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TESE DE DOUTORADO

PAPEL DA PROGRANULINA NA DOENÇA RENAL DO DIABETES E TRANSPLANTE RENAL

BRUNA BELLINCANTA NICOLETTO

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia da Universidade Federal do Rio Grande do Sul, como requisito para obtenção do título de Doutora.

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LISTA DE ABREVIATURAS

ADA	American Diabetes Association / Associação Americana do Diabetes
ADPN	Adiponectin
ANOVA	One-Way Analysis of Variances
ATG	Antithymocyte Globulin
BF%	Body Fat Percentage
BMI	Body Mass Index
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CRP	C-Reactive Protein
DEXA	Dual-Energy X-ray Absorptiometry
DKD	Diabetic Kidney Disease
DM	Diabetes Mellitus
DRC	Doença Renal Crônica
DRD	Doença Renal do Diabetes
eGFR	estimated Glomerular Filtration Rate
ER	Endoplasmatic Reticulum
ERFD	Early Renal Function Decline

ERK	Extracellular Regulated Kinase
EUA	Excreção Urinária de Albumina
FPG	Fasting Plasma Glucose
FTD	Frontotemporal Dementia
GFR	Glomerular Filtration Rate
HAS	Hipertensão Arterial Sistêmica
HOMA-IR	Homeostasis Model Assessment for insulin resistance
hsCRP	High-Sensitivity C Reactive Protein
IDF	International Diabetes Federation / Federação Internacional do Diabetes
IL-6	Interleukin-6 / Interleucina-6
IMC	Índice de Massa Corporal
IRS-1	Insulin Receptor Substrate-1
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemoattractant Protein-1
mTOR	mammalian Target of Rapamycin
NOD2	Nucleotide-binding Oligomerization Domain containing 2
PCR	Proteína C-Reativa
PGRN	Progranulin / Progranulina

PI3K	Phosphatidylinositol 3-kinase
PPARg	Peroxisome Proliferator-Activated Receptor-Gamma
SD	Standard Deviation
SOCS3	Cytokine Signaling-3
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TFG	Taxa de Filtração Glomerular
TNFα	Tumor Necrosis Factor-α
TNFR	Tumor Necrosis Factor Receptor
TNFR-1	Tumor Necrosis Factor Receptor 1 / Receptor-1 do Fator de Necrose Tumoral
UAE	Urinary Albumin Excretion

SUMÁRIO

1 APRESENTAÇÃO DA TESE

Esta tese de doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia da Universidade Federal do Rio Grande do Sul.

A tese apresenta uma introdução sobre os principais aspectos da progranulina, principalmente em relação ao diabetes mellitus tipo 2 e à doença renal. Na sequência, é apresentado o artigo de revisão já publicado que aborda detalhadamente as propriedades da progranulina nessas patologias. Após, são incluídos dois artigos originais. O primeiro, também já publicado, investiga a associação dos níveis séricos e urinários de progranulina na doença renal do diabetes mellitus tipo 2. O segundo mostra os níveis séricos de progranulina em diferentes momentos do transplante renal.

Ao final, são apresentadas conclusões relacionadas ao tema da tese, englobando aspectos da progranulina no metabolismo do diabetes mellitus tipo 2, doença renal crônica e transplante renal.

2 INTRODUÇÃO

2.1 PROGRANULINA

A progranulina (PGRN) é uma proteína de 68-88 kDa, também conhecida como proepitelina, precursor da granulina ou fator de crescimento derivado da célula PC (1). Inicialmente foi descoberta como uma glicoproteína acrossomal, chamada acrogranina, sintetizada durante a espermatogênese de roedores (2). Estudos prévios já demonstraram suas ações como fator de crescimento, atuando no remodelamento tecidual, no câncer e em doenças neurodegenerativas (3-7). Há evidências de que o RNA mensageiro da PGRN seja expressado em diversas células teciduais (8), inclusive em adipócitos (1), o que tem destacado as funções da PGRN como uma adipocina envolvida no metabolismo da glicose e da insulina (1, 9).

Desde a descoberta da leptina, em 1994, o tecido adiposo tem sido reconhecido como um órgão endócrino, onde as adipocinas por ele secretadas desempenham inúmeras funções no organismo (10). A leptina age na supressão do apetite, no controle da ingestão alimentar e no metabolismo energético (11). Quando há aumento de peso e gordura corporal, os níveis de leptina também se elevam; entretanto, uma resistência à sua ação no hipotálamo se estabelece, dificultando o desempenho de suas funções (12). Um processo semelhante a este tem sido sugerido para a PGRN (13). Estudos experimentais apontam que a administração dessa proteína no hipotálamo de roedores resulta na redução do consumo alimentar de forma dose-dependente. Apesar disso, na obesidade, uma resistência aos efeitos anorexígenos da PGRN pode contribuir para o aumento da ingestão alimentar (13).

A relação da PGRN com obesidade pode ser observada ainda em estudos que avaliaram a associação dos níveis séricos dessa adipocina com parâmetros de adiposidade corporal (14-16). Estudos prévios demonstraram uma correlação positiva da PGRN com o índice de massa corporal (IMC) (14, 15, 17, 18), percentual de gordura corporal e circunferência da cintura (15-19). Níveis aumentados de PGRN são encontrados em indivíduos com IMC ≥ 30 kg/m² (15), assim como em indivíduos com IMC ≥ 25 kg/m² (14), quando comparados àqueles com peso adequado. A distribuição de gordura corporal também parece influenciar, sendo a gordura visceral associada a maior concentração sérica de PGRN (15).

Além da relação com a obesidade, a PGRN parece estar associada à resistência insulínica. Em estudo experimental, observou-se um aumento da expressão do gene da PGRN no tecido adiposo branco de camundongos *ob/ob*, um modelo de obesidade e resistência insulínica que tem sido amplamente reconhecido e utilizado (1). Neste mesmo estudo, observou-se que camundongos nocauteados para o gene da PGRN recebendo dieta hiperlipídica apresentaram menor peso, depósito de gordura e resistência à insulina do que animais que possuíam o gene da PGRN (1). Em humanos, já foi observada uma correlação positiva entre a concentração de PGRN e o índice HOMA-IR (*Homeostasis Model Assessment for insulin resistance)* (14, 17). Além disso, estudo entre pacientes com obesidade mórbida indicou que aqueles com resistência insulínica tiveram maiores níveis séricos de PGRN (20).

A PGRN parece desempenhar suas ações principalmente através do aumento da expressão de interleucina-6 (IL-6), prejudicando a sinalização da insulina, como demonstrado em estudo utilizando células adiposas (1). Além disso, a PGRN é

capaz de se ligar ao receptor-1 do fator de necrose tumoral (TNFR-1) e induzir resistência insulínica por esta via (21). Ela também parece aumentar a atividade autofágica e levar ao estresse do retículo endoplasmático, o que também contribui para a resistência à ação da insulina (17). A PGRN ainda apresenta atividades quimiotáticas, recrutando monócitos para o tecido adiposo (15). Em humanos, estudos prévios já observaram correlação positiva dos níveis de PGRN sérica com IL-6 e também com a proteína-C reativa (PCR) (14, 15, 22, 23). Esses achados sugerem que a PGRN seja uma adipocina associada à inflamação, obesidade e resistência insulínica. Nesse sentido, a PGRN também já foi estudada em pacientes com diabetes mellitus (DM) tipo 2, onde há um aumento da sua concentração sérica (14-16, 18, 24).

