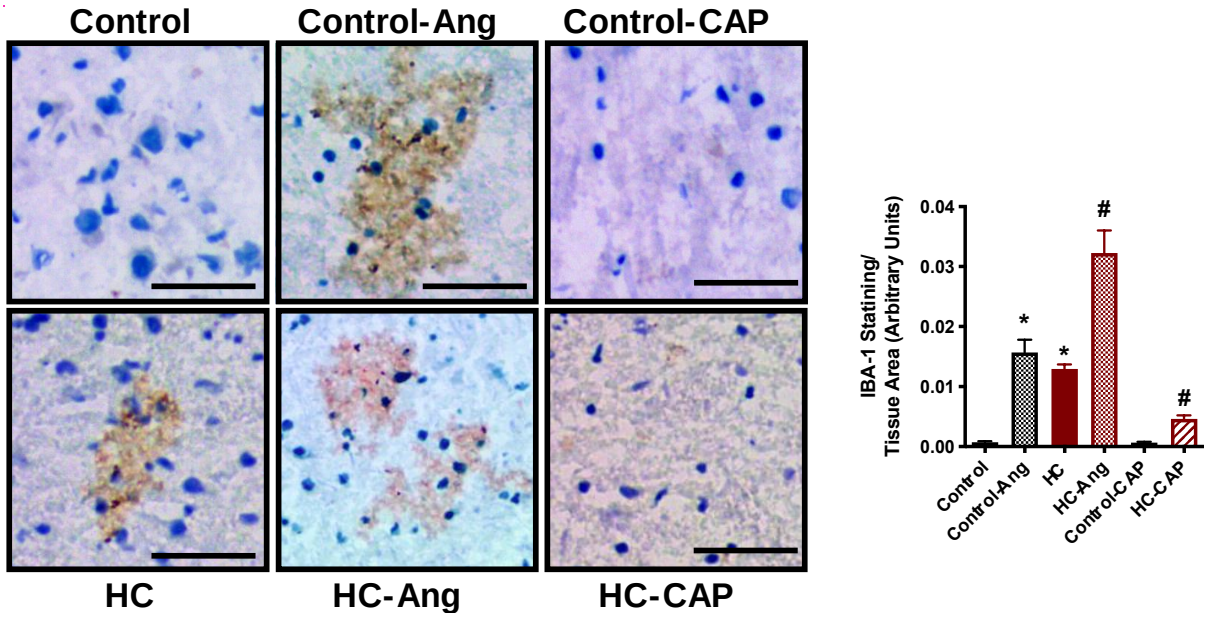

Figure 9. Representative micrographs of IBA-1 staining in the brainstem and its corresponding quantification in the different allocated groups. Scale bars are 50 μm. Depicted data represent Mean ± S.E.M of results obtained from 9 slides from 3 different rats per group. Statistical significance was determined by one-way ANOVA followed by Tukey *post hoc* test. * and # denote *P<0.05* versus response at corresponding points in control and HC groups respectively.

VII. Microscopic changes in the renal tissue

The microscopic examination of PAS- stained sections demonstrated structural/ morphological changes of the kidney glomeruli manifested by the increased Bowman capsule area to that of the total area of the renal corpuscle in all high caloric and/or Ang II treated groups which might be indicative of glomerular hypertension (Fig. 10). On the other hand, the Masson Trichrome (MTC)-stained kidney sections revealed tubulointerstitial fibrosis in HC-fed and both Ang II-treated groups (Fig. 11)

