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Assessment of guanylin peptides system in type 2 diabetes

Dissertação de Mestrado em
Tecnologia Bioquímica em Saúde

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Dissertação submetida à Escola Superior de Tecnologia da Saúde do Porto para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Tecnologia Bioquímica em Saúde, realizada sob a orientação científica da Professora Doutora Maria Benedita Sampaio Maia, professora auxiliar da FMDUP e investigadora da Unidade I&D Nefrologia – FMUP, sob a co-orientação científica da Doutora Isabel João Soares Silva, investigadora da Unidade I&D Nefrologia – FMUP, e sob a co-orientação institucional da Professora Doutora Cristina Prudêncio, professora coordenadora com agregação da ESTSP-IPP.

Setembro, 2011

"Toda a nossa ciência, comparada com a realidade, é primitiva e infantil - e, no entanto, é a coisa mais preciosa que temos."

Albert Einstein

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Resumo

Os doentes com diabetes *mellitus* tipo 2 apresentam predisposição para a retenção de sódio e são frequentemente hipertensos. No entanto, os mecanismos implicados na dificuldade do rim diabético em mobilizar o sódio são, ainda, pouco compreendidos. Os peptídeos da família das guanilinas estão envolvidos na regulação do transporte de electrólitos e água nos epitélios intestinal e renal, através da activação do receptor guanilato ciclase-C (GC-C) e subsequente libertação intracelular de GMPc. O objectivo do presente estudo foi a avaliação da actividade do sistema dos peptídeos das guanilinas (SPG) e do seu papel na regulação do balanço de sódio num modelo animal de diabetes tipo 2.

Ratinhos machos C57BL/6 foram submetidos a uma dieta com alto teor de gordura e rica em hidratos de carbono simples (ratinhos diabéticos) ou a uma dieta normal (ratinhos controlo). A expressão renal e intestinal da guanilina (GN), uroguanilina (UGN) e do receptor GC-C assim como os níveis de GMPc na urina e plasma foram avaliados nos ratinhos controlo e diabéticos, durante a ingestão de dietas normo (NS) e hiper-salina (HS).

Nos ratinhos diabéticos, durante a dieta NS verificou-se um aumento significativo da pressão arterial que foi acompanhado de redução da expressão do ARNm da GN, UGN e do GC-C no intestino e de aumento da expressão de ARNm da UGN no rim. A dieta HS induziu um aumento da expressão do ARNm da UGN no jejuno dos ratinhos controlo mas não nos diabéticos. Os ratinhos diabéticos apresentaram níveis urinários de GMPc inferiores aos controlos, em condições de dieta NS.

Em conclusão, os nossos resultados sugerem que na diabetes tipo 2 ocorre uma redução da actividade intestinal do SPG que é acompanhada por um aumento compensatório da actividade renal do SPG. A diminuição da actividade do SPG intestinal na diabetes tipo 2 deve-se não só a uma redução da expressão dos peptídeos GN e UGN, mas também a uma redução da expressão do seu receptor, GC-C. Estes resultados sugerem que o SPG pode contribuir para a sensibilidade ao sódio na diabetes.

Palavras-chave: Guanilina; uroguanilina; receptor GC-C; diabetes tipo 2; carga salina.

Abstract

Type 2 diabetes *mellitus* patients have a predisposition for sodium retention and are frequently hypertensive. However, the mechanisms implied in the difficulty of the diabetic kidney in mobilizing sodium are still poorly understood. Guanylin peptides regulate electrolytes and water transport in intestinal and renal epithelia through guanylate cyclase C (GC-C) receptor activation and subsequent cGMP intracellular release. The aim of the present study was to evaluate the activity of the Guanylin peptides system (GPS) and its role on the regulation of sodium balance in type 2 diabetes mouse model.

Male C57BL/6 mice were submitted to either a high-fat high-simple carbohydrate diet (diabetic mice) or a normal diet (control mice). The renal and intestinal guanylin (GN), uroguanylin (UGN) and GC-C receptor mRNA expression as well as urinary and plasma cGMP were evaluated in control and diabetic mice, during normo (NS) and high-saline (HS) diets.

In diabetic mice, during NS diet the increased blood pressure was accompanied by a reduced GN, UGN and GC-C mRNA expression in the intestine and increased UGN mRNA expression in the kidney. Sodium load increased UGN mRNA expression in the jejunum of the control mice, but not in diabetic. Urinary cGMP levels were reduced in diabetic mice in comparison to control mice, during NS diet.

In conclusion, our results suggest that in type 2 diabetes a down-regulation of intestinal GPS is accompanied by a compensatory increase of renal GPS activity. The blunted intestinal GPS in type 2 diabetes is due not only to a reduced expression of GN and UGN peptides but also to a reduced expression of its GC-C receptor. These results suggest that GPS may contribute to the sodium sensitivity in diabetes.

Keywords: Guanylin; uroguanylin; GC-C receptor; type 2 diabetes; sodium load.

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Abbreviations

ANP	Atrial natriuretic peptide
C	Control
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
C57BL/6	C57 black 6
D	Diabetic
EIA	Enzyme Immuno Assay
GC-C	Guanylate cyclase C
GC-A	Guanylate cyclase A
GC-B	Guanylate cyclase B
GPS	Guanylin Peptides System
GN	Guanylin
HFHSC	High fat high simple carbohydrate
HS	High-saline
mRNA	Messenger ribonucleic acid
NS	Normo-saline
STa	Heat stable enterotoxin of *Escherichia coli*
qPCR	Quantitative real-time PCR
UGN	Uroguanylin
T2DM	Type 2 diabetes *mellitus*

Introduction

Guanylin family

The existence of uroguanylin (UGN) and guanylin (GN), was foreshadowed by the discovery of an orphan receptor guanylate cyclase in opossum kidney

This receptor named guanylate cyclase C (GC-C) receptor is markedly activated by heat-stable enterotoxin (ST) peptides produced by strains of *Escherichia coli*, which cause a cholera-like form of secretory diarrhea (Field, 2003).This receptor was discovered in the intestinal, renal, hepatic, respiratory and testicular tissues (Forte *et al*., 1988, Forte *et al*., 1989, White *et al*., 1989, Krause *et al*., 1990) which suggested the activity of endogenous ST-like peptides. The identification of GN was made by Currie and co-workers that purified and sequenced a rat intestinal peptide possessing properties consistent with an endogenous ligand for the intestinal GC-C (Currie *et al*., 1992). Later, UGN was isolated from opossum urine (Hamra *et al*., 1993).

GN and UGN are closely related peptides which regulate electrolyte and water transport in intestinal and renal epithelia (Sindic and Schlatter, 2006a). These peptides are encoded by two different genes in humans, rats and mice, and are synthesized in the form of prepropeptides. In humans, these genes are located at chromosome 1 and are composed by three exons and two introns (Hill *et al*., 1995, Mägert *et al*., 1998), whereas in mice, the genes for GN peptides are located in chromosome 4 (Schulz, 1999). Cleavage of prepropeptides (preproGN – 115 amino acids; preproUGN – 112 amino acids) leads to the formation of proGN (94 amino acids) and proUGN (84 amino acids) (de Sauvage *et al*., 1992, Wiegand *et al*., 1992, Mägert *et al*., 1998, Miyazato *et al*., 1996b) but only a second cleavage of the propeptides gives origin the bioactive GN and UGN. GN and UGN are heat-stable peptides with 15 to 19 amino acids (Sindic and Schlatter, 2006a). Human and rat GN are very similar, differing only in one amino acid (Currie *et al*., 1992, Hamra *et al*., 1996a, Mägert *et al*., 1999, Sindic and Schlatter, 2005). The sequences of GN and UGN from various species are shown in Table 1.

Table 1: Differences between the amino acid sequences of GN and UGN in different species (adapted from Sindic and Schlatter, 2005)

Species	Guanylin	Uroguanylin
Human	PGT CEICAY AACT GC	NDD CEL CVNV ACTGCL
Rat/Mouse	PNT CEICAY AACT GC	TDE CEL CINV ACTGC

Human GN (15 amino acids) and UGN (16 amino acids) possess two disulfide bonds between the cysteins in positions 4 and 12 and between positions 7 and 15, that are essential for their activities (de Sauvage *et al*., 1992, Kita *et al*., 1994, Nokihara *et al*., 1997). GN peptide contains a tyrosin in position 9 from the N-terminus whereas UGN has an aspargine (Hamra *et al*., 1996a, Hamra *et al*., 1996b).

The gastrointestinal tract is the main source of GN and UGN and contains the highest levels of its respective mRNAs and mature proteins among all tissues (Beltowski, 2001). Small intestine has abundant UGN mRNAs levels whereas the large intestine is rich in GN mRNAs (Forte *et al*., 1999). In the intestine, UGN is expressed in a specific type of endocrine cell named enterochromaffin cells (Perkins *et al*., 1997, Nakazato *et al*., 1998). Human GN was detected in villus epithelial cells and Paneth cells of small intestine and in the superficial epithelial cells of large intestine (Date *et al*., 1996), namely the goblet cells of the colon (Li *et al*., 1995). The circulating GN peptides can have origin, besides the intestine, in kidneys and other tissues like adrenal glands, reproductive system, lung and pancreas (Kinoshita *et al*., 1990, Kinoshita *et al*., 1997a, Kinoshita *et al*., 1997b, Schulz *et al*., 1992, Miyazato *et al*., 1996a, Li *et al*., 1997, Nakazato *et al*., 1998).