2.2 DIABETES MELLITUS TIPO 2

O DM é uma doença caracterizada por hiperglicemia, resultante de defeitos na ação e/ou secreção de insulina (25). Dados da Federação Internacional do Diabetes (IDF, do inglês *International Diabetes Federation*) indicam que em 2015, cerca de 415 milhões de pessoas no mundo tinham DM. O Brasil foi o quarto país no ranking de prevalência de DM, com 14,3 milhões de adultos com esta doença (26). Os números aumentaram nas últimas décadas em todo o mundo e a estimativa é de que cresçam ainda mais. Para o ano de 2040, estima-se que existam 642 milhões de adultos portadores de DM no mundo (26).

Entre os tipos de DM, o tipo 2 é o mais comum, atingindo cerca 90-95% da população com diabetes. A sua etiologia é complexa; entretanto o mecanismo sugerido se associa ao efeito da glicemia elevada na função das células beta-pancreáticas, que não produzem insulina suficiente para compensar a resistência que se estabelece à este hormônio (25). Diversos fatores de risco têm sido atribuídos ao desenvolvimento de DM tipo 2, sendo os principais a obesidade e o depósito de gordura visceral (27). Outros fatores metabólicos associados com a obesidade estão intimamente relacionados com o DM tipo 2, como hipertensão arterial sistêmica (HAS), dislipidemia, inflamação, sedentarismo e fatores dietéticos (28, 29). Aspectos genéticos e de história familiar de DM também têm sido amplamente estudados e diversos genes já foram associados ao desenvolvimento do DM tipo 2 (28, 30).

Recentemente, novos marcadores ganham destaque na literatura, e diversas adipocinas parecem desempenhar um papel importante na patogênese do diabetes (31, 32), entre elas a PGRN (9, 14-16). Apesar de um estudo não ter encontrado associação entre DM tipo 2 e níveis de PGRN (33), diversas evidências reforçam essa hipótese, apontando que pacientes com DM tipo 2 apresentam maiores níveis de PGRN que os normoglicêmicos (14-16). Há evidência de que a concentração desta adipocina estaria elevada até mesmo em indivíduos com tolerância à glicose diminuída (16). Além disso, uma correlação positiva da PGRN com HbA1c, glicemia em jejum e após 2h de teste de tolerância oral à glicose foram evidenciados (14, 15, 17). Ainda assim, a obesidade parece ter um papel importante nos níveis de PGRN, uma vez que pacientes com DM tipo 2 e obesidade têm maiores concentrações de PGRN sérica que pacientes com DM tipo 2 não obesos (14).

2.3 DOENÇA RENAL

A doença renal crônica (DRC) é definida por uma redução da taxa de filtração glomerular (TFG), aumento na excreção urinária de albumina (EUA) ou ambos. Estima-se que a prevalência mundial de DRC varie entre 8 e 16%, sendo as principais causas doenças como DM e HAS, além de glomerulopatias (34).

A doença renal do diabetes (DRD), há pouco tempo conhecida por nefropatia diabética, é uma complicação do DM. A hiperglicemia associada principalmente à HAS contribui para a lesão no glomérulo renal (35, 36). A inflamação no tecido também contribui para um processo de fibrose no rim (37). Dessa forma, pode haver a passagem de proteínas na urina – em especial a albumina. Até pouco tempo, a concentração de albumina na urina era classificada como micro ou macroalbuminúria (35, 36, 38). Recentemente, a Associação Americana de Diabetes (ADA, do inglês *American Diabetes Association*) passou a classificar a albuminúria apenas como EUA normal ou aumentada (39). Além da excreção de albumina, a estimativa da TFG é importante para a avaliação da DRD. Existem evidências que alguns pacientes com DRD manifestam uma diminuição da função renal avaliada para TFG mesmo na ausência de albuminúria (35, 36).

Recentemente, a PGRN foi identificada como uma adipocina dependente da função renal. Em estudo avaliando 532 pacientes nos estágios 1 a 5 da DRC, observou-se que os pacientes no estágio mais avançado apresentaram maiores níveis séricos de PGRN, sendo a TFG um fator preditor independente dos seus níveis (23). Em outro estudo, níveis aumentados de PGRN foram observados em pacientes com DM tipo 2 e macroalbuminúria (40). Além disso, as evidências

demonstram uma associação negativa entre níveis séricos de PGRN e TFG (23, 40). Esses achados indicam que o rim possa desempenhar um papel importante na depuração da PGRN. Apesar disso, alguns estudos experimentais têm identificado a PGRN como um mecanismo de proteção renal (41-43), principalmente em situações agudas.

2.3.1 Transplante renal

O transplante é a terapia de substituição renal com melhor custo-efetividade, e proporciona aos pacientes renais crônicos melhor qualidade e expectativa de vida (44, 45). A terapia imunossupressora, principalmente os inibidores da calcineurina (ciclosporina e tacrolimus) são responsáveis, em parte, pela maior sobrevida dos pacientes transplantados e pela redução na dose de corticoesteróides. Entretanto, o uso da terapia imunossupressora pode trazer efeitos colaterais importantes (46). Algumas complicações são comumente observadas, como ganho de peso, dislipidemias, osteoporose, HAS, desenvolvimento de DM e eventos cardiovasculares. Muitas destas complicações contribuem para a redução da sobrevida do enxerto e do paciente (47-50).

O ganho de peso é um problema frequente em transplantados renais. O aumento ponderal foi evidenciado em diversos estudos (51-53), inclusive em avaliação de transplantados renais de nosso centro, onde a prevalência de sobrepeso e obesidade subiu de 6% no pré-transplante para 64% em cinco anos pós-transplante (54). Além disso, no período tardio pós-transplante renal pode haver

aumento do percentual de gordura corporal e resistência insulínica (54, 55), contribuindo para um perfil desfavorável, associado a maior risco de doença cardiovascular.

Diversas adipocinas têm sido estudadas nesse contexto. Os níveis de leptina foram avaliados em transplantados de nosso centro e uma maior concentração sérica foi observada no momento pré-transplante renal. No período imediato pós-transplante, os níveis reduziram significativamente e voltaram a aumentar em cinco anos (54). Já a concentração de adiponectina – uma adipocina com propriedades anti-inflamatórias, anti-diabéticas e anti-aterogênicas, está aumentada no pré-transplante renal e reduz significativamente após o transplante, mantendo-se reduzida em um ano (56). Em relação aos níveis de PGRN após o transplante renal, entretanto, a literatura carece de evidências.

Dessa forma, se faz importante estudar as propriedades da PGRN, suas funções no metabolismo da obesidade, inflamação, resistência insulínica e DM tipo 2, além da sua relação com a função renal. Assim, esta tese teve o objetivo de apresentar uma revisão da literatura sobre o tema, além de investigar os níveis de PGRN na DRD e no transplante renal.