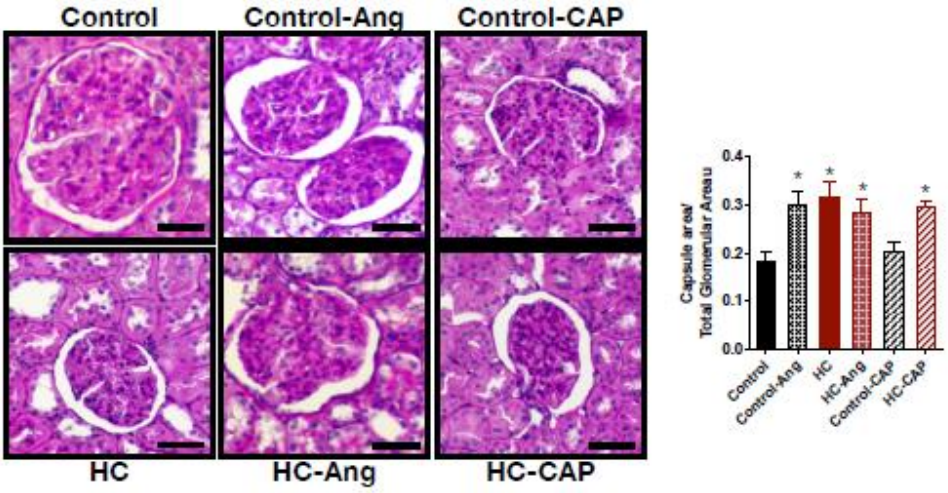

Figure 10. Representative micrographs of kidney glomeruli stained with Periodic Acid Schiff stain (PAS) and their corresponding quantification in the different groups involved: control, control-Ang II, control-cap, HC, HC-Ang II, and HC-cap. Scale bars are 25 μm. Values are represented ± S.E.M of results obtained from 9 slides from 3 different rats per group. Statistical significance was determined by one-way ANOVA followed by Tukey *post hoc* test. * denotes *P<0.05* versus response at corresponding points in control .

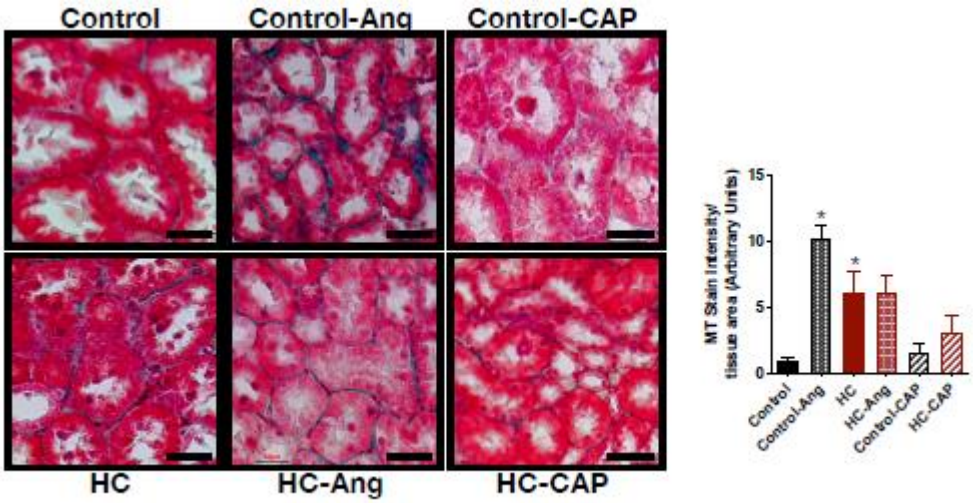

Figure 11. photomicrographs (stained with Masson's trichrome) of kidney tubules of Sprague-Dawley rats and their corresponding quantification in the different groups involved: control, control-Ang II, control-cap, HC, HC-Ang II, and HC-cap. Values are represented ± S.E.M of results obtained from 18 slides from 6 different groups. Statistical significance was determined by one-way ANOVA followed by Tukey *post hoc* test.* denotes *P<0.05* versus response at corresponding points in control.

CHAPTER IV
DISCUSSION

Tight glycemic control was insufficient in halting the progression of cardiovascular and renal diabetic complications present in many of the recently diagnosed patients. This implicates the involvement of mechanisms apart from those associated with hyperglycemia as potential initiating causes of this pathology. In the process of identifying such pathways, and as literature describes the tight relationship between metabolic syndrome, obesity, and diabetes, adipose tissue inflammation had been suggested as a principal cause standing behind the etiology of insulin resistance and thus impaired glucose control in diabetic people. As such, studying adipose inflammation is prudent to unravel the potential pathways involved in cardiovascular dysfunction early in the course of metabolic impairment and to identify molecular targets for pharmacological intervention.