In plasma, GN and UGN circulate almost exclusively as prohormone – inactive form and are freely filtered through the glomerular barrier in the kidney (Nakazato *et al*., 1994, Kuhn *et al*., 1993). Interestingly, proUGN is processed to its active natriuretic form exclusively within the renal tubules (Qian *et al*., 2008). Recent studies report that intravascularly infused proUGN induces a more effective natriuresis than UGN (Moss *et al*., 2010). The authors suggested that proUGN is converted intrarenally to an unknown natriuretic peptide that is distinct from UGN. Thus, the UGN signaling pathways in the gut and kidney appear to have diverged significantly in respect to the receptors, the ligands, and the propeptide processing mechanisms that are active in each tissue (Qian *et al*., 2011). So, urine contains UGN and low amounts of proUGN (Hamra *et al*., 1993). Regarding GN, very low amounts are found in urine; the explanation for this fact is unclear, but it was proposed that GN may be cleaved and inactivated by proteases within renal tubules (Fan *et*

al., 1997). Chymotrypsin cleaves the peptide bond in the carboxyl terminal next to the aromatic residues of GN peptides, but UGN and E. coli ST are cleavage resistant since they have asparagine residues instead of tyrosine/phenylalanine residues (Hamra *et al*., 1996a, Hamra *et al*., 1996b). The same protease converts filtered proUGN into the active peptide leading to higher UGN/proUGN ratio in urine than in plasma (Hamra *et al*., 1993). Moreover, the members of this GN peptide family display an unusual form of structural isomerism, topoisomers A and B (Skelton *et al*., 1994). The B conformation of these peptides has been considered physiologically inert because of its very low activity when tested on intestinal GC-C receptors, but it was recently discovered that human UGN-B potently elicits renal natriuresis, being this the first time that two distinct topoisomers of a single peptide have been shown to have biological activity (Moss *et al*., 2009). The differences in the metabolism of GN and UGN suggest singular functions and targets. UGN was shown to be more natriuretic than GN (Sindic and Schlatter, 2005), since Fonteles and co-workers showed that in the isolated perfused rat kidney GN mainly caused kaliuresis and diuresis with less pronounced natriuretic effect (Fonteles *et al*., 1998). In 2001, Potthast and co-workers observed that the renal expression of GN is not altered in animals on high sodium diet (Potthast *et al*., 2001). Therefore Sindic and Schlatter suggest that GN could act more as an intestinal kaliuretic peptide rather than as an intestinal natriuretic peptide (Sindic and Schlatter, 2005).

Guanylin and uroguanylin receptors, signal transduction and biological actions

GN and UGN act via activation of GC-C receptor and other not yet revealed GC-C like receptors. GC-C is mainly localized on the apical or brush border membrane of intestinal epithelial cells (De Jonge, 1975, Field *et al*., 1978, Schulz *et al*., 1990, Basu *et al*., 2010). The role of GC-C receptor in ST-induced diarrhea indicates that it may have a vital function in the maintenance of fluid-ion homeostasis in the intestine (Schulz *et al*., 1997, Mann *et al*., 1997, Basu *et al*., 2010). The importance of the GC-C gene in intestinal fluid secretion is evidenced in compromised GC-C transgenic mice that presented a reduction of intestinal fluid secretion (Schulz *et al*., 1997, Mann *et al*., 1997). Nevertheless some data suggest the existence of multiple genes coding for GC-C like receptors in mouse genome, since in GC-C–knockout mice some GC-C like receptors are still present and functionally active (Mann *et al*., 1997, Forte *et al*., 1999).

Although GC-C is the predominant receptor mediating the effects of GN peptides in the intestine, it plays only a minor role in the kidney, being possibly restricted to the proximal tubule. Along the nephron, at least three different receptors with different second messenger systems are activated by GN peptides which mediate complex effects on water and electrolytes excretion (Sindic and Schlatter, 2006a). This belief was reinforced by the recent suggestion by Moss and co-workers (Moss *et al*., 2009) that B topoisomer of human UGN elicits renal natriuresis via intestinal GC-C receptors independent pathway.

Two other members of the receptor family, guanylate cyclase A (GC-A) and B (GC-B), were previously identified and bind specifically atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP), respectively. Recently, an interaction between ANP and GN peptides was reported in isolated perfused rat kidney (Santos-Neto *et al*., 2006). These authors suggested that this interaction may play a contributory role in the regulation of kidney function in many pathophysiological states, such as in the natriuresis following ingestion of salty meals (Santos-Neto *et al*., 2006).

The GC-C receptor influences cellular function via intracellular cyclic guanosine monophosphate (cGMP). In Figure 1 various pathways directly regulated by GC-C or modified by cGMP production are shown (Basu *et al*., 2010).

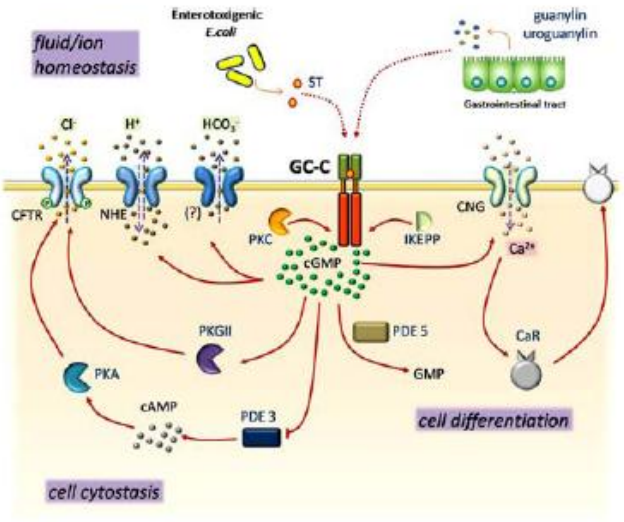

Figure 1: GC-C signaling (from Basu *et al*., 2010).

A number of studies have shown that, at the kidney level, GN, UGN and UGN-like peptide *E. coli* ST, elicits increases in urinary excretion of sodium, potassium, chloride and water (Fonteles *et al*., 1998). Activation of the GC-C receptor leads to the secretion of chloride and bicarbonate and to the inhibition of Na^+ absorption, which determines water

secretion, and at the same time induces an increase in salt and water excretion in the kidney (Fonteles *et al*., 1998). This is mediated via cGMP, that activates protein kinase G II and/or increases intracellular cAMP via inhibition of phosphodiesterase III leading to activation of protein kinase A. Protein kinase G II and protein kinase A increase the secretion of Cl^-, HCO_3^- and water via activation of the cystic fibrosis transmembrane conductance regulator (CFTR) (Sindic and Schlatter, 2006b). When compared to GN peptides, ST has a stronger and uncontrolled activation of GC-C probably because it contains three disulfide bonds instead of two present in GN and UGN (Carpick and Gariepy, 1993).

These peptides may activate different signalling pathways along the nephron and the intestinal tract. The hypothesis that UGN could serve as an endocrine axis (enterorenal axis) connecting the gastrointestinal tract to the kidney for regulation of sodium excretion (Forte *et al*., 1988) was suggested by the observations made more than three decades ago that oral administration of sodium induces a natriuresis that greatly exceeds the increase in urine sodium excretion elicited by intravenous NaCl infusion (Lennane *et al*., 1975). This hypothesis was further supported by the recent demonstration that mice deficient in UGN have blunted urinary sodium excretion responses to oral sodium loads in addition to elevated blood pressure (Lorenz *et al*., 2003), but are able to excrete sodium normally after intravenous administration of NaCl. In fact, increases in dietary sodium raise UGN mRNA levels in the intestine and kidney, suggesting that both endocrine and paracrine/autocrine actions of UGN could participate in tubular signalling mechanisms that control renal sodium transport (Beltowski, 2001).

Given that the GN peptides are involved in the regulation of electrolyte and water transport in both intestine and kidney, one can hypothesise that changes in the physiological mechanisms which regulate the production and/or secretion of GN and UGN in the kidney and/or intestine, may occur in conditions characterized by compromised sodium homeostasis. In accordance, in patients with chronic renal failure and glomerulonephritis, increases in plasma and urine concentrations of GN and UGN were reported (Fukae *et al*., 2000, Nakazato *et al*., 1996, Nakazato *et al*., 1994). On the other hand, UGN was suggested to play an important role as natriuretic factor in rats with puromycin aminonucleoside-induced nephrotic syndrome (Kikuchi *et al*., 2005). Also, plasma proGN and proUGN were found to be elevated in patients with heart failure, suggesting that this novel endocrine system may contribute to the sodium homeostasis in this pathophysiological condition (Narayan *et al*., 2010).

Sodium handling in diabetes

The World Health Organization believes that diabetes has assumed epidemic proportions and estimates that in 2025 there will be more than 300 million diabetic patients worldwide (Zimmet *et al*., 2001). Diabetes *mellitus* is known as a group of heterogeneous disorders characterized by hyperglycaemia and glucose intolerance. The causes may be insulin deficiency, impaired effectiveness of insulin action, or both (Harris and Zimmet, 1997). There are two types of diabetes, type 1 and type 2 diabetes. In type 2 diabetes, insulin resistance and relative insulin deficiency are characteristic of this pathology. The enhanced prevalence of this type of diabetes is thought to be associated with various factors such as social changes, ageing, dietary changes and an unhealthy lifestyle (WHO, 1994). Table 2 describes the several complications that can arise from establishment of diabetes.

Table 2: The major diabetes complications (addapted from IDF, 2009).

Brain and cerebral circulation Eyes		Cerebrovascular disease Retinopathy
Heart and coronary circulation		Coronary heart disease
Kidney		Nephropathy
Peripheral nervous system		Neuropathy
Lower limbs		Peripheral vascular disease
Diabetic foot		Ulceration and amputation

Type 2 diabetes *mellitus* patients have a predisposition for sodium retention and are frequently hypertensive (Landsberg, 1994, Segers *et al*., 1996). The interaction between hyperglycemia and hypertension is of outmost importance for clinical outcomes. Yet, how diabetes affects blood pressure control remains less clear. An abnormal renal sodium handling with sodium retention appears to play an important role in diabetes, even in the presence of well-preserved renal function.