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RESEARCH

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The role of progranulin in diabetes and kidney disease

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Abstract

Progranulin (PGRN) is a cysteine rich secreted protein, expressed in epithelial cells, immune cells, neurons, and adipocytes. It was first identified for its growth factor-like properties, being involved in early embryogenesis and tissue remodeling, acting as an anti-inflammatory molecule. In the central nervous system, PGRN has neurotrophic and neuroprotective actions. There is also evidence of PGRN effects on cancer, contributing to tumor proliferation, invasion and cell survival. Recently, PGRN was recognized as an adipokine related to obesity and insulin resistance, revealing its metabolic function and pro-inflammatory properties. In obesity and type 2 diabetes mellitus, PGRN levels are increased. In renal disease, there is a relevant association, however, it is not known if it could contribute to kidney damage or if it is only a route of PGRN elimination. PGRN is an emerging molecule which demands studies in different fields. Possibly, it plays distinct functions in different tissues/cells and metabolic conditions. Here, we discuss potential mechanisms and recent data of PGRN pro-inflammatory actions, regarding obesity, insulin resistance, type 2 diabetes mellitus and kidney disease.

Keywords: Progranulin, Adipokine, Obesity, Diabetes, Kidney disease, Inflammation

Background

Progranulin (PGRN) is a 68–88 kDa cysteine rich secreted protein, also known as granulin-epithelin precursor, proepithelin or PC-cell derived growth factor [1, 2]. It is encoded by *GRN* (PGRN gene) and expressed in many cell types, including epithelial cells, immune cells, neurons, and adipocytes [3]. In kidneys, PGRN is expressed by renotubular epithelia of mouse embryo [4]. In healthy adult rodents, PGRN continues to be expressed in the kidney, strongly in the transitional epithelium of the ureter; but weakly in the proximal and distal convoluted tubules of the cortex and collecting ducts of the medulla [5]. In humans, the PGRN expression in kidneys remains unknown.

The first evidence of the protein was found during guinea pig spermatogenesis, when an acrosomal glycoprotein, named acrogranin, was detected [6]; and later identified as the guinea pig equivalent of PGRN [7]. PGRN has growth factor–like properties, being involved in early embryogenesis [4], wound repair and tissue remodeling [8]. It regulates cell division, survival, and migration, mainly via extracellular regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) pathways [8]. The growth factor–like properties of PGRN could be involved in physiology of tissue repair or in diseases such as cancer [1]. By the same pathways (ERK and PI3K), PGRN contributes to tumor proliferation, invasion and cell survival [9–11]. This molecule has previously been linked to many cancer types, as breast [12], ovarian [13], cervical [14], gastrointestinal [15] and kidney cancers [16].

Progranulin is secreted in an intact form and can be cleaved into granulins by proteases [2, 3]. Granulins are small proteins of approximately 6 kDa characterized by a conserved motif of 12 cysteines and play a role in the extracellular regulation of cell function and growth [8]. It has been suggested that the full length form of the protein (PGRN) has anti-inflammatory action, while released granulins have the opposite effect, increasing the production of proinflammatory cytokines, as interleukin 8

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[1]. The intact PGRN is reported bind to tumor necrosis factor receptor (TNFR), inhibiting the binding of tumor necrosis factor-α (TNFα) and its proinflammatory signaling [17, 18]. In mouse models of arthritis, PGRN prevents inflammation [17]. In humans, increased serum levels of PGRN are observed in rheumatoid arthritis, but its relation to the pathogenesis of the disease remains unclear [19]. Elevated PGRN levels are observed in the skin of psoriasis patients [20, 21], mice dermatitis model [20, 21] and wounds [1]. Some authors suggest that PGRN has the effect of attenuating inflammation in these conditions, acting as an anti-inflammatory molecule [20, 21].

In central nervous system, PGRN has neurotrophic and neuroprotective actions [22]. It is involved in neurite outgrowth and possibly plays a role in plasticity and remodeling in the adult brain [23]. In addition, PGRN protects neurons from premature death [8] and acts in the response to stress [23] and neuroinflammation [22]. PGRN deficiency is associated with neurodegeneration and frontotemporal dementia (FTD), mainly due to mutation in *GRN* [24]. However, PGRN expression is upregulated in microglia in neurodegenerative disease [25] as FTD, especially in brain areas with a substantial pathology [26]. It is unclear if it represents a result of microglia response to injury or an active contribution to the disease progression [22].

After acute ischemia–reperfusion injury, lower PGRN levels are observed in mice brain [27] and kidney [28]; and treatment with recombinant PGRN could attenuate inflammation in this condition [27, 28]. PGRN also seems to protect against acute focal cerebral ischemia in rats by attenuation of blood–brain barrier disruption, neuroinflammation suppression, and neuroprotection [29].

Despite the reported anti-inflammatory properties of PGRN in some conditions, it seems to be a more complex molecule, revealing an opposite metabolic function. In the periphery, the intact form of PGRN has been associated with proinflammatory effects, since PGRN was recently recognized as an adipokine related to obesity and insulin resistance [3, 30]. Nothing further is known about the relationship of PGRN with its proteolytic granulins in obesity and insulin resistance.

Review

Adipose tissue, obesity and PGRN

Since the discovery of leptin in 1994, adipose tissue has been recognized as an endocrine organ, with its secreted adipokines playing many functions in the body [31]. Leptin acts on energy metabolism, regulating appetite and food intake, as an anorexigenic hormone [32]. In obesity, its levels are increased; however, leptin resistance is observed, impairing leptin functions [33]. A similar biological process is suggested for PGRN [34]. There is evidence that the administration of PGRN in the mice hypothalamus significantly suppresses fasting-induced feeding and body weight gain in a dose-dependent manner, possibly through hypothalamic neuropeptide Y and the melanocortin system [34]. However, in obesity, a resistance to the anorexigenic effects of PGRN may contribute to increased food intake [34].

In obesity, PGRN levels are increased [30, 35, 36]. Both experimental studies [35] and those performed in humans [30, 36] have reported the relationship between PGRN and adiposity. In *ob/ob* mice, a well-characterized obese and insulin resistance model, there are elevated PGRN serum levels and upregulation of *Grn* in white adipose tissue [35]. In humans, previous studies report a positive correlation between body mass index (BMI) and PGRN serum levels [30, 36, 37].

Progranulin is positively correlated to body fat percentage and waist circumference [36–38]. Fat distribution is related to PGRN levels, revealing higher PGRN serum concentration in subjects with visceral obesity [36]. It is known that visceral fat and its secreted adipokines and immune cell-derived cytokines are involved in chronic inflammation [31]. PGRN seems to play a role on this process, due its chemotactic activity, recruiting monocytes into adipose tissue as well as monocyte chemoattractant protein-1 (MCP-1) [36]. Furthermore, there is evidence that *Grn* deficient mice had significantly less infiltration of macrophages in adipose tissue [35]. Taken together, these findings suggest a proinflammatory effect of PGRN. This is supported by human studies that found a positive correlation between PGRN and C-reactive protein (CRP) [36, 39] and interleukin 6 (IL-6) [30, 39].

The effects of PGRN in obesity and inflammation have also revealed its influence on insulin resistance. A positive correlation between PGRN levels and HOMA-IR index (*Homeostasis Model Assessment for insulin resistance*) has been reported [30, 37]. In morbid obesity, patients with insulin resistance have elevated PGRN serum concentration [40].

