The Development and Progression of Renal Damage in Streptozotocin-Type1 Diabetes Mellitus under Goldblatt Renovascular Hypertension and High-Salt Condition

by

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Abstract

Under normotensive conditions, the progressive loss of renal function in diabetes mellitus is very slow. Since hypertension accelerates many forms of renal disease, we assessed the progression of nephropathy in Streptozotocin-induced type 1 diabetes mellitus under renin-mediated hypertension condition. We investigated the diabetic "salt paradox" as a modifiable susceptibility factor for renal damage. Since hyperfiltration occurs in early diabetes, the reduction of glomerular filtration rate due to an increased salt intake could be mediated by increased tubuloglomerular feedback sensitivity. We compared intact-hypertensive versus diabetic-hypertensive Long-Evans rats under normal and increased salt intake, 1 and 2.5% by weight of food eaten, respectively. Weekly 24-h blood pressure records were acquired by telemetry during the six months of the experiment. Target mean blood glucose of ~ 25 mmol/L was maintained by suboptimal insulin implants. Systolic blood pressure increased after induction of hypertension but was not affected by diabetes or increased salt intake, either alone or together. Autoregulation was highly efficient in both intact and diabetic rats. Nephropathy was scored by histology in the clipped and non-clipped kidneys at the end of the protocol. The

non-clipped kidney, which was exposed to hypertension, showed a linear pressure-dependent glomerular injury in both intact and diabetic rats. The best fit line describing the linear relationship between pressure load and injury was shifted toward lower blood pressure in diabetic rats. Over the time course of our experiments, injury was entirely pressure dependent in intact and diabetic rats. Diabetes mellitus increased the susceptibility of the kidney to injury, but independent of blood pressure. Increased salt intake affected neither blood pressure nor renal susceptibility to hypertensive injury.

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%	percent
2K1C	two-kidney one-clip
ANG II	angiotensin II
BL	body length
bpm	beats per minute
BW	body weight
BW: BL	body weight to body length ratio
CK	clipped kidney
CKD	chronic kidney disease
CTL	control week
DM	diabetes mellitus
DM1	first week of diabetes
DM2	second week of diabetes
DM3	third week of diabetes
DM-HS	diabetic high salt
DM-NS	diabetic normal salt
DT	distal tubule
ECF	extracellular fluid
ESRD	end-stage renal disease
g/24h	grams per 24 hours
GFR	glomerular filtration rate
GS	glomerulosclerosis
HT	hypertension
IN	intact
IN-HS	intact high salt
IN-NS	intact normal salt

JGCs	juxtaglomerular cells
L/min	litres per minute
MD	macula densa
mg/24h	milligrams per 24 hours
mg/kg	milligrams per kilogram
ml/24h	millilitres per 24 hours
mmHg	millimetres of mercury
mmol/L	millimols per litre
MR	myogenic response
NCK	non-clipped kidney
NO	nitric oxide
ºC	degrees Celsius
PE 50	tubing for venous catheter
PE 90	tubing for arterial catheter
PG	prostaglandins
PT	proximal tubule
RAS	renin-angiotensin system
RBF	renal blood flow
SBP	systolic blood pressure
STZ	streptozotocin
STZ-T1DM	streptozotocin-induced insulin-dependent diabetes model
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TBS	total body sodium
TGF	tubuloglomerular feedback
WK1	first week of salt treatment
WK2	second week of salt treatment
⋮	
WK14	fourteenth week of salt treatment

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Chapter 1 – Introduction

1.1 Main Causes of Chronic Kidney Disease

Chronic Kidney Disease (CKD), characterized by a progressive and permanent loss of kidney function, is a worldwide health problem (U.S. Renal Data System, 2009). There is strong evidence that Diabetes Mellitus (DM) and HyperTension (HT) are, first and second, respectively, the most common causes of chronic kidney disease (U.S. Renal Data System, 2009). The prevalence of hypertension in diabetic patients is approximately twice that of nondiabetic patients (Arauz-Pacheco *et al.*, 2002) and thus, hypertension has been generally accepted as a major contributor to the development and progression of nephropathy in both Type1 DM (T1DM) and Type2 DM (T2DM) (Ritz *et al.*, 2001; Sampanis and Zamboulis, 2008). Additionally, the incidence of End-Stage Renal Disease (ESRD) is particularly high when diabetes mellitus and hypertension coexist and exacerbate each other. Therefore, a thorough understanding of the pathophysiological mechanisms of nephropathy in diabetes remains one of the major challenges of biomedical research.

1.1.1 Diabetic nephropathy

Diabetic nephropathy (Kimmelstiel-Wilson syndrome) is characterized *clinically* by persistent hyperglycemia, hypertension, proteinuria, and progressive renal insufficiency, and *histopathologically* by different forms of glomerular, vascular, and tubulointerstitial injuries (Shafrir, 2003; Forbes *et al.*, 2007). Since hyperglycemia and hypertension are the main clinical determinants of this disease, it has been hypothesized that the complex interaction between metabolic and hemodynamic factors plays an important role in diabetes-induced renal damage. These factors are discussed next, along

with the central mechanisms responsible for the initiation and progression of nephropathy in diabetes.

1.1.1a Metabolic disorders

Persistent hyperglycemia is a necessary feature, but not sufficient in itself, for the development of diabetic nephropathy. Several studies have shown that hyperglycemia can be harmful, not only through the non-enzymatic reaction of glucose with proteins and the subsequent accumulation of advanced glycation end-products, but also through specific metabolically-driven, glucose-dependent pathways (such as oxidative stress, polyol pathway, hexosamine flux) which increase production of different types of cytokines (Adler *et al.*, 1993; Lehmann and Schleicher, 2000; Forbes *et al.*, 2007). All these pathways are activated within the diabetic renal tissue and collectively promote fibronectin-induced cell proliferation, mesangial matrix expansion and collagen synthesis, which are markers of renal injury. However, hyperglycemia alone does not fully explain these changes. Findings of diabetic lesions have been reported in the absence of hyperglycemia which suggests that factors other than long-lasting hyperglycemia may contribute to the pathogenesis of this disease (Wiwanitkit, 2009).

1.1.1b Hemodynamic disorders

Systemic and intrarenal hemodynamics abnormalities, along with the inappropriate activation of neurohormonal mechanisms, have also been proposed as major factors in the initiation and progression of nephropathy in diabetes. These factors are discussed next.

Increased systemic blood pressure

Studies that followed the natural history of T1DM in normotensive animals (Bidani *et al.*, 2007; Lau *et al.*, 2009) and humans (Jacobsen *et al.*, 1999) have revealed that the progression of kidney disease is rather slow. However, when systemic hypertension occurs, the progression of any renal disease is always accelerated despite

the underlying condition (El Nahas, 1989; Fogo, 2000). The concept that the failure of renal autoregulation leads to the transmission of systemic hypertension to the glomeruli was proposed commonly as one of the main mechanisms responsible for the development of renal injuries (Bidani and Griffin, 2004; Cupples and Braam, 2007; Bidani *et al.*, 2009)

Glomerular hyperperfusion - hyperfiltration - hypertension

Changes in glomerular hemodynamics, such as increases in Renal Blood Flow (RBF), Glomerular Filtration Rate (GFR), and intraglomerular pressure, are the primary events that occur early in the course of diabetes (Hostetter *et al.*, 1981; Vallon *et al.*, 2003).

A larger reduction of the afferent than of the efferent resistance has been initially proposed as the pathogenetic mechanism of diabetic hyperperfusion and hyperfiltration (Brenner *et al.* 1996). Mechanistically, Vallon *et al.* (2003) suggested that changes in the Proximal Tubular (PT) growth with secondary hyperfunction would better explain the glomerular hemodynamic changes in diabetes mellitus. Early in the evolution of diabetes the increased filtered load of glucose would be followed by an increased reabsorbtion of glucose, in concert with sodium, in the proximal tubules. Consequently, the reduction in the delivery of salt to the Distal Tubule (DT) would activate the TubuloGlomerular Feedback (TGF) reflex that further, would lead to vasodilation and increased GFR in order to return the distal salt delivery to its normal set point (Vallon *et al.*, 2003). However, what is incompletely understood is the diabetes “salt paradox”, which induces the reduction of the proximal tubular reabsorption and of glomerular hyperfiltration in the presence of modestly increased dietary salt (Vallon *et al.*, 2003).

Findings of an increased intraglomerular pressure in diabetes (Hostetter *et al.*, 1981; Brenner *et al.*, 1996; Fogo, 2000; Giunti *et al.*, 2006) and the prevention of glomerular injury through controlling it (Meyer *et al.*, 1987; Brenner *et al.*, 1996) induced the idea that glomerular hypertension is essential to the onset and progression of diabetic nephropathy. However, when there is only minimal glomerular enlargement, glomerular hemodynamic changes are accompanied by marginal glomerulosclerosis (Fogo, 2000). Therefore, adaptative glomerular hypertrophy, with dilated capillary loops,

was also linked to the increased susceptibility of glomeruli to the potentially harmful effects of systemic and glomerular hypertension (El Nahar, 1989; Fogo, 2000).

In conclusion, both systemic and renal hemodynamic disorders have been recognized as central to the development of diabetic nephropathy, not only through inducing structural changes (mechanical stretch, hypertrophy and hyperplasia), but also through activating pro-fibrotic and pro-sclerotic factors (El Nahas, 1989; Fogo, 2000; Shafrir, 2003, Bidani and Griffin, 2004; Kriz and Le Hir, 2005; Marin *et al.*, 2005; Giunti *et al.*, 2006).

Renin-Angiotensin System

The renin-angiotensin system (RAS), and especially its vasoactive molecule angiotensin II (ANG II), have been recognised as being strongly involved in the pathogenesis of chronic kidney diseases (Kobori *et al.*, 2007). Recent studies place an emphasis on the major influence of the local intrarenal RAS activation on the pathophysiology of nephropathy in diabetes. An inappropriate activation of this system would contribute not only to the glomerular hemodynamic alterations, but also to the glomerular hypertrophy and to tissue injury by stimulating growth factors, inflammation, immuno-modulation, and fibrogenesis (Fogo, 2000; Lehmann and Schleicher, 2000; Leehey *et al.* 2000; Wolf and Wenzel 2004; Forbes *et al.,* 2007; Kobori *et al.*, 2007). The renoprotective effects of both angiotensin-converting enzyme inhibitors and angiotensin receptor antagonists support these findings (Nagai *et al.*, 2005; Kobori *et al.*, 2007). However, the efficiency of these drugs in attenuating the progression of renal injury appears to be greater in the early rather than the later stages of sclerosis (Fogo, 2000).

1.1.1c Proteinuria

The presence of proteins in urine has been generally accepted as a characteristic feature of renal damage. Therefore, microalbuminuria is recognized as an early clinical manifestation of diabetic nephropathy in the absence of any other apparent clinical signs (Francis *et al.*, 1997; Mauer and Drummond**,** 2002; Marshall, 2004; Kriz and Le Hir, 2005). In the late stage of diabetes, the rate of decline in renal function correlates closely

with the level of proteinuria and, consequently, can be ameliorated by reducing proteinuria (Drummond and Mauer 2002; Marshall, 2004; Regeniter *et al.*, 2009).

The proteinuria in diabetic nephropathy typically has a glomerular origin. The high intraglomerular pressure, loss of the negatively charged glycosaminoglycans of the cellular basement membrane, the broadening of basement membrane pore size, or the transudation of plasma proteins into the endothelial and subendothelial space are some of the commonly proposed trigger mechanisms (Marshall, 2004). Since all these pathological changes occur together with a continuous increase in blood pressure, the presence of tubular proteinuria, along with glomerular proteinuria, predicts the end-stage renal disease risk (Marshall, 2004; Regeniter *et al.*, 2009).

1.1.1d Histological features

Diabetic nephropathy exhibits various pathological lesions in humans and other animals, such as nodular (pathognomonic lesion), focal segmental or diffuse glomerulosclerosis, extraglomerular hyaline arteriolosclerosis, and tubulointerstitial fibrosis. While some researchers inferred that mesangial matrix expansion plays the primary role in the progression of diabetic nephropathy, other researchers highlighted the role of podocytes structural changes. The former considered that the progressive expansion of the mesangial material due to the accumulation of extracellular matrix proteins, along with the thickening of the glomerular basement membrane, decreases the surface available for filtration and eventually leads to glomerular and arteriolar lesions (Drummond and Mauer, 2002; Marshall, 2004). For this reason, the change in mesangial fractional volume associated with glomerular enlargement was selected as the primary end point of early nephropathy in T1DM (Mauer and Drummond, 2002). However, recent experimental studies have highlighted podocyte changes along with a misdirected filtration as an early pathological manifestation of renal structural changes occurring in diabetes mellitus. Since podocytes normally provide structural support for glomerular capillaries, a persistent exposure of podocytes to hemodynamic and metabolic disturbances triggers structural and functional changes that ultimately lead to their loss. The hypertrophic glomerular tuft holding to the Bowman's capsule spreads a misdirected

filtration and engages extracapillary lesions through excessive plasma and protein leakage into tubular urine and interstitium. Ultimately, the presence of focal and global glomerulosclerosis, tubulointerstitial inflammation, and fibrosis indicates an accelerated decline in renal function and structure (Kriz *et al.*, 2001; Kriz and LeHir, 2005; Siu *et al.*, 2006).

In conclusion, the progressive decline in renal function in diabetes begins at the level of glomeruli and continues with subsequent tubulointerstitial injury. Therefore, any intervention in the early stage is subjected to restitution or repair (Kriz and LeHir, 2005), while in the late stage the lesions (hyalinized glomeruli along with advanced tubular atrophy and interstitial fibrosis) become irreversible (Adler et al., 1993; Shafrir, 2003).

1.1.2 Hypertensive nephropathy

Since we induced hypertension in our experiment in order to accelerate diabetic nephropathy, a brief presentation of hypertensive nephropathy is required.

Arterial hypertension *per se* is considered a major cause of progressive renal disease. However, the relationship between hypertension and kidney disease should be considered more as a "two-way causality" than a "domino-causality," since hypertension cannot be maintained in the presence of normal kidney function (Guyton, 1981); and, conversely, systemic hypertension accelerates the progression of any renal disease (Zucchelli and Zuccalá, 1998; Navar *et al.,* 1998).

Similar to diabetes mellitus, glomerular hyperperfusion-hyperfiltration-hypertension can develop in arterial hypertension, and is also accompanied by dysfunctional changes in pre- and postglomerular arteriolar resistances and renal hypertrophy (Brenner *et al.,* 1996; Keijzer *et al.,* 1988; Harrap *et al.*, 2000; Hultström *et al.*, 2008). However, it should be emphasized that glomerular hypertension has a greater influence in the development of glomerular injury than systemic hypertension. Since autoregulation defends renal blood flow as hypertension develops, a sustained increase in blood pressure results in a proportionate increase in renal vascular resistance (Keijzer *et al.,* 1988; Harrap *et al.*, 2000). As long as blood pressure remains within the

autoregulatory range and the renal blood flow autoregulation is effective, modulation of the glomerular hypertension is expected to reduce the susceptibility to hypertension-induced renal damage (Bidani and Griffin, 2004; Bidani *et al.*, 2009).

The characteristic histological lesions of nephrosclerosis (hypertensive nephropathy) involve a hypertrophic renal vascular response (myointimal hyperplasia of small renal arteries, thickened and folded glomeruli, hyaline arteriosclerosis), which in turn leads to ischemia by narrowing the vascular lumen. These changes are thought to be adaptative responses to the rise in systemic blood pressure in order to minimize the transmission of high blood pressure to the glomeruli and renal microvasculature (Luke, 1999). However, the histopathological end-points are the same as in diabetic nephropathy, focal and segmental glomerulosclerosis, plus extensive inflammatory and fibrotic tubulointerstial lesions (Meyrier, 1999; Luke, 1999). Therefore, the understanding of diabetic kidney disease is problematic due to the presence of similar renal functional and histopathological features in both diabetic and hypertensive nephropathy (Zhou and Frohlich, 2003).

1.2 Background Knowledge

Under normal physiological conditions, blood pressure and the body content of water and electrolytes are kept within narrow limits (Reinhardt and Seeliger, 2000; Eaton and Pooler, 2004). Since the mechanisms that control blood pressure and body fluid osmolarity are extremely complex, the discussion below concentrates only on the aspects relevant to this project.

1.2.1 Control of systemic blood pressure

To maintain blood pressure close to normal values, three control mechanisms are generally required: short-term (baroreceptor reflex via autonomic nervous system), intermediate-term (renin and ANG II), and long-term (kidneys). These three control

mechanisms are not independent; they are interrelated and influence each other in a feedback manner. However, the kidneys are responsible for determining the set-point for mean blood pressure by resetting the renal excretion of sodium and water and thus keeping the balance between intake and output (Guyton *et al.*, 1972; Reinhardt and Seeliger, 2000; Eaton and Pooler, 2004).

1.2.1a Baroreceptor reflex

The short-term stabilization of arterial pressure is mediated by the baroreceptor reflex. Inputs from specialized pressure sensors located mainly in the carotid arteries and aortic arch ascend to the vasomotor center from where the efferent pathways of the baroreceptor reflex send signals to the heart, blood vessels and kidneys via the autonomic nervous systems. For example, increased baroreceptor activity due to blood pressure load enhances parasympathetic activity to the heart, suppresses sympathetic tone to the heart and blood vessels, and causes a reduction of heart rate and cardiac output (which is the volume of blood pumped by the heart) in order to bring blood pressure down. These effector mechanisms usually operate fast to stabilize blood pressure. Under special conditions, when the rise in blood pressure is maintained longer, the autonomic nervous reflexes become ineffective (Guyton, 1981) and both intermediate-term and long-term regulations of blood pressure are initiated (Eaton and Pooler, 2004).

1.2.1b Renin-Angiotensin System

The Renin-Angiotensin-System (RAS) is an enzymatic and hormonal cascade that normally plays an essential role in the maintenance of the circulatory homeostasis. It starts with renin biosynthesis and secretion in the kidney JuxtaGlomerular Cells (JGCs) and ends with the generation of multiple active angiotensin peptides. However, Angiotensin II is the main active peptide of the RAS that mediates and modulates complex physiological processes in the body through binding to its high affinity membrane-bound receptors (Kobori *et al.*, 2007). The regulation of renin release from the kidney mainly involves four interdependent factors: 1) *sympathetic nerve stimulation via*

β1 adrenergic receptors, which causes renin release and leads to afferent arteriolar constriction; 2) *TubuloGlomerular Feedback mechanism* that couples the changes in the delivery of salt to the Macula Densa (MD) cells of the distal tubule inversely to the renin secretion; 3) an *intrarenal baroreceptor* mechanism that senses changes in renal perfusion pressure 4) a *negative feedback mechanism by a direct action of ANG II* on the JGCs (Persson, 2003; Eaton and Pooler, 2004; Atlas, 2007). Besides the circulating RAS generated in the JGCs (which has an important role in the regulation of blood pressure and sodium homeostasis), the locally produced ANG II manifests in various tissues and organs. Under physiological condition, ANG II is generally known as a regulator of sodium transport in the kidney, and a stimulator of renal proximal tubular reabsorption. However, under pathological condition, ANG II not only could exert an important role in renal damage through vascular and non-vascular pathways, but also could act differently from systemic RAS (Persson, 2003; Braam and Koomans, 2006; Atlas, 2007; Kobori *et al.*, 2007; DeMello and Re, 2009).

1.2.1c Pressure diuresis and natriuresis mechanism

The *pressure diuresis* (*natriuresis*) mechanism plays a key role in the long-term control of blood pressure. In an extensive series of studies, Guyton *et al.* (1972) demonstrated the importance of the kidney-volume-pressure system in regulating blood pressure and showed that this mechanism often displays infinite gain. In brief, according to Ohm's Law applied to fluid flow, blood pressure is the product of cardiac output and total peripheral resistance; therefore, any change in blood pressure can be due to changes in blood flow, vascular resistance, or both. Under normal conditions, any increase in total body sodium causes an increase in blood volume. The changes in blood volume affect blood pressure through an increase in cardiac output, followed by an opposing increase in peripheral resistance (Coleman *et al.*, 1972; Klabunde, 2005). The resulting increase in renal perfusion pressure due to blood pressure elevation induces sodium excretion via a *pressure natriuresis* mechanism, thus decreasing total body sodium and extracellular volume, and bringing the blood pressure back to control levels. However, persistence of

an excess blood volume in the circulatory system almost invariably leads to severe hypertension (Guyton, 1981).