The mechanisms implied in the difficulty of the diabetic kidney to mobilize sodium are still poorly understood. Although no single reason has emerged, a major cause was suggested to result from an increase in tubular sodium reabsorption (Vallon *et al*., 2003).

In the presence of renal insufficiency, sodium sensitivity is aggravated in diabetes and contributes importantly to the increase of blood pressure and progression of nephropathy. The activity of the GN peptides system and its role on the regulation of sodium balance in type 2 diabetes was not previously examined.

It was previously shown that mice subjected to a high fat high simple carbohydrate (HFHSC) diet present obesity, type 2 diabetes *mellitus* (T2DM) and hypertension (Rogers and Webb, 1980, Roncon-Albuquerque *et al*., 2008, Surwit *et al*., 1988). This animal model was considered one of the most reliable and clinically relevant experimental model of T2DM and was proven to be useful for mechanistic studies and as a tool for developing novel therapeutic interventions (Winzell and Ahren, 2004).

Aims

On the basis of the previous considerations, the aim of the present project was to evaluate the GN peptides system activity in diabetes using an animal model of type 2 diabetes, induced by ingestion of high-fat and high-simple carbohydrate diet in C57BL/6 mice. Given that it is described that the production of GN peptides is under the influence of sodium present in the diet, studies were performed in conditions of normal and high sodium intake. Studies were carried out to evaluate changes in 1) urinary excretion of sodium, creatinine, urea and protein; 2) blood pressure and renal function; 3) cGMP levels in urine and plasma; 4) GN, UGN and GC-C mRNA expression in the kidney and the intestine assessed by qPCR, and 5) GN, UGN and GC-C protein expression in the kidney and the intestine assessed by immunohistochemistry or western blot.

Materials and Methods

In vivo studies

Animal model of type 2 diabetes *mellitus*

Five-week-old male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Animals were kept in a controlled environment under 12:12h light-dark cycle, at a room temperature of 22±2°C. At six weeks of age C57BL/6 mice were randomly assigned to either normal diet (Teklad LM-485 Mouse/Rat Sterilizable Diet; Madison, WT), with 4.1 kcal/g composed of 5% fat, 54% carbohydrate (ground corn), 19% protein, 5% fiber, 0.31% sodium and 0.85% potassium – control mice (C), or high-fat and high-simple carbohydrate diet (HFHSC; Diet F2685; BioServ Frenchtown, NJ), with 5.4 kcal/g composed of 35% fat (lard), 35% simple carbohydrate (sucrose), 20% protein, 0.1% fiber, 0.39% sodium and 0.56% potassium – diabetic mice (D), during 12 weeks after diet initiation. The animals had free access to tap water and were fed *ad libitum* throughout the study. During this period, daily quantification of body weight, water and caloric ingestion was performed. Glucose tolerance and insulin resistance tests were performed at 12 weeks.

Blood pressure changes of these animals were characterized throughout the study. For this purpose blood pressure (systolic and diastolic) and heart rate were measured weekly in conscious restrained animals, using a photoelectric tail-cuff pulse detector (LE 5000, Letica, Barcelona, Spain). Systolic blood pressure was assessed in conscious animals. From 08h00 to 10h00, mice were placed into a semi cylindrical container with controlled temperature (36–37°C). Animals were previously conditioned to the restrainer by repeating inflation–deflation cycles for 30 min every 2 weeks from the start of the experimental protocol. Before measurements, animals were conditioned to the restrainer by repeating inflation–deflation cycles for 15 min. At 12 weeks, a minimum of five reliable systolic blood pressure measurements were obtained for each animal and the average was used for further calculations.

Glucose tolerance and insulin resistance tests were carried out at 12 weeks after diet initiation. A glucose tolerance test was performed after a 14h fast through a single intraperitoneal injection of dextrose (1.0 g/kg). Blood glucose concentration was measured before and 15, 30, 45, 60, 90, 120 and 150 min after injection using a blood glucose meter

(Freestyle Mini™ system). Insulin resistance was tested after a 6h fast through a single intraperitoneal injection of insulin, 1.5 U/kg (Actrapid® Novo Nordisk). Blood glucose measurements were made before and 15, 30, 45, 60 and 90 min after injection.

Animal experiments were performed in accordance with the European Directive number 86/609, transposed to the Portuguese Law by DL 129/92 and by Portaria 1005/92.

Metabolic studies during normal and high sodium intake

Twelve weeks after the administration of normal or high caloric diets, the animals were housed in metabolic cages (Techniplast, Buguggiate-VA, Italy) under controlled environmental conditions for the collection of 24h urine. The control and diabetic groups were subdivided: one group of animals received tap water (normal salt intake, NS) whereas the other group of animals received 1.0 % (w/v) NaCl in the drinking water (high salt intake, HS) for 3 days. Urine volume was gravimetrically determined. The daily sodium intake in normal and high sodium diets average 1 and 3 mmol, respectively.

The experimental protocol for the induction of type 2 diabetes *mellitus* animal model, subject to normal salt or high salt intake is schematically presented in Figure 2.

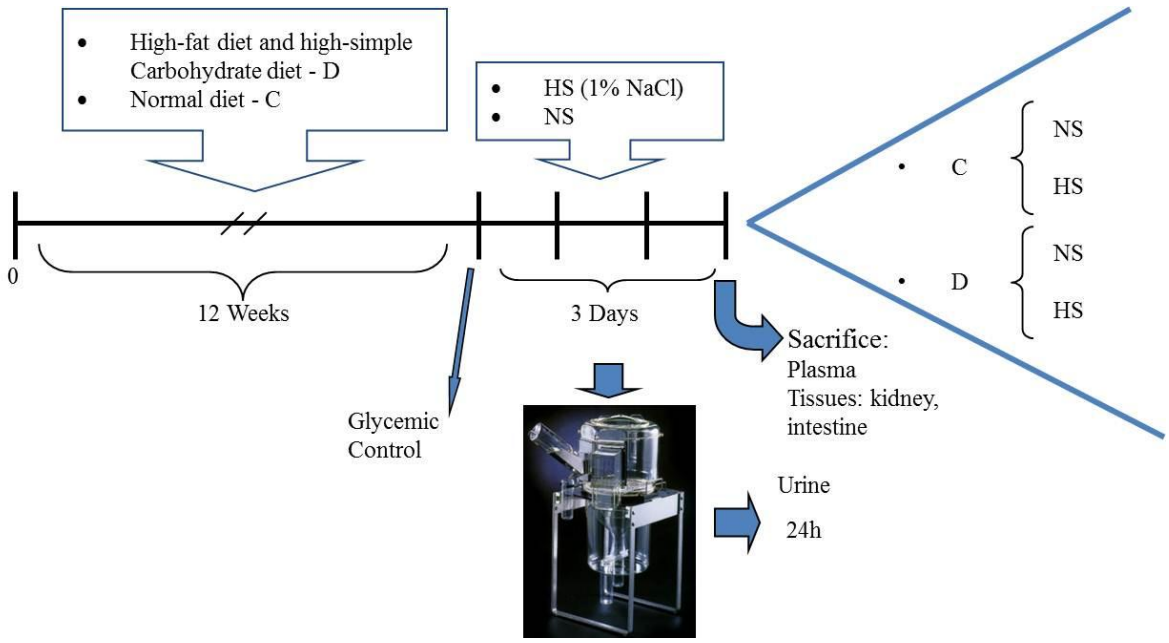

Figure 2: Type 2 diabetes *mellitus* animal model, subject to normal salt (NS) or high salt (HS) intake.

On the day of sacrifice, 72 hours after normal or high-saline oral diet, the animals were anaesthetised with sodium pentobarbital (50 mg.kg bw-1; ip). Blood was collected directly from the heart in tubes containing lithium/heparin for later determination of plasma biochemical parameters.The kidneys and intestine were rapidly removed after sacrifice through an abdominal midline incision. The kidneys were weighed, rinsed free from blood with saline solution (0.9% NaCl), decapsulated and cut in half. Half of the kidney was immediately processed for paraffin storage. The other half was divided in fragments of renal cortex, immersed in RNAlater Stabilization Reagent (Qiagen, Hilden, Germany) and stored at -80ºC. The intestine was flushed with cold saline solution and separated into the following segments: jejunum and colon. Some fragments were immersed in RNAlater Stabilization Reagent (Qiagen, Hilden, Germany) and stored at -80ºC and other were processed for paraffin storage.

***In vitro* studies**

Plasma and urine ionogram and biochemistry

Plasma cholesterol and triglycerides as well as plasma and urine levels of sodium, creatinine, urea and proteins were evaluated with an automatic analyser, Cobas Mira Plus analyser (ABX Diagnostics, Switzerland), according to ABX diagnostic reagents conditions. Total proteins in plasma samples were quantified by a colorimetric test, the Biuret reaction. In alkaline solution, cupric ions react with proteins to form a violet-coloured chelate complex. Urinary proteins were determined by a photometric test using pyrogallol red. Proteins together with pyrogallol red/molybdate form a red complex. In both colorimetric tests, the rate of colour formation is directly related to the protein concentration and is measured photometrically. Cholesterol was determined after enzymatic hydrolysis and oxidation, by an enzymatic photometric, the Trinder's reaction. In presence of peroxidase, the H_2O_2 formed effects the oxidative coupling of phenol and 4-antipyrine to form a red-coloured quinoneimine derivate. The colour intensity is directly related to the cholesterol concentration and is measured photometrically. The quantification of triglycerides was performed by enzymatic colorimetric test, GPO/PAP. Urea was measured by an enzymatic test UV/ urease/ GLDH method. The quantification of sodium was performed by ion-selective electrodes. Creatinine was measured by the Jaffé method, a colorimetric test that is based on the reaction of picric acid with creatinine in

alkaline solution to form a yellow-red-coloured complex. Creatinine clearance was calculated using 24-hour urine creatinine excretion. Fractional excretion (FE) of sodium was calculated as previously reported (Sampaio-Maia *et al*., 2005): $FE_{Na}=[(U_{Na}/P_{Na})/(U_{crea}/P_{crea})]x100$, where U_{Na} is the urinary concentration of sodium (mg/L), U_{crea} is the urinary creatinine concentration (mg/L), P_{Na} is the plasma concentration of sodium (mg/L) and P_{crea} is the plasma creatinine concentration (mg/L).
Serum insulin was quantified by ELISA, according to the manufacturer's instructions (Linco Research, St. Charles, Missouri, USA).

