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"FREE FATTY ACIDS CAUSE KIDNEY INJURY AND LIPID METABOLIC MEMORY IN OBESITY RELATED GLOMERULOPATHY"

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The highest reward for a person's toil
is not what they get for it,
but what they become by it.
(John Ruskin)

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ABSTRACT

Objective: The signaling pathways leading to renal pathology in obesity are not understood well. Growing evidence suggest that FFAs induced oxidative stress, play a role as the critical factors linking obesity with its associated complications. Our study demonstrates, for the first time, that the method of *Oliveira et al,* for coniugating palmitate with albumin, produces an ubunded palmitate fraction able to cause podocytes dysfunctions similar to the one observed in the ORG.

Methods: Conditionally immortalized human podocytes cells line were exposed to different ratio of conjugated PAL / BSA for 24h and then cells were incubated, for an additional two days, in medium without palmitate.

Results: Palmitate, at concentrations seen in obese patients, causes ROS overproduction in human podocytes. This oxidative stress induces podocytes disfunctions such as inflammation, and changes in profibrotic and lipotoxic markers. HMGB1 appears to be the main mediator of ROS damaging action, since its pharmacological inhibition prevents all ROS effects on podocytes. Moreover, palmitate is able to start a feed-back loop that causes a persistent overproduction of ROS and consequently a persistent podocyte dysfunction. So a "metabolic memory" exists for the FFAs action on podocytes, causing a persisting damage even in presence of a normalized lipid profile. *Conclusions:* The present findings may provide further insight into the underlying mechanisms that contribute to the pathogenesis of ORG.

INTRODUCTION

OBESITY

The worldwide prevalence of overweight (body mass index, BMI≥ 25 kg/m2) and obesity (BMI≥ 30 kg/m2) **(Box 1)** has risen by over 27% in the last three decades, bringing the number of affected individuals to approximately 2.1 billion (Ng et al 2013.; Qasim et al 2016). According to data provided by the World Health Organization (WHO), the number of obese people in the world has doubled since 1980 **(Fig.1)**. Problems like obesity and overweightness, were previously considered to be problems of only rich countries, but these problems are also increasing in low and middle income countries, especially in urban settlements, and are now recognized as real public health problems: in Africa the number of overweight or obese children has almost doubled from 5.4 million in 1990 to 10.6 million in 2014. In the same year, around 41 million children under the age of 5 were overweight or obese (OMS data).

Box 1 | **BMI-based definitions of obesity**

- Normal weight: BMI 18.5–24.9 kg/m^2
- Overweight: BMI 25–29.9 kg/m^2
- Obesity: BMI ≥30 kg/m^2
- Class 1 (or grade 1) obesity: BMI 30–34.9 kg/m^2
- Class 2 (or grade 2) obesity: BMI 35–39.9 kg/m^2
- Class 3 (or grade 3) or morbid obesity: BMI ≥40 kg/m^2

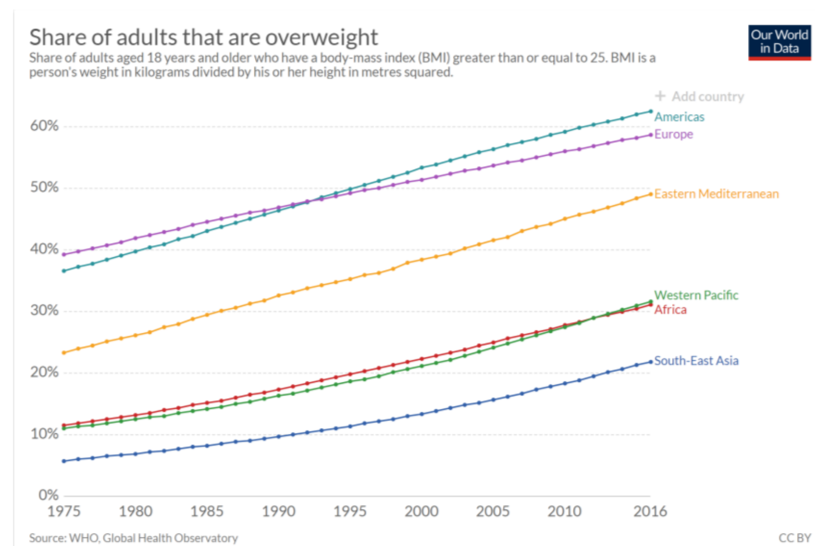

Figure 1: Share of adults who are overweight

Moreover, obesity is gaining acceptance as a serious primary health burden that impairs the quality of life because of its associated complications, including diabetes, cardiovascular diseases, cancer, asthma, sleep disorders, hepatic dysfunction, renal dysfunction, and infertility (Manna P et al 2015). Obesity-related factors cause 11% of heart failure cases in men and 14% in women by inducing haemodynamic and myocardial changes due to an increased cardiac lipotoxicity that leads to cardiac dysfunction (Ebong et al 2014). In 97 studies providing more than 2.88 million individuals and more than 270,000 deaths, relative to normal weight, both obesity (all grades) and grades 2 and 3 obesity are associated with significantly higher all-cause mortality. Grade 1 obesity overall is not associated with higher mortality. Furthermore, overweightness is associated with significantly lower all-cause mortality (Flegal et al 2013). The relative risks of all-cause mortality in overweight and obese patients with type 2 diabetes were 0.81 and 0.72, respectively, compared with the normal or non-overweight patients out of 161,984 participants from 9 studies of 13 cohorts (Liu et al 2015). In extreme cases, obesity may reduce life expectancy by 6–14 years (Kitahara et al 2014.; Qasim et al 2018). Behavioural and pharmacological interventions to manage obesity exist (Dietz et al 2015). However, these interventions seem to have little effect in tackling the epidemic. Bariatric surgery, though effective at reducing body weight and related comorbidities, and improving quality of life is invasive and is associated with a number of other complications (Piche 2015). The global food system drivers interact with local environmental and genetic factors to create a wide variation in obesity prevalence between populations (Engin 2017). The modern obesity epidemic is largely explained by environment factors, with excess energy intake and physical inactivity pinned as the main culprits. However, even in shared environments, only a subset of individuals develops obesity

(Valera et al 2015). There appears to be a differential propensity to obesity at the individual level, with biological factors such as sex, age and in utero environment contributing to this variability (Dhurandhar et al 2014) **(Fig.2)**.

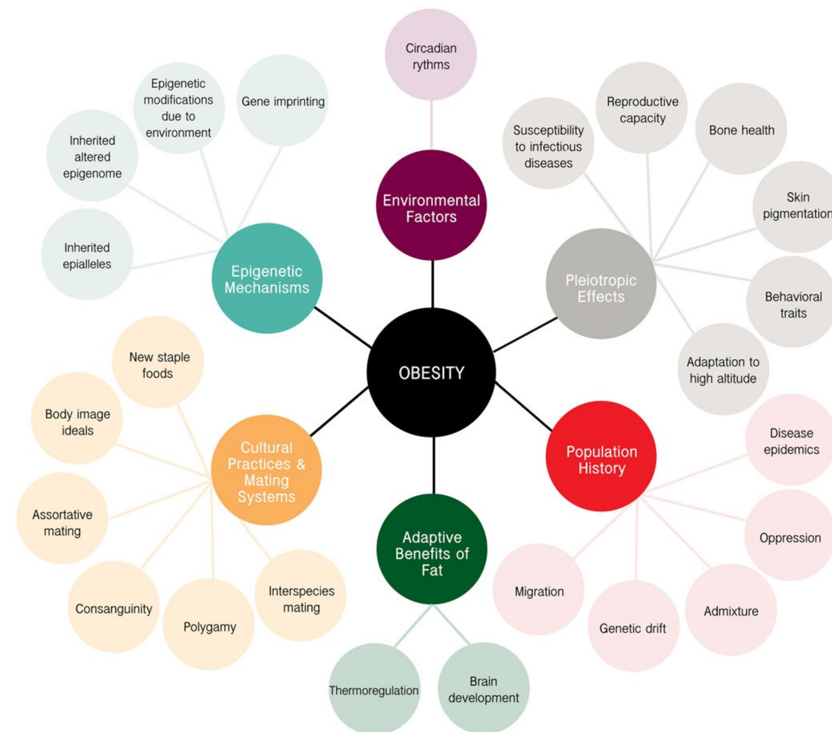

Figure 2: A thematic map outlining the biological, environmental and cultural forces that may explain the modern distribution of genetic variants predisposing to obesity and leanness in human populations.

Over the past 50 years, numerous hypotheses have emerged to explain human propensity to obesity (Genne-Bacon 2014). Neel's thrifty genotype hypothesis, for instance, posits that extra adipose tissue enabled our ancestors to survive in the face of feast–famine cycles (Neel 1962). This hypothesis, however, oversimplifies the matter: if obesity susceptibility variants were advantageous for survival, such variants would be likely fixed in humans, and obesity would be the norm in modern populations (Speakman 2008). This, however, is not the case. In fact, genetic variants conferring protection against obesity have been identified in humans (Geller et al 2004; Stutzmann et al 2007). Theories

building on the thrifty genotype hypothesis attempt to capture the complexity in the modern obesity phenomenon. The thrifty phenotype hypothesis advances the notion that inadequate nutrition in early life alters the structure and function of organs and tissues, such that energy abundance experienced later in life leads to increased risk of cardiovascular disease and hyperglycaemia (Hales et al 1992). The thrifty epigenotype hypothesis proposes that all humans possess a thrifty genotype; environmental cues, however, lead to phenotypic variability within a range of acceptable phenotypes via epigenetic modifications (Stoger 2008). Although many 'thrifty' theories exist framing adiposity as an adaptive trait, non-adaptive theories also exist. The predation release hypothesis, for instance, proposes that relaxation of predation pressures and random genetic drift contributed to the actual variability in genetic predisposition to obesity (Speakman 2008). In reality, no single theory can explain the evolutionary origins of obesity. The history of our species is complex. Since the dispersion of modern humans out of Africa over 1.8 million years ago, the environmental and social conditions faced by our species have been in constant flux. Every human population has a unique genetic history, resulting from founder effects, genetic drift, admixture events and diverse ecological challenges (Quasim et al 2018). Anyway, increased obesity rates lead to a large health and economic burden in all countries (Rtveladze et al 2014), moreover, obesity is gaining acceptance as a serious primary health burden that impairs the quality of life. According to Keaver *et al* overweightness and obesity are predicted to reach levels of 89% and 85% in males and females, respectively by 2030. This will result in an increase in the obesity-related prevalence of coronary heart disease (CHD) by 97%, cancers by 61% and type 2 diabetes by 21%. Thereupon, the direct healthcare costs will increase significantly. A 5% reduction in population body mass index (BMI) levels by 2030 is estimated to result in €495 million decrease in the

expenditures in obesity- related direct healthcare over 20 years (Keaver et al 2013) **(Table 1)** .

Direct healthcare costs by year and disease.

	Scenario 0			Scenario 1			Scenario 2		
Year	**2010**	**2020**	**2030**	**2010**	**2020**	**2030**	**2010**	**2020**	**2030**
Total costs (€ millions)	2547	4370	5400	2547	4270	5250	2547	3980	4900
CHD & Stroke	2265	4010	4970	2265	3920	4840	2265	3640	4510
Cancer	101	152	179	101	153	176	101	149	172

Scenario 0 = if BMI trends continue unabated; Scenario 1 = a 1% reduction in BMI levels relative to scenario 0 and Scenario 2 = a 5% reduction in BMI levels relative to scenario 0.

Table 1: Direct healthcare costs by year and disease.

CHAPTER I

1.1 THE RENAL PATHOLOGY OF OBESITY

Obesity affects the function of many organs. The heart is one of the main organs affected by metabolic syndrome, and obesity significantly increases the chances of cardiac dysfunction because of chronic hemodynamic burden, which causes dyspnea, edema, ongoing systemic inflammation, metabolic alterations and other related comorbidities (Abel et al 2008). Other organs such as the liver are also affected by this pathology, with lipid accumulation causing nonalcoholic fatty liver disease (Marchesini et al 2008). Lung function is also compromised by adipose tissue around the abdomen, rib cage and visceral cavity (Salome et al 1985). The kidney is also responsive to obesity. Several multicenter studies have identified a direct correlation between obesity and renal complications **(Table 2)**.

Cohort	Number of patients	Country	Result	Ref.
Dialysis patients	1957	Netherlands	Higher mortality with very high or low BMI (< 65 yr)	[157]
Kidney transplant	1810	Netherlands	Higher mortality and kidney graft failure	[158]
Native population	1924	Sweden	Higher Chronic Renal Failure	[13]
National Health and Nutrition Examination Survey III	5659	United States	Higher micro-albuminuria with metabolic syndrome	[159]
Hipertension and obesity	4585	Spain	Higher risk of renal insufficiency	[160]
Native population	2585	United States	Higher risk of kidney disease	[12]
Native population	5403	Japan	Higher risk of proteinuria	[161]
Kidney transplant	51927	United States	Lower patient and graft survival. Higher chronic graft failure and delayed graft function	[162]

Table 2: Recent major multicenter studies regarding the impact of obesity and overweight on the incidence of kidney disease, renal function prognosis and patient survival

Obesity has a multifactorial mechanism and it is also considered an independent risk factor for kidney disease (CKD) development and progression to end-stage renal disease (ESRD) (Wang et al 2008; Fox et al 2004). Several studies demonstrate that obesity-induced hypertension and diabetes are strong determinants of CKD. Analyses relating obesity and kidney transplantation revealed that in 1987, 11.6% of adults awaiting a kidney transplant were obese, and in 2001, obesity among adults rose to 25.1% (Friedman et al 2003). Concomitantly, body mass index (BMI) among patients initiating dialysis increased from 25.7 kg/m2 to 27.5 kg/m2 from 1995 to 2002 (Kramer et al 2006); and when compared with normal weight persons (BMI, 18.5-24.9 kg/m2), there is a directly proportional relationship between increased BMI and increased CKD and ESRD risk (Hsu et al 2006; Fox et al 2004). A study conducted by *Ejerblad et al* (Ejerblad et al 2006) examined the association between degrees of obesity and CKD. After making adjustments for many covariates, the investigators found a 2.8-fold increased risk of nephrosclerosis and a 7-fold increased risk of diabetic nephropathy among adults who had a BMI of 35 kg/m2 or higher compared with a lifetime highest BMI lower than 25 kg/m2 . In adults with no diabetes or hypertension, a lifetime highest BMI of 35 kg/m2 or higher was associated with a 2-fold increased risk of CKD. Conversely, obese patients had better recovery and benefitted from reduced body weight by diminishing proteinuria (Chagnac et al 2003). Obesity was recently demonstrated to accelerate IgA nephropathy progression (Kataoka et al 2012). In this scenario, obesity could be one of the few preventable risk factors for CKD development because it also mediates diabetes and hypertension, which are related to kidney disease progression (Chagnac et al 2003; Kahn et al 2006; He et al 2000) . The occurrence of obesity during early life is linked to low glomerular filtration rates (GFRs), while being overweight during adulthood doubles the chances of chronic kidney disease (Othman et al 2009). Many researchers have described the direct

impacts that obesity has on the kidneys, which includes hyperfiltration, elevated glomerular tension, and podocyte stress (Malyszko et al 2009). Some researchers have also correlated obesity-related inflammation and adipokine deregulation to kidney function. Obesity causes various structural, hemodynamic, and metabolic alterations in the kidney (Tsuboi et al 2013). Obesity-related glomerulopathy (ORG) is the best-known renal disease secondary to obesity. From observation of this unique disease state, significant knowledge has been accumulated regarding the clinico-pathological characteristics of renal injury in obesity (Kambham et al 2001; Praga et al 2001; Chen et al 2008; Tsuboi et al 2013). Importantly, however, there is a large difference between the fraction of the general population that is obese and the fraction that actually develops renal impairment. In addition, the severity of obesity-related renal impairment is not necessarily related to the severity of obesity. Thus, it is conceivable that obesity is not the only factor causing obesity-related renal injury, and that there may be additional or predisposing factors that explain the considerable differences among individuals in susceptibility to renal injury due to obesity. The mechanisms involved in ORG are complicated and integrated, especially hemodynamic changes, inflammation, oxidative stress, and apoptosis. Nephrotic proteinuria (> 3.5 g/d) is occasionally present, but typical nephrotic syndrome is characteristically absent. Furthermore, about 30% of ORG patients develop progressive renal failure or end-stage renal disease (ESRD). Hypertension and dyslipidaemia are also commonly observed in ORG patients (Xu et al 2017). Several cohort studies showed that obesity is associated with high CKD incidence and increased ESRD risk. The clinical process is indolently evolving, stable, or slowly progressive proteinuria, and 10%–33% of the patients have a possibility to develop progressive renal dysfunction and ESRD. The percentage increases at prolonged follow-ups (Kambham et al 2001 ; Praga et al 2001; Tsuboi et al 2013). Comparative studies showed that

primary focal segmental glomerulosclerosis (FSGS) has a more sudden and aggressive disease process than ORG and more easily develops to ESRD (Kambham et al 2001; Praga et al 2001). Other common clinical manifestations of ORG include hypertension (50%–75% of patients) and dyslipidaemia (70%–80% of patients) (Kambham et al 2001; Praga et al 2001; Tsuboi et al 2013).

RENAL ALTERATION IN OBESITY OR OBESITY-RELATED RENAL IMPAIRMENT

ORG is characterized by glomerulomegaly in the presence or absence of FSGS lesions **(Fig. 3)** (Kambham et al 2001; D'Agati et al 2011; Serra et al 2008) . Glomerulomegaly is identified through measuring the diameters of all glomerulus samples or those sectioned through the hilus, which is in the central part of the glomerular globe (D'Agati et al 2016).