Later, Hall *et al.* (1986) showed that an increased renal arterial pressure is essential for maintaining normal excretion of sodium and water in hypertension. They showed that pressure natriuresis and diuresis were effectively prevented and significant sodium and water retention occurred when renal arterial pressure was servo-controlled. Therefore, an increase in blood pressure normally occurs to compensate for sodium (volume)-retaining abnormalities (Granger *et al.*, 2002). However, a large body of evidence shows that, as well as increased renal perfusion pressure, the intrinsic intrarenal mechanisms efficiently mediate the pressure diuresis (natriuresis) process. The inhibition of the tubular sodium reabsorption, the modulatory effect of paracrine signalling (such as ANG II, Nitric Oxide (NO), Prostaglandins), increased renal interstitial hydrostatic pressure, and renal medullary blood flow are only a few of the mechanisms proposed that operate in concert with the pressure natriuresis mechanism to control blood pressure (Evans *et al.*, 2005).

In conclusion, pressure natriuresis and diuresis seem to be more effective at controlling blood volume (dependent upon salt and water excretion), while additional specific neurohormonal mechanisms are operative in independently controlling salt and water balance (Guyton, 1981; Eaton and Pooler, 2004).

1.2.2 Relationship between salt and blood pressure

Given that salt plays an important role not only in the body physiology, but also in blood pressure regulation, the relationship between salt and blood pressure is discussed next.

Sodium, the principal extracellular cation, has a considerable influence on serum osmolality: typically, 90% of the ExtraCellular Fluid (ECF) osmotic content is accounted by sodium and the anions that accompany it, and only 10% of the ECF osmotic content is represented by potassium, glucose, urea, and other solutes (Edelman *et al*., 1958; Eaton and Pooler, 2004).

Since blood pressure depends on volume homeostasis, and volume homeostasis depends on sodium balance, an equilibrium between sodium intake and excretion is required in order to maintain a constant Total Body Sodium (TBS) (Eaton and Pooler, 2004). Considering TBS as a controlled variable, normally, any alteration in TBS triggers complex compensatory mechanisms that reverse body fluid and blood pressure back to normal (Reinhardt and Seeliger, 2000). However, some debate still exists about the mechanisms involved in the independent control of salt and water excretion. Major changes in renal sodium and water excretion may occur without any primary change in arterial pressure and seem mediated exclusively by neurohormonal factors (Rasmussen *et al.*, 2003; Bie *et al.*, 2004).

On the other hand, during particular pathological conditions (such as diabetes or renal failure), the presence of other osmotically active solutes (such as glucose, urea, lipids, proteins, potassium) contribute to the total serum osmolarity in proportion to their concentrations, and influences sodium concentration and blood volume in an attempt to maintain the osmolarity of the extracellular compartment (Ackerman, 1990). For example, the correction of hyperglycemia-induced hyponatremia is common in hemodialysed patients; it is usually due to an inefficient osmotic diuresis associated with water shift out of the cells (Penne *et al*., 2010).

1.2.2a Salt sensitivity of blood pressure

There is strong evidence that some humans and other animals are predisposed to develop hypertension during sodium loading (Sullivan, 1991). *Salt Sensitivity* is defined as an increase in blood pressure in response to increased salt ingestion. Many factors (such as genetic predisposition, imperfect kidney excretion, neuroendocrine dysregulation) are identified in the literature as risk factors for the pathogenesis of salt sensitivity and some have common ground. Salt-sensitive individuals manifest a greater propensity to retain sodium, increase blood volume, vascular resistance and cardiac output (Koomans *et al*., 1982; Ito and Abe, 1997; Gonzalez-Albarran *et al*., *1998*; Weinberger *et al*., 2001; Haddy, 2006).

A possible role of RAS in mediating the salt sensitivity of blood pressure has also been suggested by the atypical RAS activation in normotensives versus hypertensives. For example, when salt intake is altered, normotensive humans and animals show only a minor change in blood pressure, but large compensatory changes in plasma renin activity (Rasmussen *et al.*, 2003; He and MacGregor, 2003). On the other hand, the alteration of salt intake in some hypertensives induces a major change in blood pressure with minor changes in plasma renin activity (He and MacGregor, 2003).

Moreover, ANGII influences blood pressure level not only by direct action on vascular smooth muscle and kidney, but also by acting on the sympathetic nervous system. Normally high salt intake increases the extracellular fluid volume and decreases ANGII and sympathetic activity (Bealer, 2002, Yoshimoto *et al.*, 2004***)***, while the (non-adaptative) lack of normal suppression in ANGII and/or sympathetic activity in response to increases in salt intake can produce salt-sensitive hypertension (Brooks, 1997). Therefore, RAS seems to have a substantial role in stabilizing blood pressure over a wide range of salt intakes and extracellular fluid volume fluctuations (He and MacGregor, 2003), and both the circulating RAS and the local RAS (with their possible opposing functions) should be analysed during specific pathological events (Braam and Koomans, 2006; Atlas, 2007; DeMello and Re, 2009).

In conclusion, complex neural, hormonal, or bio-chemical mechanisms are commonly activated to compensate the changes in body homeostasis, but the kidney's ability to perform its physiological functions appropriately also plays an important role in this critical stability (Navar, 1998; Reinhardt and Seeliger, 2000).

1.2.3 Autoregulation of blood flow

Under normal physiological conditions, body tissues are able to regulate their blood flow in accordance with their metabolic and functional needs (Navar, 1998; Klabunde, 2005). Early observations of a coordinated action between the "volume factor" (fluid overload) and the "constrictor factor" (peripheral vascular resistance) highlighted

the importance of the renal vascular tone adjustments in circulatory homeostasis (Coleman *et al.*, 1972; Korner, 1980; Guyton, 1981). Findings that the systemic blood pressure does not increase if there is not a concomitant increase in renal vascular resistance, and conversely, that a persistent increase in renal vascular resistance usually implies a resetting of the arterial pressure in order to maintain the blood flow to an adequate level (Guyton, 1981), raised the idea that in the "hierarchy of control schema within the body, the control of blood flow is more important than the control of arterial pressure" (Coleman *et al.*, 1972). Therefore, the *autoregulation* of renal blood flow specifically refers to the intrinsic ability of the kidney to maintain a relatively stable blood flow over a wide range of arterial pressure (Guyton, 1981; Navar, 1998; Cupples and Braam 2007; Carlson *et al.*, 2008).

1.2.3a Renal blood flow autoregulation

Under resting conditions, the kidney has a very high blood flow (2% of body mass receives ~20% of cardiac output) that sustains the normal high glomerular filtration and, consequently, high tubular reabsorption (Braam *et al.*, 1993; Cupples and Braam, 2007). While extrinsic mechanisms (such as circulating hormones or sympathetic nerves) set the level of renal blood flow (Grady and Bullivant, 1992; Brooks,1997; Cupples and Braam, 2007), the autoregulatory intrinsic mechanisms are jointly responsible for stabilizing renal blood flow, GFR and glomerular capillary pressure at constant levels despite continuous fluctuation of arterial pressure (Navar, 1998; Cupples and Braam, 2007).

Renal autoregulation operates primarily on pre-glomerular resistance and is mediated by two mechanisms: a *Myogenic Response* (MR) and *TubuloGlomerular Feedback* (TGF). The myogenic response refers to the intrinsic property of vascular smooth muscle to adjust its vascular tone in response to changes in intravascular perfusion pressure. An increase in renal perfusion pressure elicits the renal afferent arteriolar myogenic response by constricting its smooth muscles and increasing renal vascular resistance. However, the myogenic response may be modulated by vasoactive endocrine and paracrine signals (Ito and Abe, 1997; Navar, 1998; Loutzenhiser *et al.*,

2006; Cupples and Braam, 2007). The TGF mechanism also adjusts the afferent arteriole resistance, but in response to changes in the distal tubular fluid composition, and contributes not only to the regulation of the filtered load, but also to sodium homeostasis. An increase in salt delivery to the macula densa activates the TGF mechanism that reduces GFR by afferent arteriolar constriction. The TGF responsiveness may also be modulated by paracrine factors and changes in extracellular fluid volume. For example, the expansion of extracellular fluid volume diminishes the TGF sensitivity and thus allows a greater delivery of fluid and electrolytes to the distal nephron for any given level of GFR. Similarly, during hypovolemia, RAS activation and increased TGF sensitivity stimulate proximal tubular reabsorption and contribute to fluid and electrolytes conservation (Braam *et al.*, 1993; Ito and Abe, 1997; Navar, 1998; Braam and Koomans, 2006; Cupples and Braam, 2007). Since the glomerulus is a high-flow and -pressure capillary bed, it is prone to physical injury. Accordingly, the impressive renal blood flow autoregulation not only contributes to the regulation of body salt content and fluid balance, but also to the preservation of the glomerular structure and kidney function (Navar, 1998; Loutzenhiser *et al.*, 2006; Cupples and Braam, 2007).

In conclusion, MR and TGF share the same effector (afferent arteriole) and therefore, the stabilization of renal blood flow, glomerular filtration rate, and glomerular capillary pressure occurs in parallel intrinsically (Ito and Abe, 1997; Loutzenhiser *et al*., 2006; Cupples and Braam, 2007). However, MR and TGF seem to play distinct roles in regard to protection and regulatory function; TGF may be a less efficient regulator of glomerular capillary pressure than of GFR (Loutzenhiser *et al*., 2006).

1.3 Goldblatt Renovascular Hypertension

Despite a thorough understanding of the physiological mechanisms that control blood pressure, the pathophysiological aspects of hypertension are still not well understood. Two main categories are commonly described: primary (essential) and secondary hypertension. Essential hypertension (no direct cause can be identified) is encountered in about 88% of patients with elevated blood pressure but is difficult to study

because it is a multifactorial disorder. Secondary hypertension is less common, but has the advantage of having identifiable causes and therefore being much more easily interpreted (Ganong, 2005).

One of the first animal models of secondary hypertension was developed by Loesch (1933) and **Goldblatt** (1934) based on their findings that hypertension could be experimentally created by unilateral renal artery stenosis and subsequent renal ischemia (Pinto *et al.*, 1998; Glodny B and Glodny D, 2006). Since Goldblatt renovascular hypertension was induced in this experiment in order to accelerate the progression of diabetic kidney disease, the pathophysiology of Goldblatt hypertension along with its relationship with salt and renal damage progression is presented next.

1.3.1 Pathophysiology of the Goldblatt hypertension model

The **Goldblatt hypertension model** (**2K1C,** two-kidney one-clip model) was induced in our experiment for its three main advantages: 1) hypertension is initiated by increased renal renin secretion and its development is mediated by ANG II; 2) hypertension develops as a result of the kidney's inability to maintain the fluid and electrolyte balance; and, 3) hypertension is chronic in rats, as well as in humans (Navar *et al.*, 1998; Pinto *et al.*, 1998; Brands and Labazi, 2008).

In summary, the reduced renal perfusion pressure in the clipped (stenotic) kidney stimulates renin synthesis and release from that kidney. Renin activates its enzymatic cascade and ultimately generates ANG II, which in turn increases blood volume and subsequent, total peripheral resistance. Meanwhile, the non-clipped kidney which becomes renin depleted due to a progressive elevation in blood pressure, increases sodium and water excretion. However, the renin-depleted non-clipped kidney does not display an appropriate pressure-natriuretic response to the increased arterial pressure, and fails to prevent the development of hypertension (Navar *et al.*, 1998).

Since large temporal changes in plasma renin levels have been described during the development of Goldblatt hypertension, three phases were theoretically proposed (Martinez-Maldonado, 1991; Amiri and Garcia, 1997; Pinto *et al.*, 1998).

Phase I (acute phase) persists for about two to four weeks after the clipping of the renal artery, and is dependent mainly on systemic RAS activity. Therefore, in this phase, volume expansion, along with the elevated total peripheral resistance, has been inferred for the development of hypertension. Yet, numerous other systemic or local factors also concur with, or oppose, the hemodynamic actions of RAS (Martinez-Maldonado, 1991). For example, an imbalance of high levels of NO (fluid shear stress-induced nitric oxide production) compared to ANG II in the non-clipped kidney may explain the high flow and glomerular pressure in the non-clipped kidney, as well as the relative protection from Na retention during this early phase (Sigmon and Beierwaltes,1993; Turkstra *et al.,* 2000).

Phase II (moderate phase) occurs four to nine weeks after clipping the renal artery, and the renal hemodynamic responses depend on both systemic and intrarenal RAS activation in the maintenance of hypertension. Renal plasma flow in the non-clipped kidney starts to decrease, but the remaining elevated filtration fraction also suggests enhanced local renal ANGII activity in the non-clipped kidney (Tokuyama *et al.,* 2002). Greater angiotensin converting enzyme tissue activity or increased ANGII receptor sensitivity are only two of the mechanisms proposed as the cause of the locally generated ANG II (Oates, 1976; Okamura *et al.,* 1986; Amiri and Garcia, 1997).

Phase III (chronic phase) occurs more than nine weeks after clipping the renal artery. Plasma renin activity usually tends to return to near normal levels; therefore, it was assumed that factors other than the direct systemic vasoconstrictor activity of the RAS would contribute to the persistence of hypertension during this phase (Martinez-Maldonado, 1991; Amiri and Garcia, 1997). A volume-dependent phase or a particular TGF response (with the direct stimulatory action of ANG II on the non-clipped kidney) are only two of the mechanisms proposed to explain the impairment in the non-clipped kidney's ability to maintain normal rates of sodium excretion at normotensive pressures during this phase (Amiri and Garcia, 1997; Navar *et al.*, 1998). In conclusion, the 2K1C hypertension model is largely ANG II-dependent, but systemic levels do not appropriately reflect intrarenal levels and, as well, other influences can contribute to the hemodynamic changes during Goldblatt renovascular hypertension development.

In regard to the severity of the renal ischemia, our previous experiments and those of others (Möhring *et al.*, 1976; Santos *et al.*, 2005) showed that the greater the renal artery constriction, the bigger the increase in systemic blood pressure. Accordingly, a wide range of pressure values would be followed by consequent differences in the rate of progression of renal injury.

1.3.2 Relationship between salt and 2K1C hypertension

The influence of salt in the development of hypertension, along with the relationship between sodium and ANG II, has been mentioned in the previous sections. However, in studying diabetes and its interaction with hypertension in terms of renal injury, the effects of salt on the development of 2K1C hypertension were also of interest.

Contradictory findings are reported with respect to the relationship between salt and the 2K1C experimental model: salt intake in the 2K1C model did not affect, attenuate, or delay an increase in blood pressure (Möhring *et al.*, 1976; Jackson and Navar, 1986; Sato *et al.*, 1991; Lee *et al.*, 1991; Liu *et al.*, 1993). One hypothesis is that the initial retention of Na in the early phase of 2K1C is concurrent with increased plasma renin activity and volume expansion (Tobian *et al.*, 1969, Sato *et al.*, 1991). The rise of arterial pressure, along with the stimulation of natriuresis in the non-clipped kidney, maintains the body sodium at a lower level than normal; usually, rats have a stimulated RAS but a negative sodium balance (Ando *et al.*, 1990). Under high-salt intake, these animals are able to compensate for sustained sodium and water loss, and the activity of plasma RAS is normally suppressed. Therefore, on high-salt diet plasma renin activity is suppressed not only in normal rats but also in 2K1C (Möhring *et al.*, 1976; Jackson and Navar, 1986; Liu *et al.*, 1993).

On the other hand, salt sensitivity of blood pressure has been shown in ANG II-induced hypertension (DeClue *et al.*, 1978; Hall *et al.*, 1980; Ando *et al.*, 1990) when the chronic administration of ANG II blocked the normal pressure natriuresis and produced markedly elevated blood pressure. Therefore, a level of plasma ANG II (which remains constant across different levels of salt intake) along with volume expansion and

increased sympathetic nervous system activity, was hypothesised as an important factor that determines the salt sensitivity of blood pressure (Hall *et al.*, 1980; Sato *et al.*, 1991; Liu *et al.*, 1993; Brooks, 1997).

1.3.3 Renal damage in 2K1C hypertension

Hypertension-induced renal injury is commonly interpreted by the degree to which blood pressure is transmitted to the renal microvasculature (Griffin and Bidani, 2006). Renal autoregulation mechanisms provide the primary protection against the damaging effects of elevated systemic blood pressure load (Bidani and Griffin, 2004; Loutzenhiser *et al.*, 2006). While impairment of the renal autoregulation has been found in the early phase of 2K1C (that was, about four weeks after 2K1C induction), which was mainly nitric oxide dependent (Turkstra *et al.*, 2000), to the best of our knowledge there is no evidence of renal autoregulation assessments in the late stage of 2K1C. However, in the late stages of any chronic kidney disease, systemic and intrarenal nitric oxide production normally decreases (Majid *et al.*, 1998; Wever *et al.*, 1999), while local renal ANG II activity increases and triggers hypertrophic renal vascular responses to chronic elevations in renal perfusion pressure. All these changes constrict and narrow the vascular lumen and maintain RBF and GFR at lower levels, and also increase TGF responsiveness (Braam *et al.*, 1993; Luke, 1999; Bidani and Griffin, 2004).

On the other hand, some studies showed that tubulointerstitial damage precedes the glomerular injury in the non-clipped kidney in the early phase of 2K1C (Mai *et al.*, 1993; Wenzel *et al.*, 2005); others support the scenario of nephron degeneration in classic focal segmental glomerulosclerosis through glomerular tuft adhesion to Bowman's capsule and spreading of a misdirected filtrate (Kriz *et al.*, 2001; Kriz and LeHir, 2005). However, the elevated renal perfusion pressure accounts for the majority of glomerular and tubular injuries and the histopathological end-points are invariably extensive inflammatory and fibrotic glomerulo-tubular lesions (Meyrier, 1999; Luke, 1999; Polichnowski and Cowley, 2009).

1.4 Diabetes Mellitus under Hypertension and High-Salt Condition

Diabetes mellitus is a metabolic disorder characterized by inappropriately high blood sugar levels. It occurs due to either a deficiency of insulin secretion (T1DM) or impaired effectiveness of insulin (T2DM), or both (Masharani and German, 2007). Through affecting blood circulation and the nervous system, diabetes mellitus involves the entire body and, over time, this condition can easily become life-threatening.

In order to understand the diabetic pathophysiological changes, many experimental animal models have been developed. The Streptozotocin-induced insulin-dependent diabetes model (STZ-T1DM) is one that is widely used for its main advantage of a rapid and permanent hyperglycemia (O'Donnell *et al.*, 1988; McNeill, 1999).

In our research we used the diabetes mellitus-hypertension comorbid condition in order to accelerate the progression of diabetic kidney disease, and also salt as a potentially modifiable factor of renal damage susceptibility. Therefore, in this section, the impact of hypertension and salt in the progression of diabetic kidney disease is presented mainly in connection with the STZ-T1DM experimental model.

1.4.1 Relationship between diabetes mellitus and hypertension

Even after many years of research, the relationship between diabetes mellitus and blood pressure is still not well understood. While there is strong evidence that hypertension is present in the end-stage of diabetic kidney disease, there is inconsistency regarding the presence of hypertension in early diabetes mellitus. Some researchers have stated that the onset of T1DM causes a significant and sustained increase in the mean arterial pressure through changes in the intravascular volume through hyperglycemia-induced osmotic fluid shifts (Brands and Hopkins, 1996; Miller,1999); at the same time, blood pressure has also been found to be reduced or unchanged in the early stages of diabetes since pressure natriuresis and osmotic diuresis have the ability to return blood pressure to its control levels (Brands and Hopkins, 1996; Miller,1999;

Jacobsen *et al.,* 2003). The general consensus supports the statement that blood pressure is unchanged or reduced following Streptozotocin administration (McNeill, 1999; Tatchum-Talom *et al.,* 2000; Bidani *et al.,* 2007; Lau *et al.,* 2009) and a resting bradycardia has also been routinely observed (McNeill, 1999; Dall'Ago *et al.,* 2002; Lau *et al.,* 2009). The exact mechanism that mediates a reduction in the resting heart rate is still under debate, even though many hypotheses have been described, including increased parasympathetic traffic to the heart, a decline in sympathetic tone, changes in electrophysiological properties of the sinoatrial node, a hypothyroid state, and intrinsic metabolic carnitine deficiency (Shah *et al.,* 1995; McNeill, 1999; Dall'Ago *et al.,* 2002; Malone *et al.,* 2007; Gross *et al.,* 2008).