Evaluation of guanylin, uroguanylin and GC-C receptor mRNA expression in the kidney and the intestine

The relative quantification of GN, UGN and GC-C receptor mRNA expression was performed by two-step RT-qPCR (reverse transcription and quantitative real-time PCR). After collection, samples were quickly immersed in RNA later (Qiagen, Hilden, Germany) and frozen at -80ºC. Total mRNA was extracted with the trizol RNA extraction method, according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). The purity of the sample and the RNA concentration was assayed by spectrophotometry (Eppendorf AG, Hamburg, Germany). To evaluate the integrity, the RNA was separated by electrophoresis on a 1% agarose gel (SeaKem® LE Agarose; Lonza, Cologne, Germany), and then visualized with NucliStain (National Diagnostics, Atlanta, USA). Total RNA of jejunum was incubated with rDNase I (DNA-free Ambion, Austin, Texas) and DNase I buffer at 37 °C for 30 min to digest isolated traces of genomic DNA, followed by additional cleaning steps, as described in manufacturer's instructions.Reverse transcription was performed in a standard thermocycler (MyCycler, BioRad, Hercules, California, USA) with the iScript cDNA Synthesis Kit (BioRad, Hercules, California, USA). Reaction conditions were as follows: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C (hold at 4ºC).

cDNA first-strand synthesis was performed in a total reaction volume of 20 µl containing 7.5 µl of total RNA (≈750 ng), 7.5 µl of DNase/RNase free water, 4 µl 5X cDNA synthesis kit buffer, 1 µl iScript enzyme mixture (BioRad, Hercules, California, USA). The obtained cDNA was used as a template for qPCR (MiniOpticon, BioRad) using SYBR green chemistry (BioRad, Hercules, California, USA), according to the manufacturer's instructions. One µl cDNA was then subjected to a 25 µl PCR reaction

using 12.5 µl SYBR Supermix, 0.5µl of each primer (10 nm) and 10.5 µl of DNase/RNase free water. For each gene and tissue studied standard curves of graded dilutions from a randomly selected control sample were generated, for determination of efficiency of qPCR. β-actin mRNA was used as reference gene. Primers used for each gene analysed were referenced in other studies (Mann *et al*., 2010, Lorenz *et al*., 2003) or were designed with the bioinformatic tool Primer-BLAST http://www.ncbi.nlm.nih.gov/tools/primer-blast/ (Table 3).

The primers were designed according to the following: size between 17-28 bases; composition between 50-60% G+C; size of the amplified fragment (<150 bp); Tms between 55-65ºC; 3' end of primers not complementary to avoid the formation of primer dimers; primers not self-complementary to avoid hairpin-like structures. The amplification products of each primer pair were analysed by gel electrophoresis. The conditions used in the qPCR reaction are described in Table 4. Reaction specificities were verified by melting curve analysis.

Table 3: Nucleotide sequences of primers employed in qPCR.

Gene		Primer Sequence	Length	Tm (ºC)	Product lengh
UGN	Forward	5’ TGAGTTGGAGGAGAAGGAGATGTC 3’	24	55,34	87
	Reverse	5’ AAGGGCAAGGCTGGGTTATG 3’	20	54,38	
GN	Forward	5’ GAGTGACATCGCTTGCCTTTC 3’	21	54,18	78
	Reverse	5’ TGAGTTTGTTAGCCTCGTGACTTC 3’	24	55,17	
GC-C	Forward	5’ TGGAAGAACCACAAGCTCCCCA 3’	22	57.74	119
	Reverse	5’ AGCACGAGGAGGGCAATCAGC 3’	21	59.18	
β-Actin	Forward	5’ GCCCCTGAGGAGCACCCTGT 3’	20	60.18	118
	Reverse	5’ TGGCTACGTACATGGCTGGGGT 3’	22	59.66	

Table 4: Conditions employed in qPCR, for amplification of the genes GC-C, GN, UGN and β-Actin in renal cortex, colon and jejunum.

Step	T (ºC)	Time	Cycles
Initial denaturation	95	3’	
Denaturation	95	10’’	40
Annealing	60	20’’	
Extension/elongation	72	10’’	
Melting curve	65-95	0.1ºC/s	

Data acquisition and analysis was performed using the CFX manager 2.0 (BioRad, Hercules, California, USA).

Evaluation of uroguanilin, guanylin and GC-C protein expression by immunohistochemistry

Immunodetection was performed in paraffin sections, 4µm thick (Microm STS HM 340E, Walldorf, Germany). Sections were deparaffined and hydrated in alcohol decrescent concentrations. Endogenous peroxidase was blocked using 0,3% H_2O_2 in methanol for 15 or 10 minutes and then non-specific secondary antibody binding sites were blocked by incubation with rabbit anti-goat serum (Santa Cruz Biotechnology, USA) for 1h at room temperature. Thereafter, slices were incubated with polyclonal primary antibody, rabbit anti-UGN (1:25; Uroguanylin C15), GN (HMW Guanylin – N16) or GC-C (1:100; K14) (Santa Cruz Biotechnology, USA) overnight at 4ºC. Immunostaining was carried out with a goat anti-rabbit secondary biotinylated antibody (1:300; Santa Cruz Biotechnology, USA), combined with an avidin-biotin complex (Vectastain ABC kit, Vector Laboratories), followed by diaminobenzidine (DAB 97% TLC, Sigma, St. Luis, MO, USA) and hematoxylin counterstaining. Images were obtained using a Leica DM4000B microscope (Wetzlar, Germany) connected to a camera Leica DFC320. The images were processed using Leica Application Suite software (version 2.3.1 R1).

Uroguanylin, Guanylin and GC-C Semiquantitative Immunoblotting

The evaluation of protein expression in renal samples was performed by Western-blot technique. Samples were mixed in loading buffer (0,35 M tris-HCl, 4% SDS, 30% glycerol, 9,3% DTT, ph 6.8, 0,01% bromphenol blue) and boiled at 95ºC for 5 min. The samples (40µg of renal homogenates) were separated by SDS-PAGE in 12% (or 7.5% - GC-C) polyacrylamide gel. For immunoblotting, proteins were transferred to a nitrocellulose membrane and incubated with the anti-UGN, GN and GC-C polyclonal primary antibody (1:100; Santa Cruz, USA), overnight at 4ºC. The immunoblots were subsequently washed and incubated with the fluorescently labelled anti-rabbit (1:20000; IRDye800, Rockland, PA, USA) for 1h at room temperature and protected from light. The membranes were washed and imaged by scanning at 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). To determine which band or staining were specific to GN peptides, two additional blots were performed: one using primary antibody with immunizing peptide and the other using only the secondary antibody. β-actin expression was detected by specific primary antibody (1:10000, Santa Cruz Biotechnology, USA), and scanned at 700nm for normalization purpose.

Evaluation of cGMP in urine and plasma levels

The evaluation of the second messenger, cGMP, expression in urine and plasma samples was performed using cGMP assay kits (R&D Systems, Minneapolis, USA) following the manufacturer's instructions (Kim *et al*., 2005). This assay is based on the competitive binding technique in which cGMP present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled cGMP for sites on a rabbit polyclonal antibody. During the incubation, the polyclonal antibody binds to the goat anti-rabbit antibody coated onto the microplate. Following a washing step to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of cGMP in the sample.

Statistical analysis

The presented results are means ± SEM of values for the indicated number of determinations. Data were analysed with the softwares Microsoft Excel 2010 (Washington, USA) and GraphPad Prism 5 (California, USA), through the use of T-test or one way Anova followed by Tukey test when appropriate. A p value of less than 0.05 was considered statistically significant.

Results

Metabolic studies

After 12 weeks of exposure to HFHSC diet, the diabetic mice presented significantly increased body weight, plasma cholesterol and triglycerides when compared to controls, as well as hyperglycemia and hyperinsulinemia (Table 5). This was accompanied by glucose intolerance (Figure 3A) and insulin resistance (Figure 3B) in diabetic mice. Also, plasma and urinary urea were significantly reduced in diabetic mice in comparison to controls. No significant differences between groups were observed in plasma and urinary levels of proteins and sodium as well as in fractional excretion of sodium, creatinine clearance and kidney weight (Table 5).

Table 5: Body and kidney weight, metabolic balance and renal function in control (C) and diabetic (D) mice.

	C	D
Body weight, g	25.2±0.4	37.3±2.0*
Kidney weight, g	0.32±0.01	0.33±0.01
Plasma glucose, mg/dl	144.6±8.1	166.8±7.3*
Plasma insulin, ng/ml	0.62±0.04	1.02±0.16*
Plasma cholesterol, mg/dl	74.5±2.4	82.4±2.4*
Plasma triglycerides, mg/dl	25.1±2.0	36.3±1.8*
Plasma urea, mg/dl	60.2±3.0	41.0±1.9*
Urine urea, mg/24h	116.8±7.4	66.8±7.0*
Plasma protein, g/l	32.8±1.2	33.1±0.9
Urine protein, mg/24h	15.2±2.8	15.8±2.1
Plasma Na^+, mmol/l	138.0±2.2	138.6±2.7
Urine Na^+, mmol/day	0.18±0.03	0.17±0.03
FE_{Na+}, %	1.34±0.37	1.03±0.33
Ccreat, ml/min	0.09±0.03	0.12±0.03

Values are means ± SE; n = 6 to 8 experiments per group. Ccreat, creatinine clearance; FE, fractional excretion. *Significantly different from values in control mice ($p<0.05$).