Experimental studies reported that PGRN promotes IL-6 expression in adipose cells, and its elevation enhances cytokine signaling-3 (SOCS3) expression via activation of JAK-STAT signaling. This mechanism can inhibit tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), leading to insulin resistance [35]. Additional evidence reports that *Grn* deficient mice fed with high fat diet presented improved insulin sensitivity. Moreover, body weight, fat mass and size of adipocytes were lower in *Grn* deficient mice compared to the wild-type mice receiving a standard diet [35]. These findings suggest a relevant association of PGRN with obesity and insulin resistance [35], as summarized in Fig. 1.

Recently, experimental studies reported that PGRN increases autophagic activity and triggers endoplasmatic

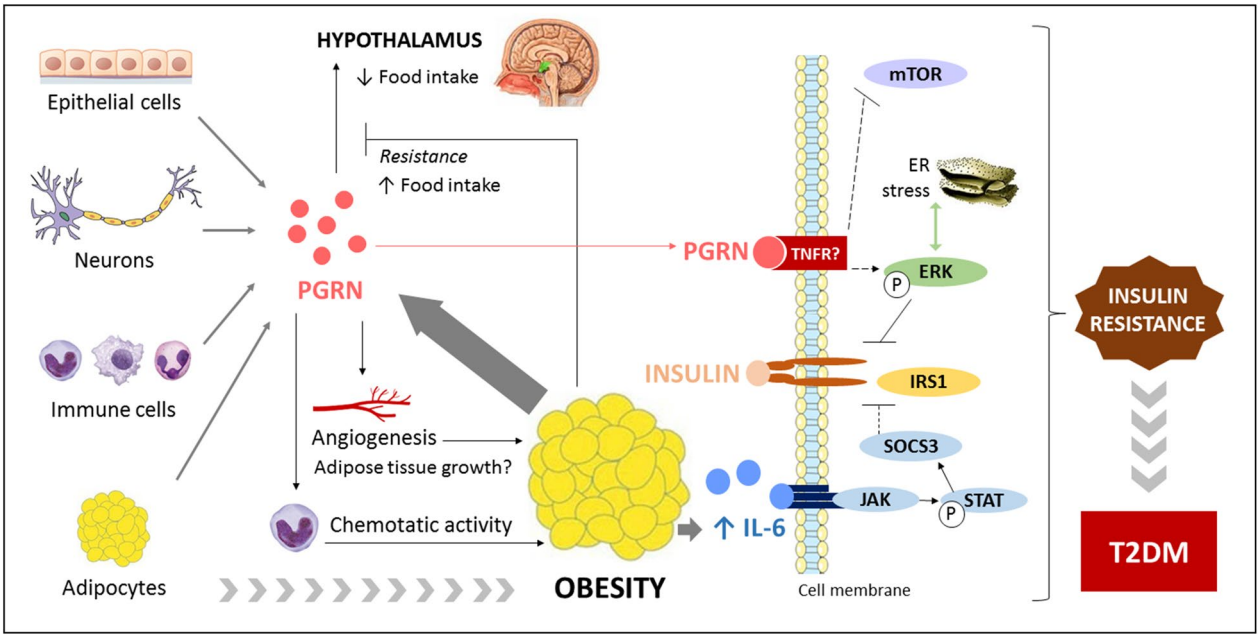

Fig. 1 Association of PGRN with obesity, insulin resistance and type 2 diabetes mellitus. *PGRN* progranulin, *IL-6* interleukin-6, *TNFR* tumor necrosis factor receptor, *mTOR* mammalian target of rapamycin, *ERK* extracellular regulated kinase, *IRS-1* insulin receptor substrate-1, *SOCS3* cytokine signaling-3, *T2DM* type 2 diabetes mellitus

reticulum (ER) stress in cultured human adipocytes, impacting on insulin signaling [37]. First, in multiple insulin-resistant cellular models there were increased levels of PGRN and autophagic imbalance. In PGRN deficient adipocytes, decreased markers of autophagy were observed. Moreover, adipocytes treated with PGRN and then stimulated with insulin revealed diminished IRS-1 and Akt phosphorylation and increased autophagic disorder. Mechanisms suggested involve ERK and mammalian target of rapamycin (mTOR) pathways [37]. ERK activation impairs IRS-1 activity and is associated with autophagy and ER stress-induced insulin resistance [41]. Likewise, inhibition of mTOR reduces insulin action and promotes autophagic disorders in adipocytes [42]. Possibly, PGRN effects are mediated by ERK activation and impaired mTOR phosphorylation [37] (Fig. 1). Moreover, there is evidence that PGRN could exert a causative role in hepatic insulin resistance, as observed in mice treated with PGRN for 21 days. Animals presented impaired glucose and insulin tolerance, and hepatic autophagy imbalance [43].

Regulation of PGRN on autophagy disorders and insulin resistance seems to be partially mediated through TNFR-1 via NF-kB signaling [37, 43]. Previous studies revealed that PGRN binds to TNFR, impairing the TNFα/TNFR interaction and suppressing chronic inflammation in mouse models of arthritis [17]. Although one study failed to demonstrate the binding of PGRN to TNFR [44], a recent publication reinforces this interaction [45]. Further evidence is required to elucidate the effects of PGRN binding to TNFR in different tissues, but it is possible that PGRN has dual roles in inflammation, exhibiting pro- and anti-inflammatory proprieties.

Some authors suggest that PGRN could be involved in the growth of adipose tissue [3]. It is known that the expansion of fat mass during obesity is followed by angiogenesis [46]; and PGRN has previously been linked to vessel formation [47]. Therefore, this adipokine could also contribute to increase adiposity (Fig. 1). However, this hypotheses needs to be confirmed by further studies.

Regarding adipogenesis, the role of PGRN is not fully understood. Its anti-adipogenic effects were reported in a previous study using porcine preadipocytes [48]. PGRN promoted ERK activation that phosphorylate peroxisome proliferator-activated receptor-gamma (PPARg) at the serine 112 site, impairing its function on adipocyte differentiation [48]. The effect of PGRN on ER stress also suppressed adipogenesis in cultured human adipose cells [37]. Moreover, Matsubara et al. [35] found that PGRN expression decreased with differentiation of 3T3-L1 adipose cells assessed by pre-adipocyte factor-1, PPARg, and fatty acid binding protein. On the other hand, a positive significant correlation between circulating PPARg and PGRN was observed in obese subjects [49].

Type 2 diabetes mellitus and PGRN

From 1988 to 2010, the total number of persons with diabetes increased by almost 75 % [50]. In 2012, there were an estimated 371 million adults living with diabetes, with 4.8 million deaths attributable to this disease [51].

Type 2 diabetes mellitus (T2DM) is characterized by a resistance to insulin action and inadequate compensatory insulin secretory response, leading to hyperglycemia [52]. Obesity and visceral fat are probably the main risk factors for T2DM [53] and are involved in its pathophysiology as well as inflammation and insulin resistance [31]. Genetic and T2DM family history are widely studied and several genes have been associated with T2DM development [54, 55].

The PGRN gene is located in the chromosome region 17q21.32. The long arm of chromosome 17 was previously linked to visceral adipose tissue, waist circumference and BMI in Hispanic families in the Insulin Resistance Atherosclerosis Family Study [56]. Moreover, linkage signals of fasting glucose was found with 17q in young European sib-pairs, suggesting an association with T2DM [57]. Other important genes are located in this chromosome region, as SOCS3 [58] and genes involved in food intake [59].