As mentioned in the previous sections, renal hyperperfusion, hyperfiltration, and glomerular hypertension are commonly associated with hyperglycemia in the early phase of diabetes (Vallon *et al.,* 1997, Miller, 1997, Hostetter *et al.,* 1981; Nakamoto *et al.,* 2008). Selective changes in vascular resistance of glomerular afferent and efferent arterioles (such as decreased afferent arteriole resistance or increased efferent arteriole resistance, or both) would be responsible not only for the specific diabetes-induced increases in GFR and glomerular capillary pressure, but also for the initiation of the later renal injuries (Hostetter *et al.,* 1981; Navar, 1998; Fogo, 2000). Therefore, it was presumed that any factor that would alter pre-glomerular resistance would influence susceptibility to diabetic renal damage.

Some studies have shown a diminished response of the dilated afferent arteriole to a variety of vasoconstrictor stimuli (Vallon *et al.,* 1997; Arima and Ito, 2003), and an attenuation of both autoregulatory mechanisms that contributed to an impaired renal autoregulation in early diabetes (Braam *et al.,* 1993; Ito and Abe, 1997; Vallon *et al.,* 2003, Arima and Ito, 2003, Brands and Labazi, 2008). In contrast, other studies have shown that higher renal vascular resistance and filtration fraction are linked to an increased TGF sensitivity in moderate to severe hyperglycemia and in long-lasting diabetes (Hostetter *et al.,* 1981; Braam *et al.,* 1993; Navar, 1998; Carmines, 2010). Activated intrarenal RAS and increased TGF sensitivity may contribute to the preservation of autoregulation in diabetes (Lau et al 2009). However, the increased

activity of the TGF mechanism at this stage is not sufficient to reduce GFR back to normal (Braam *et al.,* 1993), and RAS action on both pre- and post-glomerular resistance may contribute to the development of glomerular hypertension (Miller, 1999).

On the other hand, the experimental data indicate that increased glomerular capillary pressure is a necessary but not a sufficient condition for the initiation and progression of diabetic nephropathy. While some researchers have shown that controlling glomerular pressures and flows (Zatz *et al.,* 1986) may effectively prevent glomerular structural injury under pronounced hyperglycemia, others have shown no difference in glomerular injury between normotensive and hypertensive diabetic rats that have had a significantly elevated glomerular capillary pressure (Bank *et al.,* 1987).

In conclusion, even if systemic and glomerular hemodynamic changes observed in diabetes are considered the principal factors responsible for the development of diabetic nephropathy, the temporal changes (early and long-lasting diabetes) and the metabolic state (moderate or severe hyperglycemia) of diabetes should be taken into consideration when interpreting outcomes. Hyperglycemia and other events that occur simultaneously or progressively (such as increased urinary albumin excretion) may also play an important role in renal function decline.

1.4.2 Relationship between diabetes mellitus and salt

The influence of increased dietary salt intake on diabetes mellitus has been extensively studied in humans and animals. However, it is not a simple matter to assess sodium balance, blood volume and blood pressure control in diabetes because GFR and natriuresis change with the extent of hyperglycemia (Brands and Fitzgerald, 2002). It has been suggested that sodium retention, due mainly to enhanced proximal tubular reabsorption (increased sodium/glucose cotransport), along with increased blood volume (Hostetter *et al.*, 1981; Brøchner-Mortensen and Ditzel, 1982; O'Donnell *et al.*, 1988), play a pivotal role in the development of hypertension in T1DM (Miller, 1997; O'Hare *et al.*, 1985; Feldt-Rasmussen *et al.*, 1987; Gerdts *et al.*, 1996; Gonzales-Albarran *et al.*, 1998). But, as discussed previously, an increase in blood pressure is not always present in

diabetes; therefore, these conflicting findings in diabetes mellitus could be due to complex adjustments of neuro-hormonal mechanisms to blood pressure, exchangeable sodium, and blood volume changes (Ballerman *et al.*, 1984; Patel, 1997; Ritz *et al.*, 2001).

Moreover, a biphasic alteration of plasma RAS has been described in diabetes: a stimulation in plasma renin activity in the early stage (due to volume depletion) (Kikkawa *et al.*, 1986; Miller, 1997; Miller, 1999), and normal or suppressed plasma renin activity in the later stages (due to volume expansion caused by the osmotic effect of hyperglycemia) (Kikkawa *et al.*, 1986; Anderson and Vora, 1993; Miller 1997; Jacobsen *et al.*, 2003). Yet, activation of RAS, together with decreased GFR, always occurs in complicated T1DM (ketoacidosis) (Kikkawa *et al.*, 1986; Feld-Rasmussen *et al.*, 1987). Therefore, ANG II has been reiterated as the factor that may underlie the altered renal hemodynamics in T1DM (Arima and Ito, 2003; Giunti *et al.*, 2006). Brands *et al.* proposed local RAS activation as a counterbalancing natriuretic influence required to maintain sodium balance restoration during severe hyperglycemia. A significant diuresis and natriuresis during poor glycemic control would threaten the body with volume loss and marked blood pressure decrease if counter-regulatory systems failed to act (Brands and Fitzgerald, 2002; Brands and Labazi, 2008).

In conclusion, even if T1DM is generally accepted as a "low renin state", since plasma renin activity usually returns to normal levels in time, the local RAS seems to act independently of the systemic RAS and some organs (such as heart and kidney) could be exposed to increased ANG II action even if the plasma renin activity is suppressed (Anderson and Vora, 1993; Leehey *et al.*, 2000; DeMello and Re, 2009).

1.4.2a Salt paradox in diabetes mellitus

The assessment of the relationship between diabetes mellitus and salt becomes even more complex due to the presence of the paradoxical effect of salt. In the case of diabetes mellitus (at least in early T1DM), the kidneys are not able to increase GFR in response to a high-salt diet. In fact, GFR varies inversely with salt intake, a phenomenon

known as "*salt paradox*" (Miller, 1997; Vallon *et al.*, 1997, Vallon *et al.*, 2003; Vallon *et al.*, 2005).

There are several proposed explanations for this phenomenon, and one of the most convincing hypotheses is "the tubulo-centric" view that is based on the proximal tubule as the initiator of the paradoxical relationship between dietary salt and glomerular filtration in early diabetes (Vallon *et al.*, 2003). In brief, in early diabetes mellitus on a normal-salt diet, a primary increase in proximal tubule reabsorption is due to both increased sodium-glucose cotransport and growth of the proximal tubule. As a result, the more glomerular filtrate is reabsorbed, the less reaches the Macula Densa and that in turn leads to TGF-mediated afferent arteriolar dilatation, and increased renal blood flow and GFR. However, under a high-salt diet, there is a major decrease in proximal tubular reabsorption followed by a TGF-mediated afferent arteriolar constriction, with reduction of renal blood flow and hyperfiltration (Miller, 1997; Vallon *et al*, 2003; Vallon *et al*, 2005).

Since autoregulation operates primarily on pre-glomerular resistance and therefore renal blood flow, GFR and glomerular capillary pressure are stabilized together; the paradoxical effect of salt in early diabetes abrogating hyperfiltration and increasing TGF sensitivity could be a modifiable susceptibility factor for diabetic kidney disease. Moreover, even if the potential modulator paracrine effect of ANG II on high-salt diabetes renal response was also invoked, there are still some debates regarding its role in mediating proximal tubular sodium reabsorption in diabetes. The ANG II preglomerular effect is commonly an indirect consequence of enhanced sensitivity of the TGF mechanism (Miller, 1997; Vallon *et al*, 1997, Brands and Labazi, 2008), but during high-salt early diabetes no influence was found of ANG II on the proximal tubule (Vallon *et al*, 2003).

Therefore, during high-salt diabetes, the relative shift of resistance between afferent and efferent arterioles is due mainly to a greater activation TGF compared to the other neurohumoral regulators of GFR (Vallon *et al*, 2003). As a result, in our study we tested whether the reduction of the deleterious effect of glomerular hyperfiltration and

kidney growth would affect autoregulatory effectiveness and prevent the progression of nephropathy in high-salt diabetes mellitus.

1.4.3 Renal damage in experimental models of diabetes

The main impediment in reproducing diabetic nephropathy in animals consists of the lack of effectiveness in inducing accelerated nephropathy (O'Donnell *et al.*, 1988; Bidani *et al.*, 2007; Brosius *et al.*, 2009). Moreover, the most characteristic histopathological lesions that are held to mimic human diabetic nephropathy (glomerular hypertrophy, expansion of mesangial matrix, capillary basement membrane thickening and loss of podocytes) have been partially found in long-term moderately hyperglycemia, since more than twelve months are required to allow a consistent development of diabetic nephropathy in rodents (Bidani *et al.*, 2007; Brosius *et al.*, 2009). Focal segmental glomerulosclerosis, which is the histological characteristic feature of STZ-T1DM rodents, is not a common feature of human diabetic nephropathy (Bidani *et al.*, 2007), but glomerular basal membrane thickening and glomerular hypertrophy are usually observed in both (Bidani *et al.*, 2007)

For this reason, we decided to use Long-Evans rats since we presumed that this strain would have a high predisposition to develop diabetic renal injuries. The Long-Evans rat is the parent strain of the Otsuka Long-Evans Tokushima fatty (OLETF) rat which reliably develops T2DM and exhibit classic diabetic nephropathy (Uehara *et al.*, 1997; Lau *et al.*, 2008). In addition, a renin hypertension model was also chosen in order to precipitate the development of diabetic nephropathy since it is not only the blood pressure that induces renal injury, but also an increased renin level has been described as augmenting proteinuria and glomerular damage (Leehey *et al.*, 2000; Brosius *et al.*, 2009). Therefore, complex interactions among altered neurohumoral factors, genetic background, and blood pressure load would contribute to the differences in susceptibility to diabetic nephropathy.

1.5 Project Overview and Objectives

Our prior results, as well as the literature, show that the progressive loss of renal function and the decline of renal structure in diabetes mellitus are, at worst, very slow under normotensive conditions, and thus create difficulties in assessing susceptibility to diabetic renal disease. Therefore, we used the **hypertension-diabetes mellitus** comorbid condition to accelerate the progression of diabetic kidney disease and to test: 1) whether the presence of diabetes mellitus would increase the susceptibility to pressure-induced renal injury, and 2) whether we could modify the variance in susceptibility by manipulating salt intake.

We expected that salt might increase the blood pressure (since renal function was compromised and ANG II levels were inappropriately high), and hypertensive injuries would result from a failure of renal blood flow autoregulation. Diabetic hyperfiltration would also impair autoregulation of renal blood flow; therefore, the diabetes would increase the renal susceptibility to develop hypertensive damage. Since the diabetic "salt paradox" increases TGF sensitivity and abrogates diabetic hyperfiltration by a relative increase of preglomerular resistance in early diabetes, we predicted that the salt paradox would influence glomerular capillary pressure, autoregulatory effectiveness, and thus renal susceptibility to damage under long-lasting diabetic conditions. We therefore manipulated salt intake to test whether the resulting changes in renal hemodynamics would affect susceptibility to nephropathy in hypertensive diabetic rats.

Chapter 2 – Materials and Methods

2.1 Overview

All procedures involving animals have been approved by the University of Victoria Animal Care Committee, and are consistent with the Guidelines promulgated by the Canadian Council on Animal Care for the use of laboratory animals.

Male Long-Evans rats were acquired from Charles River (Canada) Ltd. and housed individually. The rats were allowed free access to food and water, except for specific situations as noted below. The rats were fed Purina LabDiet 5001 containing quantities of sodium and chloride equal to 0.4% and 0.67% of the food weight, respectively. Surgical preparations for the experiments were initiated when the rats were about 10 weeks of age.

The study was comprised of two components: **chronic and acute terminal**. The chronic component involved the induction of hypertension (2K1C), diabetes mellitus (T1DM), and salt treatment; it focused on the acquisition of the hemodynamic parameters by telemetry and upon metabolic assessments. The acute terminal component assessed renal blood flow autoregulatory responses and allowed for kidney harvesting for histological evaluation.

However, due to the project's complexity, this study was performed in two legs and included 35 rats (out of a total of 42), with a wide range of blood pressure values induced by different degrees of renal ischemia. The exclusion of seven rats from the study (3 out of 24 from the first leg, and 4 out of 18 from the second leg) was due to either early death or technical (battery or signal) transmitter problems.

The advantage of having a wide range of blood pressure values, which could be correlated with the resulting kidney damage, was counterbalanced by the fact that the comorbid HT-DM condition led to an early alteration of animal health, threatening

telemetry data acquisition and histological assessments. For this reason, we decided to terminate some of the rats before the target end time of the experiment (WK14), based on a number of criteria (namely, neurological symptoms such as lethargy and seizures, pulse pressure greater than 80 mmHg, proteinuria greater than 1.5g/24h, and a decrease in body weight of more than 5% between two successive biweekly measurements) that commonly predict a sudden death.

The experimental design flow chart is presented in Figure 1. The solutions, drugs, and instruments used during the experimental procedures are presented in Appendix A.

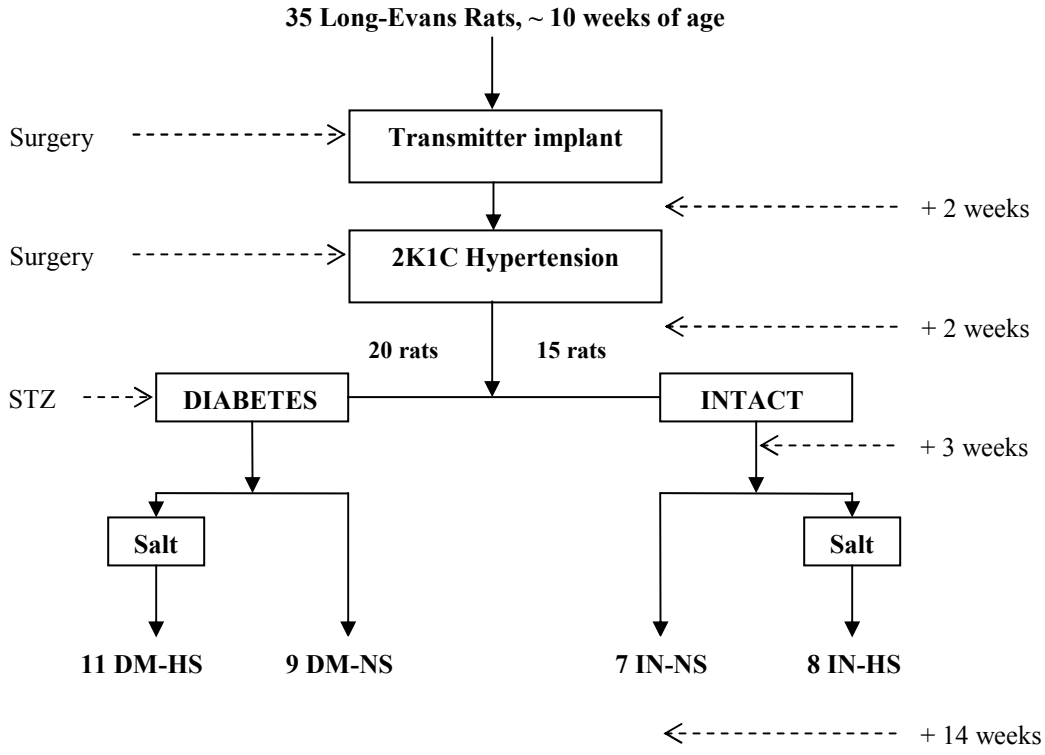

Figure 1. The experimental design flow chart comprising the four groups of rats: Intact rats with Normal-Salt intake (IN-NS), Intact rats with High-Salt intake (IN-HS), Diabetic rats with Normal-Salt intake (DM-NS), and Diabetic rats with High-Salt intake (DM-HS).

2.2 The Chronic Component of the Experiment

2.2.1 Surgical implantation of telemetry transmitters

Telemetry transmitters (model PA11-C40) from Data Sciences International (DSI, St Paul, MN, USA) were used for long-term monitoring of rat activity, blood pressures and heart rate. The major advantage of this type of data acquisition is that it allows free movement of the animal, and eliminates the stress associated with handling and restraint.

Pre-operative preparation included analgesia and sedation with Buprenorphine (Temgesic® Reckitt & Benckiser, Inc, 0.02 mg/kg,) and Medetomidine hydrochloride (Domitor®, Associated Veterinary purchasing Company *Ltd*, 0.25 mg/kg) injected subcutaneously. The lower left flank and inner thigh region was shaved, cleaned, and disinfected (Ethanol 70%, Vet Solutions Surgical Scrub) after the animal was sedated. Then the animal was placed on a homeothermic unit that maintained the rat body temperature of 37°C. The implantation of a PA11-C40 pressure telemeter having a 10 cm cannula was performed under sterile conditions and isoflurane anesthesia (1% Isoflurane carried in 0.5 L/min of Oxygen).

The skin was opened over the femoral vessels, and using blunt dissection a subcutaneous pocket was formed between the caudal edge of the rib cage and the most cranial extension of the knee's range of motion. After the transmitter was implanted in the left flank of the rat, a purse-string suture arrangement in that tissue was created in order to keep the transmitter anchored. The femoral artery was then carefully isolated from the femoral vein and the saphenous nerve. The cannula tip of the transmitter was slid into the iliac region. It was then advanced beyond the iliac bifurcation until the pressure sensing tip was situated in the abdominal aorta to the level of the renal arteries; a slack to accommodate growth was provided. After the incision was closed, the animal received Ampicillin (Associated Veterinary purchasing Company *Ltd*, 40 mg/kg) and Atipamezole hydrochloride (Antisedan®, Associated Veterinary purchasing Company *Ltd*, 0.25 mg/kg) that reversed the sedative effect of Medetomidine hydrochloride (Domitor®). The

animal was monitored until it was alert and returned to the pre-treatment physiological status.

Full recovery from surgery takes approximately 10-12 days; therefore, two weeks later, when the normal circadian rhythms were restored, a control 24-hour blood pressure record and a 24-hour metabolic assessment (comprised of water intake, food intake, and urine output) were acquired from every rat.

2.2.1a Data acquisition and analysis

Telemetry data were acquired from every rat in a 24-hour record once a week. Data were acquired at 250 Hz in 10 second bursts every two minutes throughout the day. Activity, heart rate, diastolic, systolic, and mean blood pressure were measured. We chose to report systolic rather than mean pressure because the previous analysis showed that mean and diastolic contain the same information as systolic blood pressure (Lau *et al*., 2009). In addition, using systolic pressure facilitated the comparison of our results against the long-term studies of others; it was also found to be more closely correlated with organ damage than mean or diastolic arterial pressure (Bidani and Griffin, 2004; Lau *et al*., 2009).

2.2.2 Goldblatt procedure

Two weeks after the telemetry transmitters were implanted, renovascular hypertension was induced in every rat using the two-kidney one-clip (2K1C or Goldblatt) procedure.

After shaving, cleaning, and disinfecting the right flank, the rat was prepared for the surgical intervention using the same sterile conditions and isoflurane anesthesia (2% Isoflurane carried in 0.5 L/min of Oxygen) as previously described. Through a right-flank incision, the right kidney was exposed, and the renal artery was isolated from the renal vein over a short segment using the dissecting microscope. A silver clip with a 0.22-mm internal diameter was placed around the middle portion of the right renal artery; the left

kidney was left untouched. After the incision was closed, the animal went through the same recuperation procedure as previously described.

Again, two weeks later, after recovery was complete, a 2K1C 24-hour blood pressure record and a 24-hour metabolic assessment (comprised of water intake, food intake, and urine output) were acquired.

2.2.3 Induction of Diabetes by Streptozotocin

Two weeks after the Goldblatt procedure, the rats were separated into two groups with comparable mean blood pressure: Intact (IN) and Diabetic (DM). Diabetes mellitus was induced in half of the rats by intraperitoneal or intravenous injection (tail vein) of freshly prepared Streptozotocin (Pfizer Canada Inc; 60 mg/kg in ice-cold saline) under isoflurane anesthesia (2% Isoflurane carried in 0.5 L/min of Oxygen). Blood glucose was measured biweekly in all animals using an Ascensia glucometer ACCU-Check blood glucose monitor from Boehringer; blood was acquired from the tail of conscious, unrestrained rats. When blood glucose exceeded 30 mmol/L, one half a LinPlant ® (Linshin Canada, Ltd) was implanted subcutaneously to maintain blood glucose at 20-25 mmol/L. LinPlants were renewed throughout the experiment as needed at about 4 week intervals. A 24-hour blood pressure record and a 24-hour metabolic assessment were acquired every week after inducing diabetes.