Previous work done in our laboratory revealed that early metabolic challenge in the pre-diabetic stage was associated with perivascular, epicardial, and perirenal adipose tissue inflammation with CAN affecting the parasympathetic arm of the autonomic nervous system. In addition, significant cardiovascular, renovascular, cerebrovascular, and cognitive alterations were observed [80]. Furthermore, the endothelium-dependent relaxation was also affected where Kir channels responsible for potentiating the endothelium-dependent hyperpolarization in the vessel wall were found to be down-regulated. All these insults happened in the absence of hyperglycemia, systemic inflammation, gross structural tissue damage, and sympathetic involvement. In the present study, we extended our research to investigate the status of local adipose tissue RAS during this early stage of metabolic impairment and examine its impact on

cardiac autonomic and renovascular function. As well, we examined whether the usage of an ACEI can ameliorate or reverse the changes occurring. Our results showed that all Ang II-treated groups led to a cardiac autonomic deterioration similar to that observed in prediabetic HC-fed animals. Moreover, this autonomic impairment was aggravated in the latter group (HC) after Ang II treatment as assessed by the baroreflex sensitivity. On the other hand, the impaired renovascular endothelial-dependent relaxation seen in HC-fed rats was further exacerbated with chronic Ang II infusion. Meanwhile, brainstem inflammation and renal structural damage were detected in Ang II-treated control rats and HC-fed rats but increased to higher levels upon Ang II treatment of the latter group. Interestingly, these abnormalities were ameliorated by a non-hypotensive dose of captopril.

In this regard, the HC-fed rat model a modified version of the high-fat diet model was adopted for our study. This animal model consumed 38% caloric intake as fat, which is slightly higher than the normal ADA recommendations (20-35%)[106], yet within the lower range (20-60%) of a high-fat diet model that is often used for the study of various metabolic conditions including diabetes and insulin resistance [118].

This HC-fed model provides a relatively long window of time for the study of metabolic and cardiovascular alterations occurring during early prediabetes. All salient features of metabolic syndrome and prediabetes are progressively developed including insulin resistance associated with hyperinsulinemia, impaired lipid profile (elevated non-HDL cholesterol and triglycerides), and increased fat to lean ratio. These alterations occur in the absence of hyperglycemia, hypertension, increased body weight and abnormal parameters of cardiac function. Moreover, this diet produced stable fasting hyperglycemia after 16 weeks of treatment which eventually degenerates into overt diabetes after 24 weeks of feeding.

Our present results revealed no significant differences in random blood glucose, glycosylated hemoglobin (HBA1c), and echocardiographic parameters of heart function namely ejection fraction (indicative of both systolic and diastolic function) and fractional shortening (mostly indicative of systolic function), which is consistent with the previous work done and validating the pre-diabetic nature of the rat model. Fat to lean ratio was increased in HC-fed rats indicative of adipose tissue expansion and probably AT inflammation which is also in line with the metabolic features of the rat model [80].

Non-invasive blood pressure measurement revealed an elevated systolic blood pressure in only the HC-fed rat group treated with Ang II, which was not the case with Ang II-treated control rats. The increased sensitivity of HC-fed rats to the slow-pressor dose of Ang II cannot be explained by a non-specific increase in vascular reactivity since both control and HC-fed groups developed approximately equal pressor responses to increasing doses of phenylephrine. Moreover, the impact of Ang II on the aortic medial thickness as revealed by H &E stain was similar in both groups. As such, the observed changes in renal function might be a more likely cause of the observed hypertension in HC-Ang II group. A recent study reported that early metabolic deterioration is associated with renal impairment induced by perirenal adipose tissue (PRAT) inflammation where inflammatory mediators (cytokines, macrophages) were transferred from PRAT to the renal cortical tissue in a paracrine manner and contributed to renal functional and structural pathologies [80]. Here we examined whether Ang II might have played a role in the observed phenotype.As expected and in line with previous results [80],the HC-fed rats showed functional endothelial dependent dilation to increasing doses of carbachol, however and as compared to their control counterparts the typical reno-vascular relaxation mediators including nitric oxide and prostacyclin were lost