Recently, new markers have been studied in the pathogenesis of diabetes, involving many adipokines [60, 61], such as PGRN [30, 36, 38]. There is evidence that PGRN levels are increased in T2DM when compared to non-diabetic subjects [30, 36, 38, 62]. PGRN is closely related to glucose metabolism. There is a positive correlation between PGRN and A1C, fasting plasma glucose and 2 h post-challenge plasma glucose [30, 36, 37]. Elevated PGRN concentrations are also observed in impaired glucose tolerance subjects, revealing its role in prediabetic states [38]. Moreover, a recent study evaluating T2DM patients reports that obese subjects present higher levels of PGRN [30].

The association of PGRN with T2DM is mainly explained by its role in adipose tissue and insulin resistance. PGRN promotes IL-6 expression, impairing insulin signaling [35]. Moreover, it is a chemoattractant protein that recruits monocytes into adipose tissue, promoting inflammatory response with increased cytokines levels [36] (Fig. 1).

Decrease in circulating PGRN levels can be obtained with long term diet intervention [63] and exercise training [36]. A recent study evaluated the change in PGRN levels after 24 months of dietary intervention, and showed that the decrease was sustained throughout this period, irrespective of weight stabilization or partial weight regain [63]. Another study identified a significantly decrease of ~20 % in PGRN serum concentration after a 4-week training program, only in T2DM patients [36].

Other metabolic disorders associated with T2DM have also been linked to PGRN. A positive correlation observed between total cholesterol [36], triglycerides [30, 37] and PGRN suggests a role in dyslipidemia. Patients with metabolic syndrome present higher serum PGRN concentration [37, 64] and the number of metabolic syndrome components have a significant positive correlation with PGRN levels [39]. Elevation of PGRN expression in omental adipose tissue is also observed in patients with metabolic syndrome, indicating a potential contribution of adiposity to increased PGRN serum levels [37]. Moreover, patients with metabolic syndrome also present increased autophagic activity and ER stress in adipose tissue [37]. Finally, the effects of PGRN in obesity, insulin resistance and inflammation contribute to its association with atherosclerosis [39].

T2DM is associated with poor outcomes, characterized by macro and microvascular complications. Intensity and duration of hyperglycemia exposure leads to vascular and nervous damage, resulting in organ dysfunction, such as kidney, eyes, nerves, heart and blood vessels [52, 65].

Kidney disease and PGRN

Chronic kidney disease (CKD) is defined as a reduced glomerular filtration rate (GFR), increased urinary albumin excretion (UAE), or both. The incidence and prevalence of CKD differ substantially across countries; however, the estimated worldwide prevalence is around 8–16 % [66].

Although hypertension, glomerulonephritis and other comorbidities can lead to development of renal dysfunction, diabetes is the main cause of CKD [66]. In Brazil, it is the primary kidney disease in most patients starting dialysis [67]. The prevalence of diabetic kidney disease (DKD) increases over the years after the T2DM diagnosis, affecting about 25 % of patients with 10 years of the pathology [68]. Hyperglycemia associated with hypertension can lead to glomerulus injury [69, 70]. Tissue inflammation promotes kidney fibrosis, leading to protein clearance, such as albuminuria [71]. Although increased UAE are common in DKD, some patients with T2DM present reduced GFR, even in the absence of albuminuria [69, 70, 72].

Chronic kidney disease complications include increased all-cause and cardiovascular mortality, kidney-disease progression, acute kidney injury, anaemia, mineral and bone disorders, fractures and cognitive decline [66]. When associated with diabetes, an increased risk of mortality is observed [67, 73].

Recently, PGRN was described as an adipokine dependent of renal function [74]. Five hundred thirty-two patients with stages 1–5 of CKD (according to the National Kidney Foundation classification) had their PGRN serum levels evaluated. Even after adjustment for age, sex and BMI, PGRN remained significantly different between the five subgroups of CKD, being higher in stage 5. Moreover, estimated GFR was identified as an independent predictor of PGRN circulating levels. These findings suggest that renal filtration is an important route of PGRN elimination [74]. However, the authors did not find a significant correlation between serum and urinary PGRN in a subgroup of 145 patients, which limits their conclusions. There is also an hypothesis that PGRN might contribute to the proinflammatory state frequently observed in renal disease [74].

Progranulin also seems to be involved in DKD. In a recent study evaluating eighty-four T2DM patients, increased PGRN serum levels were described in macroalbuminuric subjects [75]. The study also evaluated the presence of proliferative diabetic retinopathy and observed higher levels of PGRN in this group of patients, suggesting PGRN as a marker for diabetic microangiopathy and its severity [75].

The association of urinary PGRN levels and renal damage was investigated in seventy-four patients with type 1 diabetes mellitus (T1DM) [76]. Subjects were evaluated at baseline, when urine was collected, and after 6 years, when albuminuria and early renal function decline (ERFD, defined as a decline in cystatin C-based estimated GFR of $\geq$3.3 % per year) were assessed. Patients with both ERFD and albuminuria presented higher urinary PGRN levels at baseline than patients who maintained normal renal function and normoalbuminuria, when adjusted by age, diabetes duration, baseline albumin excretion rate, HbA1C, cystatin C and uric acid. Moreover, PGRN was significantly predictive of ERFD and albuminuria in patients with type 1 diabetes in multivariable logistic regression [76]. The study also investigated urinary levels of Tamms–Horsfall glycoprotein, clusterin and human α-1 acid glycoprotein; and concludes that a panel of these three proteins plus PGRN could be used to predict early signs of DKD [76]. Table 1 summarizes present data regarding PGRN and renal function.

There is little evidence regarding the association of PGRN and DKD in T2DM patients. The proinflammatory effects of this adipokine could be involved in the pathway of renal damage, decreasing GFR and increasing albuminuria. When CKD is established, PGRN clearance is reduced and its effects could be potentiated. However, further studies are needed to elucidate this hypothesis.

In an acute condition, such as renal ischemia–reperfusion injury, an experimental study observed lower levels of PGRN in the mice kidney [28]. Moreover, *Grn* deficient mice presented a higher elevation of serum creatinine and blood urea nitrogen, more severe morphological injury and higher inflammatory response. Administration of recombinant PGRN in vitro could

Table 1 Studies characteristics regarding PGRN and renal function

Characteristic/reference	Xu et al. [75]	Richter et al. [74]	Schlatzer et al. [76]
Patients	84 patients with T2DM and 12 health persons	532 patients with stages 1–5 of CKD	74 patients with T1DM
PGRN material	Serum	Serum	Urine
Design	Cross-sectional study	Cross-sectional study	Longitudinal study Baseline: urine collection, PGRN dosage 3 and 6-year visit: assessment of MA and ERFD
Results regarding PGRN	PGRN serum levels are increased in T2DM patients with macroalbuminuria Positive correlation between serum PGRN and urinary albumin excretion rate Negative correlation between PGRN and eGFR	PGRN serum levels are different between groups of CKD stages ↑ PGRN levels at stage 5 of CKD CKD stage or eGFR are independently associated with PGRN serum levels	Lowest PGRN levels in patients who maintained normal renal function and normoalbuminuria (n = 35) Nonsignificant increase in patients with either ERFD (n = 15) or MA (n = 16) Significant increase in patients with both ERFD and MA (n = 8) Urinary PGRN was significantly predictive of ERFD and MA in patients with T1DM
Conclusion	PGRN might be considered as a marker for diabetic microangiopathy and its severity	Renal function assessed as eGFR is a strong, independent predictor of serum PGRN PGRN serum levels significantly increase with deteriorating renal function assessed as CKD stage	A panel of 4 proteins (PGRN, Tamms-Horsfall glycoprotein, clusterin and human α-1 acid glycoprotein) could be used to predict early signs of DKD