2.2.4 Salt Treatment

After three weeks of diabetes (DM1, DM2, DM3), half of the rats from each group (IN and DM) received salt treatment. The resulting subgroups were Normal-Salt (NS) and High-Salt (HS). For the high-salt groups, the salt intake was adjusted in drinking water to be 2.5% of the food weight. This salt concentration was shown to be in the range that rats prefer in water (Lau *et al,* 2009). The salt adjustment was performed

daily, by taking into account the water and food intake during the previous 24 hours. The salt treatment was provided in these groups until the end of the experiment.

As a result, four groups of hypertensive rats have been generated: INtact Normal Salt (**IN-NS**), INtact High Salt (**IN-HS**), Diabetes Mellitus Normal Salt (**DM-NS**), and Diabetes Mellitus High Salt (**DM-HS**). This colour code is used throughout the Section Results to denote these four groups.

From this moment on, a 24-hour blood pressure record and a 24-hour metabolic assessment (comprised of water intake, food intake, and urine output) were acquired from every rat weekly, from Week 1 (WK1) to Week 14 (WK14).

2.2.5 Urinary excretion of proteins

Urinary sodium, potassium, creatinine and protein concentrations (Bradford, 1976) were determined from the 24-hour urine collections for each rat. All these measurements were performed by the Department of Laboratory Medicine of the Utrecht University in the Netherlands.

Since proteinuria is one of the criteria that can predict the progression to an advanced kidney disease and also the sudden animal death, we monitored the protein concentration in urine in our lab weekly. We used a colorimetric method, based on Albustix reagent strips for urinalysis (Albustix®, Siemens Healthcare Diagnostics Inc.). In order to increase its accuracy, we calibrated the Albustix reagent strips against rat albumin. Then, we serially diluted samples from 24-h urine collection until an apparent value between “trace” and 30 mg/dL proteins occurred. The concentration of protein in urine was finally given by the dilution factor multiplied by the value indicated by Albustix. We used this simple and quick method because our analysis showed that the assessment of proteinuria by Albustix was much closer to the Bradford protein assay, in the 0 - 30 mg/dL range.

2.3 The Acute Component of the Experiment

2.3.1 Renal hemodynamics

Renal blood flow was measured by standard protocols (Lau *et al.,* 2009) on a terminal procedure. The rats received buprenorphine (Temgesic® Reckitt & Benckiser, Inc, 0.02 mg/kg), were anesthetized by isoflurane (2 % Isoflurane carried in 0.5 L/min of Oxygen), and then placed on a homeothermic control unit that maintained the rat body temperature at 37°C. After intubation of the trachea to facilitate ventilation, cannulae were placed in the femoral artery for the measurement of perfusion pressure (PE 90) and in the femoral vein (PE 50) for infusions. Femoral arterial pressure was measured by a pressure transducer driven by a Kent TRN050 amplifier and the renal blood flow was measured by a Transonic Systems model T401 transit time ultrasound flowmeter (the probe was mounted on the left, non-clipped, renal artery). Throughout the experiment all animals received an intravenous infusion of a Ringer solution containing 2% bovine serum albumin BSA (Sigma Chemical Co.) at a rate of 0.6% of body weight per hour; the infusion rate was adjusted in diabetic rats to match the urine flow. A 60-minute equilibration period was kept before the start of the measurements and the animal was maintained under light anesthesia (1% Isoflurane carried in 0.5L/min of Oxygen) for the rest of experiment. A motorized occluder was placed on the aorta between the right and left renal arteries and was used to force blood pressure.

Renal blood flow autoregulation was assessed by a standard method pressure-ramp design (Cupples and Braam, 2007) and the range of blood pressure in which autoregulation operated was determined. First, a baseline measurement of renal blood flow and mean arterial pressure was obtained. Then, the renal perfusion pressure was reduced in steps of 10 mmHg x 1 min from 120 mmHg to 50 mmHg (120, 110, 100, 90, 80, 70, 60, 50), and then constriction was released and the renal perfusion pressure was increased back by steps of 10 mmHg x 1 min. During this procedure blood pressure and renal blood flow were recorded simultaneously.

Finally, each renal blood flow value measured at a specific pressure reduction step was normalized to the value of renal blood flow at 90 mmHg. Then, the shape of the normalized renal blood flow versus blood pressure curve (the autoregulation curve) was analyzed in each group in both flat (>90 mmHg) and linear (< 90 mmHg) portions.

2.3.2 Renal morphology

At the end of the acute phase of the experiment, both kidneys of each rat were harvested for histological analysis. After their weight was recorded, both kidneys were split and then fixed by immersion in 10% neutral buffered formalin. After embedding in paraffin, 4-μm sagittal sections were cut and stained with periodic acid Schiff (PAS) and counterstained with hematoxylin to show nuclei. The slides were examined by a renal pathologist (Dr. A.B. Magil, St Paul's Hospital, Vancouver) in single blind fashion. Glomerular and tubulointerstitial lesions in a cross-section of the kidney were examined to detect the presence of diabetic and hypertensive lesions. The glomerular changes were expressed as a percentage of segmental or global sclerosis, along with an estimation of tubular dilatation, atrophy and interstitial fibrosis. The percentage of segmental or global sclerosis was scored as being *segmental* if less than half of the glomerulus was affected and *global* when more than half of the entire glomerulus was affected. Tubular atrophy/ interstitial fibrosis was scored as a percentage of the cortical tubulointerstitial system showing these changes (>50% = 3+, 25-50% = 2+, 5-25% = 1+) and tubular dilation as percentage of cortical tubules showing dilation (>50% = 3+, 25-50% = 2+, 5-25% = 1+).

2.3.3 Anthropometric measurements

Body weight (g) was monitored biweekly throughout the course of the experiment. At the end of the experiment, body length (cm) and body weight to body length ratio were determined in order to estimate indirectly the animal body composition and nutritional status (Simson and Gold, 1982; Kuhlmann *et al*., 2007).

2.4 Statistical Method

The data were analyzed using the SPSS statistical software package (Field, 2005). Statistical comparisons between groups were performed using Student's *t* – test, analysis of variance (ANOVA) with or without repeated measures, analysis of covariance (ANCOVA), and linear regression. The Independent Student's *t*-test was used to compare the difference between the sample means. Analysis of variance (One-way ANOVA) was also used to test the differences among the means of the four groups (IN-NS, IN-HS, DM-NS, and DM-HS) as well as their evolution in time. Analysis of covariance (ANCOVA) was used to test whether certain factors (such as salt, diabetes, blood pressure) had an effect on the dependent variables after removing the variance of a number of quantitative covariates. Linear regression analysis was performed to analyze the relationship between a dependent variable (such as glomerulosclerosis) and an independent variable (such as blood pressure). Data are presented as means ± SE, and $p \leq 0.05$ is considered to indicate a statistical significant difference.

Chapter 3 – Results

3.1 Anthropometric Measurements

3.1.1 Body weight

Figure 2 shows the evolution of the mean body weight in all four groups. At baseline measurements (weeks CTL and 2K1C), body weight was not statistically different among the groups.

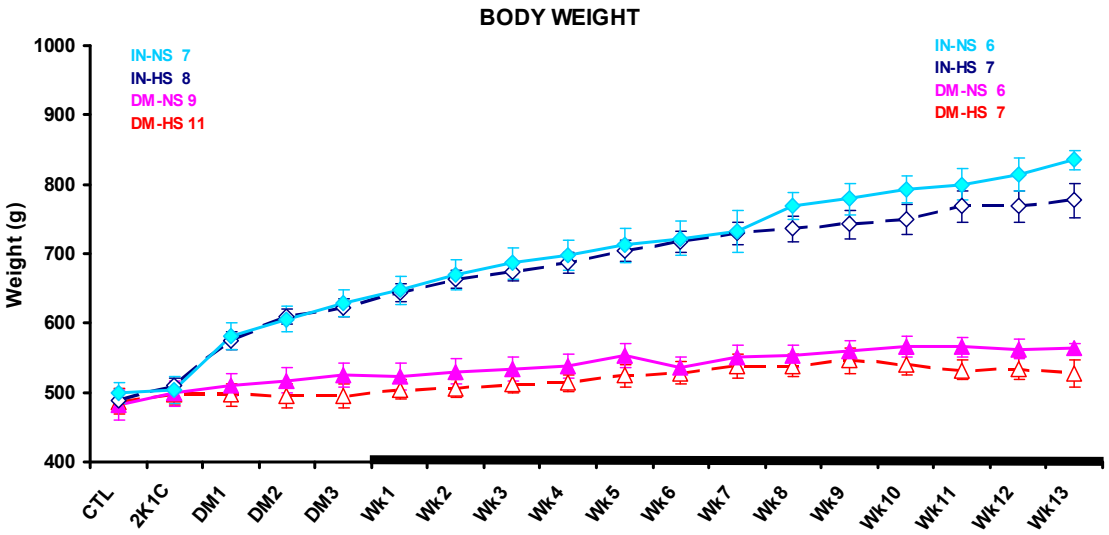

Figure 2. The evolution of body weight from the initial (CTL, 2K1C) measurements, through induction of diabetes (DM1, DM2, DM3) and increased salt intake (WK1 to WK13) in the four groups of rats. IN-NS group is represented by the solid light blue line and solid diamonds; IN-HS is represented by the dashed dark blue line and open diamonds; DM-NS is represented by the solid pink line and solid triangle; DM-HS is represented by the dashed red line and open triangles. The top of the graph shows the number of rats in each group at the beginning (CTL) and close to the end of the experiment (WK12). Each point represents mean value ± SE. Mixed design ANOVA test confirmed that the body weight in diabetic and intact groups diverged over time.

To assess the body weight evolution within and between rat groups, we have performed a mixed design ANOVA test with body weight as repeated-measure variable. The test revealed that, on average, the body weight significantly increased over time. After inducing diabetes (week DM1), the body weight in diabetic and intact groups diverged ($p < 0.01$): the body weight of the diabetic rats had only a small rise over time, while the body weight of the intact rats increased steadily. However, neither mixed design ANOVA, nor ANCOVA with systolic blood pressure as the covariate revealed any influence of salt on body weight for intact or diabetic rats ($p > 0.05$).

3.1.2 Body length and body weight to body length ratio

The body length and body weight to body length ratio, along with kidneys weight, are shown in Table 1. The body length and the body weight were measured at the beginning of the terminal procedure in order to estimate how diabetes mellitus affected the growth and nutritional status. Both body length and body weight to body length ratio were smaller in the diabetic rats compared to the intact rats ($p < 0.01$). Independent *t*-tests and ANCOVA with systolic blood pressure as the covariate revealed no significant differences in body length or body weight to body length ratio between IN-NS and IN-HS, or between DM-NS and DM-HS.

Table 1. Body length (BL), body weight to body length ratio (BW: BL), kidneys weight in all four groups at the terminal procedure (7 IN-NS, 8 IN-HS, 10 DM-NS, and 12 DM-HS rats.

	IN-NS	IN-HS	DM-NS	DM-HS
Body length (BL), cm	28.2 ± 0.2	28.3 ± 0.1	27.6 ± 0.3‡	26.5 ±0.4‡
BW:BL	27.8 ± 1.3	27.2 ± 1.0	20.3 ± 0.5‡	20.4 ± 0.5‡
Left kidney weight, g	2.9 ± 0.1	3.0 ± 0.2	3.5 ± 0.1†§	3.3 ± 0.2†
Right kidney weight, g	1.8 ± 0.2	1.8 ± 0.3	2.1 ± 0.2	2.1 ± 0.3

Values are means ± SE. The numbers shown in parentheses represent the number of rats from each group. † $p < 0.05$, diabetic rats compared with intact rats, ‡ $p < 0.01$, diabetic rats compared with intact rats; § $p < 0.01$, diabetic normal-salt rats compared with intact normal-salt rats.

3.2 Kidney Weight

Kidney weight was measured at the end of the terminal procedure in order to assess kidney hypertrophy and, indirectly, the GFR (Vallon *et al.*, 1997) in the long-term experiment. Independent *t*-test analysis revealed that the left (non-clipped) kidney weight was larger in diabetic rats than in intact rats ($p < 0.05$). However, one-way ANOVA performed on all four groups showed that the left kidney weight was significantly larger only in the DM-NS group with respect to the IN-NS group. The right (clipped) kidney weight did not differ among the groups ($p > 0.05$). No significant differences were found between IN-NS versus IN-HS groups, or between DM-NS versus DM-HS groups for any of the considered parameters.

3.3 Metabolic Measurements

3.3.1 Blood glucose

Figure 3 shows the mean blood glucose in all four groups at weekly intervals (values were averaged over two measurements taken biweekly). To statistically assess the blood glucose evolution, a mixed design ANOVA test was performed in order to quantify the differences between the four rat groups while subjecting them to repeated measures. At baseline (CTL and 2K1C), blood glucose did not differ among the four groups; it remained low and stable, around 5 mmol/l for both the IN-NS and IN-HS groups throughout the experiment. The blood glucose increased in both diabetic groups after inducing diabetes ($p < 0.01$), and remained elevated around the 25 mmol/l target throughout the experiment. Adding salt to the diet had no effect on blood glucose in either diabetic or intact rats.

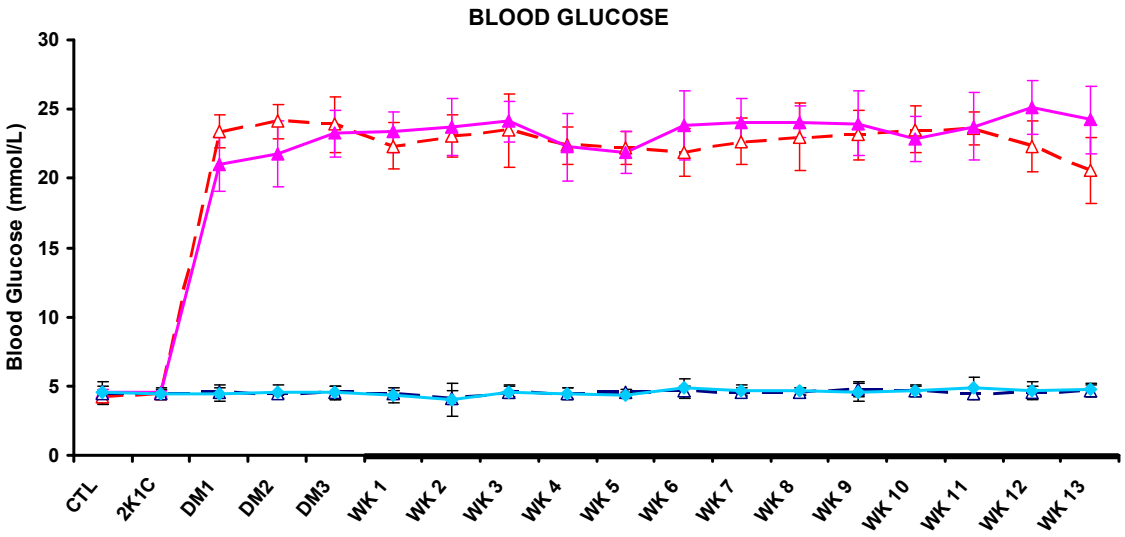

Figure 3. The evolution of the blood glucose from the initial (CTL, 2K1C) measurements, through induction of diabetes (DM1, DM2, DM3) and increased salt intake (WK1 to WK13) in the four groups of rats. IN-NS group is represented by the solid light blue line and solid diamonds; IN-HS is represented by the dashed dark blue line and open diamonds; DM-NS is represented by the solid pink line and solid triangle; DM-HS is represented by the dashed red line and open triangles. The points represent mean values ± SE. Mixed design ANOVA test confirmed that the blood glucose in diabetic and intact groups differed significantly over time.

3.3.2 Evolution of water intake, food intake and urine output

Figure 4 shows average water intake, urine output, and food intake over 24-hour metabolic assessments in all four groups throughout the experiment. A mixed design ANOVA revealed that all metabolic variables of diabetic rats were significantly larger than those of intact rats after DM1. Salt treatment had no influence on metabolic variables within each intact or diabetic group, except for weeks WK1 and WK2, when water intake and urine output were both higher in the DM-HS group compared to the DM-NS group ($p < 0.05$).

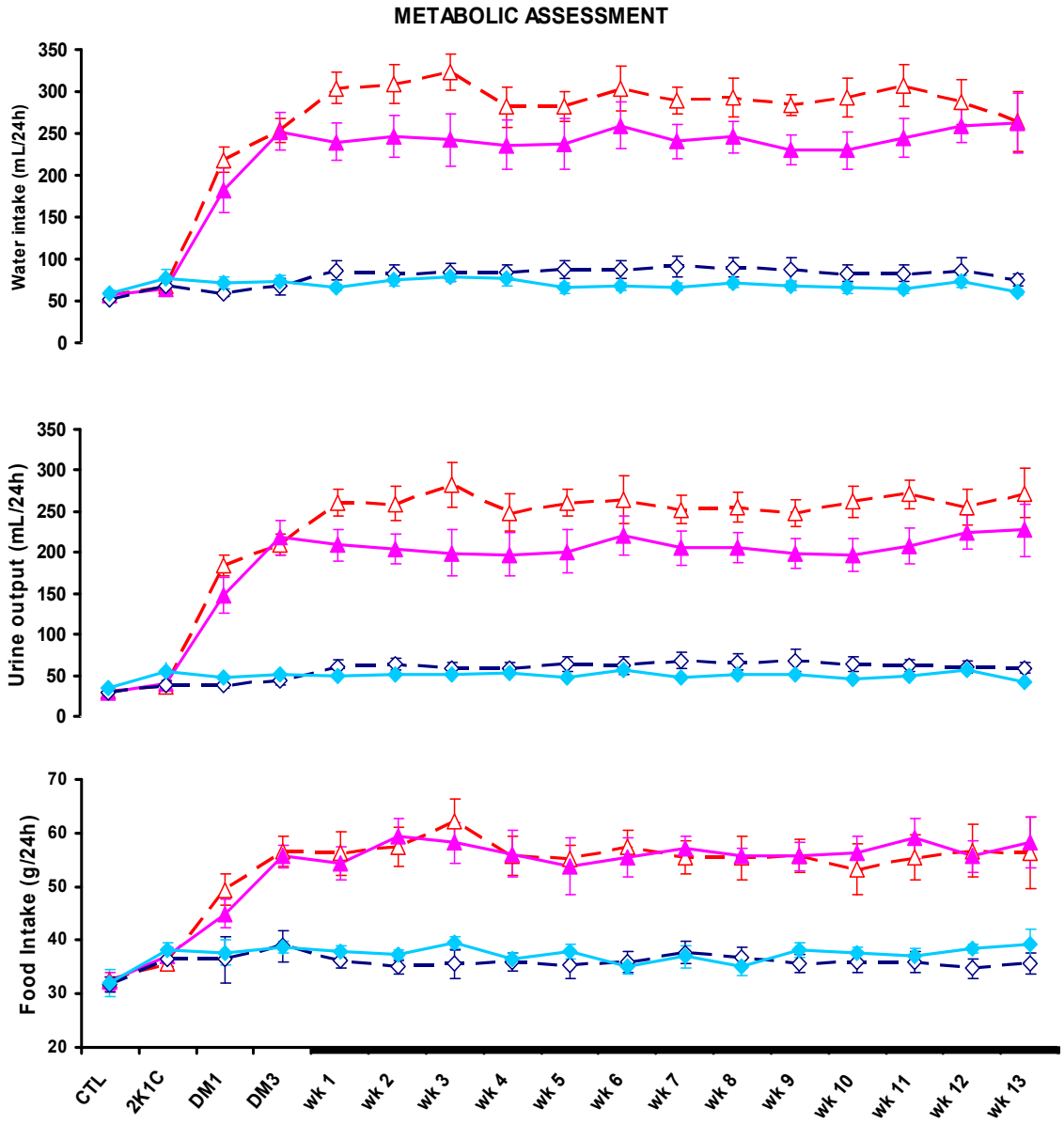

Figure 4. The evolution of water intake, urine output, and food intake from the initial (CTL, 2K1C) measurements, through induction of diabetes (DM1, DM2, DM3) and increased salt intake (WK1 to WK13) in the four groups of rats. IN-NS group is represented by the solid light blue line and solid diamonds; IN-HS is represented by the dashed dark blue line and open diamonds; DM-NS is represented by the solid pink line and solid triangles; DM-HS is represented by the dashed red line and open triangles. Points represent mean values ± SE. Mixed design ANOVA revealed that all metabolic variables of diabetic rats were significantly larger than those of intact rats after DM1.