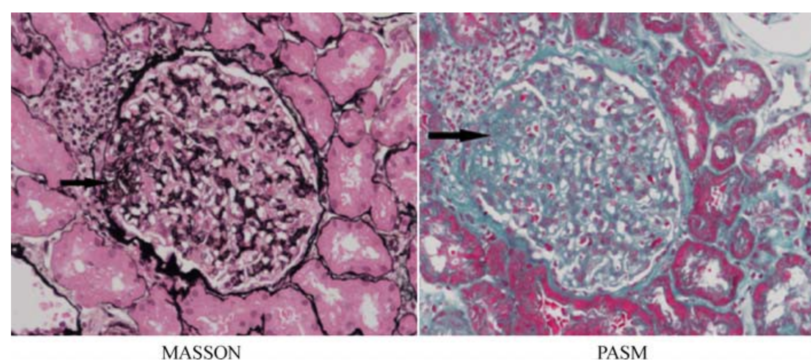

Figure 3: Glomeruli of patients with ORG. Glomerulomegaly is present, and increased capillaries number is observed. Capsular space is restricted, and segmental sclerosis sites are located near the vascular pole (magnification 200X).

Glomerulomegaly is accompanied by mesangial proliferation, matrix accumulation, and hypertrophied podocytes with milder foot process fusion (de Vries et al 2014). FSGS is defined as a segmental consolidation of the glomerular tuft by extracellular matrix and/or hyaline, resulting in capillary obliteration (D'Agati et al 2016). FSGS lesions are predominantly perihilar and typically

observed in hypertrophied glomeruli (Praga et al 2001). Perihilar lesions might also contain other glomerular globe parts. Exclusively perihilar lesions are observed in 19% of ORG biopsy samples, and a mixture of perihilar and peripheral lesions in 81% (Kambham et al 2001). This observation indicates that the ultrafiltration pressure at the afferent end of the glomerular capillary bed is greater than that at the efferent end, and this difference in ultrafiltration pressure leads to afferent arteriole reflex dilation (Chagnac et al 2000). In contrast to primary FSGS, which shows diffuse effacement, ORG-related FSGS presents an irregular mild foot process effacement under an electron microscope. Furthermore, the experimental models of ORG showed that glomerular tuft volume increases exponentially in relation to body weight gain in wild-type Fischer intact rats kept on an ad libitum diet (Fukuda et al 2012). The numerical density of podocyte decreases as the renal mass and glomerular diameter increase, thereby inducing the extension of podocytic processes and covering the expanded area. This expansion can cause podocyte detachment, which induces loss in protein selectivity and formation of denuded areas. The loss of protein selectivity and presence of denuded areas trigger matrix deposition and inflict podocyte injury, finally causing glomerulosclerosis (Liu et al 2010; Matsusaka et al 2011). In addition, lipids are deposited in mesangial cells, podocytes, and proximal tubular epithelial cells (Bobulescu et al 2014). The loaded lipids in the mesangial cells induce structural damage and function loss. Lipid deposition in podocytes leads to insulin resistance and apoptosis, while accumulation of nonesterified fatty acid (NEFA)-bound albumin causes atrophy and interstitial fibrosis in tubular cells (Stefan et al 2016).

1.2 MECHANISMS OF ORG

The pathophysiologic mechanism surrounding CKD development in obese patients remains unclear, but many events such as altered renal hemodynamics, insulin resistance, hyperlipidemia, inflammation and oxidative stress must be linked to ESRD **(Fig.4)**.

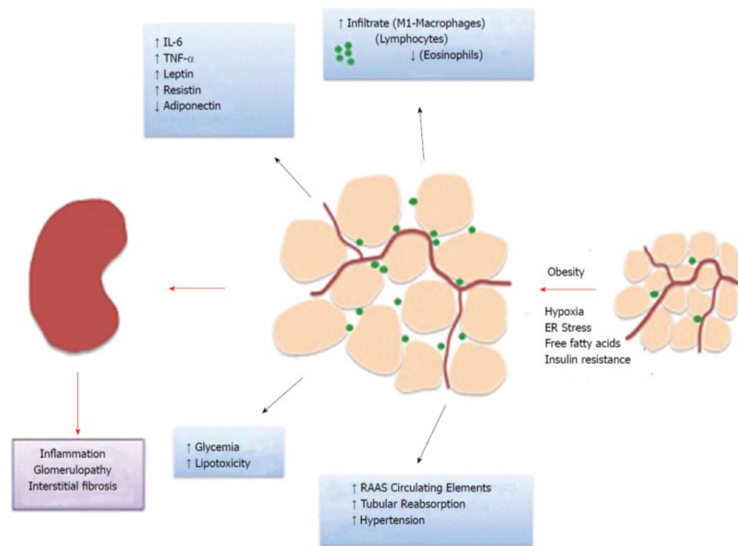

Figure 4: Main factors involved in obesity-induced inflammation, metabolic stress and hemodynamic disorder that participate in kidney function impairment.RAAS: Renin-angiotensin-aldosterone system; ER: Endoplasmic reticulum; TNF-α: Tumor necrosis factor alpha

The mechanisms involved in ORG are complex. Adipose tissue is unbalanced in terms of lipid accumulation in renal cells, and the effects of obesity-associated diseases, such as hypertension, diabetes, dyslipidemia, insulin resistance, and obstructive sleep apnea (OSA), contribute to ORG occurrence. ORG primarily contributes to renal injury through multiple effectors such as adipokines, lipids, reninangiotensin-aldosterone system (RAAS), sympathetic nervous system (SNS), inflammation, oxidative stress, and apoptosis **(Fig.5)** (Felizardo et al 2014).

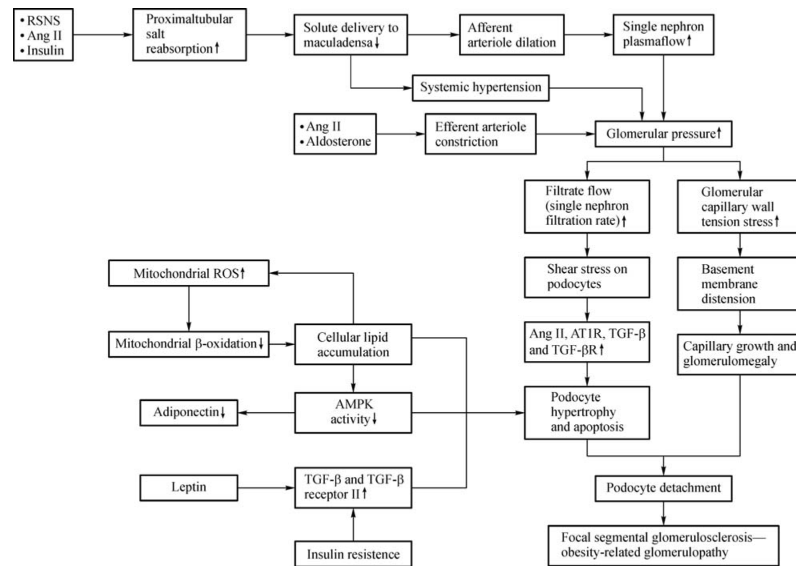

Figure 5: Ang II, renal sympathetic nervous system, and insulin can cause proximal tubular salt reabsorption that increases glomerular pressure, and efferent arteriole constriction has the same effect. Increase of glomerular pressure leads to the increase of filtrate flow, intensified wall tension, and hypertrophy and apoptosis of podocytes, finally resulting in obesity-related glomerulopathy. Leptin and insulin resistance can promote TGF-β and TGF-β receptor II activities that aggravate podocyte apoptosis. Increase of mitochondrial ROS limits mitochondrial β-oxidation and causes cellular lipid accumulation, which causes a further rise of mitochondrial ROS in return. Lipids can damage mitochondria and decrease AMPK activities, resulting in podocyte apoptosis. Furthermore, adiponectin deficiency can decrease AMPK activity. Ang II, angiotensin II; RSNS, renal sympathetic nervous system; TGF-β, transforming growth factor β; TGF-βR, TGF-β receptor; AT1R, type 1 angiotensin II receptor; AMPK, AMP kinase.

HEMODYNAMIC CHANGES

Hemodynamic alterations such as higher renal plasma flow, GFR and filtration fraction were linked to obesity. This was found when comparing the levels in obese and non-obese patients (Chagnac et al 2000; Pecly et al 2006). The effect of BMI on renal hemodynamics was also proven by another study in which GFR and effective renal plasma flow (ERPF) were evaluated within a high-sodium diet. According to this study, ERPF and the GFR were statistically increased when individuals were exposed to a high-sodium diet compared to another group that was exposed to a low-sodium diet without a change in filtration fraction (FF).

However, increased sodium intake-induced changes in the GFR and FF were significantly greater in people with a BMI ≥ 25 kg/m2 (Krikken et al 2007). Previous animal experiments and intervention studies in obese subjects have demonstrated that renal plasma flow (RPF) and glomerular filtration rate (GFR) both increase with obesity (Felizardo et al 2014; Henegar et al 2011; Reisin et al 1987; Chagnac et al 2000). Renal tubular overload in obesity is characterized by an increase in the filtration fraction (GFR/RPF), and may stimulate sodium and water reabsorption in the proximal tubules, resulting in decreased pre-glomerular vascular resistance via the tubuloglomerular feedback mechanism (Vallon et al 1999). A dilation of the glomerular afferent arterioles leads to a further increase in the GFR (glomerular hyperfiltration). Although the origin of such a vicious circle between increased salt reabsorption in the tubules and glomerular hyperfiltration remains unclear, such alterations in renal hemodynamics may constitute the most important pathophysiological basis for the renal abnormalities of obesity **(Fig. 6)**. Consistent with this hypothesis, cetazolamide, a carbonic anhydrase inhibitor that inhibits salt reabsorption in the proximal tubule, has been shown to reduce GFR in obese nondiabetic subjects (Zingerman et al 2015).

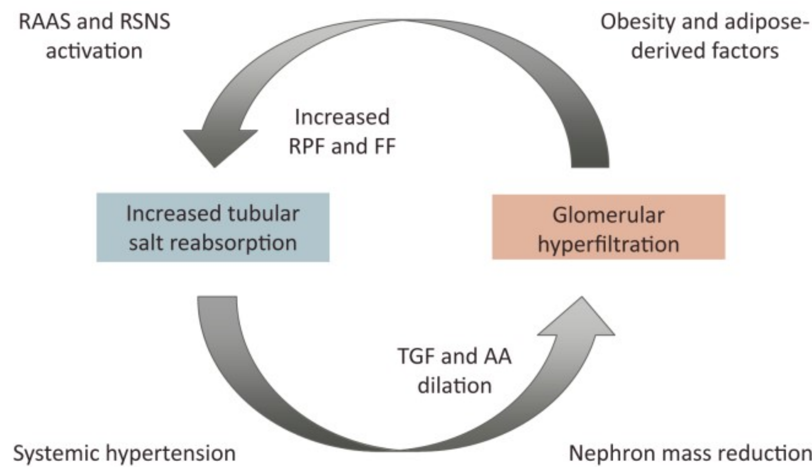

Figure 6: Hemodynamic abnormalities and factors promoting obesity-related renal injury. Renal plasma flow (RPF) and glomerular filtration rate (GFR) increase in obesity. Such renal tubular overload in obesity is characterized by an increase in the filtration fraction (FF: GFR/RPF) and may stimulate sodium and water reabsorption in the proximal tubules, decreasing preglomerular vascular resistance via tubuloglomerular feedback (TGF). A dilation of the glomerular afferent arterioles (AAs) leads to a further increase in GFR, that is, glomerular hyperfiltration. Various factors associated with obesity, including adipose-derived factors, activation of the renin_angiotensin_aldosterone system (RAAS) and renal sympathetic nervous system (RSNS), systemic hypertension, and nephron mass reduction, constitute and promote this vicious circle.

The hemodynamic effects of overweight on kidney function and albuminuria are enhanced with hypertension, which itself is a clinical complication of obesity. *Chagnac et al* (Chagnac et al 2008) demonstrated that glomerular hyperfiltration could have a relevant role in development of hypertension in obese patients by increasing postglomerular oncotic pressure and proximal tubular sodium reabsorption. As an individual gains weight, renal mass as well as the glomerular diameter increase (Kasiske et al 1985). Podocytes are highly specialized cells that support the glomerular basement membrane (GBM) and play an important role in the glomerular filtration barrier via their foot processes. With glomerular hypertrophy, podocytes must cover a larger area by expanding these processes. If this podocyte enlargement is not proportional to glomerular hypertrophy, this adaptation could cause podocyte detachment and consequently a loss in

selectivity of serum protein selectivity (Wiggins et al 2005; Kriz et al 1995) . Considering that podocytes are cells with limited capacity for cell division and replacement, proteinuria may be detected as is commonly observed in obese patients. Supporting this hypothesis, individuals who reduced their body mass also had significant reductions in proteinuria (Chagnac et al 2003; Afshinnia et al 2010). Extensive studies demonstrate that a lack of podocytes covering the GBM results in the formation of denuded areas, which trigger matrix deposition resulting in glomerulosclerosis in experimental models as well as in human biopsies (Mundel et al 2002; Kriz et al 2002; Laurens et al 1994; Barisoni et al 1999). As kidney injury persists, kidney fibrosis becomes an inevitable outcome in which epithelial-mesenchymal and endothelial-mesenchymal transition events generate matrix-producing fibroblasts in the interstitial space that contribute to renal fibrosis. Accumulation of matrix elements caused by the fibrotic process progressively alters normal kidney architecture by contraction and increased stiffness, resulting in disrupted blood flow supply and nephron function (Liu et al 2010; Kaissling et al 2013). Once a number of podocytes are injured, a vicious cycle starts in which other podocytes also become damaged, accelerating podocyte deterioration and glomerulosclerosis (Matsusaka et al 2011). The extensive loss of glomeruli imposes excessive stress on the remaining glomeruli because of hemodynamic alterations and glomerular hypertrophy, which can subsequently cause further sclerosis of the remaining glomeruli (Nagata et al 1992). This could explain the progressive spreading of glomerular damage in later disease stages in which patients develop chronic renal failure (Matsusaka et al 2011). The approach of using new agents to avoid podocyte lesions in different models of acute and chronic kidney disease resulted in less matrix deposition and consequent glomerulosclerosis (Van Beneden et al 2011; Pereira et al 2011).

RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

In obesity, the renin-angiotensin-aldosterone system (RAAS) is commonly activated and is one of the strongest links to renal injury. All of the major components necessary to generate angiotensin II (Ang II) are found in the kidney (Zhuo et al 2011). The RAAS is a well-known mechanism that regulates blood pressure, fluids and electrolyte balance (Kobori et al 2007), and its activation impairs normal pressure natriuresis, increases renal tubular sodium reabsorption, and causes volume expansion. Physical compression of kidneys by visceral adipose tissue in obesity exacerbates these responses and increases blood pressure, leading to hypertension in obese subjects. RAAS effects are obtained when angiotensinogen (AGT), the precursor of bioactive angiotensin peptides, is cleaved by both renin and angiotensin converting enzyme (ACE) to generate Ang II. Ang II, which is the active peptide and is the main effector of RAAS, possesses a dual role in physiology. Ang II helps maintain long-term blood pressure and blood volume in the body; conversely, it has also been considered to be a multifunctional cytokine that plays a role in cell proliferation, hypertrophy, superoxide production, inflammation and extracellular matrix deposition (Rüster et al 2011). Ang II plays an endocrine role, and its participation in the development of obesity was evidenced by several studies in which AGT, ang II and ang II receptor-deficient mice were protected against high-fat diet-induced obesity (Massiera et al 2001; Yvan-Charvet et al 2005; Kouyama et al 2005). Moreover, angiotensin II promotes the production of transforming growth factor-β (TGF-β) and leads to renal fibrosis and podocyte apoptosis (Felizardo et al 2014). Taken together, Ang II directly and indirectly enhances capillary filtration pressure and promotes proteinuria, which is one of the most important factors involved in renal disease progression. Ang II is also involved in nephrin dephosphorylation during podocyte apoptosis (Ren et al

2012), which is a protein that is part of the slit diaphragm and binds to the adjacent nephrins of other podocytes. Human adipose tissue expresses all of the RAAS components, including angiotensin, ACE, renin and the AT1 and AT2 receptors. Consequently, the AGT produced by adipose tissue contributes significantly to circulating AGT levels. In humans and mice, a strong relationship has been observed between increased AGT gene expression and obesity (Yasue et al 2010), supporting a role for adipose AGT in hypertensive obese patients. Weight reduction reduced blood pressure through systemic RAAS suppression and decreased AGT, renin and aldosterone levels in adipose tissue and plasma (Engeli et al 2005). Mice with adipose tissue-restricted AGT expression were normotensive, whereas when adipose AGT was overexpressed, the mice became hypertensive (Massiéra et al 2001). Ang II is also involved in adipocyte metabolism because it influences leptin and adiponectin release. Once leptin levels are increased, Ang II promotes a number of cellular processes that attenuate leptin signal and contribute to leptin resistance, which is common in obesity (Myers et al 2010). Conversely, adiponectin was upregulated when RAS was blocked by an ACE inhibitor or Ang II receptor blocker suggesting Ang II participation in the inhibition of adiponectin release (Furuhashi et al 2003). Not only AGT but also aldosterone levels are increased in obese patients. Aldosterone is a mineralocorticoid hormone that is produced in the adrenal glands in response to Ang II and a high extracellular potassium concentration, which increases blood pressure via sodium retention in the collecting duct. Aldosterone is correlated with increased blood pressure (Bochud et al 2006) and can also be produced by adipocytes through pathways that are dependent on the Ang II-ATI receptor axis and calcineurin signaling (Briones et al 2012). Aldosterone is correlated also at independent pathways of Ang II, in which adipocytes secrete factors that may stimulate the adrenal gland and increase circulating aldosterone levels, resulting in mineralocorticoid

receptor activation and increasing blood pressure and hypertension (Ehrhart-Bornstein et al 2004). Aldosterone binds to cytosolic mineralocorticoid receptors and promotes cell signaling pathways, endothelial dysfunction, inflammation and fibrosis independently and in concert with Ang II (Nishiyama et al 2006). Moreover, Ang II activates the mineralocorticoid receptor in the absence of aldosterone and promotes kidney injury (Kawarazaki et al 2012; Luther et al 2012). Blocking the mineralocorticoid receptors with antagonists attenuates obesity-induced hypertension and glomerular hyperfiltration (de Paula et al 2004). Many clinical trials have been performed to mitigate the effects caused by RAAS. Multiple pharmacological strategies are used to treat CKD patients to diminish proteinuria and blood pressure. These strategies comprehend the use of RAAS-blocking agents alone or combined with ACE inhibitors, angiotensin-receptor blockers, direct renin inhibitors and mineralocorticoidreceptor antagonists (Lambers Heerspink et al 2013). The combination of a pharmacological therapy with reduced sodium intake was a better choice to diminish blood pressure and proteinuria than combined therapies (Slagman et al 2011). Attempts to antagonize aldosterone receptors demonstrated promising results to diminish glomerulosclerosis (Aldigier et al 2005). In summary, the obesity-RAAS-hypertension axis is closely related to renal disease, as the increased release of adipose tissue derived-RAAS elements into the circulation can alter hemodynamic homeostasis. Increased Ang II, AGT and aldosterone levels promote increased tubular reabsorption, leading to arterial hypertension and renal vasodilation. These events contribute to glomerular hypertension, which is an important factor in glomerulosclerosis and CKD progression.