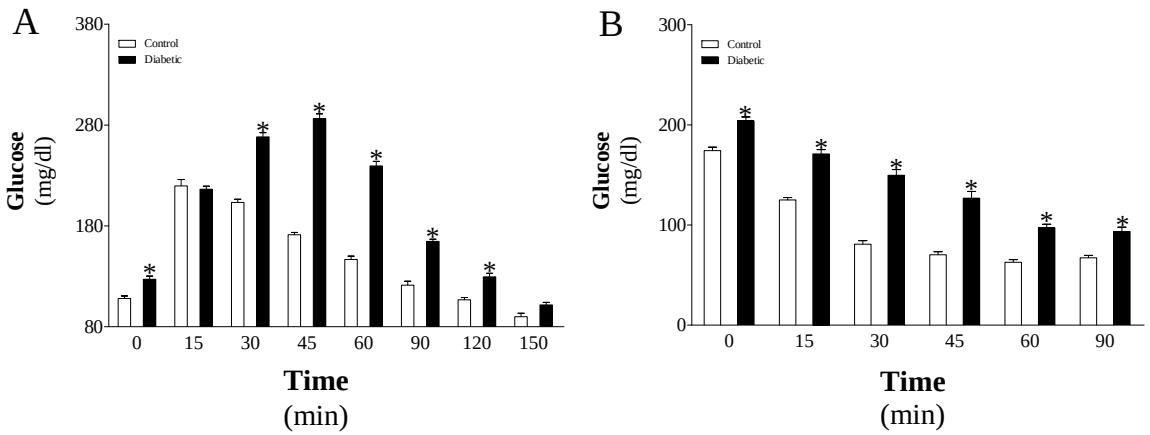

Figure 3: Glucose tolerance test (A) and insulin resistance test (B) in control and diabetic mice. Symbols represent means of 6 to 8 experiments per group, and error bars represent SE. *Significantly different from control mice ($p<0.05$).

In addition, diabetic mice presented a significant increase in systolic and diastolic blood pressure (Figure 4).

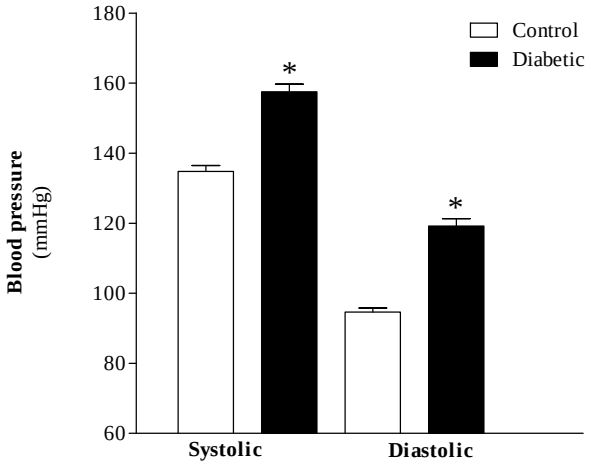

Figure 4: Systolic and diastolic blood pressure in control and diabetic mice. Bars represent means of 6 to 8 experiments per group and error bars represent SE. *Significantly different from values in control mice ($p<0.05$).

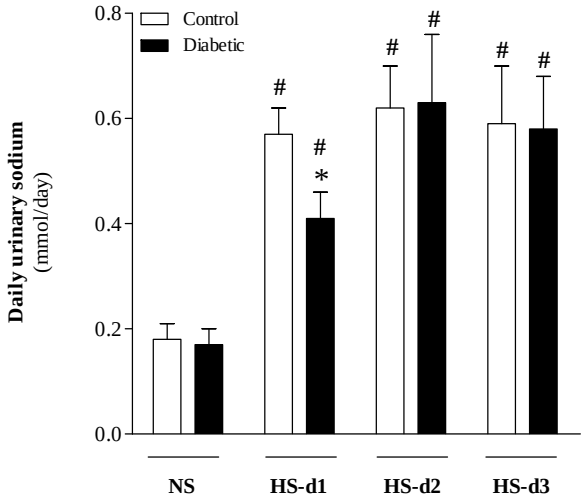

Figure 5: Daily urinary levels of sodium during normal (NS, day 0) or high (HS) sodium intake on days one (HS-d1), two (HS-d2) and three (HS-d3) days, in control (C) and diabetic (D) mice. Bars or symbols represent means of 6 to 8 experiments per group and error bars represent SE. *Significantly different from values in control mice (C) ($p<0.05$). #Significantly different from values in normal sodium (NS) intake mice ($p<0.05$).

Figure 5 represents the daily urinary sodium excretion during the 3 days that mice were subjected to high sodium intake (HS). During the first 24 hours of HS diet, the diabetic mice presented an urinary sodium excretion lower than control mice. However, during the last 2 days of HS diet, the urinary excretion was similar between control and diabetic mice. During the last day of HS diet, the control and diabetic mice presented a significant increase in urinary sodium levels as well as in fractional excretion of sodium (Table 6). Creatinine clearance did not differ between NS or HS diets.

Table 6: Renal function and sodium handling in control (C) and diabetic (D) mice, during normo-saline (NS) or high-saline (HS) diet.

	NS		HS	
	C	D	C	D
Plasma Na^+, $mmol.l^{-1}$	138.0±2.2	138.6±2.7	141.9±1.8	138.3±1.3
Urine Na^+, $mmol.day^{-1}$	0.18±0.03	0.17±0.03	0.59±0.11#	0.59±0.09#
FE_{Na+}	1.34±0.37	1.03±0.33	2.44±0.32#	3.00±0.40#
Ccreat, $ml.min^{-1}$	0.09±0.03	0.12±0.03	0.14±0.02	0.10±0.01

Ccreat, creatinine clearence; FE, fraccional excretion. *Significantly different from control mice ($p<0.05$). #Significantly different from mice subject to normo-saline oral diet ($p<0.05$).

Evaluation of cGMP in urine and plasma levels of type 2 diabetes mouse model

Urinary cGMP levels in diabetic mice were lower than in control mice, although differences only achieved statistical significance during NS diet (Figure 6). Neither control nor diabetic mice demonstrated an increased level of urinary cGMP in response to a HS diet (Figure 6). Plasma cGMP levels were similar between control and diabetic mice during NS and HS diets, and were not altered in response to a sodium load.

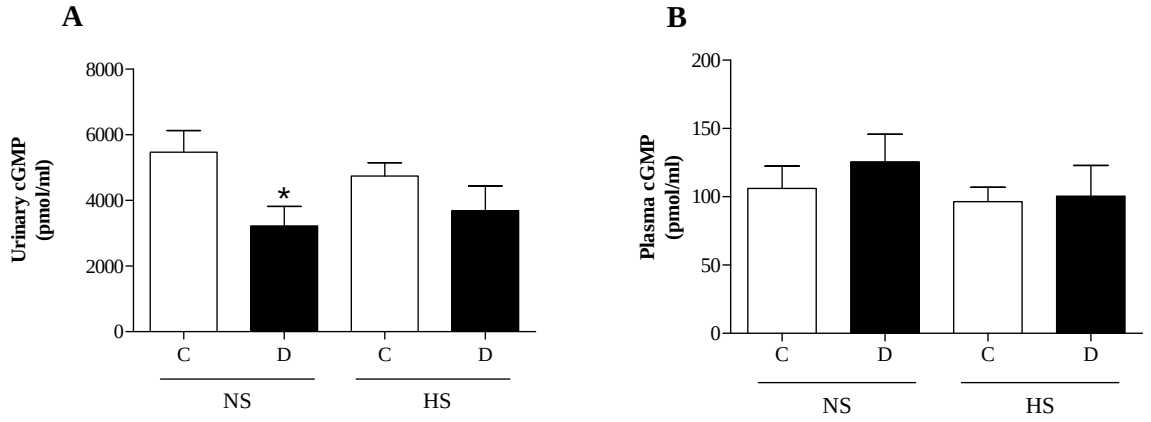

Figure 6: cGMP levels in urine (A) and plasma (B) of control (C) and diabetic (D) mice, during normal (NS) and high-saline (HS) diet. *Significantly different from control values ($p<0.05$).

Evaluation of guanylin, uroguanylin and GC-C receptor mRNA expression in the kidney and the intestine

In Figure 7 is depicted one example of the results obtained with CFX manager 2.0 (BioRad, Hercules, California, USA), for qPCR.

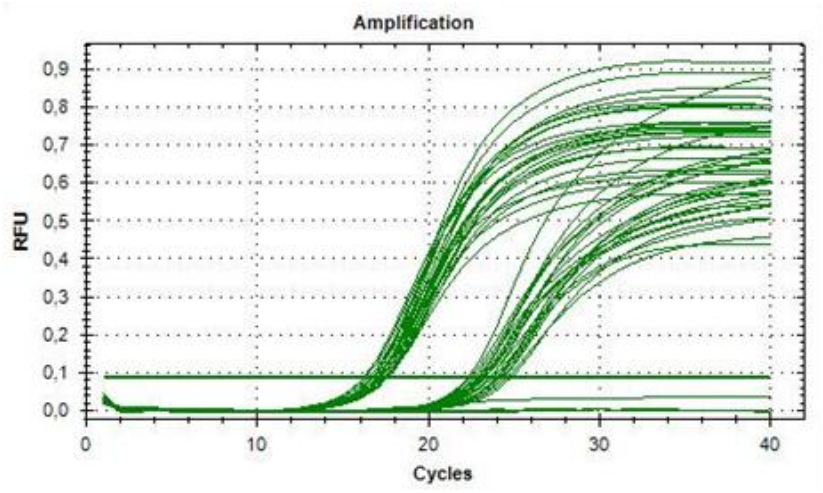

Figure 7: Amplification curves of *β-actin* and *UGN* genes in colon, by qPCR.