3.4 Salt Intake and Renal Function Curve

Table 2 shows the salt intake in g/24h in all four groups at 2K1C (after inducing hypertension), DM1 (in the first week of diabetes), and WK1 (in the first week of salt treatment), which was calculated from food and water intake. Salt intake did not differ among the four groups at 2K1C. After inducing diabetes (DM1), the diabetic rats ingested more water and food than intact rats, which implicitly increased their salt intake, with the same proportion ($p<0.05$). There were no significant differences between the normal-salt and high-salt groups at DM1, in either intact or diabetic rats. From WK1, salt treatment was provided to high-salt groups by adding salt in drinking water, such as the salt intake in IN-HS and DM-HS groups was approximately 2.5 times larger than in the IN-NS and DM-NS groups, respectively ($p<0.01$).

Table 2. Salt intake (g/24h) in all four groups at weeks 2K1C (after inducing hypertension), DM1 (in the first week of diabetes), WK1 (in the first week of salt treatment).

	2K1C	DM1	WK1
IN-NS	0.38±0.02	0.38±0.02	0.38±0.01
IN-HS	0.36±0.01	0.36±0.04	0.97±0.06 ‡
DM-NS	0.37±0.02	0.45±0.03†	0.54±0.03
DM-HS	0.36±0.01	0.49±0.03†	1.50±0.10 ‡

Values are means ± SE. † $p < 0.05$, diabetic rats compared with intact rats at week DM1; ‡ $p < 0.01$, high-salt groups compared to normal-salt groups at week WK1.

The renal function curve in Figure 5 describes the relationship between salt intake and blood pressure. The renal function curve for high-salt intact and high-salt diabetic rats was obtained through the normalization of salt intake at weeks DM1 and WK1 to the week CTL value. In both the intact high-salt rats (n=8) and the diabetic high-salt rats (n=11), the blood pressure dependence on dietary salt intake was weak over a 2.5-fold range.

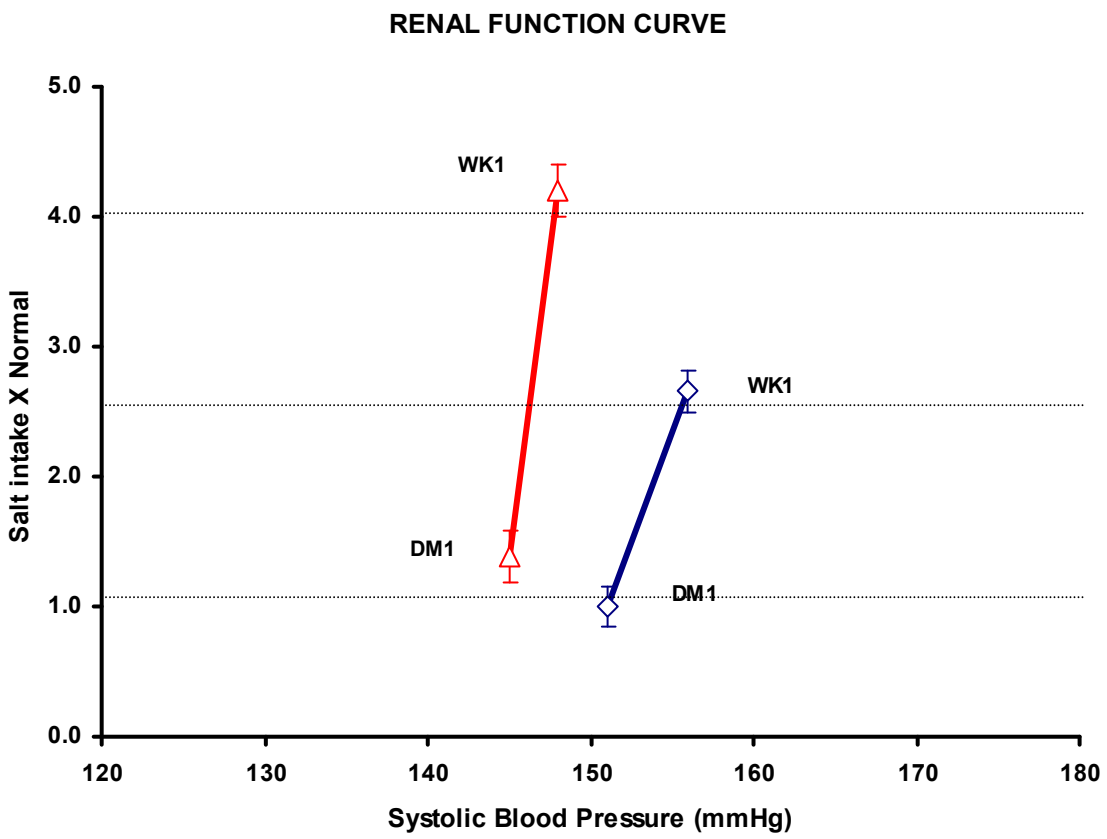

Figure 5. Renal function curve acquired from week DM1 to week WK1 in intact high-salt rats (solid dark blue line) and high-salt diabetic rats (solid red line).

3.5 Hemodynamic Measurements

3.5.1 Heart rate

Figure 6 presents the evolution of heart rate and systolic blood pressure. The mean heart rates at CTL and 2K1C did not differ significantly between groups. To statistically assess the heart rate evolution, a mixed design ANOVA test was performed on the four rat groups while subjecting them to repeated measures. On average, the heart rate decreased over time after the induction of hypertension ($p < 0.05$). The induction of

diabetes resulted in a rapid and reliable reduction of heart rate ($p < 0.01$), and remained significantly lower in diabetic rats for the rest of the experiment. There were not any significant differences between the DM-NS and DM-HS groups, or between IN-NS and IN-HS; this means that we did not detect any effect of salt on heart rate.

3.5.2 Systolic blood pressure

A mixed design ANOVA revealed that the average systolic blood pressure was not significantly different among the four groups during the experiment. The absence of a statistically significant increase in systolic blood pressure due to salt treatment indicated that the blood pressure was salt resistant. Despite this inconclusive result, it was important to analyse the slope of the systolic blood pressure evolution over time, since this could predict the future development of glomerular damage. Since the highest pressure animals tend to drop out first, affecting the statistics, we calculated the slopes using linear regression only on points from 2K1C up to WK5, when all the rats were still alive. We determined that while there was only a small difference in the slope of the systolic blood pressure between the IN-NS and IN-HS groups (1.66±0.25 mmHg/week versus 1.60±0.27 mmHg/week, respectively), the DM-NS group showed a much steeper slope in contrast to the DM-HS group (2.21±0.22 mmHg/week versus 1.22±0.16 mmHg/week, respectively).

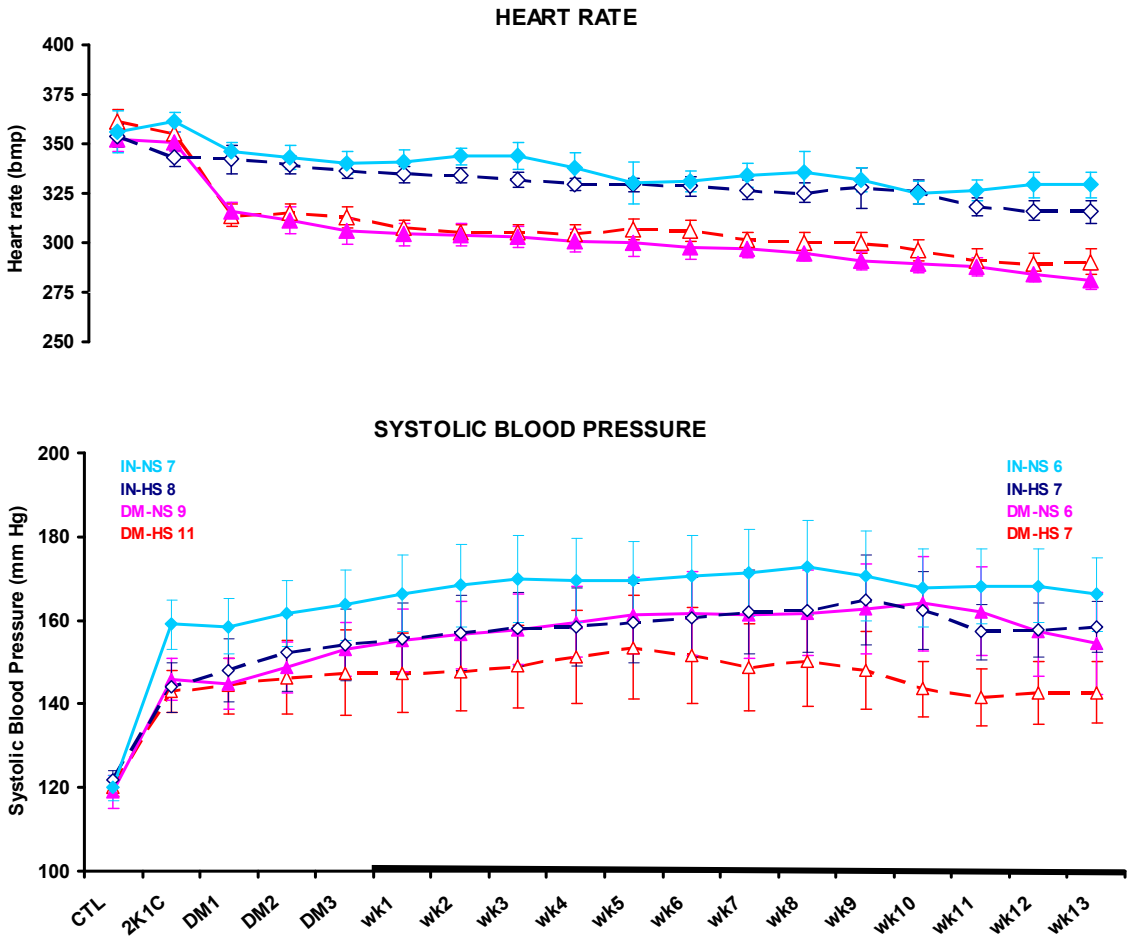

Figure 6. Evolution of heart rate and systolic blood pressure from the initial (CTL, 2K1C) records, through induction of diabetes (DM1, DM2, DM3) and increased salt intake (WK1 to WK13) in the four groups of rats. IN-NS is represented by the solid light blue line and solid diamonds; IN-HS is represented by the dashed dark blue line and open diamonds; DM-NS is represented by the solid pink line and solid triangle; DM-HS is represented by the dashed red line and open triangles. The top of the graph shows the number of the rats for each group at the beginning (CTL) and close to the end of experiment (WK12). The points represent mean values ± SE. Mixed design ANOVA test confirmed the reduction of the heart rate in diabetes and the salt resistance of blood pressure.

3.6 Survival of Rats through the Protocol

Table 3 shows the survival of rats in each group at three different moments (WK5, WK8, and WK12) through the protocol. Based on a number of criteria (neurological symptoms such as lethargy and seizures, pulse pressure greater than 80 mmHg, proteinuria greater than 1.5 g/24h, and a decrease in body weight of more than 5% between successive biweekly measurements) that commonly predict sudden death, some of the rats were terminated earlier. On average, the long-term survival rate of diabetic rats was poorer. At WK12, 13 out of 15 intact rats were alive compared to 13 out of 20 diabetic rats. A Chi-Square analysis performed to statistically quantify this result revealed Expected Counts lower than 5, which violates the main assumption of the Chi-Square test. Therefore, this test is not powerful enough to confirm the effect of diabetes on the long-term survival rate.

Table 3. The survival of rats in each group at three different moments (WK5, WK8, and WK12) through the protocol.

	WK5	**WK8**	**WK12**
IN-NS	7 rats	7 rats	6 rats
IN-HS	8 rats	8 rats	7 rats
DM-NS	9 rats	8 rats	6 rats
DM-HS	11 rats	10 rats	7 rats

3.7 Proteinuria

Proteinuria is recognized as a marker of renal damage. Figure 7 presents the progression of proteinuria over time in all four groups at hypertension induction (2K1C), established diabetes (DM3), five weeks after inducing salt treatment (WK5) when all rats were still alive, and close to the end of experiment (WK12). Despite a progressive increase of proteinuria over time, the independent *t*-test or one-way ANOVA did not find a significant difference among the four groups (except IN-HS versus DM-HS at WK12).

However, ANCOVA analysis using systolic blood pressure as covariate revealed that proteinuria was higher in diabetic rats than in intact rats ($p < 0.05$) at each of those times. It revealed no significant changes in proteinuria due to salt treatment within either intact or diabetic groups.

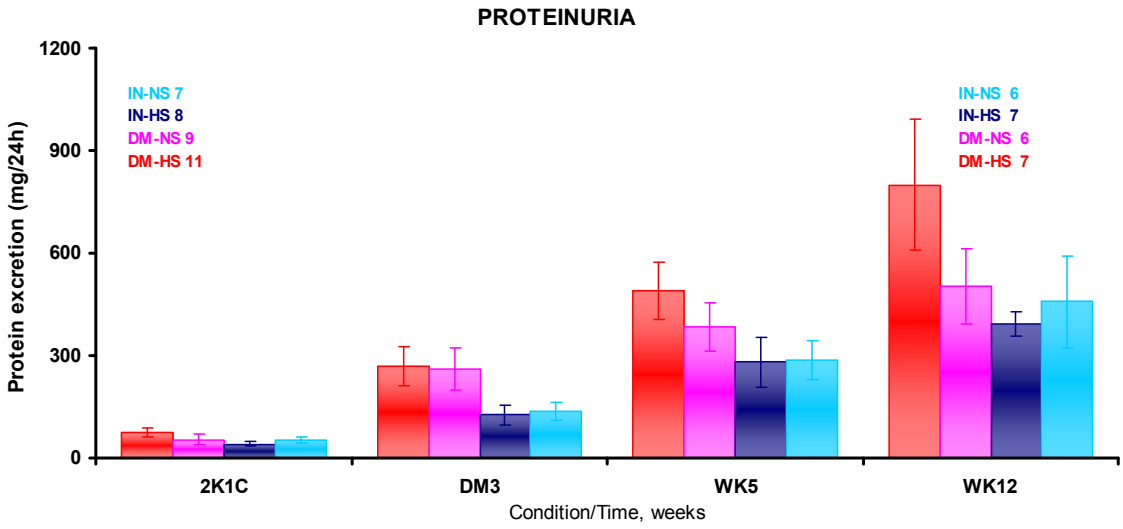

Figure 7. The mean proteinuria in all four groups at four different moments during the experiment (2K1C, DM3, WK5, WK12). IN-NS is represented by the solid blue bar; IN-HS is represented by the solid dark blue bar; DM-NS is represented by the solid pink bar; DM-HS is represented by the solid red bar. The top of the graph shows the number of the rats for each group at the beginning (CTL) and close to the end of experiment (WK12). ANCOVA analysis using systolic blood pressure as covariate revealed that proteinuria was higher in diabetic rats than in intact rats.

3.8 Renal Autoregulation

Figure 8 shows the steady-state renal blood flow autoregulation. Each point represents the average of normalized renal blood flow at each pressure reduction step. The normalization was performed for each individual rat with respect to its renal blood flow at 90 mmHg. It is apparent that the RBF was independent of perfusion pressure at pressures above 90 mmHg, and dependent on perfusion pressure at lower pressures. Post hoc ANOVA indicated that there were no significant differences in RBF among the four groups above 90 mmHg; below 90 mmHg, it also indicated that the pressure-flow relationships differed at 60 mmHg ($p<0.1$, one-tailed) and at 50 mmHg ($p<0.05$,

one-tailed) in DM-NS versus IN-NS. Inspection of Figure 8 shows that this effect arises from stronger autoregulation at low pressures in DM-NS rats than in the other groups.

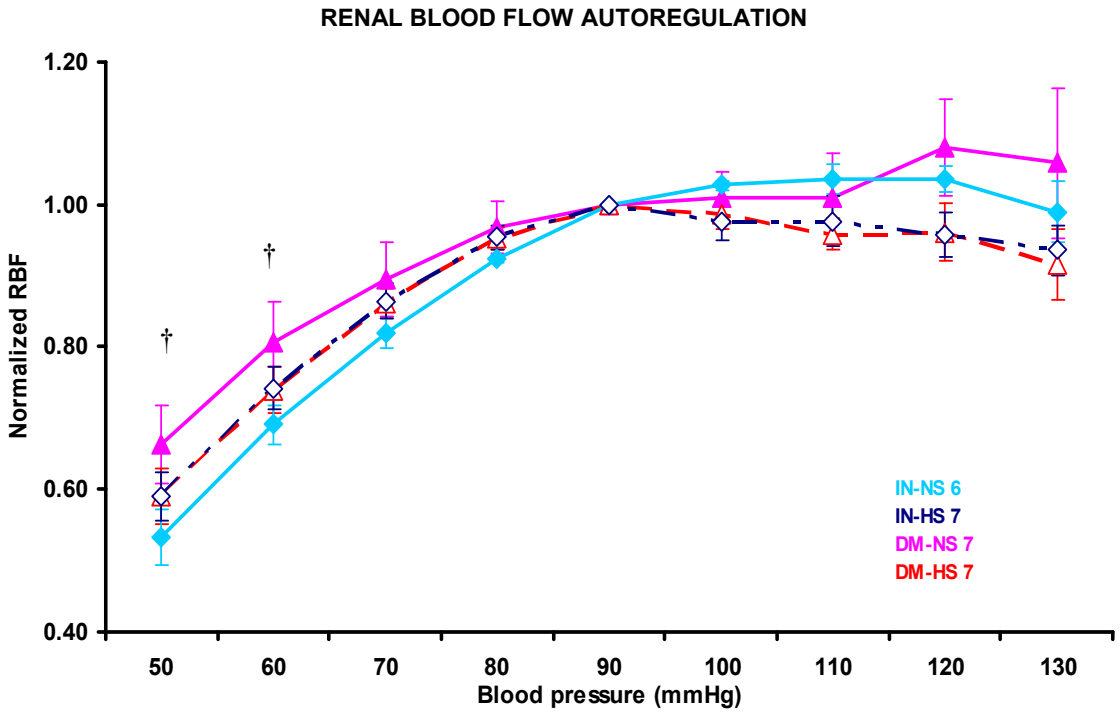

Figure 8. Steady-state renal autoregulation in all four groups during the terminal procedure. The points represent averages of normalized renal blood flow at each discrete pressure step. The pressure-flow relationships differed at 50 and 60 mmHg. Values are mean ± SE. The number of rats from each group is shown at the bottom of the graph.† $p < 0.05$ (one-tailed) DM-NS compared to IN-NS rats.

Table 4 shows the renal blood flow at baseline blood pressure on the terminal procedure. A two-way ANOVA test assessing the renal blood flow response to diabetes and to increased dietary salt was performed. It was revealed that the baseline renal blood flow was higher in intact rat groups than in diabetic rat groups ($p < 0.05$). No significant differences in renal blood flow were revealed between the normal salt groups and the high salt groups.

Table 4. The renal blood flow at baseline blood pressure during the terminal procedure.

	IN-NS	IN-HS	DM-NS	DM-HS
BP (mmHg)	144±8	131±4	144±8	131±5
RBF (ml/min)	10.9±2.2†	10.7±1.3†	8.2±1.1	8.3±1.3

Values are means ± SE. † $p < 0.05$ (ANOVA), intact rat groups compared with diabetic rat groups.

3.9 Glomerulosclerosis

The clipped kidney which was not exposed to hypertension showed minor glomerular lesions that did not differentiate between intact and diabetic rats. The non-clipped kidney which was exposed to hypertension showed substantial, pressure-dependent glomerular injury (Table 5). We found that the glomerular damage of the non-clipped kidney was significantly higher in diabetic rats than in intact rats ($p < 0.05$). By plotting the glomerular injuries of the non-clipped kidneys versus pressure load, we found that the glomerulosclerosis was linearly related to systolic blood pressure (Figure 9). However, the diabetic groups shifted the best-fit line to the left, to lower blood pressure, indicating increased renal susceptibility to injury.

Table 5. The percentage of glomeruli with segmental/global sclerosis (% GS) in non-clipped (NCK) and clipped (CK) kidneys reported to the average of systolic blood pressure (SBP) in the last three weeks before the terminal procedure for each group of rats.