OBESITY AND DIABETES IN RENAL DISEASE

Obesity is an important risk factor for hypertension and type 2 diabetes development, which are the leading causes of end-stage renal disease. The relationship between obesity, diabetes and kidney disease is very close because obesity and diabetes alter renal function, leading to renal disease. These renal alterations in both cases include anatomical, physiological and pathological changes **(Fig.4)**. Physiological and hemodynamic alterations are largely responsible for the subsequent anatomical and histopathological modifications. Among the major hemodynamic changes in obese and/or diabetic patients are increased GFR and intraglomerular capillary pressure (Thomson et al 2004; Hostetter 2003). Such alterations lead to diabetic nephropathy, increases in kidney weight and size, increased glomerular size, podocyte hypertrophy and mesangial matrix expansion (Kopple et al 2011). Diabetes-related renal injuries can be grouped into five stages that comprise the remodeling that occurs throughout diabetic nephropathy. These stages are summarized in **Table 3**. Although obesity and diabetes per se are responsible for renal injury, some other factors usually present in these conditions significantly aggravate renal damage such as blood pressure, hyperlipidemia, hyperglycemia, genetic factors (Dronavalli e3t al 2008) and inflammation. Some of these conditions are described in the following sections.

Stages	Features
1 and 2	Hyperfiltration and renal hypertrophy
3	Microalbuminuria and hypertension as clinical features. As histological features: arteriolar hyalinosis, glomerular basement membrane thickening and mesangial matrix expansion
4 (Diabetic Nephropathy)	Proteinuria, nephrotic syndrome and decreased GFR
5	End-stage renal disease

Table 3: Summary of the most important changes in the kidney during diabetes

HYPERGLYCEMIA AND INSULIN RESISTENCE

Vascular alterations in diabetes are largely due to increased blood glucose levels. Hyperglycemia promotes microvascular injury by several mechanisms. The most important mechanisms are as follows: increased intracellular advanced glycated end product (AGE) formation; interaction between AGEs and their receptors, with consequent disruption of cell signaling and function; constant protein kinase C activation (Koya et al 1998); and increased hexosamine pathway activity (Kolm-Litty et al 1998). Renal endothelial and mesangial cells are susceptible to such hyperglycemia induced changes (Brownlee 2005). Because of AGE-driven structural changes in extracellular matrix proteins, metalloproteinases lose their ability to degrade the matrix efficiently, which causes basement membrane thickening (Thomas et al 2005). In the mesangium, AGE-induced changes include increased pericyte apoptosis and increased vascular endothelial growth factor expression, and these changes in turn cause glomerular hyperfiltration (Wendt et al 2003). Because hyperglycemia causes severe damages to the kidneys and other organs, several studies were developed to demonstrate the importance of glycemic control in order to prevent diabetic nephropathy. Insulin resistance results in renal hemodynamic changes, mainly glomerular hyperfiltration, hypertension, and excessive sodium reabsorption. Insulin resistance causes renal damage, including endothelial dysfunction, increased vascular permeability, protein traffic, mesangial hyperplasia, renal hypertrophy, and enhanced endothelial cell proliferation (De Cosmo et al 2013; Chen et al 1987). Some studies showed that insulin activities in podocytes play an essential part in glomerular function and morphology, cytoskeleton remodelling, and survival (De Cosmo et al 2013). Insulin resistance also causes metabolic syndrome, hyperinsulinaemia, adipocytokine dysregulation, and low-grade inflammation (Redon et al 2015; Ye 2013).

ABNORMAL LIPID METABOLISM

Dyslipidemia is an important component of metabolic syndrome and is often directly related to obesity and diabetes. Patients with diabetic nephropathy usually have several changes in their lipid profile (Shoji et al 2001), and the presence of increased blood lipid levels is a risk factor for albuminuria (Rutledge et al 2010). Several studies have demonstrated a correlation between triglyceride and cholesterol levels with renal function markers. Ravid and colleagues (Ravid et al 1995) observed a significant and positive correlation between total cholesterol and albuminuria in type 2 diabetic patients in a five-year cohort. Similarly, *Klein et al* (Klein et al 1999) noted that type 1 diabetic patients with elevated total cholesterol and low HDL levels also had higher incidence of renal failure. Although these studies demonstrate significant correlations between dyslipidemia and impaired renal function in diabetic subjects, little is known about the mechanisms by which the increased lipid profile causes kidney damage. Studies have demonstrated lipid deposits in the glomeruli and in the mesangium of obese individuals, suggesting that these lipids may cause kidney damage and lipotoxicity (Griffin et al 2008). This glomerular lipotoxicity would be because of renal sterol-regulatory element-binding protein (SREBP-1 and 2) expression, whereas lipotoxicity causes tubulointerstitial fibrosis and inflammation in the proximal tubule epithelial cells (Jiang et al 2005). Furthermore, alterations in the coagulation-fibrinolytic system, increased atherosclerosis and endothelial cell damage can also cause or aggravate diabetic nephropathy (Misra et al 2003). Lipids, also, can damage mitochondria and decrease AMPK activity, thereby resulting in podocyte apoptosis. Furthermore, lipid accumulation increases mitochondrial ROS, which causes further amassing of lipids in return (Xu et al 2015). Thus, the importance of lipid control in the maintenance of kidney function in diabetic patients has been postulated (Fried et al 2001).

MITOCHONDRIAL DYSFUNCTION

Szeto et al. demonstrated mitochondrial dysfunction is the main cause of renal pathology induced by high-fat diet (HFD) (Szeto et al 2016). Given that the kidney is an organ that demands continuous high-energy provision, mostly from mitochondrial fatty acid β-oxidation (FAO), lipid overload and impairment FAO lead to a disturbance in fatty acid uptake and utilization, further aggravating lipid accumulation in kidney cells and tissue (Tang et al 2017). Renal lipid deposition and downregulated FAO are often present in both obese mice and humans (Herman-Edelstein et al 2014). In previous research, reduction of AMPactivated protein kinase (AMPK) activity was demonstrated to be a downstream consequence of mitochondrial dysfunction (Tang et al 2017). Adiponectin-AMPK pathway downregulates both inflammation and profibrotic pathways in both ORG and diabetic kidney disease (Sharma et al 2014; Declèves et al 2014). AMPK regulates not noly NFkB activation but also NADPH oxidases (Sharma et al 2014). AMPK activation can decrease mesangial matrix expansion and lower the levels of profibrotic and proinflammatory markers, such as TGF-β1, tumor necrosis factor-α (TNF-α), and monocyte chemoattracting protein (MCP-1) (Declèves et al 2014; Dugan et al 2013). Mitochondrial dysfunction generates reactive oxygen species (ROS), which limit mitochondrial β-oxidation and causes cellular lipid accumulation, which results in a further increase in mitochondrial ROS levels. Lipids can damage mitochondria and decrease AMPK activity and thus can promote podocyte apoptosis and damage.

INFLAMMATION

Adipose tissue is known for its roles in lipid storage, thermogenesis and metabolic regulation. However, in recent years, focus has been given to its endocrine properties such as

cytokine and adipokine secretion **(Fig.4)**. In literature its known that obesity and diabetes are conditions that present a state of low-grade inflammation (Felizardo et al 2014). Significant evidence supports the concept of adipose tissue as an immunomodulatory organ. Adipose tissue harbors a considerable amount of immune cells such as macrophages, lymphocytes and eosinophils. In obesity, the frequency of infiltrated cells rises, and they acquire a pro-inflammatory profile (Xu et al 2003). Excess free fatty acids that are present in obesity activate diverse inflammatory pathways involving endoplasmic reticulum stress (Ozcan et al 2006), tolllike receptor (Shi et al 2006; Tsukumo et al 2007), inflammasome and nuclear factor-κB (NF-kB) signaling activation (Vandanmagsar et al 2011; Stienstra et al 2011). In parallel, adipose tissue becomes hypoxic with adipocyte hypertrophy, which induces a change from aerobic to anaerobic glycolysis and lactate production. With obesity, adipocyte hypertrophy and hypoxia induce cell death and resident immune cell activation, which in turn promotes inflammatory cell recruitment (Cinti et al 2005). Chronic adipose inflammation, which forms from the imbalance between proinflammatory and anti-inflammatory factors, is a major factor for ORG (Nolan et al 2013). Macrophages constitute the principal population of resident and recruited cells in adipose tissue, which have a role in maintaining tissue homeostasis by assisting with the clearance of dead cells and debris. Because of lipid accumulation and adipocyte cell death, non-inflammatory tissue-resident M2 type macrophages and recruited monocytes undergo proliferation and macrophage M1 polarization (Trayhurn et al 2007; Lumeng et al 2007; Prieur et al 2011). These cells in turn secrete higher levels of inflammatory cytokines such as TNF-α, IL-6 and MCP-1 and lower levels of anti-inflammatory mediators such as arginase 1(Lumeng et al 2007;. Prieur et al 2011; Wentworth et al 2010), IL-4-expressing eosinophil counts also decrease with obesity, this factor contributes to inflammation (Wu et al 2011). Furthermore, CD8+ and CD4+ Th1 lymphocyte counts also increase while

Treg numbers reduce with obesity. In accordance, B cell pro-inflammatory immunoglobulin G2c (IgG2c) production also participates in cell activation (Winer et al 2011; Schipper et al 2012; Nishimura et al 2009; Winer et al 2009) . Proinflammatory cytokines are also produced by the renal parenchyma in response to hyperglycemia as well as vasoactive peptides such as angiotensin II and endothelin (Ruiz-Ortega et al 2002). These molecules activate signaling second messengers such as protein kinase C, MAP kinase and NF-κB, which alter the gene expression of several cytokines and growth factors. Increased TNF-α levels reduce the expression of nephrin and podocin, causing podocytopathy (Ikezumi et al 2008). Similarly, IL-6 promotes adhesion molecule expression, which increases oxidative stress (Patel et al 2005), and IL-6 receptor blockade can inhibit the progression of proteinuria, renal lipid deposition and mesangial cell proliferation (Tomiyama-Hanayama et al 2009). An additional important growth factor for renal injury is transforming growth factor (TGF)-β, which induces podocyte apoptosis, extracellular matrix synthesis and mesangial cell proliferation, thus exacerbating the development of the glomerular lesions associated with diabetes and obesity (Ziyadeh et al 2004). While many studies demonstrate the effect of metabolism on the immune system, studies have demonstrated that the reverse also happens; immune cell activation in adipose tissue is a determinant of obesity-linked metabolic changes such as insulin resistance (Han et al 2013). For example, in response to inflammatory mediators, adipose tissue also down regulates glucose transporter GLUT4 expression, which increases insulin resistance. Obese individuals have high levels of leptin, which binds to specific receptors in mesangial cells. Leptin upregulates TGF-β and TGF-β receptor II, thus inducing an increment of type I and type IV collagen fibers in the mesangium and promoting the formation of fibrosis. Leptin binds to obRb receptors in the hypothalamus and overactivates SNS, which induces renal hemodynamic changes and renal damage (Young et al

2013; Nasrallah et al 2013). Obese individuals have low concentrations of adiponectin. As an anti-inflammatory and insulin-sensitizing factor, adiponectin activates AMPKs to protect podocyte functions and structures by reducing podocyte permeability (Rutkowski et al 2013).

1.3 GLOMERULAR PODOCYTES

Podocytes (or visceral epithelial cells) are terminally differentiated cells lining the outer surface of the glomerular capillaries. As a major component of the ultrafiltration apparatus, podocytes have a complex cellular architecture consisting of cell body, major processes that extend outward from their cell body, forming interdigitated foot processes (FPs) that enwrap the glomerular capillaries (Pavenstädt et al 2003). Podocyte FPs comprise a functioning slit diaphragm (SD) in between (Reiser et al 2000; Fukasawa et al 2009), a meshwork of proteins actively participating in podocyte signaling (Huber et al 2005; George et al 2012; Grahammer et al 2013). Podocytes form the glomerular filtration barrier together with the opposing monolayers of fenestrated endothelium in the vascular space (Satchell et al 2009) and glomerular basement membrane (GBM) in between (Kretzler 2002 ; Farquhar 2006). This three-layer filtration barrier serves as a size-selective and charge-dependent molecular sieve by facilitating the filtration of cationic molecules, electrolytes, and small and midsized solutes but restricting the passage of anionic molecules and macromolecules (Tryggvason et al 2005; Menon et al 2012). It is important to bear in mind that those layers should be arranged with decreasing selectivity, with the SD being the least selective filter; otherwise, retained plasma proteins would routinely accumulate behind the filtration slits of podocytes (Haraldsson et al 2008). This elegant structure has to oppose hydrostatic pressure in the glomerular capillary, which is the natural driving force behind macromolecular filtration. If podocytes are injured,

mutated, or lost, the elaborate structure of podocytes is physically altered—a process termed 'foot process effacement', which is found in many proteinuric kidney diseases.

1.4 FREE FATTY ACIDS (FFAs)

Lipid disorders, mainly hypertriglyceridemia, are common in patients with all stages of chronic kidney disease (CKD) including diabetic nephropathy (DN) (Senti et al 1992; Attman et al 1993; Kwan et al 2007; Arnadottir et al 1995) . It has been reported that lipid abnormalities in renal disease contribute to the process of glomerulosclerosis with progressive renal dysfunction (Wheeler et al 1994; Kees-Folts et al 1993; Thabet et al 1993; Srivastava et al 2014; Yukawa et al 1999). One of the major pathogenic mediators in ORG and its complications is dyslipidemia, resulting in high saturated free fatty-acid (FFA) concentrations. Two of the most common fatty acids in humans are the long-chain saturated FFA palmitate (C16:0) and the monounsaturated oleate (C18:1) (Oliveira et al 2015). In particular, palmitate is the most abundant saturated FFA in the plasma of humans and rodents accounting for ~ 25% of total fatty acids (Wheeler et al 1994; Kees-Folts et al 1993). Western diets rich in fatty acids are associated with increased levels of plasma cholesterol, hepatic steatosis, and a greater risk of cardiovascular disease (Siri-Tarino et al 2010; Roden 2006). High levels of circulating FFAs, in particular of saturated FFAs, are implicated in insulin resistance and pancreatic b-cell dysfunction, and are predictive of diabetes development (Cnop 2008 ; Paolisso et al 1995; Blaak 2003; Giacca et al 2011). *In vitro* exposure to high levels of FFAs leads to lipotoxicity, causing cellular dysfunction and death (Cnop 2008; Schaffer et al 2003).

1.5 OXIDATIVE STRESS INDUCED BY FFAs

Saturated FFAs in the cytosol induce reactive oxygen species (ROS) production as observed in pancreatic β-cells, hepatic cells and skeletal muscle cells (Yuan et al 2010; Yuzefovych et al 2010; Srivastava et al 2007). In muscle cells, palmitate stimulates superoxide generation through the mitochondrial electron transport chain and NADPH oxidase activities (Lambertucci et al 2008). However, the mechanisms underlying how dyslipidemia accelerates CKD progression remain unclear. ROS are essential signaling molecules that regulate physiological cell functions (Trachootham et al 2008). However, the overproduction of ROS in pathologic conditions has detrimental consequences, causing organellar stress, injury and cell death (Wehinger et al 2015; Liu et al 2015). Palmitate is a potent inducer of ROS in a number of cell types, including pancreatic β cells (Carlsson et al 1999; Sato et al 2014), cardiomyocytes (Liu et al 2015; Joseph et al 2016), vascular smooth muscle cells (Brodeur et al 2013), endothelial cells (Yamagishi et al 2002), skeletal muscle cells (Taheripak et al 2013), glomerular mouse podocytes (Xu et al 2015), hepatocytes (Gao et al 2010) and adipocytes (Davis et al 2009). Increased mitochondrial fatty acid oxidation has been proposed as the main process leading to ROS generation in lipotoxicity **(Fig.6)**.

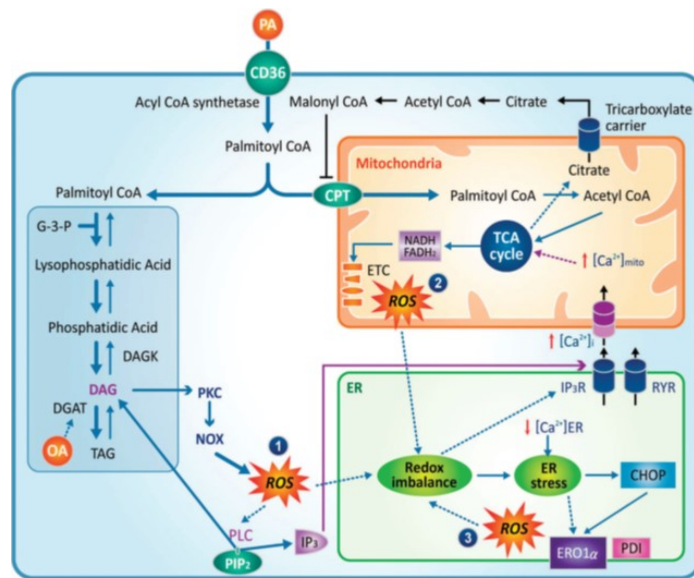

Figure 6: Palmitate induces ROS overproduction. (1) Increased β-oxidation, (2) DAG-PKC-NOX, (3) CHOP-ERO1α and PDI under ER stress.ROS produced by palmitate triggers PLC activation, ER Ca2+ release, ER stress and mitochondrial dysfunction, which, in turn, aggravate ROS generation. CHOP, CCAAT-enhancer-binding protein homologous protein; DAG, diacylglycerol; ERO1α, ER oxidoreductin 1 alpha; NOX, NADPH oxidase; PDI, protein disulfide isomerase; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species.