In renal cortex, GN mRNA expression did not present significant differences between control and diabetic mice, either during NS or HS diet (Figure 8). On the other hand, UGN mRNA expression was increased in renal cortex from diabetic mice when compared to control, during NS diet whereas during HS diet differences between control and diabetic mice did not achieve statistical significance. Additionally, no significant differences were observed between mice subject to NS or HS diets in respect to GN and UGN mRNA expression in renal cortex. GC-C mRNA expression was not detected in renal cortex, using qPCR.

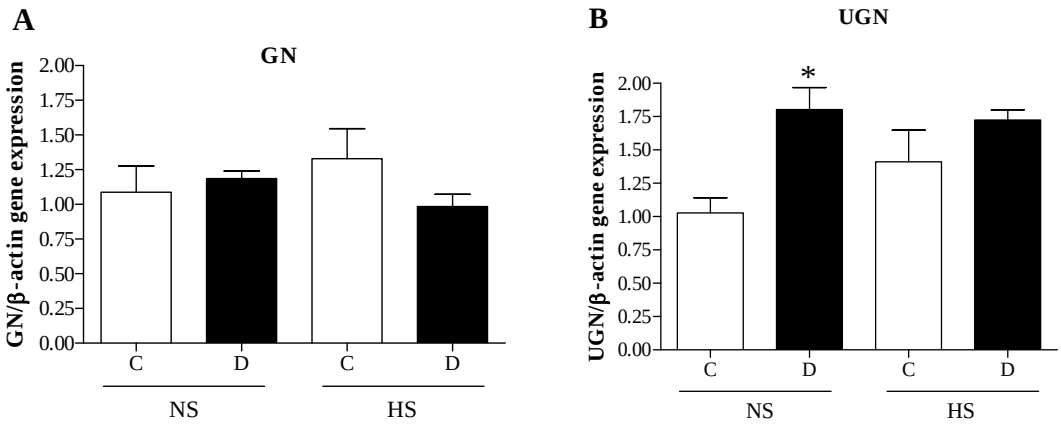

Figure 8: GN (A) and UGN (B) gene expression in renal cortex from control (C) and diabetic (D) mice, during normo-saline (NS) or high-saline (HS) oral diet. *Significantly different from control mice ($p<0.05$).

In jejunum, a significant decrease of GC-C gene expression was observed in diabetic mice when compared with control, both in NS and HS conditions. This was accompanied by a reduction in GN and UGN gene expression in diabetic mice during HS oral diet but not during NS diet. Sodium load did not alter GC-C or GN gene expression in control and diabetic mice, but increased UGN mRNA expression in control mice (Figure 9).

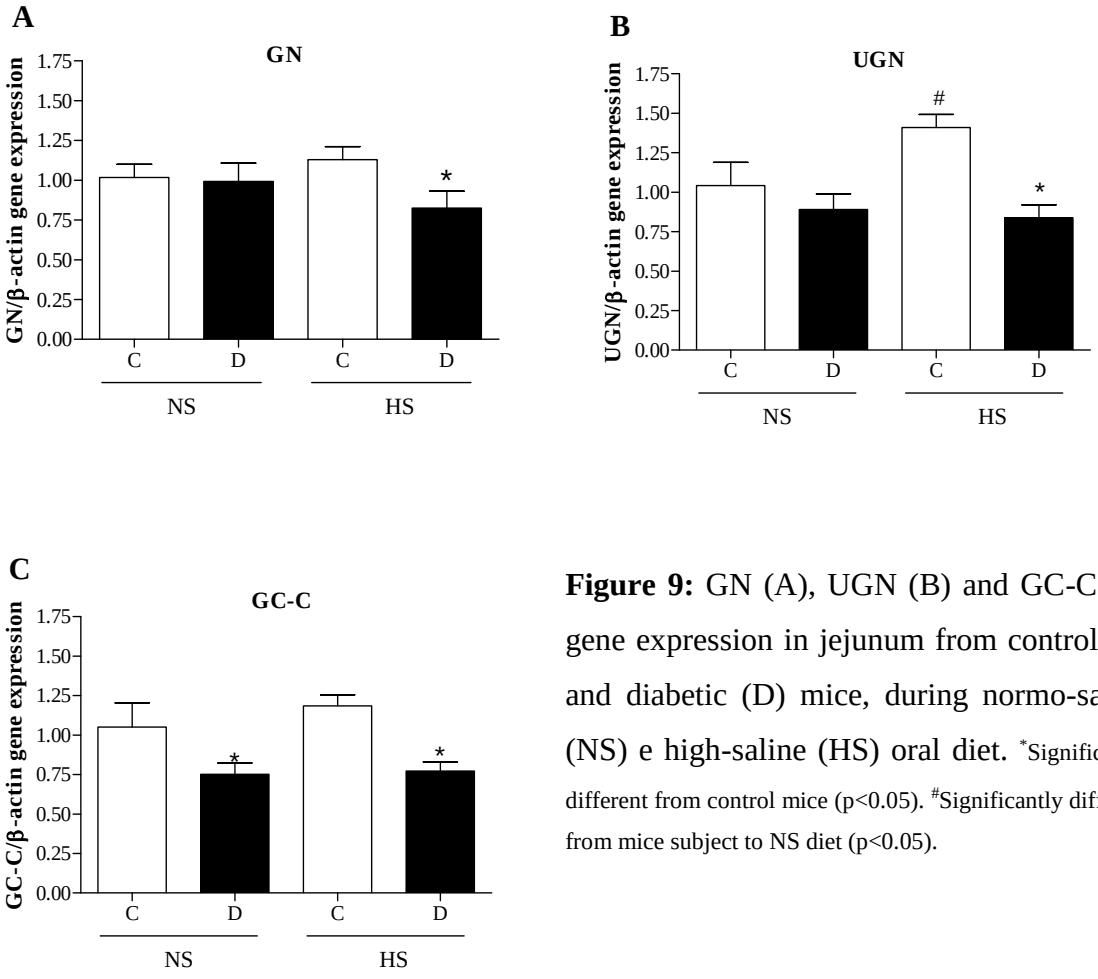

Figure 9: GN (A), UGN (B) and GC-C (C) gene expression in jejunum from control (C) and diabetic (D) mice, during normo-saline (NS) e high-saline (HS) oral diet. *Significantly different from control mice ($p<0.05$). #Significantly different from mice subject to NS diet ($p<0.05$).

In colon (Figure 10), a significant decrease of GC-C mRNA expression was observed in diabetic mice when compared with controls, both during NS and HS conditions. GN mRNA expression showed the same pattern of expression as GC-C, presenting a significant decrease of gene expression in diabetic mice during both NS and HS diets. UGN mRNA expression only showed a significant decrease in diabetic mice subjected to NS oral diet. Sodium load did not alter mRNA of GC-C, GN and UGN in either control or diabetic mice.

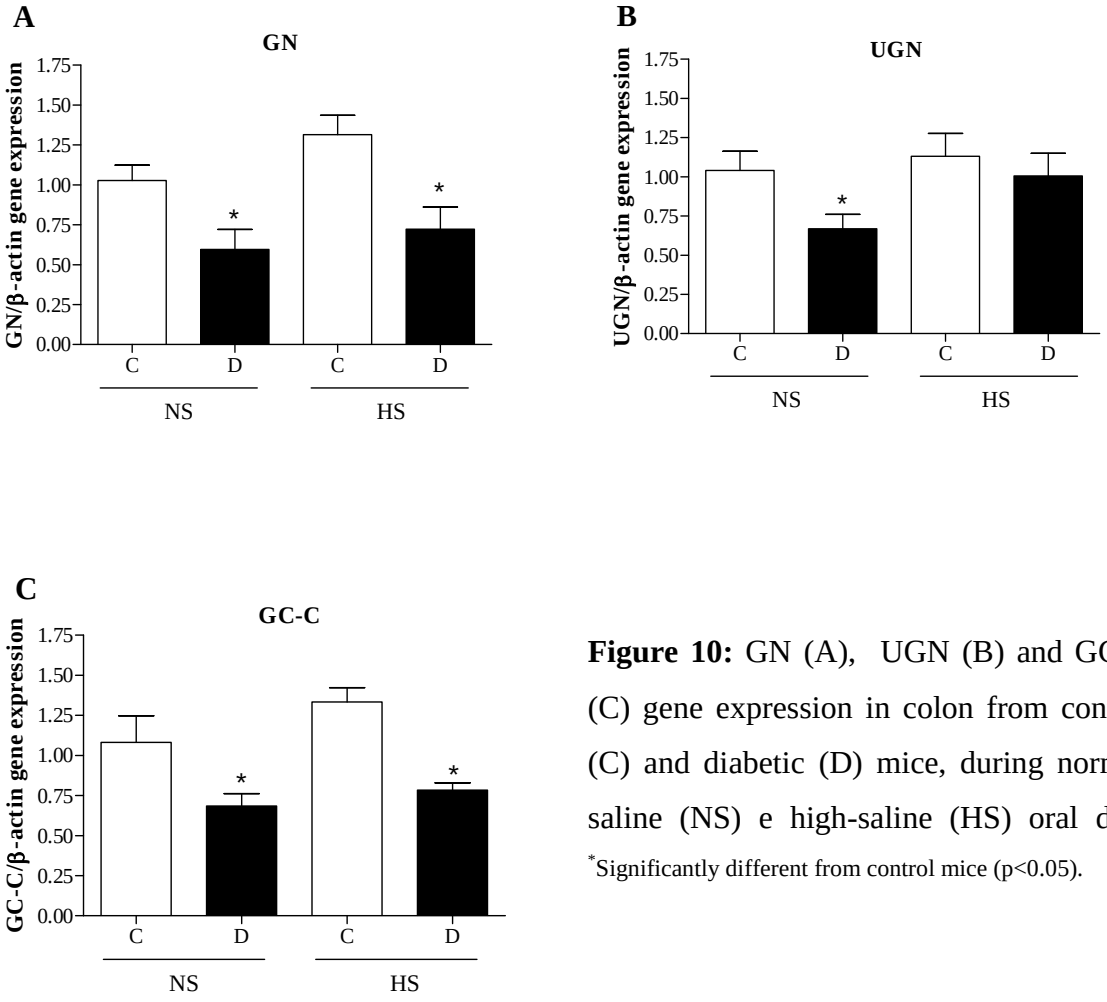

Figure 10: GN (A), UGN (B) and GC-C (C) gene expression in colon from control (C) and diabetic (D) mice, during normo-saline (NS) e high-saline (HS) oral diet. *Significantly different from control mice ($p<0.05$).