CKD chronic kidney disease, *T1DM* type 1 diabetes mellitus, *PGRN* progranulin, *ERFD* early renal function decline, *eGFR* estimated glomerular filtration rate, *MA* micro- or macroalbuminuria, *DKD* diabetic kidney disease

Table 2 Metabolic conditions associated with pro- or anti-inflammatory effects of PGRN

Proinflammatory	Anti-inflammatory
Obesity	Wound repair
Insulin resistance	Psoriasis
T2DM	Central nervous system
Dyslipidemia	Arthritis
Metabolic syndrome	Acute ischemia–reperfusion injury

T2DM type 2 diabetes mellitus

attenuate inflammation after renal ischemia–reperfusion injury at least in part associated with a nucleotide-binding oligomerization domain containing 2 (NOD2)-mediated immune response [28]. Therefore, PGRN plays a protective role and has an anti-inflammatory effect in the kidney after renal ischemia–reperfusion injury [28].

Conclusions

Progranulin is an emerging molecule which demands studies in different fields. Previous data have identified PGRN as a pro- and anti-inflammatory protein. Possibly, it plays distinct functions in different tissues/cells and metabolic conditions, as reported in Table 2. It was previously demonstrated that expression of PGRN in intact skin is low, but in injured skin, it raises significantly [1]. In addition, PGRN exerts anorexigenic effect in lean state, but a resistance is observed in obesity, leading to increased food intake [34]. Moreover, in acute condition of ischemia–reperfusion injury, PGRN plays an anti-inflammatory effect [27–29], while in obesity (a chronic condition), it is associated with insulin resistance and inflammation [35].

It is not fully understood if PGRN is a cause or consequence of some conditions. PGRN could be involved in the pathogenesis of obesity and T2DM, and become a target for metabolic disorders prevention or treatment. In renal disease, it is not known if it could contribute to kidney damage or if it is only a route of PGRN elimination. In the last case, PGRN could be used as a marker of renal disease. Further studies are necessary to elucidate these questions and investigate the crosstalk between pro- and anti-inflammatory PGRN proprieties in different tissues and conditions, in order to clarify the action mechanisms of this potential molecule.

Abbreviations

PGRN: progranulin; ERK: extracellular regulated kinase; PI3 K: phosphatidylinositol 3-kinase; FTD: frontotemporal dementia; TNFR: tumor necrosis factor receptor; TNFα: tumor necrosis factor-α; BMI: body mass index; MCP-1: monocyte chemoattractant protein-1; CRP: C-reactive protein; IL-6: interleukin 6; HOMA-IR: Homeostasis Model Assessment for insulin resistance; SOCS3: cytokine signaling-3; IRS-1: insulin receptor substrate-1; ER: endoplasmatic reticulum; mTOR: mammalian target of rapamycin; PPARg: peroxisome proliferator-activated receptor-gamma; T2DM: type 2 diabetes mellitus; CKD: chronic kidney disease; GFR: glomerular filtration rate; UAE: urinary albumin excretion; DKD: diabetic kidney disease; T1DM: type 1 diabetes mellitus; ERFD: early renal function decline; NOD2: nucleotide-binding oligomerization domain containing 2.

Authors' contributions

BBN and LHC wrote the manuscript. Both authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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5 ARTIGO ORIGINAL I

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RESEARCH ARTICLE

Serum and Urinary Progranulin in Diabetic Kidney Disease

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Abstract

Progranulin has been recognized as an adipokine related to obesity, insulin resistance and type 2 diabetes mellitus (T2DM). There are scarce data regarding progranulin and kidney disease, but there are some data linking diabetic kidney disease (DKD) and increased progranulin levels. We aimed to better describe the relationship between serum and urinary progranulin levels and DKD in T2DM. This is a case-control study including four groups of subjects: 1) Advanced DKD cases: T2DM patients with estimated glomerular filtration rate (eGFR) <60 mL/min/1.73m^2; 2) Albuminuric DKD cases: T2DM patients with urinary albumin excretion (UAE) ≥30 mg/g creatinine and eGFR ≥60 mL/min/1.73m^2; 3) Diabetic controls: T2DM patients with UAE <30 mg/g creatinine and eGFR ≥60 mL/min/1.73m^2; and 4) Non-diabetic controls: individuals without T2DM. Progranulin was determined by enzyme-linked immunosorbent assay. One hundred and fourteen patients were included (23 advanced DKD cases, 25 albuminuric DKD cases, 40 diabetic controls and 26 non-diabetic controls). Serum progranulin was increased in advanced DKD compared to other groups [70.84 (59.04–83.16) vs. albuminuric cases 57.16 (42.24–67.38), diabetic controls 57.28 (42.08–70.47) and non-diabetic controls 44.54 (41.44–53.32) ng/mL; p<0.001]. Urinary progranulin was decreased in advanced DKD cases compared to albuminuric cases [10.62 (6.30–16.08) vs. 20.94 (12.35–30.22); diabetic controls 14.06 (9.88–20.82) and non-diabetic controls 13.51 (7.94–24.36) ng/mL; p = 0.017]. There was a positive correlation between serum progranulin and body mass index (r = 0.27; p = 0.004), waist circumference (r = 0.25; p = 0.007); body fat percentage (r = 0.20; p = 0.042), high-sensitive C reactive protein (r = 0.35; p<0.001) and interleukin-6 (r = 0.37; p<0.001) and a negative correlation with eGFR (r = -0.22; p = 0.023). Urinary progranulin was positively associated with albuminuria (r = 0.25; p = 0.010). In conclusion, progranulin is affected by a decrease in eGFR, being at a higher concentration in serum and lower in urine of DKD patients with T2DM and eGFR <60 mL/min/1.73m^2. It is also associated with markers of obesity and inflammation.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Progranulin (PGRN) is a 68–88 kDa protein, also known as acrogranin, proepithelin, granulin-epithelin precursor or PC-cell derived growth factor [1, 2]. It is expressed in many cell types, including immune cells, epithelial cells, neurons, and adipocytes [3]. It was first identified for its growth factor like properties, being involved in early embryogenesis and tissue remodeling, acting as an anti-inflammatory molecule [4, 5]. In the central nervous system, PGRN performs neurotrophic and neuroprotective actions [6]. There is also evidence of PGRN effects on cancer, contributing to tumor proliferation, invasion and cell survival [7–9].

Recently, PGRN was recognized as an adipokine related to obesity and insulin resistance, revealing its metabolic function and pro-inflammatory properties [3, 10]. Elevated serum levels of PGRN are found in obese patients, with a positive correlation with body mass index (BMI) [10, 11]. Moreover, PGRN is associated with body fat, mainly with abdominal depot [11, 12]. Visceral adiposity, in turn, is strongly related to chronic inflammation, due to its secretion of adipokines and immune cell-derived cytokines [13].