The oxidation of palmitate delivers excess electrons to the electron transport chain (ETC), which thus causes superoxide overproduction (Lambertucci et al 2008; Nakamura et al 2009; Boudina et al 2007). There are, however, conflicting data in the literature showing that the acceleration of β-oxidation actually relieves oxidative stress, and the inhibition of mitochondrial fatty acid uptake aggravates ROS production (Choi et al 2011; Namgaladze et al 2014). The molecular mechanisms for cellular ROS generation by palmitate, therefore, remain to be fully elucidated. Palmitate-induced superoxide cannot be fully eliminated by the addition of the complex III inhibitor antimycin A, revealing that ROS are also generated through sources other than the ETC (Lambertucci et al 2008). In chondrocytes, a mixture of oleate and palmitate enhances ROS production and induces cell apoptosis, mainly by upregulating the protein levels of NOX4 (Fu et al 2016). Notably, NOX4 is expressed in mitochondria and contributes to mitochondrial ROS production (Das et al 2014; Ago et al 2010).

AIMS OF THE STUDY (1)

The signaling pathways leading to renal pathology in obesity are not well understood. Growing evidence suggest that FFAs induced oxidative stress plays a role as the critical factor linking obesity with its associated complications (Manna et al 2015). Podocytes are susceptible to saturated FFAs (Sieber et al 2010). In particular, palmitate is the most abundant saturated FFA in the plasma of humans and rodents and accounts for ~ 25% of total fatty acids (Wheeler et al 1994; Kees-Folts et al 1993). FFAs are present in blood at concentrations from ∼100 μM to >1 mM. Since FFAs have low solubility in aqueous solutions, such as blood plasma and interstitial fluid, they need to bind proteins to increase their concentration in vascular and interstitial compartments. Albumin acts as main fatty acid binding protein in extracellular fluids (van der Vusse 2009). Because FFAs are bound to albumin, the total plasma FFAs concentration does not directly reflects the physiologically active FFAs concentration. The albumin-bound FFAs cannot bind to proteins, or serve as substrate for FFAs-utilizing enzymes. Only FFAs, that are soluble monomers in the aqueous phase, can be transported across membranes, bind to specific sites on proteins and function in enzymatic reactions. The soluble unbound FFAs (FFAus) are a tiny fraction of the total FFAs (bound and unbound). *In vitro* studies used FFAs-albumin complexes to generate FFAus, presumed to be at physiologic concentrations. However, because FFAus concentrations increase exponentially with increasing ratios of FFAs to albumin, especially above mole ratios of 4:1, reliable FFAus levels can be assumed only (Huber et al 2017).

Recently *Oliveira et al* have proposed a new method to generate FFAs-albumin complex that assure the formation of physiopatological concentrations of FFAus (Oliveira et al 2015). Therefore, the aims of this part of my PhD thesis has been to

evaluate, in human conditioned immortalized podocytes cell line, the capacity of phisyopathological concentrations of palmitate-albumin complex generated by the method of *Oliveira et al* to induce:

- intracellular ROS production;
- podocytes ER stress;
- podocytes inflammation.

CHAPTER II

2.1 HIGH MOBILITY GROUP BOX 1 (HMGB1)

STRUCTURE AND ORIGIN OF HMGB1

HMGB1 is a highly conserved chromatin-associated factor present in the nucleus of almost every cell type (Goodwin et al 1973). In mammals, there are three members of the HMGB family, HMGB1, HMGB2, and HMGB3. Evolutionary studies suggest that the organization of genes coding for the HMGB proteins is very conserved among multicellular animals (99% omology between mammals) and is unknown outside Metazoans. Structurally, HMGB1 is a 25 kDa protein composed of 215 amino acids organized in two positively charged DNA-binding structures, named A and B boxes and a negatively charged C-tail, composed of 30 glutamic and aspartic acids. The A and B boxes are helical structure, partly covered by the tail, which is folded over the protein. HMGB1 has two nuclear localization signals (NLS1 and NLS2) and two unusual nuclear export signals (NESs) that imply the continuous shuttling between nucleus and cytoplasm even if the concentration in the first compartment is much higher (in the range of micro-molar) than in the second **(Fig.7)**. Box A has anti-inflammatory properties, since it is an antagonist for HMGB1 binding to its receptor RAGE, and inhibits HMGB1 cytokine effects *in vivo*. On the contrary, box B has pro-inflammatory effect and contains the binding sites for different receptors, including TLR4 and RAGE **(Fig.7)** (Andersson et al 2000). HMGB1 has three conserved cysteines in position 23, 45 (Box A) and 106 (Box B) that are susceptible to reduction or oxidation depending on the conditions of the compartment in which HMGB1 is localized and that influences HMGB1 functions **(Fig.7)**. The translocation from nucleus to

cytoplasm requires post-translationally modification. It is still known that hyperacetylation (20% of the residues of HMGB1 are lysines) and phosphorylation are very important for HMGB1 export and secretion (Bonaldi et al 2003; Youn et al 2006). In particular the 9 isoform of protein kinase C (cPKC) catalyzes the phosphorylation of HMGB1 (Oh et al 2009) and very recently it has been shown that sirtuin SIRT1 deacetylases HMGB1 increasing its translocation to the cytosol (Rabadi et al 2014). Because HMGB1 lacks of the signal peptide, its secretion doesn't occur via the classical ER-Golgi route but requires specific endolysosomes that direct this protein towards secretion (Gardella et al 2002) in a process Ca2+-dependent (Oh et al 2009).

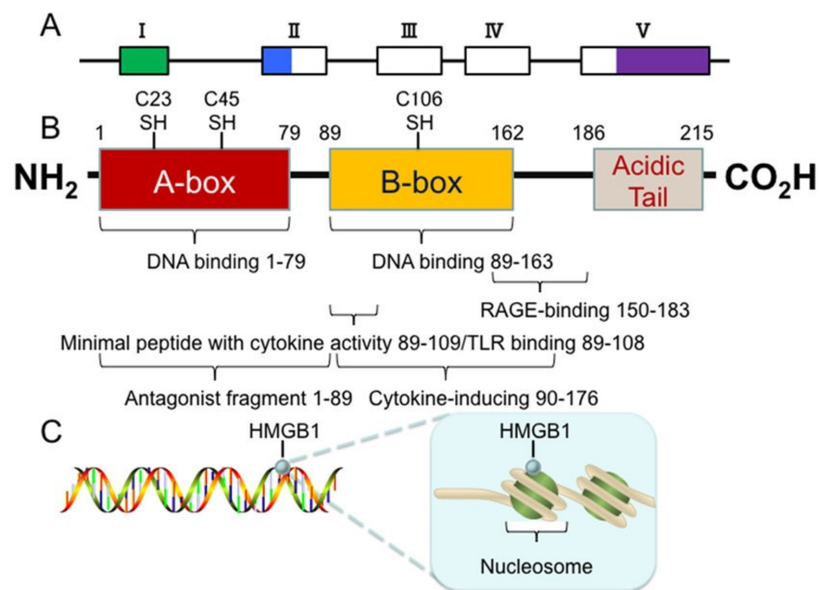

Figure 7: Structure of the HMGB1 protein. (A) The 5 exons of human HMGB1 gene are indicated by boxes (hollow for translated regions and solid for un-translated regions). (B) The human HMGB1 protein has 215 amino acid residues and is composed of three domains: A box, B box and an acidic C-terminal tail. There are three redox-sensitive cysteine residues at positions 23, 45, and 106, which regulate HMGB1 function in response to oxidative stress. (C) The human HMGB1 is loosely and transiently associated with nucleosomes. In this location, HMGB1 is important for spatial segregation and nuclear homeostasis.

2.2 FUNCTIONS OF HMGB1

NUCLEAR FUNCTION

In the nucleus HMGB1 binds and bends the minor groove of DNA in a no sequence-specific manner, regulating chromatin structure, gene expression and gene transcription (Agresti et al 2003). An interesting peculiarity of HMGB1 is its high dynamism, ability to interact with a new nucleosome every second by scanning the DNA in search for the right binding site (Scaffidi et al 2002). The release of HMGB1 after exposure to an inflammatory stimulus reduces the histones content in macrophages, demonstrating the strong relationship between HMGB1 and histones (De Toma et al 2014). It has been previously demonstrated that in activated macrophages, HMGB1 can be secreted gleaning from the nuclear pool (Bonaldi et al 2003) and the depletion of HMGB1 affects macrophages response to inflammation (De Toma et al 2014). Indeed, in Hmgb1-/- fetal liver-derived monocytes (FLDMs) many chemokine transcripts involved in chemotaxis, motility, cell adhesion and response to stress stimuli were upregulated, demonstrating that the release of HMGB1 from activated macrophages led to a chromatin rearrangement caused by nucleosomes and histones loss that contribute and regulate the inflammatory response (De Toma et al 2014). As a nuclear factor, HMGB1 has many other roles: it helps the enhanceosome formation, stabilizes nucleoprotein complexes and is involved in chromatin remodeling and gene transcription thus regulates the activity of several DNA-binding factors **(Fig.8)** (Lotze et al 2005). HMGB1 is also able to bind different members of the onco-suppressor gene p53 family, including two splicing variants of the tumor suppressor factor p73, α and β. Both Box A and Box B can interact with p73 and the formation of a p53/p73-HMGB1 can enhance the recruitment of both p53 and p73 to the Bax and Mdm2 promoters, facilitated by the DNA bending activity of

HMGB1 (Stros et al 2002). McKinney and Prives proposed that HMGB1 binds and bends DNA to form an optimal configuration that is more easily recognized by the C-terminus of p53 (McKinney et al 2002).

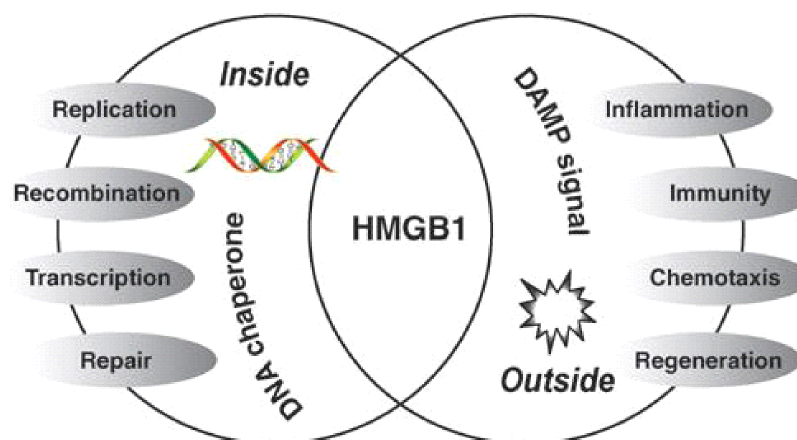

Figure 8: Functions of high-mobility group box 1 (HMGB1). HMGB1 is present in almost all metazoans and plants. As a DNA chaperone, HMGB1 participates in DNA replication, recombination, transcription, and repair. HMGB1 is passively released from necrotic cells and is actively secreted by inflammatory cells, mediating the response to inflammation, immunity, chemotaxis, and tissue regeneration (Tang et al 2012).

EXTRACELLULAR FUNCTION

HMGB1 was discovered around 40 years ago and studied for many years for its nuclear role but in the last decade this protein has aroused great interest for another novel function. In certain conditions HMGB1 can translocate from the nucleus to the cytosol to be secreted and the various functions played by extracellular HMGB1 are still matter of studies since the first evidence discovered in 1999 (Wang et al 1999) **(Fig.7)**. Wang *et al.* described for the first time the effect of a late mediator of endotoxemia released in murine macrophage-like RAW 263.7 cells stimulated for 18 hours with LPS. Notably, this factor was HMG-1 and its neutralization with an anti-HMG-1 antibody

increased the survival rate of LPS-treated mice from 30 to 70%. (Wang et al 1999). This was the first evidence that this nuclear factor can be released in the extracellular space, triggering inflammation. Even though HMGB1 is abundant in the nucleus of most cells, the secretion happens only by certain cell types and at given maturation states (Rubartelli et al 2007). In fact, it has been demonstrated that HMGB1 can be secreted by activated monocytes after translocation to the cytosol in response to inflammatory stimuli, acting as others pro-inflammatory cytokines **(Fig. 9)**. Unlike IL-1β and TNF-α (that are early mediators of inflammation), HMGB1 is a late mediator of inflammation because it can be detected only at a delayed time point after monocytes activation (Gardella et al 2002) and its secretion by activated immune cells is an active process that requires exocytosis of secretory lysosomes (Rovere-Querini et al 2004). Extracellular HMGB1 has numerous positive functions, induces stem cells proliferation and migration (Palumbo et al 2004), EPC homing to ischemic tissue (Chavakis et al 2007), neurite outgrowth (Hori et al 1995), monocytes/macrophages and dendritic cells activation and migration (Venereau et al 2012; Yang et al 2007; Dumitriu et al 2007), endothelium activation (Treutiger et al 2003), plays a role in skeletal muscle regeneration (De Mori et al 2007; Vezzoli et al 2011) and moreover ameliorate cardiac function (Kitahara et al 2008). On the other hand, there are studies showing the deleterious effects that HMGB1 can have in some areas like diabetes (Wang et al 2014), ischemia-reperfusion (Andrassy et al 2008) or sepsis and autoimmune diseases (Ulloa et al 2006). This means that HMGB1 has different roles in different contexts that must be further investigated.

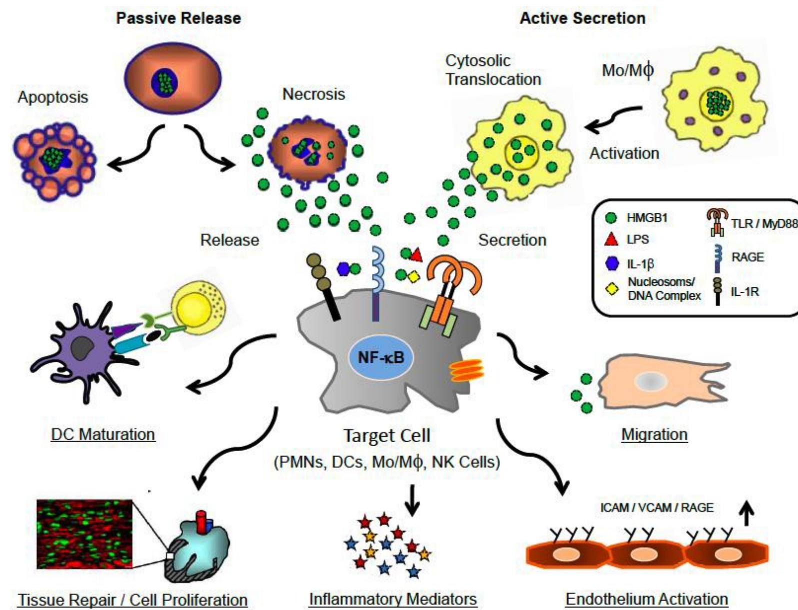

Figure 9: Extracellular role of HMGB1: HMGB1 can be releases in a passive way from necrotic cells or in an active way from activated inflammatory cells, especially monocytes (Mo) and macrophages (MΦ) and requires the cytosolic translocation. Apoptotic cells do not release HMGB1. Once released HMGB1 binds different receptors on several cell types, exerting various functions such as dendritic cells (DC) maturation, migration, endothelium activation, secretion of inflammatory mediators and cell proliferation, important for tissue repair.

2.3HMGB1 AS AN EFFECTOR OF THE INFLAMMATORY STATE

The secretion of HMGB1 in the extracellular space is controlled by a calcium dependent mechanism (Oh et al 2009) and plays an important role since it has been demonstrated that monocytes and macrophages secrete HMGB1 following stimuli inflammatory induced with lipopolysaccharide (LPS), a tumor necrosis factor (TNF-) and / or interleukin-1 (IL-1) (Wang et al 2014). Following its release, HMGB1 interacts with a series of receptors placed on cell membrane in particular with:

- RAGE receptors (Receptor of Advanced Glycation Endproduct) the interaction of HMGB1 with this type of receptor **(Fig.10)** induces the activation of the transcription of NF-kB, which has as its effector the Tumor Necrosis

Factor-Alpha (TNF-α) which, interacting with the its receptor activates the JNK and IKK kinase- responsible for the phosphorylation and inactivation of Insulin Receptor Substrate 1 and 2 (IRS1 and IRS2). This inactivation causes insulin resistance (Chen et al 2016).

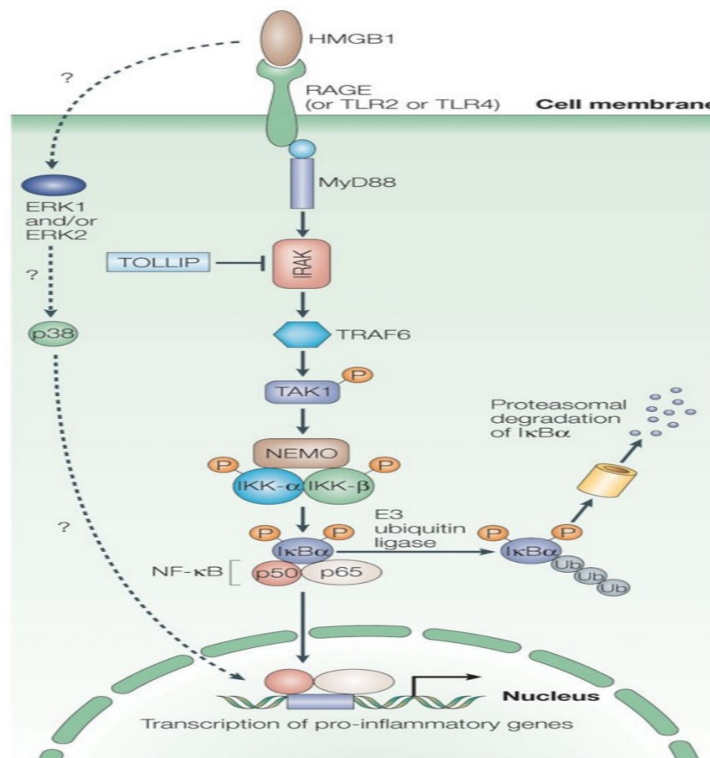

Figure 10: RAGE signal transduction pathway in response to receptor interaction with HMGB1 edmhypothetical p38 transduction pathway (Lotze et al 2005)

- TLR-2 and TLR-4 receptors (Toll-Like receptor 2 and 4): **(Fig.11)** interaction with these receptors leads to kinase activation activated mitogen (MAPK), which lead to the release of cytokines proinflammatory like tumor necrosis factor alpha (TNF-), interleukin 1 beta (IL-1), interleukin 1 alpha (IL-1), interleukin 6 (IL6) and the inflammatory protein of macrophages (Andersson et al 2000; Degryse et al 2001; Stemmer et al 1997).