Evaluation of uroguanylin protein expression in the kidney and the intestine

Two different approaches were used to evaluate GN peptides protein expression in kidney and intestine, namely immunohistochemistry and western-blot. Using immunohistochemical analysis it was possible to observe a positive staining for UGN in renal and colon tissues (Figures 11 and 12) and GC-C receptor in colon tissue (Figure 13). Given that this method does not allow the accurate quantification of proteins expression, it was used only as qualitative assay. Thus, we concluded that UGN is present in the kidney and colon of control and diabetic mice and GC-C receptor is present in the colon of both groups of animals. Although GC-C receptor was analyzed in the kidney using immunohistochemistry, no satisfactory results were obtained, so conclusions could not be withdrawn. In addition, the immunohistochemical analysis was not performed in the

jejunum portions of the intestine. In respect to GN several difficulties arose during the optimization processes and results could not be obtained. Using western-blot technic no consistent results were obtained for any of the molecules tested. Figure 14 shows representative images of the optimization of protein expression evaluation in renal tissue from control and diabetic mice subjected to normal or high-saline diet, by western blot. The fact that GN peptides have a very low molecular weight together with the poor quality of the antibodies available for these peptides in the initial phase of the study contributed to the difficulties of these approaches. Due to time limitations, no further analyses were performed.

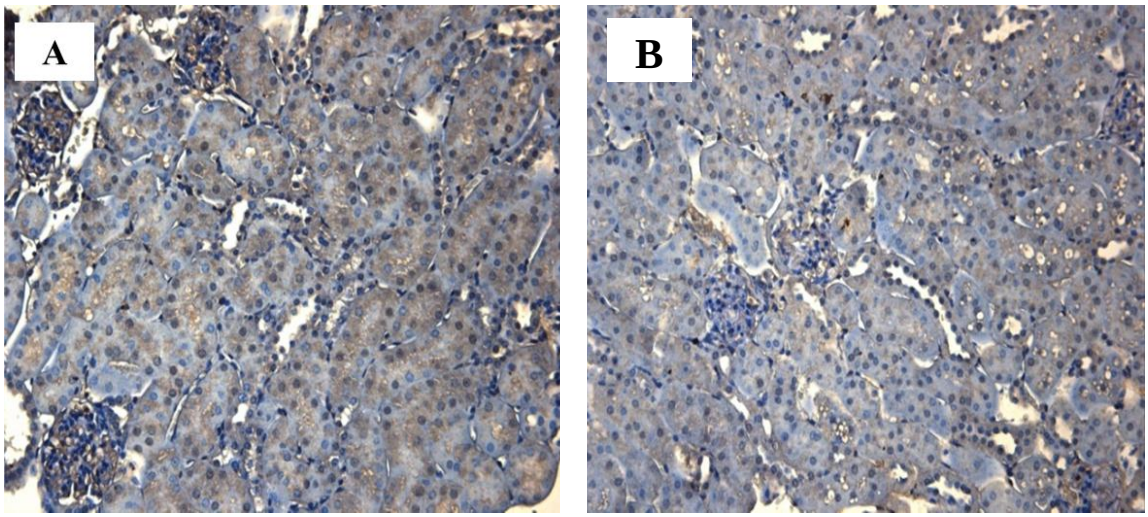

Figure 11: Representative light microscopy images of immunohistochemical analysis of kidney paraffin slices positively stained for UGN from control (A) and diabetic (B) mice (400x).

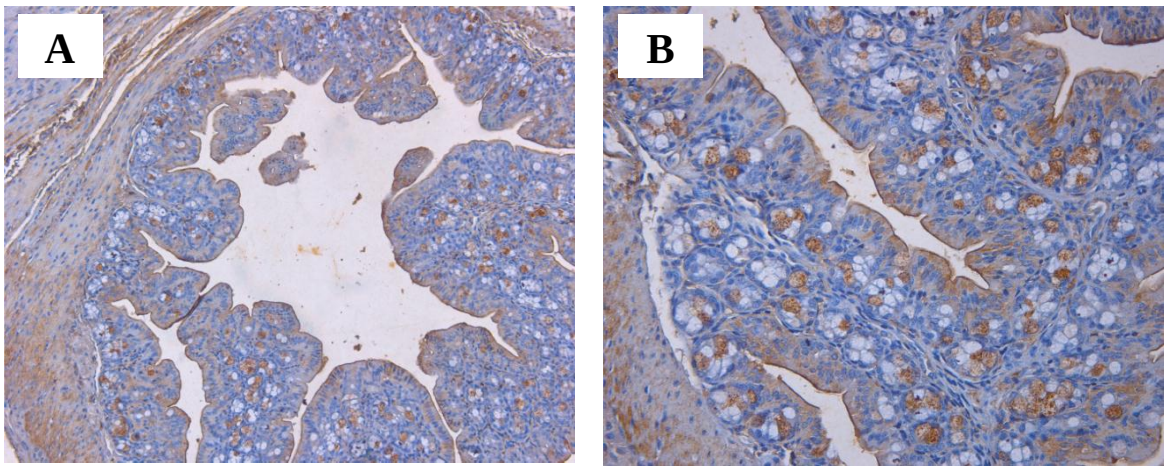

Figure 12: Representative light microscopy images of immunohistochemical analysis of colon paraffin slices positively stained for UGN from control mice (A - 100x; B - 200x).

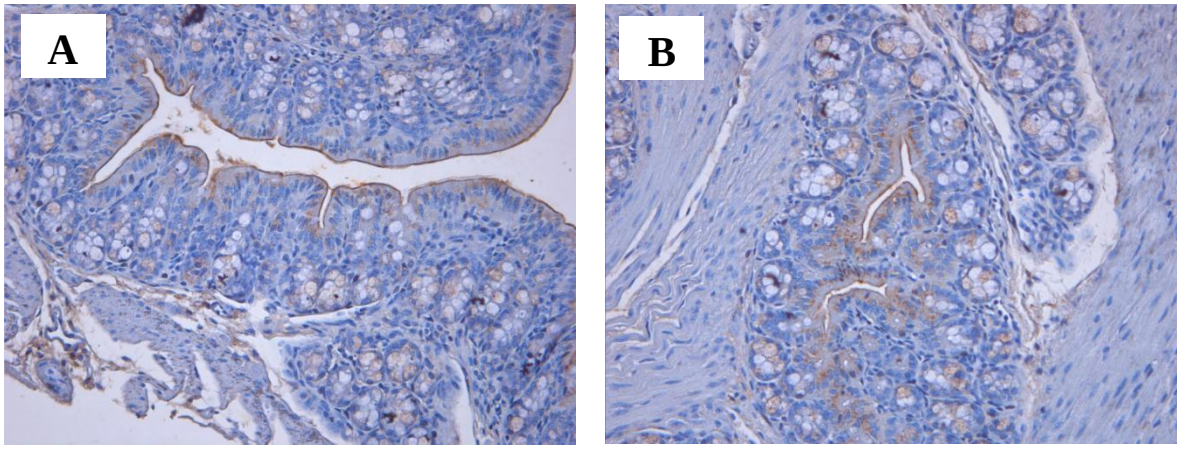

Figure 13: Representative light microscopy images of immunohistochemical analysis of colon paraffin slices positively stained for GC-C from control mice (A - 200x; B - 400x).

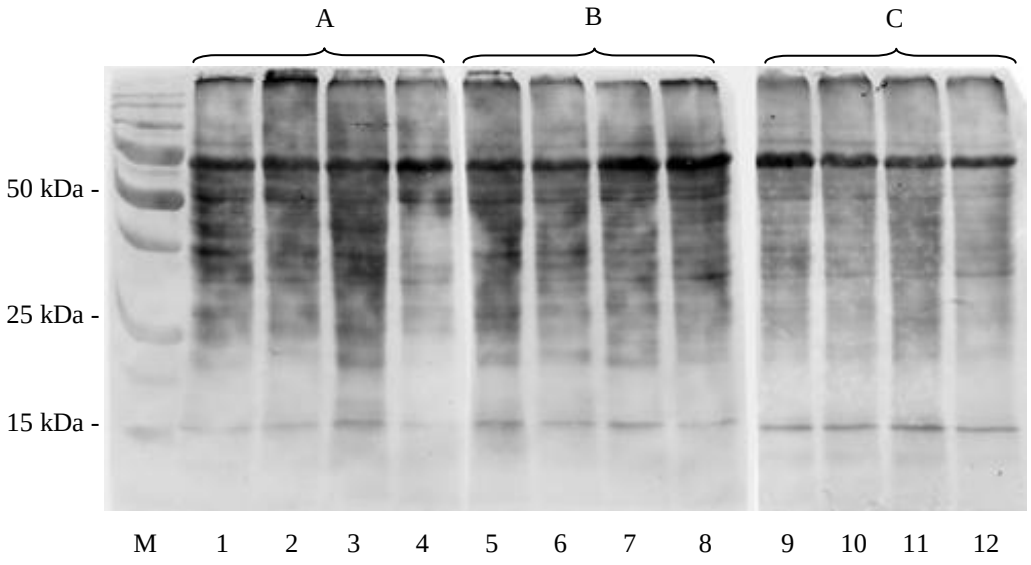

Figure 14: Representative images of GN protein expression analysis by western-blot in renal cortex. M – Protein marker; 1, 5, 9 – NSC; 2, 6, 10 – NSD; 3, 7, 11 – HSC; 4, 8, 12 – HSD. A – Membrane was incubated with primary GN antibody and secondary antibody; B – Membrane was incubated with primary GN antibody blocked with immunizing peptide and secondary antibody; C – Membrane was incubated only with secondary antibody.