PGRN seems to promote interleukin-6 (IL-6) expression, leading to insulin resistance [14]. In humans, there is a positive correlation between serum PGRN and IL-6 and HOMA-IR index (Homeostasis Model Assessment for insulin resistance) [10, 15]. Increased PGRN serum levels have also been described in patients with type 2 diabetes mellitus (T2DM) [10–12].

Diabetic kidney disease (DKD) is a common complication of diabetes [16], associated with cardiovascular disease [17]. Recently, increased serum PGRN was observed in macroalbuminuric patients with T2DM [18]. The theoretical role of PGRN in DKD could be found in a recent review [19]. Moreover, PGRN was described as a renal function-dependent adipokine, since elevated serum levels were observed in patients at stage 5 of chronic kidney disease (CKD) [20]. Urinary levels of PGRN in patients with T2DM and DKD remain unknown. However, in patients with type 1 diabetes, PGRN concentration in urine was predictive of early renal function decline and albuminuria [21].

Therefore, the main objective of this study was to investigate the association of serum and urinary PGRN levels with DKD in T2DM. We also aimed to evaluate the factors associated with PGRN levels.

Materials and Methods

Design and patients

One hundred and fourteen patients were included in this case control study. Patients with T2DM and no exclusion criteria were invited to participate in the study. Two study groups were included: 1) Advanced DKD cases: T2DM patients with estimated glomerular filtration rate (eGFR) <60 mL/min/1.73m^2 and 2) Albuminuric DKD cases: T2DM patients with urinary albumin excretion (UAE) ≥ 30 mg/g creatinine and eGFR ≥ 60 mL/min/1.73m^2. Once cases were included, controls were sought based on similar age, gender and BMI and were divided into another two control groups: 1) Diabetic controls: patients with T2DM with UAE <30 mg/g creatinine and eGFR ≥ 60 mL/min/1.73m^2 and 2) Non-diabetic controls: individuals without diabetes and eGFR ≥ 60 mL/min/1.73m^2. All groups consisted of outpatients attending the Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil) between October 2013 and November 2014. Exclusion criteria were age below 18 years old, cancer, pancreatitis, acute infections, secondary T2DM, dialysis, transplantation, pregnancy and alcohol or drug abuse. Fourteen patients refused to participate in the study.

The diagnosis of T2DM and increased UAE was based on American Diabetes Association criteria [22, 23]. Two of three spot urine samples were considered for classification of increased

UAE. The eGFR was assessed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [24].

This study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre and all subjects received adequate information about the study and gave their written informed consent.

Clinical, anthropometric and biochemistry assessment

Demographic and clinical data were collected using a standard questionnaire and review of medical registry, including the following variables: age, gender, T2DM duration, arterial hypertension, systolic and diastolic blood pressure and the use of antidiabetic, antihypertensive and antilipidemic medications. Hypertension was defined by blood pressure $\geq$140/90 mmHg or antihypertensive medication use.

All anthropometric evaluations were performed by the same dietitian and consisted of weight, height, and waist circumference. Body weight (kg) and height (m) were assessed in order to calculate BMI (kg/m^2). Waist circumference was measured at the midpoint between the lowest rib and the iliac crest, using a flexible, inelastic measuring tape. Body composition was measured with a direct segmental multiple-frequency bioelectrical impedance analysis method (InBody 230; Biospace, Seoul, Korea) to assess body fat percentage (BF%) and trunk fat (kg). The measurements were performed with the patient fasting, without shoes, wearing light clothing, in a stable condition [25].

Blood and spot urine samples were taken after 12-hour overnight fasting. High-sensitivity C reactive protein (hsCRP), fasting plasma glucose, total cholesterol, HDL-cholesterol, triglycerides, proteinuria, albuminuria and urinary creatinine were determined using standard local laboratory techniques. Serum creatinine was measured by the Jaffe method (Modular P, Roche Diagnostic, Mannheim, Germany) traceable to isotope dilution mass spectrometry [26]. HbA1c was measured by the HPLC method (Bio-Rad Variant™ II Turbo analyzer), as standardized by the National Glycohemoglobin Standardization Program (http://www.ngsp.org/certified.asp) and aligned with the International Federation of Clinical Chemistry [27]. The Clinical Pathology Department participates in an HbA1c External Quality Assurance Program with excellent performance. LDL-cholesterol was calculated using the Friedewald formula when triglyceride levels were lower than 400 mg/dL.

Blood and urine were centrifuged, and samples obtained were stored in duplicates at -80°C for later PGRN and IL-6 analysis. The PGRN concentration was determined in serum and urine samples, using the Human Progranulin Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). Of all samples (serum and urine), 30.7% were performed in duplicates. The assay sensitivity was 0.54 ng/mL and assay range was 1.56–100 ng/mL, whereas the inter-assay coefficient was less than 10% for serum and urine samples. The IL-6 concentration was assessed in serum samples by Human IL-6 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). Duplicates were performed in 34.2% of serum samples. The assay sensitivity was 0.7 pg/mL, with a range between 3.12–300 pg/mL and inter-assay coefficient less than 6.5%.

Statistical analyses

The sample size calculation was based on previous studies [10, 12, 20], in which we observed an approximate difference of one standard deviation in PGRN serum levels between groups of interest, and a correlation of 0.3 between serum PGRN and some biochemical markers. Therefore, considering $\alpha = 0.05$ and $\beta = 0.10$ errors, the total sample estimated was 113 individuals.

Data were analyzed using the Statistical Package for Social Sciences version 20.0 program (SPSS, Chicago, IL). After assessing normality of continuous variables by the Shapiro Wilk test,

the study groups were compared by One-Way Analysis of Variances (ANOVA) with Levene and Tukey or Kruskal-Wallis with Dunn tests, as appropriate. Data with normal distribution are presented as mean ± SD, whereas data with asymmetric distribution are presented as median (interquartile range). Categorical variables were compared among groups by Chi-square test and they are reported as absolute numbers and percentages. Correlations were tested by Spearman's correlation coefficient, since the serum and urinary PGRN variables presented an asymmetric distribution. Multivariate linear regression analyses were performed, using serum or urinary PGRN as dependent variables. The independent variables included in each model were selected if they were correlated with PGRN using Spearman's correlation coefficient and had no collinearity. Normal distribution of residuals was accepted in multivariate linear regression analyses. Only valid cases were included in each analysis. The level of statistical significance was established as 5%.

Results

Clinical characteristics

Patients' demographic and clinical characteristics are shown in Table 1. Insulin was the main hypoglycemic agent used by advanced cases patients, while other T2DM groups were treated mainly with oral agents, such as metformin and glibenclamide. Patients with diabetes also presented more hypertension and use of anti-hypertensive medication and statins than the non-diabetic group. BMI and body composition assessed by BF% and trunk fat were similar in the four study groups. Waist circumference was lower in non-diabetic subjects when compared to advanced DKD patients. Non-diabetic subjects had higher LDL-cholesterol, while advanced DKD cases had worse HDL-cholesterol and triglyceride levels. Elevated IL-6 was also observed in the advanced DKD group (Table 1).