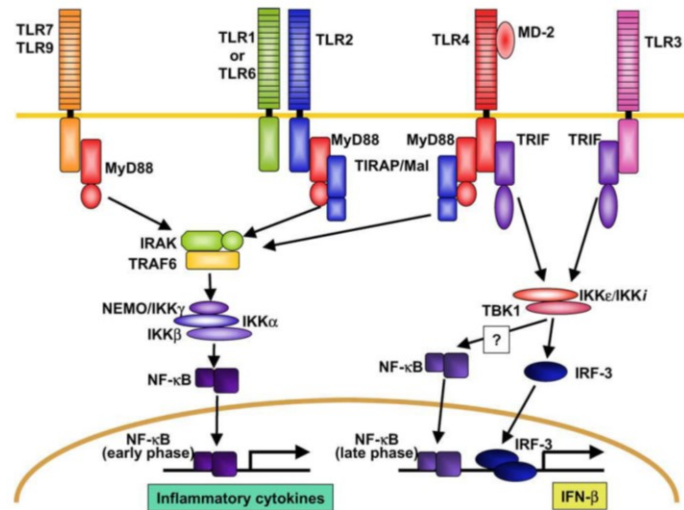

Figure 11: Signal transduction pathway of Toll-like family receptors: TLR2 and TLR 4 are the receptors that they interact with HMGB1 once secreted in the extracellular medium

By virtue of the presence of an active release mechanism and a system receptor / signaling it is reasonable to consider HMGB1, not simply as a structural factor of DNA remodeling, but also as a molecule of physiological importance. Recent studies have highlighted how the release of HMGB1 from the nuclei of activated macrophages and / or from necrotic cells, covers an important role in the context of the immune system acting as an adjuvant immune (Andersson et al 2000). This process can take place through activation of dendritic cells, macrophages and T cells; furthermore its biological role on progenitor cells gives it a role important in the regeneration and repair of damaged tissues (Bianchi et al 2007). There HMGB1 secretion by macrophages has a central role in the progression of the inflammatory state (Gardella et al 2002; Scaffidi et al 2002) and until recently it was believed that this was an event exclusively for the immune system. However, it has been noted that the release of HMGB1 may also occur at the expense of endothelial tissue subjected to a stimulus of inflammatory nature (Mullins et al 2004); this study showed how in

human umbilical cord endothelial cells (HUVEC), in the presence of an inflammatory stimulus, such as the lipopolysaccharide (LPS), there is a cellular relocation of HMGB1 which moves from the nucleus to the cytoplasm (Bianchi) and subsequently to the extracellular space where it exercises the function of a pro-inflammatory cytokine inducing the secretion of factors such as tumor necrosis factor- (TNF-) and nuclear factor kappa B (NF-kB). The progression of the inflammatory state is therefore not borne exclusively from the cells of the immune system. Given this particular relevance, HMGB1 has proven to be a potential therapeutic target in experimental models for infections and inflammatory disorders such as sepsis, trauma, cancer and rheumatoid arthritis (Gardella et al 2002; Ulloa et al 2006; Marrack et al 2004). The action of HMGB1 it is of particular interest at the gastrointestinal level since it increases both the permeability of the ileal mucosa and bacterial translocation in lymphoid nodes mesenteric through a mechanism that is dependent on the formation of nitric oxide (NO) (Sappington et al 2002; Yang et al 2005).

2.4 HMGB1 IN DIABETIC KIDNEY DISEASE

Emerging studies have implicated high mobility group box 1 (HMGB1) protein as a key facilitator and potential biomarker of renal inflammation (Venereau et al 2016) . The multiprotein complex called the inflammasome, which is a large caspase-1-activating protein complex, plays a regulatory function in tissue inflammation, through the cleaving of pre-inflammatory cytokines into mature forms and by mediating the extracellular release of HMGB1 from activated immune cells (Vande Walle et al 2011) . The NLRP3 inflammasome, (nucleotide-binding domain, leucine-rich-containing family, pyrindomain-containing-3), for example, is expressed in circulating monocytes as well as in

resident tissue macrophages, and plays a significant role in renal tissue injury. Inflammasome activation leads to the local expression of pro-inflammatory molecules like interleukin (IL)-18 and IL1β, which in turn activate the cellular release of HMGB1 that may have a causal effect on glomerular injury. While the precise mechanisms triggered by HMGB1 to promote injury and scarring remains unclear in DKD, a plethora of interlocked mechanisms are likely at play. A few studies have shown that blocking the release of HMGB1 or using anti-HMGB1 antibody significantly decreases the severity of colitis and gut barrier dysfunction (Dave et al 2009; Yang et al 2006) . A growing body of evidence also indicates that HMGB1 plays an important role in glomerulonephritis, autosomal dominant polycystic kidney disease, and kidney disease (Bruchfeld et al 2008; Sato et al 2008). Glycyrrhizin (GLYZ), a glycol-conjugated triterpene extracted from the licorice plant, *Glycyrrhiza glabra* , demonstrated anti-inflammatory properties and is considered to inhibit the cytokine activity of HMGB1 with amelioration of inflammation during acute/chronic phases of hepatitis, myocarditis, and liver injury (Ogiku et al 2011; Zhai et al 2012) . A recent study showed that GLYZ protects renal ischemia reperfusion injuries in rabbits (Subramanian et al 1999).

AIMS OF THE STUDY (2)

It's known how high mobility group box 1 protein (HMGB1) is a potential biomarker of renal inflammation. HMGB1, a nuclear DNA binding protein, is released under pathological conditions and locally acts as one of potent damage-associated molecular patterns (DAMPs) to produce tissue injury and chronic inflammation. HMGB1 synthesis and its secretion is increased in obese patients in different types of tissue especially in the adipose tissue. In literature it is known that HMGB1 has a role in the pathogenesis of different type of kidney disease as chronic kidney disease and diabetic nephropaty (Chen et al 2016). It's known that palmitate at concentrations seen in the serum of obese patients induces podocytes dysfunctions, and podocyte insulin resistance, all events that are involved in the reduction of kidney function. All these effects are mediated by the palmitate induced increase of ROS generation. In letterature it has been shown how HMGB1 is secreted via ROS-dependent mechanism (Min et al 2017).

We hypothesized that short exposures to concentrations of palmitate associated with obesity can increase HMGB1 expression. Therefore, the aim of this part of my PhD thesis has been to evaluate, in a human podocytes conditioned immortalized cell line:

- The effect of physiopathological concentration of palmitate on HMGB1 expression;

- The role played by HMGB1 expression in causing palmitate induced podocytes dysfunctions.

CHAPTER III

3.1 “METABOLIC MEMORY”

The first definition of metabolic memory came from several studies that showed that changes in microcirculation due to hyperglycaemia were relatively reversible if an early and adequate control of blood glucose was achieved. Studies conducted on a large scale (Nathan et al 2005; Holman et al 2008) have shown that early intensive glycemic control decreases the risk of diabetic microvascular complications. The first study where metabolic memory was postulated was the 1987 report from Engerman et al., who evaluated the extent of the arrest in the development of diabetic retinopathy derived from improved glycemic control. Later clinical trials in diabetes also provided a picture of the phenomenon called “metabolic memory” in greater detail. Different mechanisms are involved in metabolic memory, including mitochondrial DNA damage, protein kinase C activation, and the polyol pathway, increased production of advanced glycation end products (AGEs), AGE receptor overexpression, increased anion superoxide formation, mitochondrial protein glycation, and hexosamine flux alterations (Ceriello 2012). However, targeting these mechanisms with new therapies has had limited success in slowing down the progression of diabetes complications (Russell et al 2015).

3.2 THE HYPERGLYCEMIC AND UREMIC “MEMORY EFFECT”

In diabetes hyperglycemic memory there is a model to explain the long-lasting effects that short term hyperglycemic spike may have on kidney tissue (Chen et al 2013). It has been shown that transient exposure to pathological concentration of glucose causes persistent increases in inflammatory gene expression

during subsequent periods of normal glycaemia, by inducing epigenetic modifications (Rao et al 2009). The persistent adverse effects of prior sustained exposure to hyperglycemia persisting for years after hyperglycemia has been ameliorated is defined as "metabolic memory" (Giacco et al 2015). A key factor in the genesis of "metabolic memory" is the increase of intracellular ROS production induced by hyperglycemia. In endothelial cells transient hyperglycemia can activate a multi-component feedback loop which maintains increased levels of mitochondrial ROS production for days after glucose concentration is normalized (Guido et al 2012). This continued mitochondrial overproduction of ROS despite normalization of glucose concentration would explain the persistence of the epigenetic changes induced by the initial hyperglycemic spike that otherwise will be rapidly reversed by histone demethylases and histone methyltransferases (Ding et al 2017). Accumulating evidence supports the concept that even prior exposure to hyperglycemia for hours causes persistent ROS lasting for days of subsequent normal glucose, triggering vascular dysfunction and damage (Costantino et al 2015; Brasacchio et al 2009) by inducing epigenetic modifications (Giacco et al 2015; Wegner et al 2014) . Similar to glucose, urea at disease relevant concentrations has a direct vascular toxicity (Massy et al 2016; Giardino et al 2017), inducing endothelial dysfunction by increasing intracellular ROS production through the activation of both mitochondrial and cytosolic ROS generating mechanisms (D'Apolito et al 2015). D'Apolito *et al.* have shown that transient exposure to concentrations of urea associated with chronic renal failure may cause a persistent increase in mitochondrial ROS production in arterial endothelial cells during subsequent days of normal uremia. This continuous persistent mitochondrial ROS overproduction after dialysis would cause a progressive accumulation of pathologic changes in arterial endothelial cells, promoting the progression of atherosclerotic lesions into

unstable plaques. Thus, similar to hyperglycemia, pathological concentrations of urea could induce long lasting effects on arterial cells. Such a phenomenon could explain the failure of clinical treatment to reduce CVD morbidity and mortality in the CRF population. Together, these results show that the urea-induced mitochondrial damage initiates a vicious cycle of ROS production that continues to self-propagate even after the urea insult is terminated. This persistent ROS production causes an increased expression of inflammatory markers in endothelial cells that also last for two days after the urea is removed **(Fig.12)**. The study of D'Apolito *et al* proves that dysfunctional mitochondria are the source of the increased ROS production that persists when the urea is removed. Moreover the data shown in the work do not exclude the possibility that a cytosolic source of ROS could also be persistently activated by the transient exposure to urea, and thus also contribute to the self-propagating cycle of ROS production observed in absence of the initial urea stimulus. The study also did not investigate how long the urea memory effect can last, and what mechanisms are involved and what mechanisms are involved (D'Apolito et al 2018).

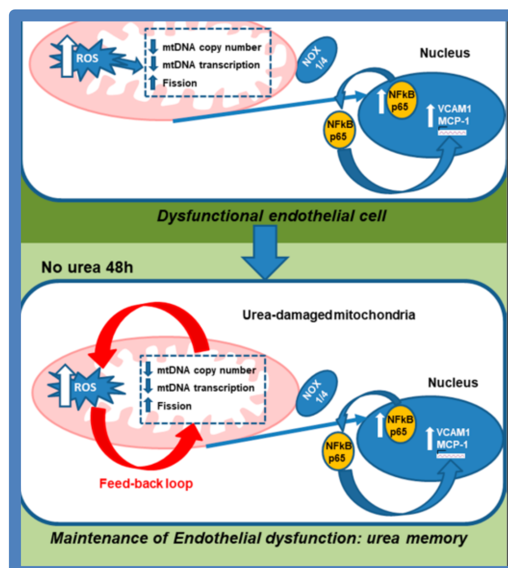

Figure 12: Scheme showing that urea-induced mitochondrial damage initiates a vicious cycle of ROS production that continues to self-propagate after the urea insult is terminated.

3.3 THEORETICAL BASIS FOR “METABOLIC MEMORY”

AGEs (non-enzymatic glycation end products), glycation of mitochondrial proteins, and oxidative stress have been found to explain, at least in part, the “glycometabolic theory”. Although hyperglycemia remains a hallmark in the pathophysiology of chronic diabetes complications, it is now clear that therapies should also address a number of factors only partially related to glycemic control (Pugliese et al 2008; Stirban 2009). These factors appear to be related to an imbalance between oxidative stress and antioxidant capacity, which could be the link between hyperglycemia and the multiple biochemical cascades that lead to diabetes complications (Giacco et al 2010). Two temporally-separated phases may be highlighted behind the role of AGEs in the genesis of microvascular damage. In the early years of the disease, a linear relationship between hyperglycemia, increased oxidative stress, and excessive AGE formation could be hypothesized. Later, a persistent respiratory chain protein glycation and DNA damage in the mitochondria could generate a hyperglycemia-independent vicious cycle (Ceriello et al 2009), in which oxidative stress is self-supporting, and AGEs ‘feed’ this process. The effects of this metabolic imbalance could include changes in the composition and structure of the extracellular matrix, mediated by inflammatory processes induced by receptor binding of AGEs or oxidative stress (Jax 2010). Subsequent fibrosis and the extension of the extracellular structures interfere with capillary blood flow, reducing capillary density in particular (Jax 2010). These structural changes could cause endothelial dysfunction and then atherosclerosis. Recently, epigenetic mechanisms have been hypothesized to be a crucial interface between genetic and environmental factors to explain metabolic memory (Reddy et al 2011). Hyperglycaemia can induce a variety of epigenetic changes that persist for days after the normalization of glucose levels (Brasacchio et al 2009; Miao et al 2004;

Reddy et al 2015; Al-Haddad et al 2016), mainly through the involvement of inflammatory genes (Brasacchion et al 2009; Miao et al 2004). Among the epigenetic mechanisms studied in metabolic memory, DNA methylation and post-translational histone modifications (PTHMs) are the most extensively investigated. In particular, high glucose levels can alter the activity of PTHMs and DNA methyltransferases, with irreversible changes over time. These modifications may explain the long-term harmful effects of metabolic memory (Brasacchion et al 2009; Miao et al 2004; Reddy et al 2015; Al-Haddad et al 2016). Additional epigenetic mechanisms have been identified. Gene expression regulation could be influenced by non-coding RNAs, including microRNAs (miRNAs), which may be the key regulators in metabolic memory modulation. More than 2000 human miRNAs have been identified to date, making them one of the most abundant classes of epigenetic regulatory molecules (Baek et al 2008). Another factor which may have an important role in metabolic memory is low-grade inflammation. Inflammation plays a key role in diabetes mellitus and its vascular complications, and prolonged inflammation could mediate metabolic memory. Epigenetic mechanisms can regulate inflammatory gene expression and cardiovascular disease susceptibility—even under non-diabetic conditions—and may be accentuated by diabetes, leading to vascular complications and metabolic memory. All environmental factors that promote the development and progression of diabetes mellitus trigger an inflammatory response, promoting inflammation-mediated insulin resistance and endothelial dysfunction (Reddy et al 2011; Thompson et al 2013; Guarner et al 2015). Necrosis factor-kappa (NF-k) plays a key role in mediating inflammatory gene expression that has been well-evaluated. Diabetic conditions can promote inflammatory gene expression via NF-k activation and enhance monocyte binding to endothelial and vascular smooth muscle cells, and subsequently promote monocyte-to-macrophage differentiation (Reddy et al 2015). The NF-

activation induces the expression of inflammatory cytokines involved in vascular inflammation, which stimulates the generation of endothelial adhesion molecules, proteases, and other mediators (Guarner et al 2015). The Toll-like receptor signaling is another important factor that links inflammation and oxidative stress and is a pathogenic contributor to hypertension, insulin resistance, and obesity (Thompson et al 2013). Also, epigenetic mechanisms may activate inflammatory gene expression in vascular cells and monocytes. Gene induction by proinflammatory agents was associated with increased histone lysine acetylation in endothelial and vascular smooth musclecells (Reddy et al 2015). These findings reinforce the idea that the mechanisms of metabolic memory may be interdependent and simultaneous. Finally, another important mechanism that leads to metabolic memory is the above cited non-enzymatic glycosylation of proteins, where Maillard reaction and other following reactions lead to the formation of AGEs, changing protein structure andfunction (Goldin et al 2006). In summary, the following four basic mechanisms have been proposed to play a role in metabolic memory: oxidative stress, non-enzymatic glycation of proteins, epigenetic changes, and chronic inflammation (Testa et al 2017) **(Fig.13)**.

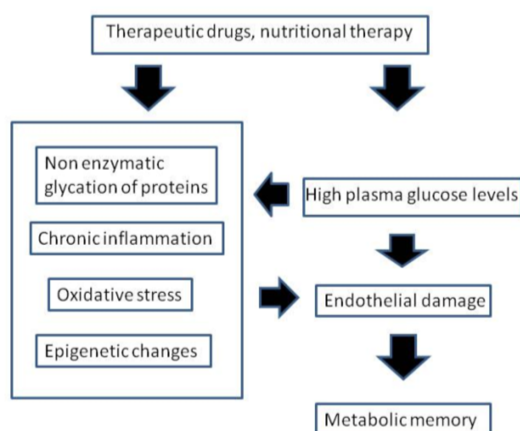

Figure 13: Interrelationship among high glucose levels, oxidative stress, non-enzymatic glycation of proteins, epigenetic changes, chronic inflammation, endothelial damage.