Groups	%GS NCK	%GS CK	SBP
IN-NS	25 ± 6	1.8 ± 1.6	174 ± 9
IN-HS	14 ± 4	1.9 ± 0.8	166 ± 9
DM-NS	31 ± 6	1.1 ± 1.0	167 ± 10
DM-HS	35 ± 7	1.2 ± 0.6	160 ± 11

One-way ANOVA did not reveal any significant differences in glomerulosclerosis among the four groups. ANCOVA analysis (with blood pressure as covariate) performed to determine the impact of salt on glomerular damage within each of the intact and diabetic rats groups also revealed no significant effect in either intact or diabetic rats. The visually apparent differences in Figure 9 between IN-NS and IN-HS slopes (0.54 ± 0.18 and 0.22 ± 0.14 %/mmHg, respectively) and between DM-NS and DM-HS slopes (0.58 ± 0.16 and 0.62 ± 0.15 %/mmHg, respectively) correspond to a one-tailed test significant with a 90% confidence interval.

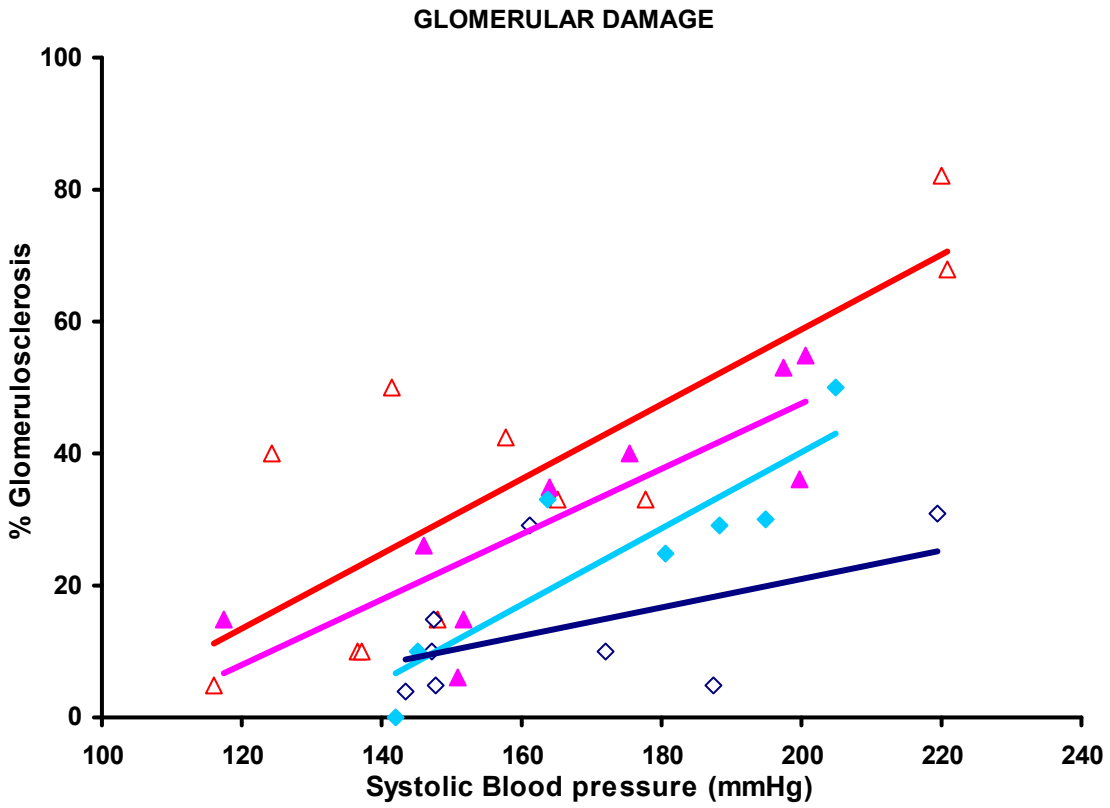

Figure 9. The line of best fit of glomerulosclerosis in the non-clipped kidney versus systolic blood pressure (averaged over the last three weeks before terminal procedure). IN-NS is represented by the solid light blue line and solid diamonds; IN-HS is represented by the solid dark blue line and open diamonds; DM-NS is represented by the solid pink line and solid triangle; DM-HS is represented by solid red line and open triangles. ANCOVA with blood pressure as covariate revealed no effect of salt in either intact or diabetic rats. Diabetic groups exhibit a left shift of the glomerulosclerosis best fit line versus intact groups.

Histological examination pictures are provided in Appendix B. A sample of non-clipped kidney for each group is presented in Figure 10. This figure reveales different stages of kidney injury and various morphological aspects in both intact and diabetic rats, such as segmental glomerular lesions, glomerulosclerosis with tuft retraction, arteriolar sclero-hyalinosis, tubular dilation, tubular atrophy, and interstitial fibrosis. All these morphopathological aspects were greater in diabetic rats. In contrast, Figure 11 shows the clipped kidneys from the same rats, which have preserved structure of the glomeruli and reinforces that the damage in non-clipped kidney are mainly pressure dependent and diabetes exacerbates these renal injuries.

Chapter 4 – Discussions

Under normotensive conditions, the progressive loss of renal function in diabetes mellitus is typically very slow (Jacobsen *et al.,* 1999; Bidani *et al.,* 2007; Lau *et al.*, 2009). Hypertension normally accelerates renal disease of all other etiologies and is also present in the evolution of diabetes (Navar *et al.*, 1998; Zucchelli and Zuccala, 1998; Fogo, 2000). In order to assess the incidence and rate of progression of diabetic renal injury under an established hypertensive condition, the present study combined renin-mediated hypertension (2K1C) and Streptozotocin-induced diabetes mellitus, and used salt as a potential modulator of renal and cardiovascular dysfunction.

We hypothesised that under an established hypertensive condition diabetic hyperfiltration would impair autoregulation of renal blood flow; therefore, the diabetes would increase the susceptibility to pressure-induced renal damage. The modulator effect of salt on hypertension and diabetes was also investigated. We tested whether the renal hemodynamics changes due to salt would affect susceptibility to nephropathy in hypertensive-diabetic rats. Specifically, two questions were of interest: Would salt increase the blood pressure and, therefore, become a risk factor for renal damage under diabetic conditions? Or would the paradoxical effect of salt reduce diabetic renal susceptibility to damage by abrogating hyperfiltration and increasing the TGF sensitivity?

In the following discussion, the variables of interest for our study are presented: blood pressure, anthropometric assessment, organ weight, heart rate, renal autoregulation, and glomerular damage.

4.1 Evolution of Blood Pressure

Systolic blood pressure is closely correlated with organ damage (Bidani and Griffin, 2004). Therefore, in order to address the susceptibility of the diabetic kidney to

pressure-induced injury, we closely monitored systolic blood pressure during the entire experiment. As discussed in Subsection 3.5.2, systolic blood pressure increased continuously after the 2K1C procedure, with a different slope in each of the four groups. The onset of poor glycemic control caused a small and transient decrease in systolic blood pressure in the first week after STZ administration.

Most recent studies using the telemetric acquisition of 24-h blood pressure have also shown that blood pressure either remained stable or progressively reduced (Hicks *et al.*, 1998; Tatchum-Talom *et al.*, 2000; Bidani *et al.*, 2007; Lau *et al.*, 2009) following the induction of STZ-T1DM under normotensive conditions. To our knowledge, the combination of STZ-T1DM, 2K1C hypertension, and telemetric blood pressure acquisition has not been reported previously.

The effect of high-salt treatment on blood pressure development appears to be relatively slight in our hypertensive intact rats; these findings are consistent with the literature on salt loading in 2K1C (Mohring *et al.,*1976; Jackson and Navar, 1986; Lee *et al.*, 1991; Liu *et al.*, 1993). However, according to our previous experiments, the Long-Evans rats also showed a salt-resistant blood pressure under normotensive conditions (Lau *et al.*, 2009). Therefore, it is still an open question as to whether the preservation of the ability to excrete sodium was a rat-strain characteristic, or the salt concentration was able to efficiently suppress the neurohormonal mechanisms (such as plasma renin angiotensin and sympathetic nervous system) responsible for fluid and electrolyte conservation (Jackson and Navar, 1986; Liu *et al.*, 1993; Bealer, 2002; Yoshimoto *et al.*, 2004). As a result, the pressure natriuresis effect, along with the ANG II-effect on tubular salt reabsorption (DeClue *et al.*, 1978; Ando *et al.*, 1990; Liu *et al.*, 1993; Sato *et al.*, 1990; Vallon *et al.*, 2003; Braam and Koomans, 2006), could provide an explanation for our findings in regard to the 2K1C hypertensive rats.

The effect of dietary salt on blood pressure in diabetes mellitus seems to be even more difficult to assess experimentally since sustained hyperglycemia can induce important natriuresis (Brands and Fitzgerald, 2002) and, therefore, influence the blood pressure level. On the other hand, while small changes in animal salt loading limit the detection of salt-dependent hypertension, large salt changes may reduce food or fluid

intake and could corrupt the experiment (Lau *et al.*, 2009). The literature regarding the effect of salt on blood pressure in diabetes mellitus also reports contradictory results (Feldt-Rasmussen *et al*. 1987; Gerdts *et al*. 1996; Miller, 1997; Lau *et al.*, 2009).

Under our specific experimental conditions, despite no statistically significant differences between the two diabetic groups, the diabetic normal-salt group rapidly overrode the blood pressure reduction produced as a result of STZ administration, while the diabetic high-salt group had only a very slight and slow increase of blood pressure.

In the early phase of diabetes characterized by volume depletion due to osmotic diuresis, RAS activation may counterbalance the natriuresis in order to maintain a virtually unchanged blood pressure (Brands and Labazi, 2008). The evolution of blood pressure encountered in STZ-T1DM would indicate that in the early stage of diabetes the natriuretic influence induced by an elevated GFR was slightly affected by a sodium-retaining influence of ANG II, but more affected at a later stage. We also found no blood pressure dependence on dietary salt intake in the diabetic rats. These findings would be consistent with a suppressed systemic RAS due to a relative volume expansion and with the shift of the pressure natriuresis curve to the left.

4.2 Anthropometric Assessment

As shown in Subsection 3.1, body weight increased steadily in intact rats during the experiment. Salt did not induce any changes in the mean body weight of the 2K1C intact rats; this result is similar to other findings reported in the literature (Liu *et al.*, 1993). Since a weight gain during salt treatment is a good indicator of an increased extracellular fluid volume and salt sensitivity (Haddy, 2006), the absence of a significant weight gain due to salt treatment encountered in this experiment is consistent with the salt-resistant blood pressure findings.

The body weight of the rats that received Streptozotocin and developed a poorly controlled hyperglycemia increased continuously, although this increase was markedly smaller than in the intact rats. However, an interpretation of the relationship between body weight and extracellular fluid volume in diabetic rats is difficult to be made due to

the changes in body composition following the onset of the disease. The body weight-to-length ratio, which describes the growth and the nutritional status better than the body weight (Simson and Gold, 1982; McNeill, 1999), satisfactorily predicted a larger reduction in the body mass of our diabetic rats. That was consistent with the slight reduction of growth (body length) and substantially reduced body weight in the diabetic rats compared to the intact rats.

Protein malnutrition usually develops during the course of chronic kidney disease (Kuhlmann *et al.*, 2007). Since food intake was greater in the diabetic groups than in the intact groups, their wasting of protein energy can only be related to an impaired metabolism and chronic inflammation. Therefore, the complex changes in body composition that occur in chronic kidney disease become more evident in the presence of diabetes mellitus, and are also reflected in an earlier alteration of animal health and predisposition to sudden death.

4.3 Organ Weight

Kidney hypertrophy normally accompanies an increased GFR (Hostetter *et al.*, 1981; O'Donnell *et al.*, 1988; Vallon *et al.*, 1997), and both glomerular hyperfiltration and kidney growth are seen as risk factors for diabetic renal injuries (Vallon *et al.*, 2003). Short (four-week) experiments reported an increased GFR and kidney weight in normal and 2K1C hypertensive rats with chronic salt loading (Vallon *et al.*, 1997; Jackson and Navar, 1986), and also in diabetic rats with a normal salt diet (Vallon *et al.*, 1997). However, kidney hypertrophy also depends on the degree of metabolic control in diabetes (Hostetter *et al.*, 1981; O'Donnell *et al.*, 1988; Vallon *et al.*, 1997). Each maneuver that attenuates kidney growth in diabetes (insulin therapy, severe hyperglycemia, or renal vasoconstriction in response to high-salt diet) is expected to reduce GFR by preventing hyper-reabsorption and workload (Hostetter *et al.*, 1981; Miller, 1997; Vallon *et al.*, 2003). Thus, a blunted increase in kidney weight was found in diabetic rats with chronic salt loading (Vallon *et al.*, 1997).

In all four groups of this experiment, the clipped kidneys that were not exposed to an increased blood pressure and flow weighed less than the non-clipped kidneys, and these weights were independent of the regimen (salt or diabetes). Even if the non-clipped kidneys weighed more in diabetic rats than in intact rats, a statistical significant difference was found only between the diabetic normal-salt and the intact normal-salt groups. There were no significant differences in non-clipped kidney weight between the normal-salt and high-salt groups in either intact or diabetic rats, despite tendencies for an increased kidney weight in the intact high-salt group and a decreased kidney weight in the diabetic high-salt group.

The lack of consistency with the literature can be attributed to the much longer duration of our experiment. A progressive increase in kidney weight was found in moderate and severe hyperglycemia in the early phase of diabetes (Vallon *et al.*, 1997), but no significant differences were found between diabetic and control rats in diabetes later stages (McNeill, 1999). Therefore, the absence of a significant effect of high salt intake on kidney weight can be explained by an attenuation of the hemodynamic difference between the groups due to the chronic experiment (Hill *et al.*, 2010). The suboptimal insulin therapy or the level of hyperglycemia could also blur the differences between diabetic groups. While the diabetic rats that do not receive insulin manifest marked hyperfiltration and renal hypertrophy, incomplete insulin therapy decreases the diabetic hyperfiltration and renal hypertrophy (Biederman *et al.*, 2005).

4.4 Evolution of Heart Rate

As mentioned in Subsection 3.5.1, the heart rate decreased over time in all four groups, but the decrease was more important in the diabetic groups after the administration of STZ. This finding is in accordance with the literature (McNeill, 1999; Bidani *et al.*, 2007; Lau *et al.*, 2009). If the initial bradycardia occurred primarily by the activation of the baroreflex control mechanism, which is triggered by the blood pressure load, the important bradycardia in STZ-T1DM could be mainly explained by a chronic autonomic nervous system deficiency with increased parasympathetic and defective

sympathetic function (Thomas and Bannister, 1980; Hicks *et al.*, 1998; Dall'Ago *et al.*, 2002; Gross *et al.*, 2008). The absence of any significant differences in heart rate between normal-salt and high-salt in either intact or diabetic groups would explain the lack of a significant relationship between blood pressure and salt encountered in this experiment.

4.5 Renal Autoregulation

Renal autoregulation operates primarily on pre-glomerular resistance through myogenic response and TGF mechanism (Cupples and Braam, 2007). In the face of blood pressure fluctuations, an effective renal autoregulation is required to preserve the glomerular structure, and to maintain the normal renal excretory function and volume homeostasis (Cupples and Braam, 2007; Loutzenhiser *et al*., 2006).

Pressure-induced renal injury is highly dependent on RBF autoregulation since the rate of progression of glomerular disease in hypertension is inversely related to the effectiveness of autoregulation (Loutzenhiser *et al*., 2006; Bidani *et al.,* 2009). However, during long-lasting shifts of renal perfusion pressure, neurohormonal compensatory mechanisms modulate the renal vascular resistance in order to accommodate such shifts. Therefore, hypertensive kidneys can fail to preserve normal renal blood flow values, and there is also a resetting of pressure levels at which autoregulation is optimal (Characopos, 1983; Cupples, 1993; Sorensen et al., 2000; Bidani *et al.,* 2009; Hill *et al*., 2010). In the long-term, despite preserved blood flow autoregulation, these changes can lead to maladaptative renal vascular function (López-Novoa *et al*., 2010).

To evaluate the hypertensive kidney's vulnerability to damage as diabetes advanced, the RBF autoregulatory response to acute changes in renal perfusion pressure was assessed at the end of experiment. Contrary to our initial hypothesis that a hypertensive-diabetic condition would impair autoregulation, we found that the autoregulation of RBF was effective in all four groups. In addition, the renal autoregulation was extended to lower blood perfusion pressure in diabetic-normal salt group, and that was displayed by a left shift in the low limit of autoregulation.

The presence of an impaired RBF autoregulation in the early phases of 2K1C (Turkstra *et al*., 2000) and diabetes (Carmines, 2010), which could trigger inevitable vascular pathological changes, cannot be omitted from analysis. However, in the late phases of both diseases, Hill *et al.* (2010) also showed effective autoregulation in the presence of ANG II in isolated perfused kidneys from rat models of diabetes and hypertension at four months of experiment. Besides, preservation of cerebral blood flow autoregulation was found in long-standing hypertensive patients with increased vulnerability to reduced cerebral perfusion (Birns *et al.,* 2009).

Moreover, in the later stages of any chronic kidney disease, there is an imbalance between ANG II and NO that contributes to the renal pathophysiology (Singh *et al.,* 2008). While systemic and intrarenal NO production normally decreases, ANG II production increases and exerts deleterious effects on renal structure and function (Majid *et al.,* 1998; Wever *et al.,* 1999; Singh *et al.,* 2008). The intrarenal ANG II activation that increases the TGF sensitivity could not be effective in preserving renal structure; an elevated GFR with normal or lower RBF indicates a relative shift of resistance from afferent to efferent arterioles, which in turn increases glomerular hypertension and, therefore, physical injury (Navar 1998; Hostetter *et al*., 1981). On the other hand, an increased TGF sensitivity seems to be more effective in stabilizing RBF, since that would adjust the filtration and reabsorption to the demands of fluid and electrolyte homeostasis (Just *et al*., 1998; Loutzenhiser *et al.,* 2006). Therefore, an up-regulated intrarenal RAS and a reduced NO generation (Lau *et al.,* 2009) could both increase the gain of TGF and be potential mediators of preserved autoregulation.

We also hypothesised that the paradoxical effect of salt in diabetes would reduce GFR and produce a greater activation of the TGF mechanism, improving renal autoregulation. Such a mechanism would provide better protection against damage for the diabetic kidney. After four months of diabetes and salt, an effect of the salt on diabetic renal autoregulation was apparent, because the left shift of the autoregulation curve seen in the diabetic normal-salt group was abrogated by salt. That is consistent with our previous experiment on the normotensive diabetic rats under the same salt regimen. But, as it will be discussed below, it did not affect the progression of nephropathy.

4.6 Glomerular Damage

Before speaking about renal damage in the diabetic-hypertension condition, it is worth mentioning that both metabolic and hemodynamic factors are fundamental for the initiation and progression of renal damage; hyperglycemia without hypertension does not easily lead to kidney injury and as we will discuss next, hypertension without hyperglycemia manifests less severe kidney injury.

In the evolution of our experiment, both the 2K1C and STZ-T1DM experimental models experienced similar renal hemodynamic (increased pressure and flow) and nonhemodynamic (ANG II) changes. The renin-dependent hypertension model (2K1C), which is mediated mainly by the elevated activity of the RAS, displayed pressure-induced glomerular damage in the non-clipped kidney. Since a high-dietary Na intake was described as decreasing plasma RAS (Jackson and Navar, 1986; Liu *et al.*, 1993), a high-salt treatment was presumed to lessen the 2K1C renal damage by diminishing the ANG-mediated effects on renal function in this model of hypertension. In our case, there was indeed a tendency toward diminished renal glomerular and vascular lesions in the intact high-salt group compared to the intact normal-salt group, but that was not found statistically significant. While there is strong evidence in the literature of increased glomerular damage in the 2K1C model, the assessment of vascular and renal damage under long-term high-salt 2K1C conditions is inconclusive and contradictory, reporting from minimal to pronounced glomerular and vascular lesions (Jackson and Navar, 1986; Liu *et al.*, 1993). However, all these results should be interpreted by taking into consideration the duration of the experiment, the degree of pressure achieved, the limitation of tail cuff blood pressure measurements, and the amount of salt intake (Tobian and Hanlon, 1990).