Discussion

To our knowledge this is the first study to characterize the GPS in type 2 diabetes. In comparison to control mice, diabetic mice presented reduced GN, UGN and GC-C mRNA expression in the intestine and increased UGN mRNA expression in the kidney. This represents a down-regulation of intestinal GPS which is accompanied by a compensatory increase of renal GPS activity.

Given that diabetes presents whole body complications, an animal model was employed taking in consideration the 3 R's policy (Refinement, Reduction and Replacement) during animal related procedures and experiences planning. The exposure of C57BL/6 mice to high-fat high-simple carbohydrate diet was accompanied by obesity, hyperglycemia, glucose intolerance, insulin resistance, hyperinsulinemia and hypertension as already described by others (Rogers and Webb, 1980, Roncon-Albuquerque *et al*., 2008, Surwit *et al*., 1988). This animal model is considered a reliable and clinically relevant experimental model for type 2 diabetes pathophysiologic studies (Winzell and Ahren, 2004).

In the present study, the intestinal mRNA expression of GN, UGN and GC-C receptor was evaluated in a proximal (jejunum) and a distal (colon) portion given that previous studies in rodents have shown that UGN levels are relatively high in the jejunum and decrease progressively towards the large intestine, whereas GN shows the opposite pattern (Miyazato *et al*., 1996a, Whitaker *et al*., 1997, London *et al*., 1997).

During normal salt intake, both GN and UGN expressions were down-regulated in diabetic colon. In addition, when a sodium load was given to the mice, a reduction of the peptides gene expression was also observed in diabetic jejunum. This down-regulation of jejunal GPS after a sodium load may be related not to the reduced absolute expression of GN peptides in diabetic mice but instead to the up-regulation of the GN peptides in control mice, which has been described to occur after an oral sodium load (Potthast *et al*., 2001). However, in our data only the UGN gene expression increased significantly in control mice in response to an oral sodium load.

In contrast to the intestine, the UGN gene expression was increased in the diabetic kidney during normal salt intake, suggesting a renal compensatory response to the reduced intestinal production of GN and UGN peptides. This result reinforces the occurrence of complementary functions between the intestine and the kidney with GPS playing a role in renal-intestinal cross-talk. Moreover, renal GN peptides mRNA expression did not respond

to oral sodium load. This result suggests that renal GPS may contribute to the dysfunctional sodium handling in diabetes.

Although in our animal model of type 2 diabetes no differences were observed in respect to urinary sodium excretion and fractional excretion of sodium, the diabetic mice showed an elevated blood pressure in comparison to controls. The increase of UGN gene expression in diabetic kidney may contribute to sodium excretion; however, the particular influence of this system when viewed together with all natriuretic system of the kidney may be small. In accordance with this view are the reports of Narayan and co-workers showing that despite elevated plasma proGN and proUGN and increased urinary bioactivity of UGN, a reduced natriuretic response to an oral salt load is observed in subjects with heart failure when compared with healthy subjects (Selektor and Weber, 2008, Narayan *et al*., 2010).

Potthast and co-workers found an increase in renal mRNA expression of UGN after oral administration of 1% NaCl as drinking solution for 3 days (Potthast *et al*., 2001). This result was not obtained in our study. The blunted response of GN or UGN mRNA expression to oral sodium load was also described by other author (Carrithers *et al*., 2002). These authors justified the absence of response to high sodium load with the fact that the NS-group of animals received higher sodium levels than equivalent normal human levels. Thus this NS intake was sufficient to stimulate GN mRNA expression masking the effect of a HS intake (Carrithers *et al*., 2002). The existence of controversial results on the effect of HS in the regulation of the GN peptides system justifies the need for further studies to clarify the effect of oral sodium intake on peptide synthesis and/or secretion and the resultant changes in intestinal and renal transport of sodium.

GN and UGN peptide actions are mediated by GC-C receptor in the intestine. Interestingly, GC-C mRNA expression was significantly reduced in both jejunum and colon of diabetic mice, either during normal or high sodium intake, reinforcing the down-regulation of the intestinal GPS in diabetes. Although we were able to detect GC-C receptor mRNA in the intestine, it was undetectable in the kidney. These observations are consistent with previous studies (Qian *et al*., 2011) and support the well-established hypothesis that UGN signalling in the kidney involves a receptor that is distinct from GC-C (Carrithers *et al*., 1999, Carrithers *et al*., 2004, Sindic and Schlatter, 2005, Sindice *et al*., 2002). However, previous gene expression studies have reported GC-C expression in the rat kidney (Carrithers *et al*., 2000). Qian and co-workers suggest that this variability reported in the literature may reflect differences in the handling or feeding of experimental

animals (Qian *et al*., 2011). However if GC-C is not the main receptor in the kidney, it may regulate an indeterminate component of renal function present in some conditions (Qian *et al*., 2011). A candidate for GN peptide receptor, GC-C independent, was described by Lehner and co-workers (Lehner *et al*., 2007). In this study was observed that GN peptides activate PLA_2 (Phospholipase A_2) and inhibit ROMK (Renal outer medullary potassium channel) channels in HEK293 (Human embryonic kidney cells) cells transfected with the human GPR14 receptor (G-protein coupled receptor), which is present in mouse and human cortical collecting duct (Lehner *et al*., 2007).

To further characterise the GN peptides system and better understand its possible regulatory pathways we evaluated the levels of cGMP, GC-C receptor second messenger, in urine and plasma in experimental diabetes. In our study we found reduced cGMP levels in urine of diabetic mice, despite the increase in renal UGN mRNA expression. In the kidney, UGN seems to activate other receptors than GC-C receptor, with cGMP-independent signaling pathways (Potthast *et al*., 2001, Qian *et al*., 2011, Carrithers *et al*., 2004). Our results are in agreement with the suggestion of Qian and co-workers that UGN does not stimulate renal synthesis or urinary excretion of cGMP (Qian *et al*., 2011). Thus, the reduced levels of cGMP in urine may be related to other signaling pathways that may be altered in the diabetic kidney.

Another hypothesis that may justify the reduced levels of urinary cGMP observed in parallel to the increased renal UGN expression is UGN resistance in diabetic kidney. A mechanism that may be similar to the ANP resistance observed in the nephrotic syndrome and diabetes, which occurs after ANP binding to its receptors in the collecting duct and appears to result from the activation of a phosphodiesterase responsible for the catabolism of cGMP, the second messenger of ANP (Valentin *et al*., 1996)

The present work showed altered GPS gene expression in diabetic mice, however this does not directly translate to the expression/activity of the corresponding proteins. Given that these peptides suffer a strong post-transcriptional regulation, the parallel evaluation of GN peptides protein expression in the tissues is critical to fully understand this system. An attempt to evaluate protein levels was performed by western-blot and immunohistochemistry but no satisfactory results were obtained. The factors underlying this unaccomplished goal may be related to the small size of bioactive peptides and the quality of commercially available antibodies. Another limitation of the study was the fact that GN and UGN were not measured in plasma and urine. At time of the study no specific immunoassays for these small molecules were available for mice. So, future work will

include the determination of protein expression in the analyzed tissues as well as in plasma and urine.

Conclusion

Our results suggest that in type 2 diabetes a down-regulation of intestinal GPS is accompanied by a compensatory increase of renal GPS activity. The blunted intestinal GPS in type 2 diabetes is due not only to a reduced expression of GN and UGN peptides but also to a reduced expression of its GC-C receptor. These results suggest that GPS may contribute to the sodium sensitivity in diabetes.

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Publications, communications and prizes

Panel Communication

- May 28, 2011 - Simões-Silva L, Quelhas-Santos J, Moreira-Rodrigues M, Soares-Silva I, Sampaio-Maia B. Expressão génica da guanilina, uroguanilina e do receptor guanilato ciclase-C no cólon de ratinhos com diabetes tipo 2. In "IV Jornadas de Análises Clínicas e Saúde Pública", ESTSP-IPP, Porto.
- June 25 - 28, 2010 - Simões-Silva L, Quelhas-Santos J, Fernandes-Cerqueira C, Moreira-Rodrigues M, Pestana M, Sampaio-Maia B. Reduced renal uroguanylin expression in type 2 diabetes. In XLVIII ERA-EDTA Congress/II DGfN Congress, Munich.

Publication in conference proceedings

- **2010** - Simões-Silva L, Quelhas-Santos J, Fernandes-Cerqueira C, Moreira-Rodrigues M, Pestana M, Sampaio-Maia B. Reduced renal uroguanylin expression in type 2 diabetes. NDT Plus. 2010: 3: Supl 3: iii111

Prize

- **2011** - Expressão génica da guanilina, uroguanilina e do receptor guanilato ciclase-C no cólon de ratinhos com diabetes tipo 2. Simões-Silva L, Quelhas-Santos J, Moreira-Rodrigues M, Soares-Silva I, Sampaio-Maia B. First prize for panel communication – Scientific Committee of "IV Jornadas de Análises Clínicas e Saúde Pública", ESTSP-IPP, Porto.