AIMS OF THE STUDY (3)

It's known that palmitate at concentration seen in the serum of obese patients induces podocytes dysfunctions, and poocyte insulin resistance, all events that are involved in the reduction of kidney function. All these effects are mediated by the palmitate induced increase of ROS generation. In diabetes hyperglycemic memory is a model to explain the long-lasting effects that short term hyperglycemic spike may have on kidney tissue (Chen et al 2008). On the other hand, it has been shown that transient exposure to pathological concentrations of urea causes persistent increases in inflammatory gene expression during subsequent periods of normal uremia, by inducing pathologic changes in arterial endothelial cells (D'Apolito et al 2018). A key factor in the genesis of "metabolic memory" is the increase of intracellular ROS production induced by uremia. In endothelial cells, for example, transient exposure to urea can activate a multi-component feedback loop which maintains increased levels of mitochondrial ROS production for days after urea concentration is normalized (D'Apolito et al 2018). This continued mitochondrial overproduction of ROS despite normalization of urea concentration would explain the persistence of the progression of atherosclerotic lesions induced by the initial uremic spike (D'Apolito et al 2018). Like urea, palmitate can damage kidney function by increasing intracellular ROS production in the target tissues.

Therefore we hypothesized that short exposure to concentrations of palmitate associated with obesity can causes a persistent increase in mitochondrial ROS production in podocytes. This continued mitochondrial ROS overproduction may induce an accumulation of pathologic changes in the cells, causing a permanent podocytes dysfunctions.

So, the aims of this part of my PhD thesis has been to evaluated, in a human conditioned immortalized podocytes cell line:

- The capability of phisyopathological concentrations of palmitate to induce a persistent increase of ROS concentration;
- The capability of phisyopathological concentrations of palmitate to induce a persistent podocytes inflammation;
- The capability of physiopathological concentrations of palmitate to increase, persistently, HMGB1 expression.

MATERIALS AND METHODS

CELLs CULTURE CONDITIONS

The conditionally immortalized human podocytes cell line were purchased from University of Bristol. Conditionally immortalized human podocytes cell line were developed by transfection using large T antigen (SV40) that allows cells to proliferate at the "permissive" temperature of 33°C. When transferred to the "non-permissive" temperature of 37°C results in the inactivation of large T antigen with minor changes in gene expression (Stamps et al 1994). Podocytes, then, enter growth arrest and express markers of differentiated *in vivo* podocytes, including the novel podocytes proteins, nephrin, podocin, CD2AP, and synaptopodin and known molecules of the slit diaphragm ZO-1, alpha-, beta-, and gamma-catenin and P-cadherin (Saleem et al 2002).

The cells were grown on dishes in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100ug/mL penicillin/streptomycin (Sigma), 1g/L D-Glucose Solution (Gibco) and Insulin-Transferrin-Selenium (Gibco) in an atmosphere containing 5% CO_2 at 33 °C. To induce podocyte differentiation, the cells were cultured at 37 °C, when cells were 40%-60% confluent, for 14 days, changing medium 3 times per week and then the following experiments were performed.

PREPARATION OF FFAs SOLUTION

Palmitate (sodium salt, Sigma) was dissolved in 50% (volume/volume) ethanol to a concentration of 50 mM. This stock solution was diluted in medium containing 0.75 or 1% (weight/volume) free fatty acid (FFA)-free BSA (Roche, Germany) to a final concentration of 0.5 mM for 1 h at 37°C (Oliveira et al 2015).

ADENOVIRAL VECTOR

UCP-1 was cloned into the shuttle vector pAd5CMVK-NpA, and adenoviral vector. This is an empty control virus that was prepared by the Gene Transfer Vector Core at the University of Iowa, as described previously (D'Apolito et al 2010). Podocytes were infected with UCP-1 or control adenovirus at an MOI of 500, 4 h before addition of either medium, containing 0.75% or 1% (weight/volume) free fatty acid (FFA)-free BSA.

INIBITION OF NADPH OXIDASE ACTIVITY

Cells were cultured in the presence or absence of NADPH oxidase inhibitor GKT137831 (Cayman Chemical, Ann Harbor, MI) (Aoyama et al 2012, Green et al 2012) GKT137831 was dissolved in 0.1% ethanol and was added to the cells 20 min before palmitate addition at concentrations of 10 μM..

INHIBITION OF HMGB1

Cells were cultured for 20 min with or without the HMGB1 inhibitor glycyrrhizin (Sigma), dissolved in DMSO, at final concentration of 130 μM before palmitate addition (Wang et al 2014).

MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS) IN PODOCYTES

Pretreated cells in 96-well plates were incubated with 10 mmol/L CM-H2DCFDA (Molecular Probes-Life Technology, Brooklyn, NY) for 45 min at 37 °. The intracellular formation of ROS was measured at excitation/emission wavelengths of 485/535 nm using a Wallac 1420 Fluorescent Plate Reader.

RT REACTION AND REAL-TIME QUANTITATIVE PCR

Total RNA from treated cells was extracted using the RNeasy Mini Kit (Ambion), following the manufacturer's instructions. The mRNA was reverse transcribed by SuperScript IV First Strand Synthesis System (Life Technology, Brooklyn, NY). Experiments were performed in optical 96-well reaction plates on CFX96 Touch™ Real-Time PCR Detection System (BiorRad) according to the manufacturer's instructions and Real-Time PCR Detection System was performed with Sso Advanced TM Universal SYBR® Green Supermix (BioRad). Melting curves were analyzed to ensure that fluorescence signals solely reflected specific amplicons. Expression levels of BiP/GRP78, sXBP1, Nf-kB subunit p65, MCP-1, TGF-β, SREBP1c, HMGB1, CytB, ND1, ND4 and ND4L mRNA were normalized to WT-1 levels in the same sample. The following used primers are validated and purchased by Qiagen, Primm and Invitrogen:

Xbp1s	FW 5′ TGCTGAGTCCGCAGCAGGTG 3′
	RV 5′ GCTGGCAGGCTCTGGGGAAG 3′
BiP/GRP78	FW 5' CGTGGAATGACCCGTCTGTG 3'
	RV 5' CTGCCGTAGGCTCGTTGATG 3'
p65	FW 5' AACAACAACCCCTTCCAAGTT 3'
	RV 5' GTTCACTCGGCAGATCTTGAG 3'
MCP-1	FW 5' TCATAGCAGCCACCT 3'
	RV 5' AGCTTCTTTGGGACA 3'
TGF-β	Assay mix Qiagen (QuantiTect™)
SREBP1c	FW 5' GGAGGGGTAGGGCCAACGGCCT 3'
	RV 5' CATGTCTTCGAAAGTGCAATCC 3'
HMGB1	FW 5' GGCTGACAAGCGTCGTTATG 3'
	RV 5' TTGCAACATCACCAATGCAT 3'
CytB	FW 5'CCAAATCACCACAGGACTAT 3'
	RV 5' GATGGCGGATAGTAAGTTTG 3'
ND1	FW 5' CACATCTACCATCACCCTCT 3'
	RV 5' GTCATGATGGCAGGAGTAAT 3'

ND4	FW 5’ TCTTCTTCGAAACCACACTT 3’
	RV 5’ AAGTACTATTGACCCAGCGA 3’
ND4L	FW 5’ AGCATTTACCATCTCACTTCT 3’
	RV 5’ GCATTGGAGTAGGTTTAGGTT 3’
WT-1	FW 5’ TGAATTAGTCCGCCATCACA 3’
	RV 5’ ACCAACTCTTCCAGGCACAC 3’

PCR conditions were as follows: 7 min at 95 °C and 45 cycles of 30 s at 95 °C and 30 s at 60 °C. WT-1 was chosen as the internal control as a marker of mature podocytes.

STATISTICAL ANALYSIS

Data were analyzed using 1-factor ANOVA to compare the average of all groups. The Turkey-Kramer multiple-comparisons procedure was used to determine the significant difference between the medium pairs. A p value <0.01 was considered statistically significant.

RESULTS

1.1 Palmitate induces reactive oxygen species (ROS) production in conditioned human podocytes cell line through the activation of mitochondrial and cytosolic mechanisms

Since palmitate, at concentration similar to that seen in patients with diabetic nephropathy (DN), can induce ROS production in cultured mouse podocytes cell line (MPC5) (Jiang et al 2017), we evaluated whether an increased unbound of palmitate could also induce ROS generation in conditioned human podocytes cell line. Human podocytes were exposed to different ratios of conjugated PAL / BSA for 24h. The concentration of 0.5 mM of PAL / 1% BSA, produced an unbound palmitate concentration of 20 nM comparable to the concentration seen in healthy humans; this concentration, as expected, did not affected ROS production in human podocytes (**Fig.1**, bar 1). Instead, when the cells were exposed to PAL/ 0.75% BSA conjugate, corresponding to a physiopathological concentration of unbound FFAs of 27 nM, ROS production was increased significantly (SEM 1,5 ± 0,01) compared to the control PAL/ 1% BSA conjugate (**Fig. 1**, bar 4). Mannitol, used as an osmotic control, had no effect on intracellular ROS generation (data not shown).
Next, we investigated the mechanisms by which palmitate increased ROS production. It has been shown that both mitochondrial and cytosolic mechanisms can participate in intracellular ROS generation (D'Apolito et al 2010; Susztak et al 2006; Menini et al 2006). To evaluate the role played by mitochondria in the induction of ROS by palmitate in human podocytes, we overexpressed uncoupling protein 1 (UCP-1), a specific protein uncoupler of oxidative phosphorylation which collapses the proton electrochemical gradient that drives superoxide production (Choi et al 2007) . Overexpression of UCP-1 prevented

ROS production in cells exposed to 27 nM palmitate (**Fig. 1** , bars 5).

These findings demonstrate that physiopathological palmitate concentrations stimulate ROS generation in human podocytes through mitochondrial mechanisms.

Next, we explored the involvement of cytosolic mechanisms in the induction of ROS production in human podocytes by palmitate. Since NADPH oxidases are major cytosolic sources of superoxide in podocytes (Greiber et al 1998), we treated immortalized human podocytes with the most specific Nox1/4 inhibitor currently available, GKT137831 (Aoyama et al 2012; Green et al 2012). As shown in **Fig.1**, bar 6, GKT137831 also prevented ROS production in cells exposed to PAL / 0.75% BSA conjugate.

Together, these findings demonstrate that physiopathological palmitate concentrations stimulate ROS generation in human podocytes through both mitochondrial and cytosolic mechanisms.

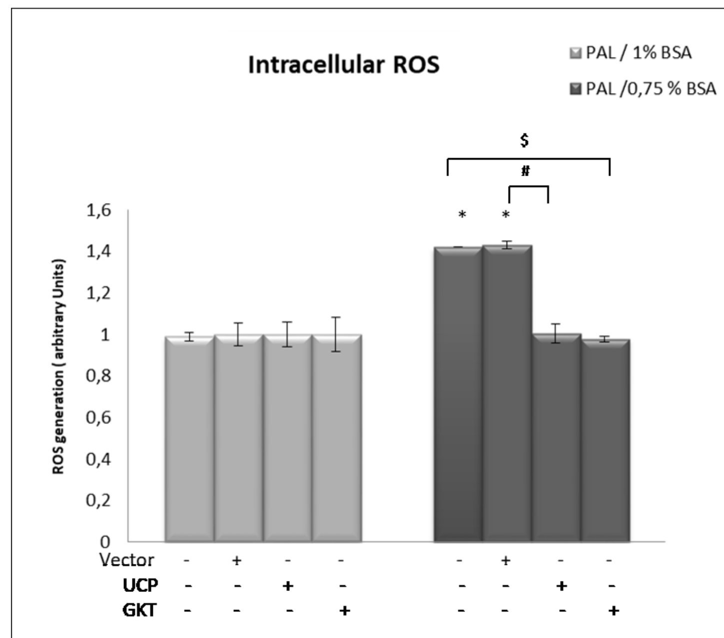

Figure 1: **Palmitate induces reactive oxygen species in human podocytes.**

ROS production was measured, after 24h, in human podocytes incubated with different ratio of conjugated PAL/BSA or after infection with adenoviral vectors expressing UCP-1 or overexpression of NADPH oxidase inhibitor GKT137831. Each bar represents the mean ±SEM of 3 separate experiments. *p ≤ 0.01 compared to ctrl. # p≤ 0.01 cells transfected with the empty vector compared to cells over expressing UCP-1, $ p≤ 0.01 cells not transfected or not treated with the inhibitor compared to cells treated with GKT13781.

1.2 FFAs induce endoplasmic reticulum (ER) stress in human podocytes cell line

ER stress is one potential molecular mechanism of lipotoxicity, and here we examined whether palmitate induced ER stress in human podocytes cell line. Human podocytes were exposed to different ratios of conjugated PAL/BSA for 24h. After treatment with PAL / 0.75% BSA, we evaluated the mRNA gene expression levels of the ER stress induced spliced form of mRNA for the transcriptional activator Xbox binding protein 1 (XBP-1) and the ER chaperone immunoglobulin heavy chain-binding protein/glucose-regulated protein 78-kDa (BiP/GRP78) that resulted as increased by SEM 2.7 ± 0,04 (**Fig. 2** A bar 2) and by SEM 1.4 ± 0,03 (**Fig.2** B bar 2) respectively.

Overexpression of GKT137831 inhibitor prevented ER stress in cells exposed to conjugated PAL/BSA (**Fig. 2** A-B bars 4). These data suggest that palmitate induced cytosolic ROS production activated ER stress in human podocytes cell line.

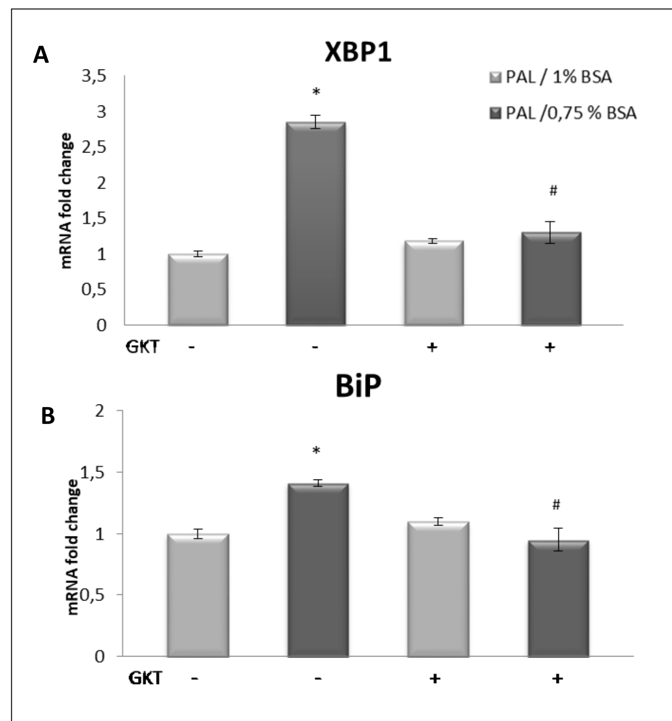

Figure 2: Palmitate induces ER stress in human podocytes. ER stress was evaluated in cells with different ratio of conjugated palmitate/BSA by measuring both BiP mRNA expression and mRNA sXBP1 alternative splicing, before or after treatment with NADPH oxidase inhibitor GKT137831. Each bar represents the mean ± SEM of 3 separate experiments. *p ≤ 0.05 compared with control, # p ≤ 0.05 cells treated with PAL but not not treated with the inhibitor compared to cells treated with GKT13781.

1.3 Palmitate-induced ROS causes pro-inflammatory changes in human podocytes cell line

In order to evaluate if the production of ROS, induced by physiopathologicals concentrations of plamitate, causes podocytes inflammation, we evaluated, in our model, the mRNA expression of both major inflammatory mediators: NFκB subunit p65 and monocyte chemoattractant protein-1 (MCP-1),

NFkB-specific inflammatory target gene. **Fig.3** shows that, in cells exposed to pathologicals concentrations of palmitate for 24h, mRNA gene expression levels of subunit p65 and MCP-1 were increased by SEM 2.5 ± 0,03 (A) and by SEM 3 ± 0,04 (B respectively proving that the palmitate/BSA coupled complex, made by the method of *Oliveira et al,* is able to induce podocyte inflammetion. All these palmitate effects were significantly reduced when the cells were treated with GKT137831 inhibitor proving that palmitate induces podocytes inflammation by increasing ROS production.

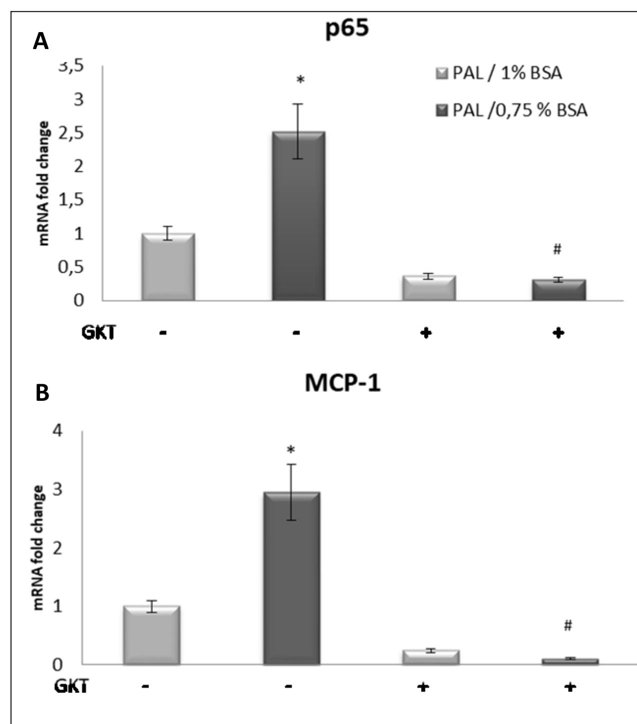

Figure 3: **Palmitate induced ROS causes podocytes inflammation.** (A.) NFκB subunit p65 and (B.) Monocyte chemoattractant protein-1 (MCP-1), serum pro-inflammatory cytokines were evaluated in cells treated with physiopathological concentrations of palmitate for 24h by measuring mRNA expression. All these effects were prevented by overexpression of GKT137831. Each bar represents the mean ± SEM of 3 separate experiments. *$p \leq 0.05$ compared to ctrl, # $p \leq 0.05$ cells treated with PAL but not not treated with the inhibitor compared to cells treated with GKT13781

1.4 Palmitate-induced ROS causes renal fibrosis and lipotoxicity

Then, we further investigated the effect of ROS induced by palmitate on renal fibrosis and renal lipid accumulation.