The diabetic rats also displayed pressure-dependent renal injury. In addition, they showed an enhanced susceptibility to hypertensive renal damage. By plotting the glomerular injuries versus pressure load, we found that the glomerulosclerosis was linearly related to blood pressure load, but the line of best fit for the diabetic rats shifted to the left. That indicated a lower blood pressure threshold for inducing renal damage in

diabetic rats; results also matched in the published literature (Bidani and Griffin, 2004). Therefore, the non-clipped kidney, which was exposed to high blood pressure, manifested glomerular lesions whose severity was pressure dependent and exacerbated by STZ-T1DM. The clipped kidney, which was protected from increased blood pressure, displayed minimal glomerular injuries despite exposure to elevated ANG II levels. Also, preserved renal mass and the absence of atrophy indicates that they were still functional.

The difficulty in reproducing diabetic nephropathy in rodents is well known. A spontaneously lower ambient blood pressure in STZ-T1DM was also postulated as a potential contributor to the slow development of overt diabetic nephropathy (Bidani *et al*., 2007). In our case, although we had an increased blood pressure, we found the same type of histological lesions (focal and segmental glomerulosclerosis) in both the intact and diabetic rats, but with a higher degree in diabetes. However, we cannot exclude that most of the diabetic nephropathy lesions in humans are probably hypertensive nephropathy because as both diseases progress a common renal phenotype develops (López-Novoa *et al*., 2010). We also chose the Long-Evans rats in this experiment (the parent strain of the Otsuka Long-Evans Tokushima fatty rats) because we presumed that genetic susceptibility may play a role in a predisposition to develop diabetic renal injuries. Otsuka Long-Evans Tokushima fatty (OLETF) rats reliably exhibit classic diabetic nephropathy lesions.

One of our research hypotheses was that increasing salt intake will protect the diabetic kidney from damage; we found that salt had very little effect on the diabetic rats. It did not make the blood pressure worse (actually, high-salt reduced blood pressure), did not impair renal autoregulation, and glomerular vascular injury was not significantly different from that of the diabetic-normal salt group. For this reason, we have rejected the hypothesis that an increased salt intake would be renal protective in diabetic rats. Therefore, the salt paradox in later stages of diabetes is not a modifiable susceptibility factor for renal damage.

In conclusion, our results show that the elevated renal perfusion pressure was the main determinant in the development of kidney damage. However, in the long term, diabetes aggravated the effect of high blood pressure. Similar findings have been reported

in humans and diabetic rats with prior unilateral nephrectomy or exposure to the 2K1C procedure (Berkman and Rifkin, 1973; Mauer *et al.,* 1978), or with ANG II-induced hypertension (Polichnowski and Cowley, 2009). Also, our findings regarding significant renal injury despite the preservation of renal autoregulation (the only mechanism that protects kidneys from damage) requires a more thorough analysis on the role of neurohumoral activation in modulating hemodynamic and structural changes.

Initial changes in the glomerular hemodynamic (increased pressure and flow) that occur in the early phases of these diseases would increase the afferent arteriolar diameter in the non-clipped kidney by reducing renal vascular resistance; thus, allowing a greater transmission of blood pressure to the glomeruli. An impaired autoregulation in early phases of both 2K1C and STZ-T1DM was also described (Turkstra *et al.,* 2000; Carmines, 2010). The cumulated effect of hyperperfusion and hyperfiltration translates systemic hypertension into intrarenal hypertension (López-Novoa *et al*., 2010). Kidney growth and stretch-induced changes initiate production of cytokines, growth factors, and ANG II; also, damage progresses to the glomerular tuft and the filtration barrier. The concurrent loss of the glomerular basement membrane and podocytes' integrity (misdirected filtration and peritubular filtrate spreading) led to disruption of the glomerular structure (Kriz *et al.,* 2001; Kriz and LeHir, 2005).

However, under these pathophysiological changes, compensatory mechanisms act in parallel to restore the body system equilibrium. In the long term, vascular remodeling (hypertrophy of resistance vessels in response to chronic elevations in renal perfusion pressure) underlie renal functional changes. An excess of ANG II vasoconstrictor influence (mediated by TGF) would increase renal vascular resistance and decrease RBF. Increased oxygen consumption, generation of reactive oxygen species, and ANG II deleterious effects play an important role in renal damage progression. Ultimately, the presence of focal and global glomerulosclerosis, tubulointerstitial inflammation, and fibrosis indicates an accelerated decline in renal function and structure (Kriz *et al.,* 2001; Kriz and LeHir, 2005; Bidani *et al.,* 2007; Hultström *et al.,* 2008; Nakamoto *et al.,* 2008).

Chapter 5 – Conclusions and Future Directions

In order to assess the development and progression of diabetic renal damage under an established hypertensive condition, we combined the renin-mediated hypertension and Streptozotocin-induced type 1 diabetes mellitus. We used salt as a modulator of renal and cardiovascular dysfunction, to investigate the diabetic "salt paradox" as a modifiable susceptibility factor for renal damage. A six-month experiment was performed on a set of 35 Long-Evans rats divided in four groups: IN-NS, IN-HS, DM-NS, and DM-HS. Weekly 24-h blood pressure records were acquired by telemetry. Mean blood glucose of ~ 25 mmol/L was maintained by suboptimal insulin implants. Nephropathy was scored by histology in the clipped and non-clipped kidneys at the end of the protocol. Our findings can be summarized as follows:

- Systolic blood pressure increased after induction of hypertension but was not affected by diabetes or increased salt intake, either alone or together.
- We found an effective autoregulation in all groups. The DM-NS group displayed a better autoregulation at low perfusion pressure, which means the RBF remained constant at lower pressure in this group.
- We found that the glomerular damage was pressure dependent in both intact and diabetic groups, but both DM-NS and DM-HS groups exhibited an increased susceptibility of the kidney to injury, but independent of blood pressure.
- Salt did not change the renal susceptibility to hypertensive injury, which means that the salt paradox in later stages of diabetes is not a modifiable susceptibility factor for renal damage.

The particular aspect of this experiment was the effective renal blood flow autoregulation in the presence of glomerular damage. These findings open new questions

about the potential factors that can modulate the renal blood flow autoregulation under specific pathological conditions. New insights into the role of renal autoregulation in preserving the kidney structure and maintaining the circulatory homeostasis would be an interesting topic for future work.

Excessive activation of intrarenal RAS is believed to play an important role in the pathophysiology of hypertension and organ injury. Particular to our experiment, we believe that ANG II can be one of the causes that may induce (directly or indirectly by modulating other factors) the left shift in the renal autoregulation curve in DM-NS group. It is known that high salt inhibits RAS. In our experiment, the left shift in the renal autoregulation curve was abrogated by salt treatment. However, the glomerular damage was not significantly different between DM-NS and DM-HS groups. Therefore, we can conclude that there also other dominant factors involved in these changes.

As a result, it would be interesting to design an experiment to sustain or infirm the role of ANG II in the left shift of RBF in DM-NS group. Angiotensin-converting enzyme blockers could be able to mitigate the excessive ANG II, shifting the autoregulation curve back to the right.

Finally, the *in vivo* assessment of renal blood flow would bring new insights into the renal blood flow maintenance under physiological and pathological conditions. That way, the reduction of the anesthesia effect, along with renal and extrarenal losses during the terminal procedure, would improve the experimental assessment of renal blood flow. By understanding the renal hemodynamics in diabetes mellitus and hypertension, we might be able to prevent the progression of renal damage.

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Appendix A – Solutions, Drugs, and Instruments

The solutions, drugs, and instruments used during the experimental procedures are provided below in alphabetical order:

- 10% neutral buffered formalin
- Acoustic Gel
- Albustix reagent strips for urinalysis (Albustix® from Siemens Healthcare Diagnostics Inc.)
- Ampicillin (Associated Veterinary purchasing Company Ltd, 40 mg/kg)
- Ascensia glucometer ACCU-Check blood glucose monitor (Boehringer)
- Atipamezole hydrochloride (Antisedan®, Associated Veterinary purchasing Company Ltd, 0.25 mg/kg)
- Betadine topical antiseptic (Associated Veterinary purchasing Company Ltd)
- Bovine Serum Albumine (10%, pH 3)
- Buprenorphine (Temgesic® Reckitt & Benckiser, Inc, 0.02 mg/kg)
- Ethanol 70%
- Heparin saline
- Homeotermic control unit
- Isoflurane for inhalational anesthesia (Associated Veterinary purchasing Company Ltd)
- LinPlant® (Linshin Canada, Ltd) 7-mm long insulin implant with a diameter of 2 mm (fabricated under sterile conditions from mono-component human insulin)
- Medetomidine hydrochloride (Domitor®, Associated Veterinary purchasing Company Ltd, 0.25 mg/kg)
- Metabolic cages for rats
- Oxygen

- Physiological saline (0.9% solution)
- Potassium chloride (saturated solution)
- Pressure Transducer driven by a Kent TRN050 amplifier
- Ringer's lactate solution
- Silver Clip 0.22 mm internal diameter
- Small Animal Ventilator (Kent Scientific Corporation)
- Sodium chloride (5% solution)
- Streptozotocin powder (Pfizer Canada Inc)
- Surgical instruments
- Telemetry transmitters (model PA11-C40) from Data Sciences International (DSI, St Paul, Minnesota, USA)
- Tracheal intubation catheter
- Transonic Systems model T401 transit time ultrasonic flowmeter
- Tubing for venous and arterial catheters (PE 50, 90)
- Vet Solutions Surgical Scrub

Appendix B – Kidney Histological Examination Images

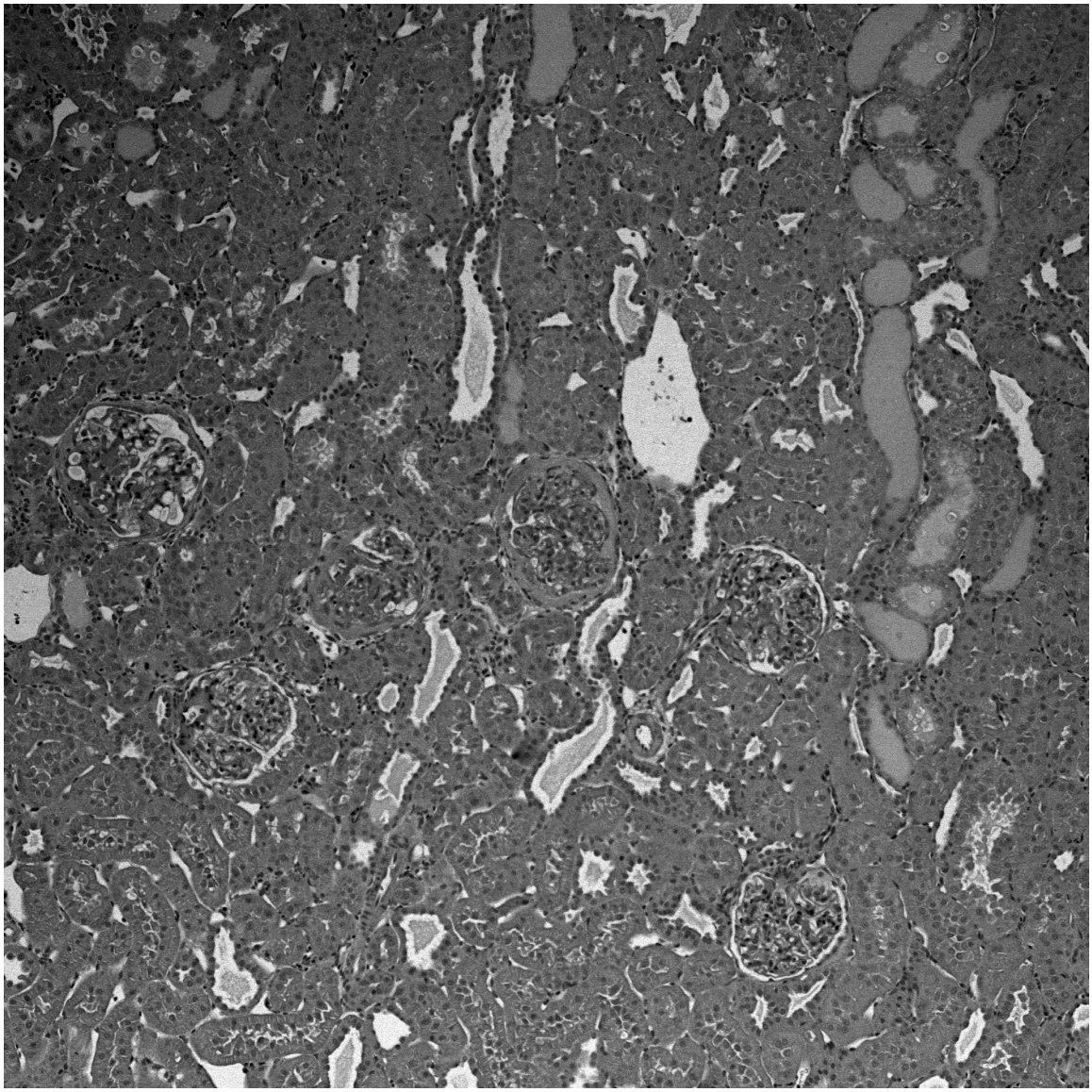

Figure 10 A. Image from confocal microscopy showing an IN-NS left (non-clipped) kidney (rat LE119). Periodic acid Schiff stain. Magnification 100X.

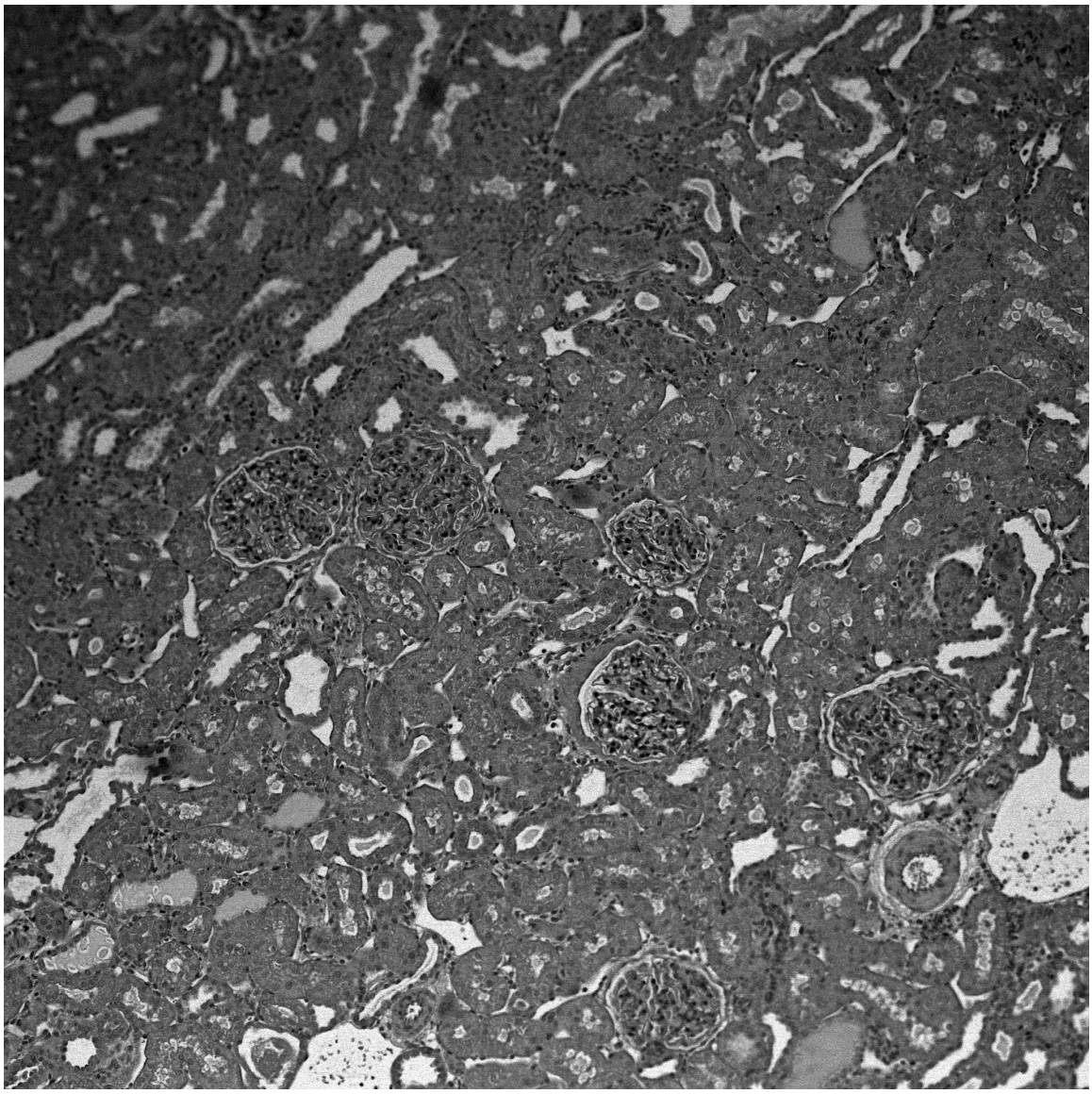

Figure 10 B. Image from confocal microscopy showing an IN-HS left (non-clipped) kidney (rat LE124). Periodic acid Schiff stain. Magnification 100X.

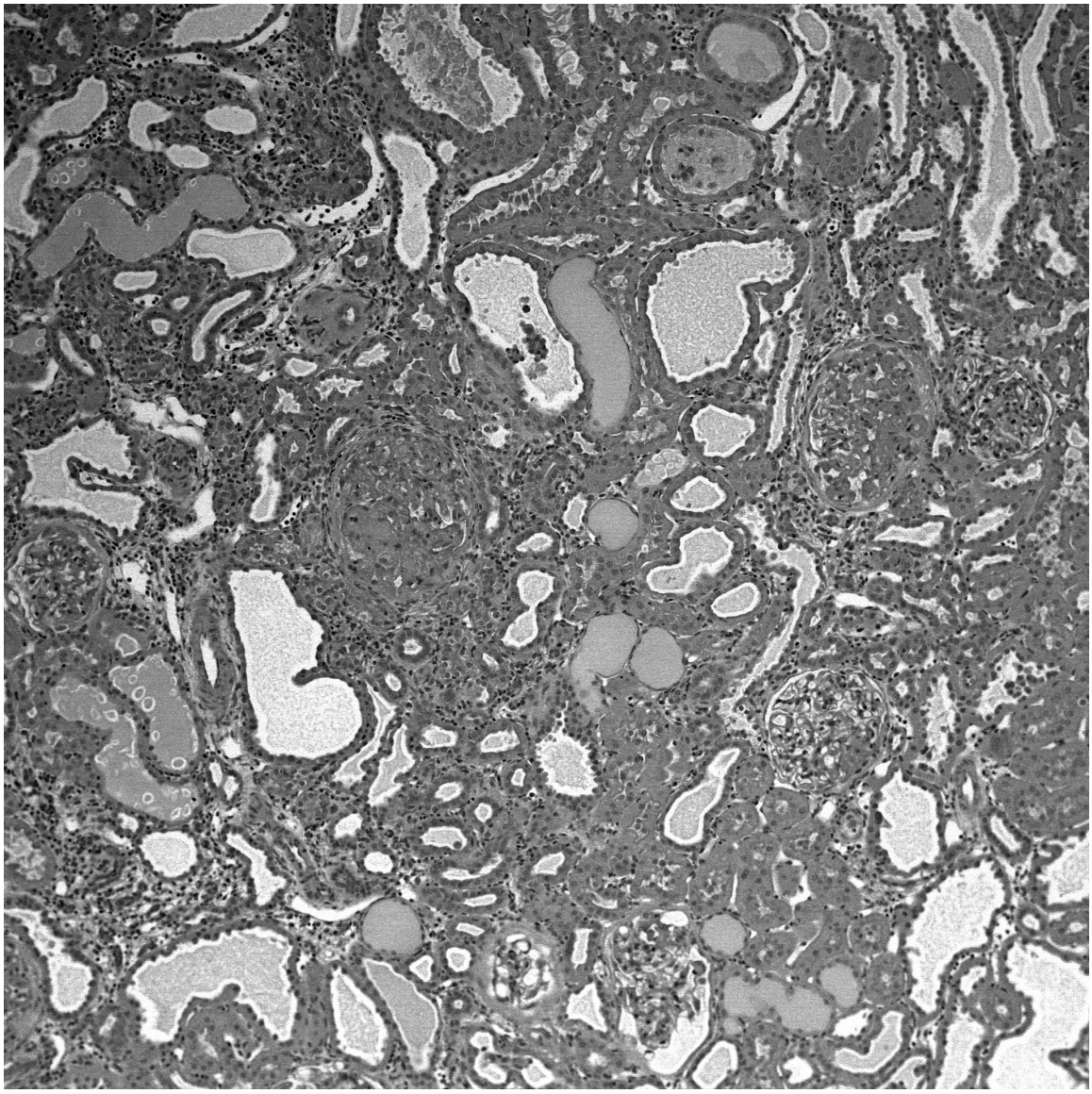

Figure 10 C. Image from confocal microscopy showing a DM-NS left (non-clipped) kidney (rat LE122). Periodic acid Schiff stain. Magnification 100X.