and the observed CCh- mediated dilatory responses- based on a recent study - can be attributed to an adaptive mechanism that involved increased generation of an arachidonic acid derivative, epoxyeicosatetranoic acid (EET) (fig.7D) [80].This compensatory vasodilatory effect -that was EET- dependent- was completely lost in HC-Ang II treated group as revealed by their corresponding dose-response curve which flipped into a pure constrictive phenotype (fig.7E). This vasoconstrictive phenotype was paradoxically reduced once LNAME and diclofenac the respective endothelial vasodilator blockers of nitric oxide and prostacyclin were added. These findings could be best explained by the fact that Ang II in the HC-Ang II rats might have either shifted the metabolism of arachidonic acid towards formation of a vasoconstrictor eicosanoid, 20-hydroxyeicosatetraenoic acid (20-HETE) instead of EET, and the former is well known to play a role in the regulation of vascular tone, kidney function and the evolution of hypertension [119] or that Ang II may have up-regulated or increased the activity of the enzyme thromboxane synthase that catalyzes the formation of the vasoconstrictor mediator thromboxane A2 (TXA2) from PGH2 and this could be further supported by the reduced vasoconstrictive responses to CCh once a cyclooxygenase inhibitor (COXI) was added. COXIs prevent the synthesis of both prostaglandins and thromboxanes. It is noteworthy mentioning that despite the maintained vasodilatory responses to increasing doses of CCh in the NC-Ang II rats, its dose-response curve showed a reduced vasodilatory tendency in response to the successively increased CCh doses which might confirm the Ang II effect previously mentioned and indirectly indicate the presence of an endogenous Ang II component that might have been up-regulated with all HC- fed animals (fig.7B). Moreover, HC rats treated with captopril had an improved reno-endothelial function which further suggests the increased formation of Ang II in HC rats. Based on all of the above, the hypertension observed with HC-Ang II rats is

probably reno-vascular in origin and has to do with the ability of Ang II to elevate the generation of vasoconstrictor mediators, as such the HC kidneys were producing less vasodilators and therefore they were more sensitive to the effect of exogenous Ang II as compared to their controls. Furthermore, the Ang II infused aggravated the already compromised reno-vascular endothelial function causing a marked vasoconstrictor effect particularly on the efferent renal arteriole [120]. This probably led to increased GFR and hyperfiltration possibly underlying the increased area of the Bowmans space exaggerated in the HC-fed rats treated with Ang II suggestive of glomerular hypertension[121]. Furthermore, the same group revealed increased tubular fibrosis that might be indicative of early chronic kidney disease [122]. It is noteworthy that a previous study on the same rat model reported that endothelial dysfunction of renal blood vessels in rats commenced during the prediabetic stage under the effect of a high caloric diet[80], which was also seen in our results as well, moreover it indicated the presence of proteinuria and hyperfiltration [80]. Finally, as captopril ameliorated the renovascular insult observed in HC-fed rats and changed the response to a pattern comparable to that of the controls. It is worth mentioning based on the literature, low doses of RAS blockers are sufficient to improve the metabolic changes taking place [41]. Moreover, long-term treatment with an ACEI was associated with increased insulin sensitivity and reduced oxidative stress secondary to its impact on fat mass reduction [41].

Significantly, when compared to other allocated groups, the Ang II- treated rats had a reduction in caloric intake and body weights (Figure 2B). This weight loss can be best explained by the possible central effects of Ang II. Paradoxically, adipose- derived Ang II exerts a fat-building effect peripherally that would be counteracted centrally potentially by decreasing energy storage mainly through reducing food intake and increasing energy

expenditure. Specifically, it was found to enhance BAT thermogenesis and WAT lipolysis via increasing sympathetic signaling to these adipose pools leading to negative energy balance [27]. Moreover, centrally induced Ang II resulted in increased hypothalamic expression of the corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) which together mediate anorexigenic effects[27]. One study done by Brink *et al* reported that Ang II caused weight loss through a pressor independent mechanism whereby serum Ang II caused a reduction in the level of the insulin-like growth factor 1 (IGF1) which is responsible for growth-promoting effects. In this study, Ang II –infused hypertensive rats lost 18-26% of their body weight during the first week of Ang II infusion compared to their sham counterparts, moreover, treatment with losartan but not with hydralazine was able to reverse the changes in both body weight and circulating IGF1 which indicated that Ang II-mediated its weight loss effect through the AT1 receptors independently of its vasoconstricting one [123]. Indeed, this central effect of Ang II might be related to the observed CAN. Ang II infusion was associated with brainstem inflammation in control rats that was exaggerated in HC-fed rats. Furthermore, the central effect of Ang II could have underlied the sympathovagal imbalance at the core of CAN in these conditions.