Chronic kidney diseases (CKD), are characterized by the accumulation of extracellular matrix (ECM) components and lipids in the glomeruli (glomerular fibrosis, glomerulosclerosis) and the tubular interstitium (tubulointerstitial fibrosis) (López-Hernández et al 2012; Muller et al 2018). Transforming growth factor beta (TGF-β) has been recognized as an important mediator of renal fibrosis, renal parenchyma degeneration and loss of function associated with CKD. The sterol regulatory element-binding protein-1c (SREBP-1c) pathway has been shown to mediate the renal accumulation of triglyceride and cholesterol (Jiang et al 2005).

Therefore, we evaluated the mRNA expression levels of both TGF-β and SREBP1c in our model of immortalized human podocytes cell line. After 24h of treatment, with different percentages of conjugated FFA, we observed (**Fig. 4**) that TGF-β mRNA expression and SREBP1c increased respectively by SEM 1.7 ± 0,02 (A) and by SEM 2.2 ± 0,01 (B). All these results were significantly decreased when the treatment was associated with GKT137831 inhibitor proving that palmitate induces renal fibrosis and lipotoxicity by increasing ROS production.

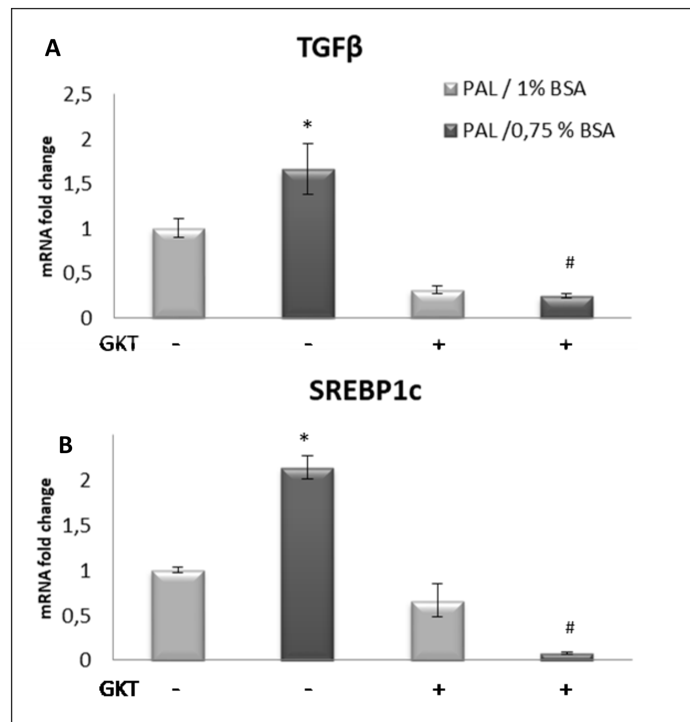

Fig.4: **Palmitate induced ROS causes renal fibrosis and lipotoxicity.** (A) Transforming growth factor-β (TGF-β) and (B) Sterol regulatory element binding protein1c (SREBP-1c) were evaluated in cells treated with PAL / 0,75% BSA conjugate for 24h by measuring mRNA expression. All these effects were prevented by overexpresion of GKT137831. Each bar represents the mean ± SEM of 3 separate experiments. *p ≤ 0.05 compared to ctrl, # p ≤ 0.05 cells treated with PAL but not not treated with the inhibitor compared to cells treated with GKT13781

2.1 Palmitate increases HMGB1 expression in human podocytes

In literature HMGB1 is considered to be important in the pathogenesis of a wide range of kidney disease (Chen et al 2016) Therefore, we next decided to evaluated the HMGB1 mRNA gene expression in the human podocytes treated with palmitate conjugated with the method of *Oliveira et al* with different percentage of BSA for 24h (Oliveira et al 2015). As shown in **Fig.5**, in podocytes treated with PAL / 0.75% BSA there were an increase by SEM 2 ± 0,02 of HMGB1 mRNA expression level and the overexpression of GKT137831 inhibitor prevented this increase on HMGB1 expression proving that it is mediated by ROS production.

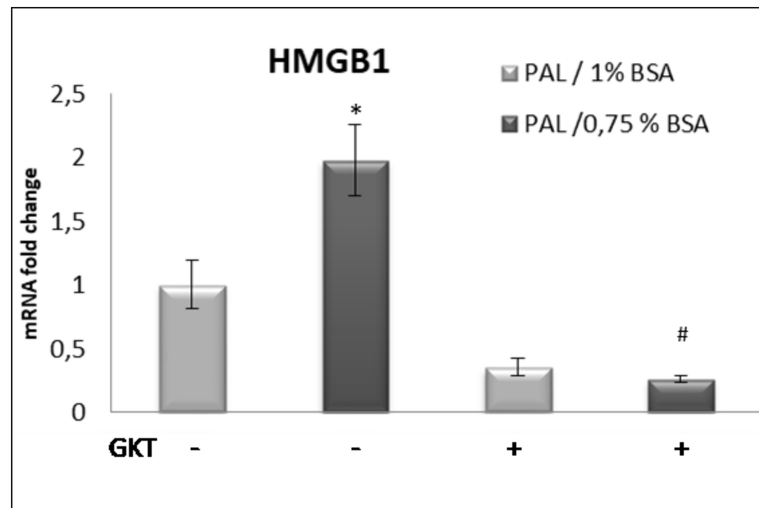

Figure 5: **Palmitate induces increase in HMGB1 expression.** The expression of high mobility group box 1 protein (HMGB1) was evaluated in cells treated with palmitate by measuring mRNA gene expression. The increase in the mRNA expression was prevented by GKT137831 inhibitor. Each bar represents the mean ± SEM of 3 separate experiments. *$p \leq 0.05$ compared to ctrl, # $p \leq 0.05$ cells treated with PAL but not not treated with the inhibitor compared to cells treated with GKT13781.

2.2 Palmitate causes podocytes dysfunction by increasing HMGB1 expression.

To understand the role of HMGB1 in causing palmitate-induced glomerulophaty, we treated podocytes for 24h with PAL / 0,75% BSA and we measured the mRNA expression of inflammatory, fibrosis and steatosis genes; after that, we stimulated cells for 30min with 130 µM Glycyrrhizin (GLYZ), a natural anti-inflammatory and antifungal factor, which inhibits HMGB1 chemoattractant and mitogenic activities through direct binding to HMGB1 (Mollica et al 2007). As shown in **Fig.6**, the increase of podocytes inflammation (A-B), fibrosis (C) and lipotoxic effect (D), induced by physiopathologicals concentrations of palmitate, was prevented by Glycyrrhizin inhibitor proving the key role played by HMGB1 in ORG and suggesting a regolatory role of HMGB1 on inflammation, steatosis and fibrosis.

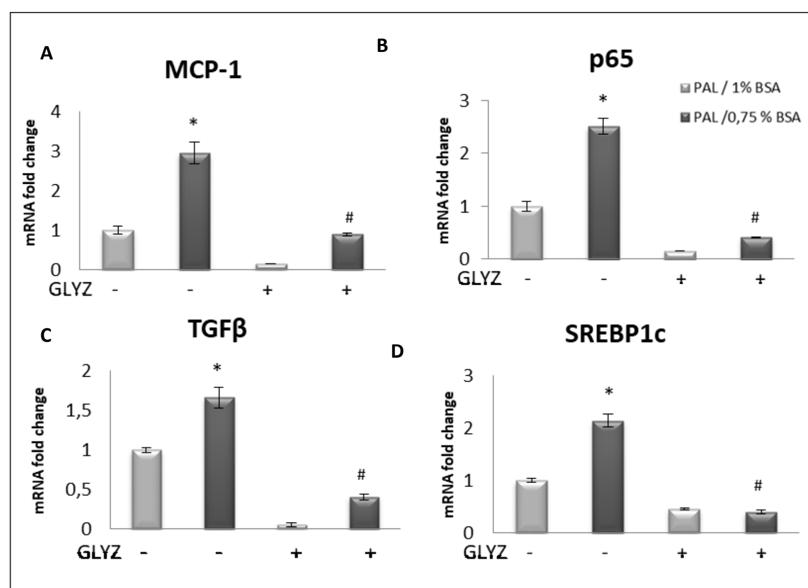

Figure 6: **Palmitate causes podocytes dysfunction by increasing HMGB1 expression.** (A)Monocyte chemo attractant protein-1 (MCP-1) (B), NFκB subunit p65 , serum pro-inflammatory cytokines, (C) Transforming growth factor-β (TGFβ) and (D) Sterol regulatory element binding protein1c (SREBP-1c) were evaluated in cells treated with palmitate for 24h by measuring mRNA expression. All the increased effects were prevented by Glycyrrhizin 130µM for 30 min. Each bar represents the mean ± SEM of 3 separate experiments. *$p \leq 0.05$ compared to ctrl, # $p \leq 0.05$ cells treated with PAL but not not treated with the inhibitor compared to cells treated with GLYZ.

3.1 Palmitate induces reactive oxygen species production in conditioned human podocytes cell line that persist over the time

To evaluate if obese related concentrations of palmitate induce a persistent ROS overproduction when the stimulus of palmitate was removed, podocytes were exposed for 24h to PAL / 0.75% BSA conjugate and, then, maintained in culture for another 24h or 48h in medium, without palmitate, containing BSA as control. At each time point, ROS production was measured as described in methods. As shown in **Fig.7**, in cells exposed to 27 nM of palmitate for 24h, the ROS production increased by SEM 1.5 ± 0,01. Mannitol, used as an osmotic control, had no effect on the intracellular ROS production (data not shown). This raise

of ROS production persisted, without significant change, until two days after that the stimulus of palmitate was removed. In conclusion, 24h of exposure to PAL / 0,75% BSA, were sufficient to cause an increase in ROS production that persisted for subsequent 24h or 48h in the absence of palmitate.

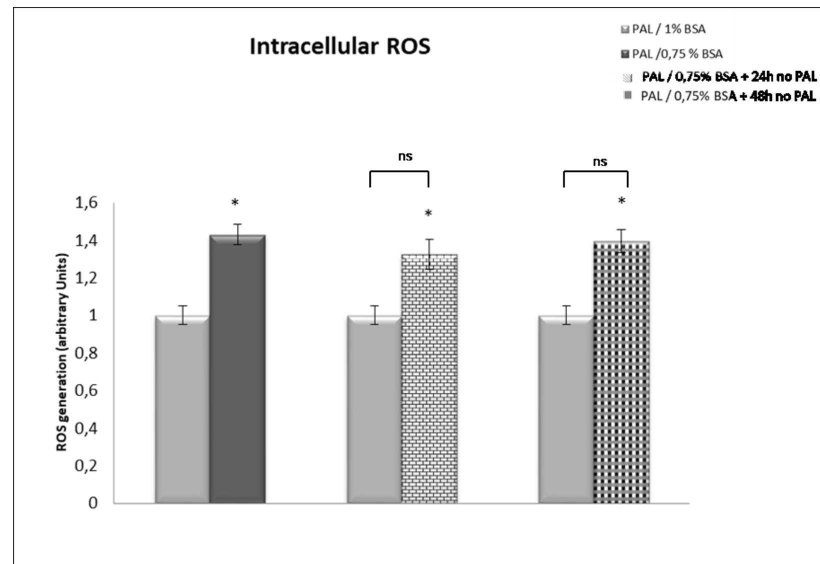

Figure 7: **Palmitate induces reactive oxygen species in human podocytes that persist over the time.** ROS production was measured in human podocytes incubated with different ratios of conjugated PAL / BSA for 24h and, then, maintained in colture for another 24h or 48h in normal medium (no PAL). Each bar represents the mean ± SEM of 3 separate experiments. *p ≤ 0.01 compared to ctrl, #p ≤ 0.01 treated cells deprived of the PAL compared with treated cells .

3.2 Cytosolic and mitochondrial ROS generating mechanisms initiate the persistent ROS production induced by transient exposure to physiopathological concentrations of palmitate in human podocytes cell line

Since FFAs increases ROS production in human podocytes through the activation of both mitochondrial and cytosolic ROS generating mechanisms (see *1.1*), we investigated how the inhibition of either affected the persistent ROS generation induced by transient PAL / 0,75% BSA conjugate. To evaluate the role played by mitochondria in the generation of persistent

ROS production induced by palmitate, we overexpressed uncoupling protein 1 (UCP-1) for two days after the palmitate was removed. As shown in **Fig.8** bar10, overexpression of anti-oxidant prevented the persistent ROS production induced by prior exposure to palmitate.

Next, we explored the involvement of cytosolic mechanisms in the activation of the persistent ROS production induced by transient palmitate exposure. We treated podocytes with the specific NADPH Oxidase 1/4 (Nox1/4) inhibitor GKT137831. As shown in **Fig.8** bar 11 , GKT137831 also prevented the persistent ROS production induced by transient palmitate. Together, these findings demonstrate that both mitochondrial and cytosolic ROS generating mechanisms are involved in initiating the persistent ROS over-production induced by transient exposure to palmitate.

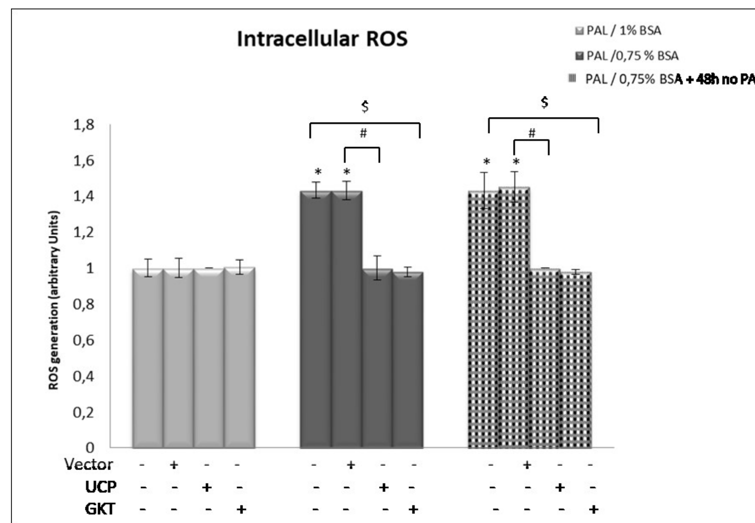

Figure 8: Cytosolic and mitochondrial ROS generating mechanisms start the persistent ROS production induced by transient exposure to palmitate in human podocytes cell line. In the indicated groups, podocytes were infected with UCP-1 (MOI 500), or control empty adenoviral vectors before transient exposure to PAL / 0,75% BSA, or incubated for 24 h in PAL / BSA conjugate in the presence of the NADPH oxidase inhibitor GKT137831. ROS levels were measured by CM-H2DCFDA. Data are the mean ± S.E. from 3 independent experiments. * $p \leq 0.01$ cells treated with PAL / 0,75% BSA compared to PAL / 0,1% BSA, # $p \leq 0.01$ cells over expressing UCP-1 compared to cells transfected with the empty vector, $ $p \leq 0.01$ cells treated with GKT13781 compared to cells not transfected or not treated with the inhibitor.

3.3 FFAs induced mitochondrial dysfunction are responsible for the persistent mitochondrial ROS production in podocytes after transient exposure to high FFAs concentrations

Next we investigated which ROS generating mechanisms activated through transient incubation with FFAs induce persistent ROS production in podocytes after the initial palmitate pro-oxidative stimulus has been removed. The mitochondria are the major source of cellular ROS, and mitochondrial damage result in a pathological increase of ROS production (Turrens et al 2003). Demaged mitochondria showed an impaired integrity and function of electrotransport chain system, caused by a reduced transcription of mtDNA-encoded proteins (Malik et al 2013). Therefore, to investigate the consequence of the transient exposure to FFA on mitochondrial function, we evaluated the expression of mitochondrial genes ND1, ND4, ND4L and Cytocrome b (CytB) in podocytes treated with palmitate as described. Cytocrome b a subunit of complex III of mitochondrial respiratory chain while, ND1, ND4L and ND4 encode subunits of Complex I that has been recently recognized to be the mitochondrial source of deleterious ROS (Bleier et al 2015). Complex I derived ROS can readily react with mitochondrial DNA or other matrix components vulnerable to oxidative damage. In contrast, Complex III-derived ROS serve as second messengers in cellular signaling (Bleier et al 2015). In the cells incubated with high palmitate for 24 hours, the expression of CytB was reduced by 0,7 ± 0,02 fold (**Fig. 9** A bar 3) compared to the control. At the same time, palmitate reduced the expression of ND1, ND4 and ND4L and were reduced by 0,5 ± 0,01; 0,6 ± 0,03 and 0,4 ± 0,02 fold respectively (**Fig.9** B-C-D bars 3). The palmitate-induced reduction in the expression of each one of these mitochondrial genes lasted unchanged for 2 days after the FFA was removed (**Fig.9** A-B-C-D bars 5). This indicates that transient high palmitate can persistently

compromise the electron transport system. These long lasting effects of palmitate on expression of proteins encoded in the mitochondrial genome were reversed by GKT137831 (**Fig.9** A-B-C-D bars 6). These results suggest that high palmitate induces mitochondrial dysfunction that is responsible for the long lasting production of ROS when the pro-oxidative stimulus has been removed.