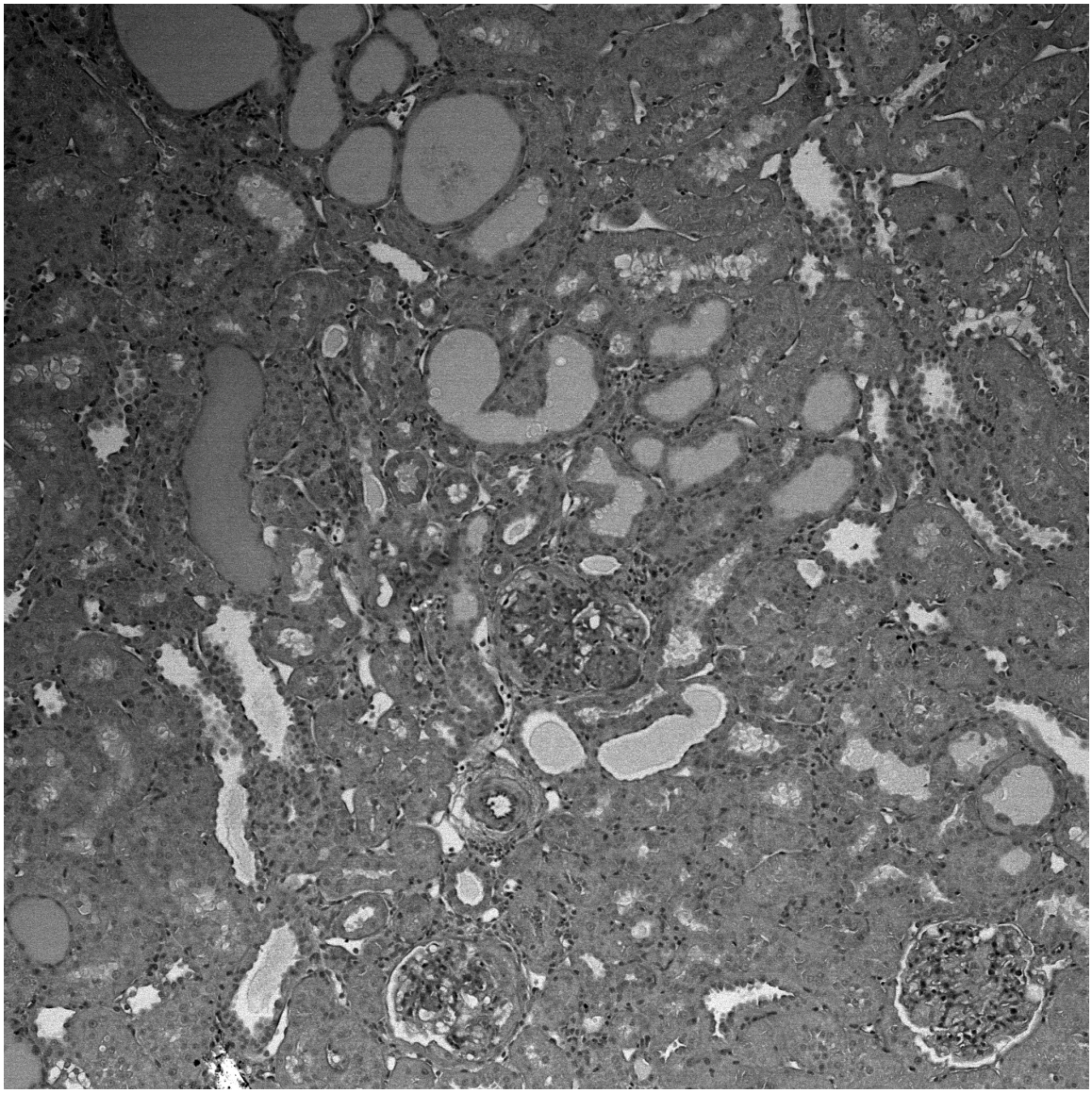

Figure 10 D. Image from confocal microscopy showing a DM-HS left (non-clipped) kidney (rat LE126). Periodic acid Schiff stain. Magnification 100X.

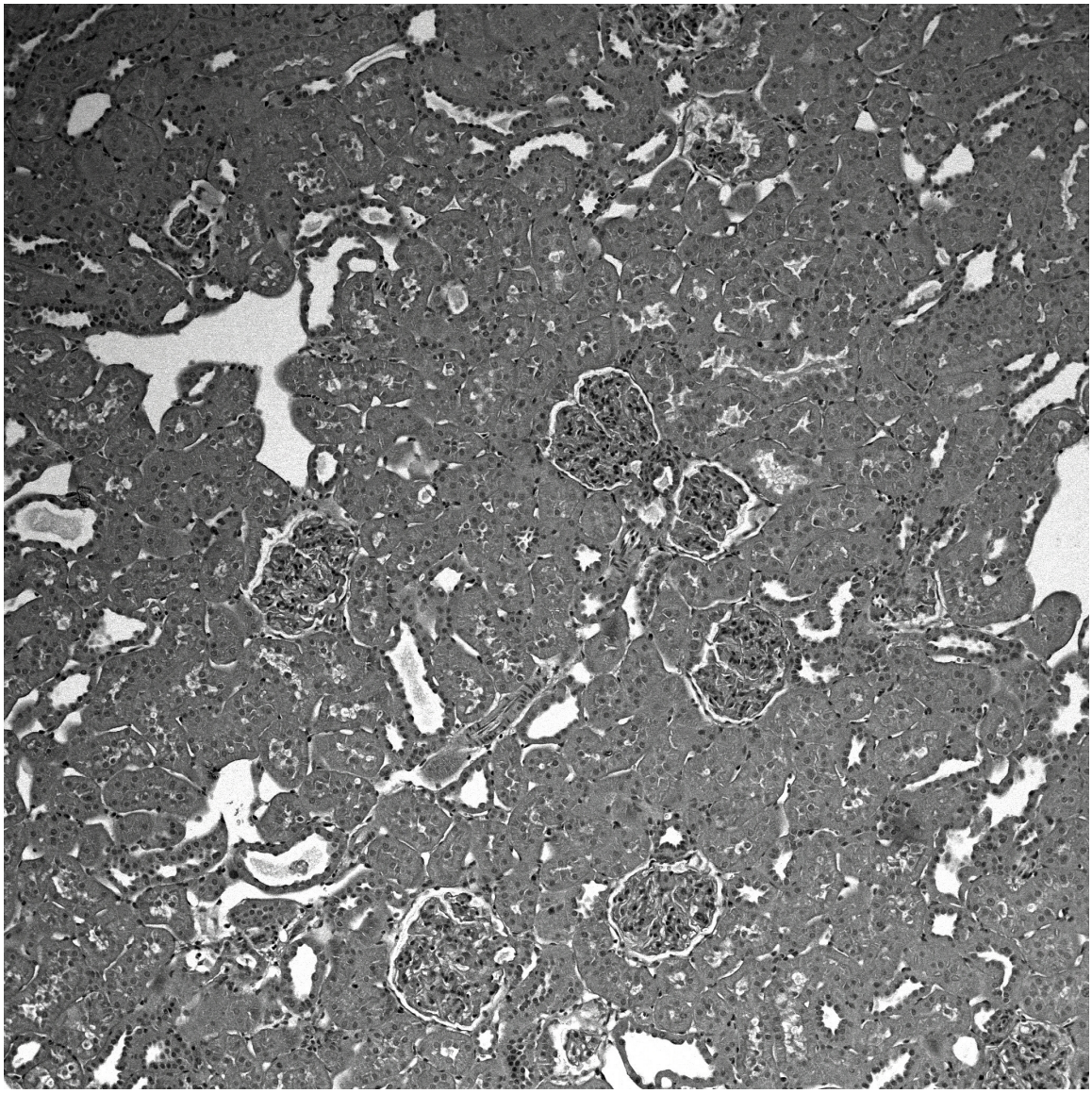

Figure 11 A. Image from confocal microscopy showing an IN-NS right (clipped) kidney (rat LE119). Periodic acid Schiff stain. Magnification 100X.

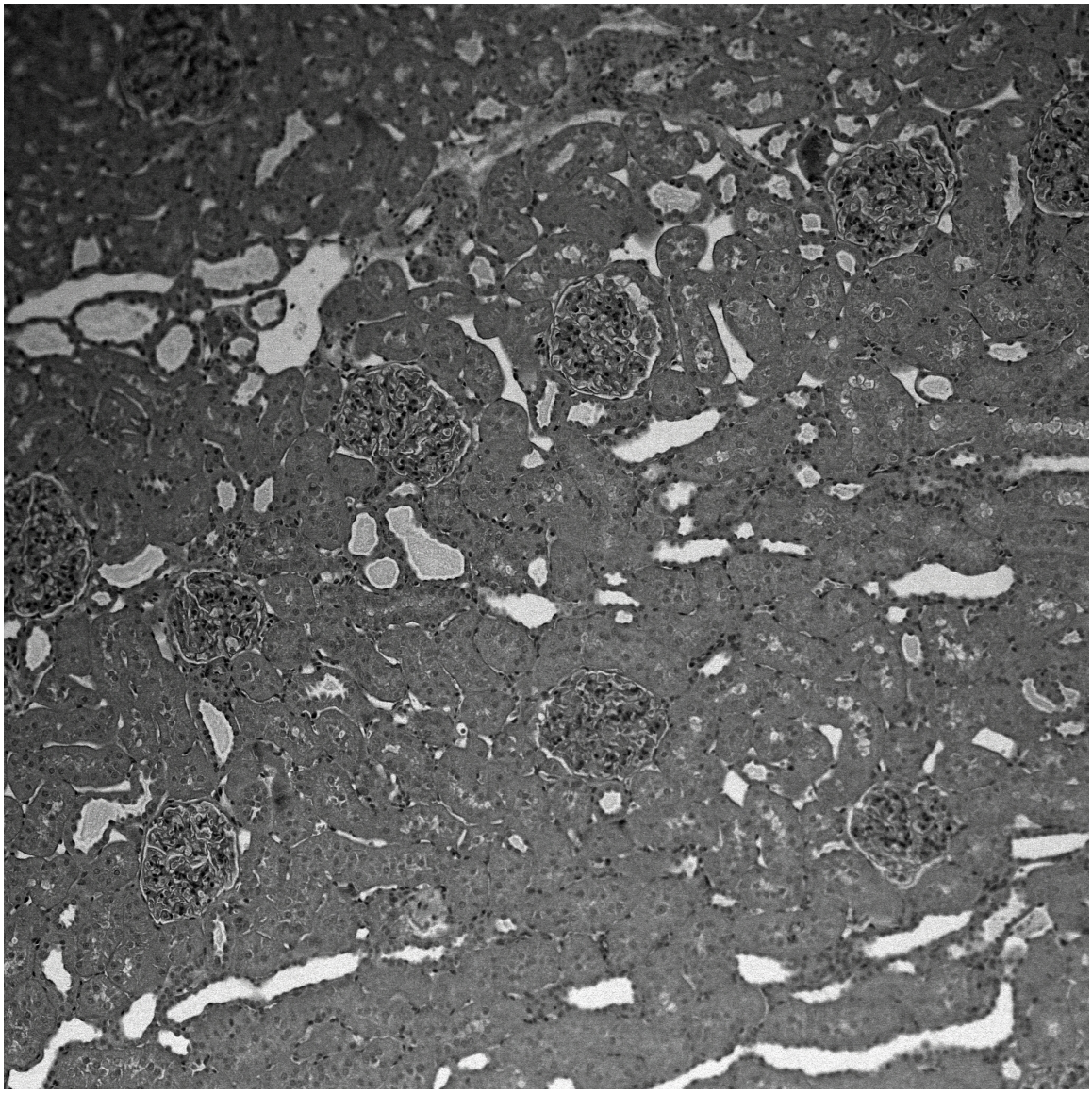

Figure 11 B. Image from confocal microscopy showing an IN-HS right (clipped) kidney (rat LE124). Periodic acid Schiff stain. Magnification 100X.

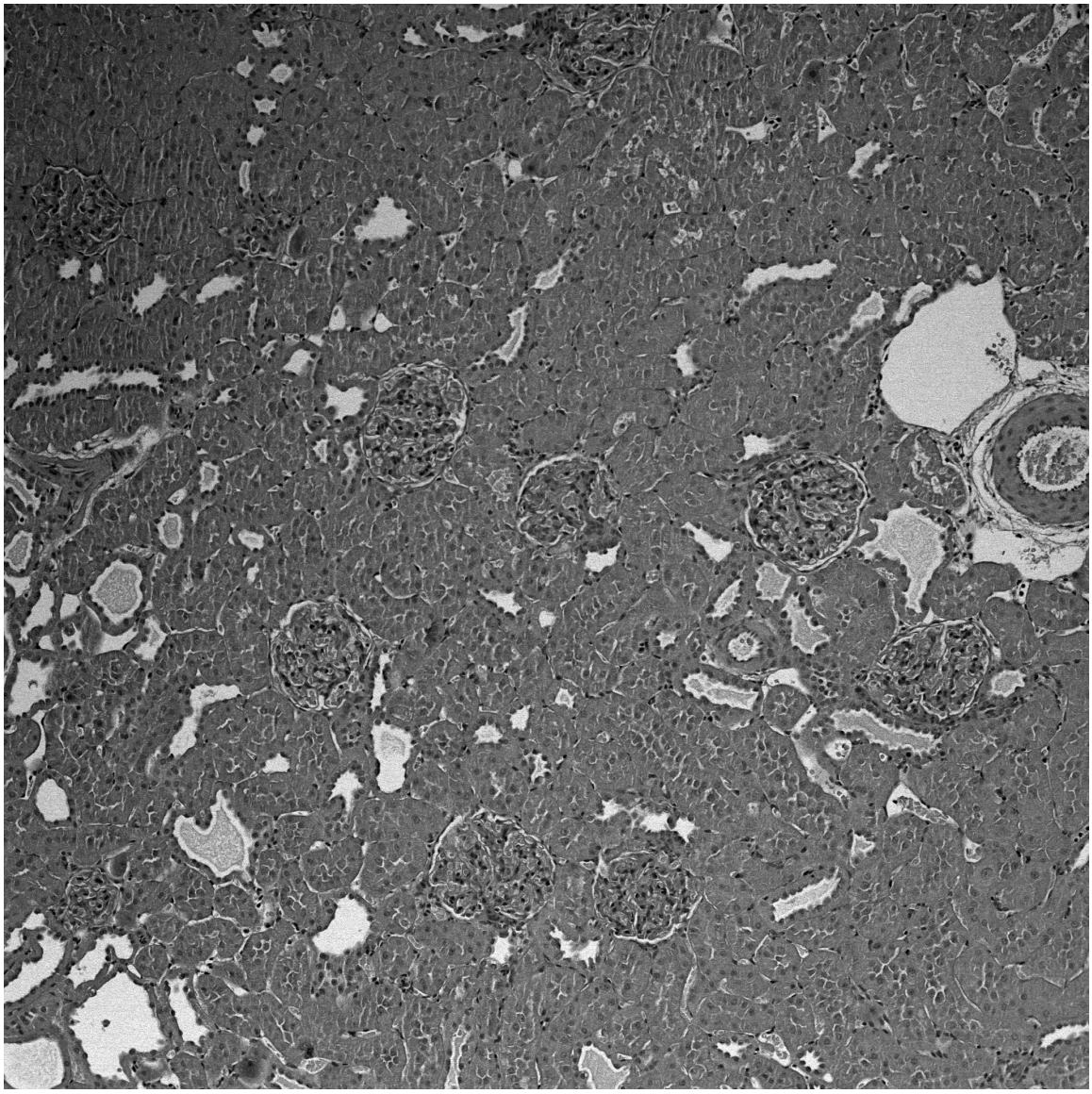

Figure 11 C. Image from confocal microscopy showing a DM-NS right (clipped) kidney (rat LE122). Periodic acid Schiff stain. Magnification 100X.

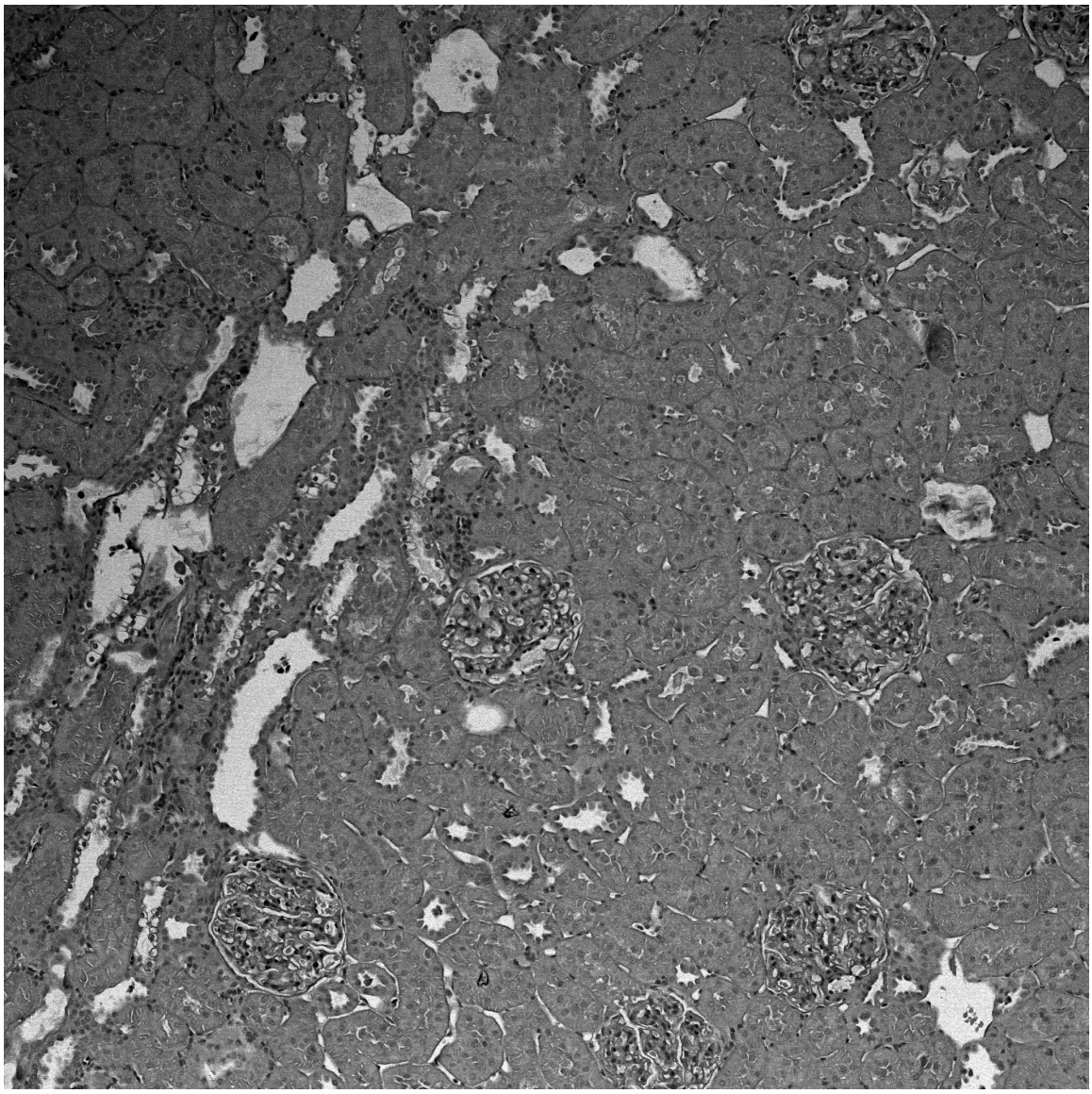

Figure 11 D. Image from confocal microscopy showing a DM-HS right (clipped) kidney (rat LE126). Periodic acid Schiff stain. Magnification 100X.