Whereas the insult (cardiac and renal) was aggravated in HC-fed rats upon Ang II infusion, control rats seemed to respond to Ang II in a manner recapitulating that observed in HC-fed rats. As such, it might seem plausible that adipose tissue inflammation in HC-fed could have precipitated the observed cardiac autonomic and renal phenotype through increased Ang II production or sensitivity.Thus, we proceeded to characterize whether there is a contribution of endogenous Ang II during this early stage of metabolic impairment. Significantly, treatment with captopril reversed the cardiac autonomic deterioration and was able to restore the

baroreceptor sensitivity to values comparable to that of control rats. Moreover, captopril treatment ameliorated the renovascular phenotype in HC-fed rats. This treatment was also associated with a positive impact on the inflammatory and structural alterations observed in the kidney and brainstem, respectively. On this basis, one can conclude the presence of an upregulated endogenous Ang II whose production is basically triggered by PVAT inflammation induced by the high caloric fat-based diet. Captopril, in turn, disrupted the production of this *de novo* octapeptide and thus alleviated the insult. Nevertheless, recent research indicated that the overproduction of Ang II by a fat pool is related to an imbalance in the two counteracting arms of the RAS system that is the ACE/Ang II/ AT1R arm versus the ACE2/Ang 1-7/Mas receptor axis. Several studies demonstrated that mice lacking AGN, AT1, or AT2 receptors were protected from diet-induced obesity and AT expansion while those lacking the Mas receptor featured excess abdominal fat mass associated with increased adipose tissue AGN expression, which infers the crucial role played by Ang 1-7 in terms of regulation of Ang II formation in the AT[40]. Accordingly, any deterioration or dysregulation of Ang 1-7 levels could alter that of Ang II and result in their overproduction. Excess Ang II formed promotes lipid storage via both AT1 and AT2 receptors, which synergistically result in down-regulation of lipolysis and up-regulation of lipogenesis, respectively, leading to AT expansion, hypertrophy, and inflammation[40]. In WAT, the Ang II –AT1 R axis is pro-inflammatory mostly through triggering MCP-1, IL-6 and IL-8 release from adipose cells via NF-kb – dependent mechanism. Moreover, Ang II also stimulates NADPH oxidase that increases ROS formation, which in turn stimulates the NF-kb pathway. On the other hand, the Ang1-7/ Mas axis exerts anti-inflammatory effects via reducing NF-kb signaling and enhancing adiponectin secretion from the primary adipocytes[41].

In conclusion, our present work outlines the involvement of local RAS system in the PVAT and PRAT inflammation during early metabolic derangement. The resultant Ang II is involved in the pathology of CAN and kidney impairment. These conditions can be targeted early in the course of the metabolic disease to avoid further cardiovascular and renal (nephropathy) diabetic complications. This is possible using a non-hypotensive dose of an ACEI (captopril) that can prevent or slow the progression of these metabolic-induced anomalies potentially by interfering with adipose inflammation.

CHAPTER V

LIMITATIONS AND FUTURE DIRECTIONS

Invasive and some of the non-invasive experiments were done in the presence of anesthesia which may affect the hemodynamic measurements and might contribute to additional stress to the animal. Alternatively, continuous telemetric blood pressure measurements could be used. Furthermore, our work was done on male rats only. Gender-driven differences should be taken into account as there are well-established variations in terms of adipose pools distribution in females versus males. Whereas females tend to accumulate fat in subcutaneous –gluteal and femoral depots, the visceral fat is the most common form of energy storage in males. These differences might affect how the adipose tissue RAS is regulated during early metabolic impairments especially in females where the presence of estrogen is known to down-regulate ACE.

Future research would also be required to identify the temporal relation between dysregulation in ACE/ACE2 expression and the subsequent development of PVAT inflammation where the extent of protein expression of the following: ACE, ACE2, Ang II, and Ang 1-7 would be determined, compared and analyzed in PVAT of HC-rats. We can also intervene pharmacologically with the Ang 1-7 pathway and examine its effect on PVAT inflammation, renal and cardiovascular function like using a Mas receptor agonist AVE 0991. Moreover, we can check the impact of previously used drugs with established pleiotropic anti-inflammatory effect on ACE/ACE2 expression like statins, metformin, and pioglitazone. We can also allocate another group under the condition of HC/Ang II /Captopril and examine the effect of the concomitant use

of an ACEI in the presence of chronic Ang II infusion on the vasculature and renal function. Further molecular data are required to test for ROS, local and systemic markers of inflammation.

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