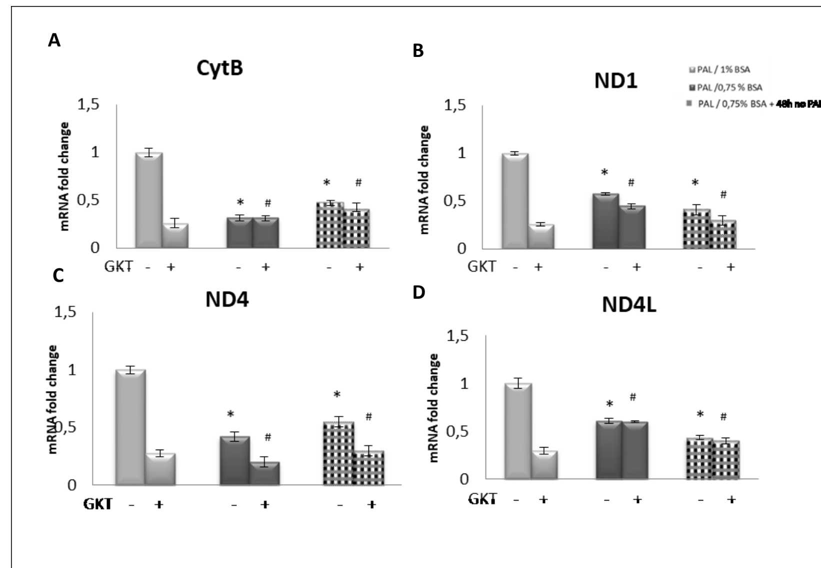

Figure 9: Transient exposure to high FFAs causes a long lasting reduction of mitochondrial electron transport chain protein expression. Podocytes were exposed to high FFAs for 24 hr or to high FFAs for 24 hr followed by 2 days of subsequent incubation in media alone . In the indicated groups, podocytes were treated with GKT137831 during the transient exposure to palmitate. The mRNA expression of **(A)** CytB, **(B)** ND1, **(C)** ND4 and **(D)** ND4L were measured by Real time PCR. Data are the mean ± S.E. from 3 independent experiments. * $p<0.05$ compared with cells not treated with high FFAs.

3.4 Transient high FFAs causes podocytes cells dysfunction lasting for 2 days despite palmitate normalization

In diabetes, it has been shown that the persistent rise of ROS production, induced by transient hyperglycemia in endothelial cells, caused a persistent increase in the expression of pro-

atherogenic genes during subsequent periods of normal glycaemia (Giacco et al 2015).

To determine whether the persistent ROS production induced by transient palmitate can causes similar effects in podocytes dysfunction, the expression of the major inflammatory mediator NFκB subunit p65 and monocyte chemoattractant protein-1 (MCP-1) was measured in cells exposed to PAL / 0,75% BSA conjugate for 24h and then maintained for two more days in media without palmitate but containing BSA as control. As shown in **Fig.10** A, bar 5, the 2,5-fold increase in NFκ-B specific inflammatory target genes p65 mRNA expression, induced by exposure to PAL for 24h, persisted unchanged for two days in the cells deprivate of the palmitate (bar 5). Similarly, transient palmitate induced a persistent increase in the expression of the MCP-1. Infact, the 3-fold increases in MCP-1 remained increased for two days after that palmitate was removed (**Fig. 10** B, bar 5). On the other hand, in **Fig.10** C, it's shown that TGF-β gene expression increased by 1.7 fold and (D) HMGB1 by 2 fold (as shown in *1.4-2.1)*. This increase expression is mainteined after that palmitate was removed. These data indicate that palmitate induced a persistent activation of NFκB and a persistent increase of HMGB1 and TGF- β expression. To test the hypothesis that the persistence of these changes in gene expression are due to the pesistant increase of ROS production caused by palmitate-induced dysfunction, cells exposed to PAL / 0,75% BSA were treated for 24h with GKT137831 inhibitor during the initial exposure to palmitate. NADPH Oxidase 1/4 (Nox1/4) inhibitor completely reversed the palmitate-induced changes in NFκBp65, MCP-1, TGF-β and HMGB1 gene expression (**Fig. 10** A-B-C-D, bars 6). These results demonstrate that persisting changes in podocytes cell gene expression are mediated by ROS over production that continued in the absence of palmitate.

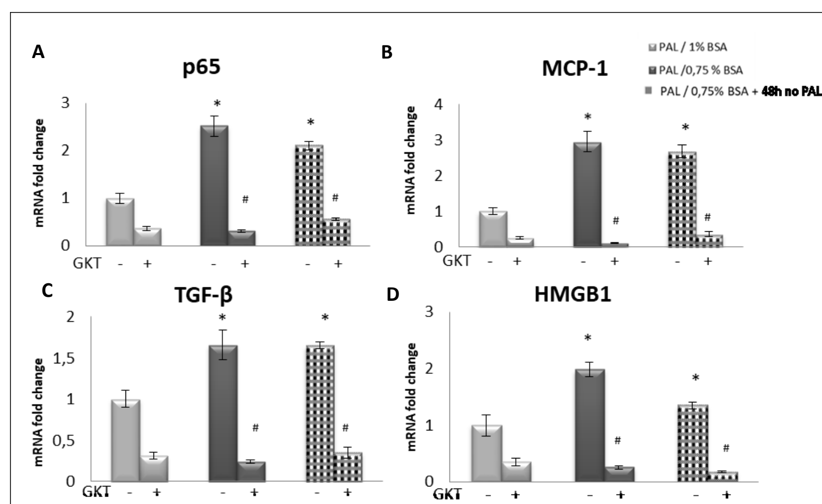

Figure 10: Transient exposure to FFAs causes podocytes cells dysfunction that lasts for 2 days after the palmitate is removed mRNA levels of NFκB subunit p65 , monocyte chemoattractant protein-1 (MCP-1), TGF-β and intracellular HMGB1 expression was evaluated in cells exposed to high palmitate for 24h or for 24h followed by 2 day of subsequent incubation in media alone . In the indicated groups, podocytes were treated with GKT137831 during the transient exposure to palmitate . Each bar represents the mean ± SEM of 3 separate experiments. *$p \leq 0.05$ compared to ctrl, # $p \leq 0.05$ cells treated with PAL in presence of the inhibitor GKT137831 compared to cells treated with PAL but not not treated with the inhibitor.

DISCUSSION

In this study we investigate, for the first time in human conditioned immortalized podocytes cell line, the effect of phisyopathological concentration of palmitate coniugated to the albumin following the method of *Oliveira et al.* Podocytes are cells in the Bowman's capsule of the kidneys that wrap around capillaries of the glomerulus. These cells play an important role in glomerular function and together with endothelial cells of the glomerular capillary loop and the glomerular basement membrane, they form a filtration barrier. If podocytes are injured, mutated, or lost, the elaborate structure of podocytes is physically altered and this results in many proteinuric kidney diseases (Pavenstädt et al 2003). It has been reported that lipid abnormalities in renal disease contribute to the process of glomerulosclerosis with progressive renal dysfunction (Wheeler et al 1994; Kees-Folts et al 1993; Thabet et al 1993; Srivastava et al 2014; Yukawa et al 1999). One of the major pathogenic mediators in ORG and its complications is dyslipidemia, resulting in high saturated free fatty-acid (FFA) concentrations. Palmitate is the most abundant saturated FFA in the plasma of humans and rodents and accounts for ~ 25% of total fatty acids (Wheeler et al 1994; Kees-Folts et al 1993). Saturated FFAs in the cytosol induce reactive oxygen species (ROS) production as observed in pancreatic β-cells, hepatic cells and skeletal muscle cells (Yuan et al 2010; Yuzefovych et al 2010; Srivastava et al 2014). Palmitate is a potent inducer of ROS in a number of cell types, including cardiomyocytes (Liu et al 2015; Joseph et al 2016), endothelial cells (Yamagishi et al 2002), glomerular mouse podocytes (Xu et al 2015) and adipocytes (Davis et al 2009).

The low solubility of long-chain FFAs in aqueous solutions represents one of the major limitations for *in vitro* and *in vivo*

studies. To overcome this problem, FFAs can be conjugated to albumin, allowing the preparation of solutions in the physiological concentration range. The fraction of unbound FFAs accessible for cellular uptake depends on the ratio of total FFAs to albumin (Spector 1975). Thus, the biological effect of FFAs can be augmented by increasing the FFA concentration or by decreasing the bovine serum albumin (BSA) concentration. The unbound FFA concentration is also determined by the relative affinities of the FFAs for albumin (Spector 1975). Physiologically, FFAs are linked to BSA in the serum and only a small part is free. Whilst, in pathological conditions the unbounded fractions of FFAs increases generating cellular dysfunctions. Until now, in experimental condition, different doses of PAL were incubated with a fix dose of BSA producing an unknown amount of free PAL. So, following the protocol suggested by *Oliveira et al*, in our experiments we manually prepared the PAL/BSA conjugate maintaining unvaried palmitate concentration and by varying the BSA percentage. In this protocol the amount of unbounded FFAs obtained reproduced the limit of physiopathological conditions of obese patients (under normal physiological conditions, an average of 2 FFA molecules are bound to each albumin molecule in the circulation, corresponding to concentrations of unbound FFAs less than 20 nM) (Oliveira et al 2015) . From literature its known that palmitate, at concentration similar to that seen in patients with diabetic nephropathy (DN), can induce ROS production in cultured mouse podocyte cell line (MPC5) (Jiang et al 2017) . Since that mouse podocytes doesn't preserve the human cell type main metabolism, we use an *in vitro* model, instead, consisting of human conditionally immortalized podocytes incubated with palmitate. Our data demonstrate that physiopathological palmitate concentrations stimulate ROS generation in human podocytes through mitochondrial and cytosolic mechanisms

(Fig.1) and this palmitate induced cytosolic ROS production activated ER stress in human podocytes cell line (Fig.2).

Adipose cells release a series of adipokines, such as TNF- α, leptin, adiponectin, interleukins (IL)-6, IL-10, MCP-1, plasminogen activator inhibitor (PAI) -1, resistin, and CRP, and promote chronic low-grade inflammation in obese patients. These lipid-mediated inflammations lead to renal structural and functional changes in obesity cases (Manabe 2011). Chronic adipose inflammation (which forms from the imbalance between proinflammatory and anti-inflammatory factors) is a major factor for ORG (Nolan et al 2013). Therefore we wanted to evaluate, in our model, if the production of ROS induced by physiopathological concentration of plamitate can cause podocytes inflammation. We analized the mRNA gene expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) that is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages (Deshmane et al 2009), the gene expression of the subunit p65 that is one of the five components that form the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factor family; as part of the NF-κB signaling pathway, p65 is typically involved in the body's inflammatory response (Velaei et al 2017), the mRNA expression of transforming growth factor-β (TGF-β) that is considered a major culprit for renal cell injury in progressive CKD (Casalena et al 2012) and contributes to both renal cell apoptosis and renal fibrosis and the mRNA gene expression of sterol regulatory element binding protein1c (SREBP-1c) that belongs to the basic helix-loop-helix-leu-cine zipper family of transcription factors and plays a key role in regulating fatty acid synthesis and could also participate to the hepatic steatosis (Ferré 2007). Our data suggests that in cells exposed to physiopathological concentrations of palmitate for 24h, mRNA gene expression levels of MCP-1 and subunit p65 were increased (Fig.3) such as the mRNA gene expression of TGF-β

and SREBP-1c (Fig.4) and all these palmitate effects were significantly reduced when the cells were treated with GKT137831 inhibitor proving that palmitate induces podocytes inflammation by increasing ROS production.
High mobility group box 1 protein (HMGB1), a nuclear DNA binding protein, is released under pathological conditions and locally act as one of potent damage-associated molecular patterns (DAMPs) to produce tissue injury and chronic inflammation. HMGB1 synthesis and its secretion is increased in obese patients in different types of tissue from adipose tissue (Wang et al 1999) and it is is considered to be important in the pathogenesis of a wide range of kidney disease (Chen et al 2016).
A number of studies have reported the increased levels of serum HMGB1 in ORG patients or models and showed its positive correlation with the severity of the disease. Second, decreasing HMGB1 levels by HMGB1 specific inhibitors can significantly decrease the release of related cytokines and reduce the inflammatory reaction in kidney disease (Chen et al 2016). The exact function of HMGB1 and its mechanism still needs to be elucidated. We demonstrated that HMGB1 mRNA gene expression, in the human podocytes treated with palmitate conjugated with different percentage of BSA for 24h, was increased and the overexpression of GKT137831 inhibitor prevented this increase on HMGB1 expression proving that it is mediated by ROS production (Fig.5). Morover its known taht glycyrrhizin (GLYZ), a natural anti-inflammatory and antifungal factor, demonstrated anti-inflammatory properties and is considered to inhibit the cytokine activity of HMGB1 with amelioration of inflammation during acute/chronic phases of hepatitis, myocarditis, and liver injury (Ogiku et al 2011; Zhai et al 2012). A recent study showed that GLYZ protects renal ischemia reperfusion injuries in rabbits (Subramanian et al 1999). To understand the role of HMGB1 in causing palmitate-induced glomerulophaty, we measured mRNA expression of

inflammatory, fibrosis and steatosis genes in treated podocytes stimulated with Glycyrrhizin for 30min. Our results show that the increase of podocytes inflammation, fibrosis and lipotoxic effect, induced by physiopathologicals concentrations of palmitate, was prevented by Glycyrrhizin inhibitor proving the key role played by HMGB1 in ORG and suggesting a regolatory role of HMGB1 on inflammation, steatosis and fibrosis (Fig.6).
Although the mechanisms by which HMGB1 is released and the signaling pathways it activates require further elucidation, evidence suggests that modulating HMGB1-mediated signalling that it may constitute a new strategy for the treatment of kidney diseases. Further animal and cell studies are required to evaluate how extracellular and intracellular HMGB1 are implicated in the pathogenesis of different kidney diseases.
In literature its known that the persistent adverse effects of prior sustained exposure to hyperglycemia persisting for years after hyperglycemia has been ameliorated is defined as "metabolic memory"(Giacco et al 2015). Accumulating evidence supports the concept that even prior exposure to hyperglycemia for hours, causes persistent ROS lasting for days of subsequent normal glucose, triggering vascular dysfunction and damage (Costantino et al 2015; Brasacchio et al 2009) by inducing epigenetic modifications (Giacco et al 2015; Wegner et al 2014). In the present study we demostrated that, transient exposure to FFAs can induce a persistent production of ROS that lasts for 48 hours after the pro-oxidative stimulus is removed. Thus, a cellular memory for palmitate-induced oxidative stress exists in podocytes. Both mitochondrial and cytosolic ROS generating mechanisms are involved in initiating the persistent ROS over-production induced by transient exposure to PAL/BSA conjugate (Fig.7-8). Infact both the overexpression of UCP-1 and the use of NOX4 inhibitor can prevent the effect of transient exposure to high palmitate on the long lasting intracellular ROS production. Although NADPH oxidase 4 (NOX4) has been hypothesized to be a direct source

of increased ROS in the diabetic kidney (Jha et al 2016), it appears to act indirectly in preventing mitochondrial ROS production. NOX4 is constitutively active. However, it associates with mitochondria and inhibits mitochondrial biogenesis (Bernard et al 2017). Knockout of NOX4 increases mitochondrial biogenesis and maximal respiratory capacity dramatically, which would prevent substrate-driven increased mitochondrial ROS production (Bernard et al 2017). So, inhibition of NOX4 may be relieving these effects, allowing more and better functioning mitochondria (it increases mitochondrial respiratory reserve), which would reduce mitochondrial production of ROS. On the other hand, the direct inhibition of mitochondrial production of ROS (e.g. UCP1) would reduce ROS independent of NOX4 (Giedt et al 2012).

Since ROS have a very short half-life, their increased concentration after two days without palmitate suggests that transient exposure to palmitate, activates self-maintaining ROS generating mechanisms that induces an increased of intracellular ROS production even when the initial pro-oxidative stimulus was removed. The observed persistent ROS over-production is the result of FFA effects on mitochondrial function. Several studies have demonstrated that oxidative mtDNA damage can further increase the generation of superoxide by impairing the integrity and the function of electron transport chain (Brasacchio et al 2009; Giardino et al 2017; Bernard et al 2017). In particular, in our cells transient high palmitate significantly reduces the transcription of the ND1, ND4 and ND4L subunits of Complex I, which have been recently recognized to be the mitochondrial sources of deleterious ROS (D'Apolito et al 2018). Complex I derived ROS can readily react with mitochondrial DNA or other matrix components vulnerable to oxidative damage. In contrast, Complex III-derived ROS serve as second messengers in cellular signaling (D'Apolito et al 2018). So, palmitate is able to activate a multi-component feedback loop, which causes a persistent ROS production. Next ,we have shown that this

persistent increase in mitochondrial ROS production, during subsequent days of normal palmitate, is responsible for a persistent increase in the expression of the major inflammatory mediator NFκB subunit p65, the monocyte chemoattractant protein-1 (MCP-1), the TGF-β and the HMGB1 in the cells transient exposed to high palmitate. Other pathological conditions such as diabetes (Giacco et al 2015) or uremia (D'Apolito et al), have been shown to produce a similar effect by inducing metabolic memory. In particular, the long lasting effects of transient hyperglycemia on the vascular bed has been demonstrated to be caused in part by hyperglycemia induced epigenetic modifications (Assam El-Osta et al 2008). Epigenetic changes refer to a variety of covalent modifications on histone proteins, and to methylation/demethylation of cytosine nucleotides in genomic regions that regulate gene expression (Barros et al 2009). It has been shown that hyperglycemia-induced ROS cause different epigenetic modifications in endothelial cells, including DNA methylation and histone modifications. These change expression of both protein –coding genes and non-coding RNAs such as microRNA (miRNA) (Xinyuan et al 2017). Although many of these epigenetic changes in cells can be rapidly reversed, in diabetes they lasted for a long time because they were sustained by a hyperglycemia-induced increase of ROS production that persisted for days after normalization of glucose concentration (Xinyuan et al 2017).

Our study demonstrates for the first time that the method of *Oliveira et al* for coniugating palmitate with albumin produces an ubunded palmitate fraction able to cause podocyte dysfunctions similar to the one observed in the ORG. In particular, concentration of unbunded palmitate similar to the one saw in patients with ORG, causes ROS overproduction in human podocytes. This oxidative stress causes podocytes disfunctions such as inflammation, and changes in profibrotic and lipotoxic markers. In our study, HMGB1 appears to be the

main mediator of ROS demaging action on podocytes. In fact, the pharmacological inhibition of HMGB1 prevents all ROS effects on podocytes. The limit of this part of our study is to not have performed, at the moment, the silencing of HMGB1 to prove definivetly is key role in the pathogenesis of ORG. Moreover, in our study, palmitate appears to be able to start a feed-back loop that causes a persistant overproduction of ROS and consequently a persistent podocyte dysfunction. So we establish that a metabolic memory exists for the FFAs action on podocytes, causing a persisting damage even in presence of a normalized lipid profile. More studies have to be performed to better understand several aspects of our results.

In conclusion, the present findings may provide further insight into the underlying mechanisms that contribute to the pathogenesis of ORG.

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