Serum and urinary PGRN concentrations

Serum PGRN was increased in advanced DKD patients compared to the other groups [(70.84 (59.04–83.16) vs. albuminuric DKD cases 57.16 (42.24–67.38), diabetic controls 57.28 (42.08–70.47) and non-diabetic controls 44.54 (41.44–53.32) ng/mL; $p<0.001$] (Fig 1A). There was no difference in PGRN serum levels between albuminuric DKD cases, diabetic controls and non-diabetic groups. However, the nominal values for the diabetic groups seemed to be higher than for the non-diabetic group. To evaluate whether this was due to sample size effect, we analyzed diabetic controls and albuminuric DKD patients together versus subjects without diabetes. PGRN serum levels in the first group [57.16 (42.62–69.18) ng/mL; n = 65] were significantly higher than non-diabetic subjects [44.54 (41.44–53.32) ng/mL; n = 26; p = 0.014].

Urinary PGRN was decreased in advanced DKD cases when compared to albuminuric DKD patients [10.62 (6.30–16.08) vs. 20.94 (12.35–30.22); diabetic controls 14.06 (9.88–20.82) and non-diabetic controls 13.51 (7.94–24.36) ng/mL; p = 0.017] (Fig 1B). The other groups presented similar levels of PGRN in urine. Urinary PGRN levels were not available for seven patients, so this analysis was performed including 107 individuals.

Correlations

The relationships of serum and urine PGRN were evaluated using Spearman's correlation test (Table 2). Taking the entire group, the serum PGRN correlated with BMI (r = 0.27; p = 0.004), waist circumference (r = 0.25; p = 0.007), BF% (r = 0.20; p = 0.042); hsCRP (r = 0.35; $p<0.001$), IL-6 (r = 0.37; $p<0.001$), albuminuria (r = 0.25; p = 0.008) and proteinuria (r = 0.24; p = 0.010). A negative correlation with eGFR (r = -0.22; p = 0.023) was observed (Table 2). In multivariate

Table 1. Clinical and laboratory characteristics of study subjects.

	Non-diabetic controls (n = 26)	Diabetic controls (n = 40)	Albuminuric DKD cases (n = 25)	Advanced DKD cases (n = 23)	P value
Age (years)	58.8 ± 10.8	59.8 ± 8.2	63.3 ± 7.9	61.5 ± 9.8	0.290
Male gender, n (%)	12 (46.2)	19 (47.5)	11 (44.0)	12 (52.2)	0.952
Diabetes mellitus duration (years)	-	14.9 ± 9.9	14.2 ± 7.8	18.0 ± 9.1	0.307
Antidiabetic agents, n (%)					
Insulin	-	22 (55.0) [a]	18 (72.0) [ab]	23 (100) [b]	0.001
Metformin	-	34 (85.0) [a]	25 (100) [a]	4 (17.4) [b]	<0.001
Glibenclamide	-	16 (40.0) [a]	10 (40.0) [a]	2 (8.7) [b]	<0.001
Statin use, n (%)	4 (15.4) [a]	27 (67.5) [b]	21 (84.0) [b]	22 (95.7) [b]	<0.001
Anti-hypertensive medication use, n (%)	10 (38.5) [a]	37 (92.5) [b]	25 (100) [b]	23 (100) [b]	<0.001
Hypertension n (%)	12 (52.2) [a]	38 (95.0) [b]	25 (100) [b]	23 (100) [b]	<0.001
Systolic blood pressure (mmHg)	130.8 ± 14.7	137.2 ± 21.4	139.5 ± 14.2	144.7 ± 20.6	0.087
Diastolic blood pressure (mmHg)	78.6 ± 10.1	80.5 ± 12.3	79.5 ± 11.9	82.9 ± 13.2	0.645
Body mass index (kg/m^2)	28.7 (25.5–32.0)	30.8 (26.8–35.8)	31.8 (27.4–36.7)	30.9 (28.0–38.5)	0.212
Waist circumference (cm)	99.8 ± 13.3 [a]	105.1 ± 13.7 [ab]	109.2 ± 12.0 [ab]	111.0 ± 18.9 [b]	0.036
Body fat %	36.50 ± 9.44	36.47 ± 9.20	36.53 ± 11.11	37.66 ± 11.84	0.976
Trunk fat (kg)	15.42 ± 5.82	16.27 ± 5.42	16.20 ± 5.16	16.19 ± 6.58	0.954
Fasting plasma glucose (mg/dL)	90.0 (84.3–94.0) [a]	141.5 (114.8–170.5) [b]	166 (94.5–227.5) [b]	139 (97–178) [b]	<0.001
HbA1c (%)	5.6 (5.3–5.7) [a]	7.9 (6.9–9.2) [b]	8.7 (7.6–9.4) [b]	7.9 (7.2–9.4) [b]	<0.001
HbA1c (mmol/mol)	38 (34–39) [a]	63 (52–77) [b]	72 (60–79) [b]	63 (55–79) [b]	<0.001
Total cholesterol (mg/dL)	188 (165.8–215.8)	172.5 (145.3–193.8)	172 (151–200.5)	170 (147–213)	0.102
LDL-cholesterol (mg/dL)	122.4 (101.1–142.9) [a]	102.1 (79–124.4) [ab]	92.4 (79.4–99) [bc]	89 (72–122.5) [bc]	0.002
HDL-cholesterol (mg/dL)	46.0 (38.8–51.3) [a]	40.5 (35.0–45.8) [ab]	37.0 (30.0–44.0) [b]	36.0 (30.0–44.0) [b]	0.004
Triglycerides (mg/dL)	127.5 (84.3–168.5) [a]	139.0 (96.0–192.8) [ab]	167.0 (122.5–298.5) [bc]	223.0 (148.0–288.0) [c]	0.001
hsCRP (mg/dL)	3.34 (1.81–10.80)	3.44 (1.13–8.06)	2.71 (1.78–7.04)	6.06 (1.89–18.56)	0.382
IL-6 (pg/mL)	3.12 (3.12–3.17) [a]	3.12 (3.12–3.94) [a]	3.12 (3.12–4.06) [a]	7.35 (4.18–10.27) [b]	<0.001
eGFR (mL/min/1.73m^2)	97.2 (78.7–109.8) [a]	95.6 (86.1–115.8) [a]	98.0 (88.0–104.5) [a]	23.0 (17.0–33.6) [b]	<0.001
Albuminuria (mg/L)	7.4 (3.0–12.3) [a]	10.7 (4.58–18.93) [a]	100.5 (63.55–181.5) [b]	459.2 (186.2–1561) [b]	<0.001
UAE (mg albumin/g creatinine)	5.87 (3.78–8.46) [a]	7.32 (4.24–16.12) [a]	81.92 (43.02–168.13) [b]	718.7 (157.8–2142) [b]	<0.001
Proteinuria (mg/L)	80 (40–180) [a]	60 (70–108) [a]	250 (180–350) [b]	880 (400–2290) [b]	<0.001

hsCRP: high-sensitivity C reactive protein; IL-6: interleukin-6; eGFR: estimated glomerular filtration rate; UAE: urinary albumin excretion.

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linear regression, serum PGRN was inversely and independently associated with eGFR (beta = -0.28; p = 0.006) (Table 3). The interaction between diabetes and eGFR was analyzed in the multivariate model, and no association was observed (beta = 0.25, p = 0.761).

To identify associations between serum PGRN and other covariates in patients with preserved renal filtration, we performed Spearman's correlation test excluding the advanced DKD group. Serum PGRN remained associated with BMI (r = 0.32; p = 0.002), waist circumference (r = 0.28; p = 0.007), BF% (r = 0.29; p = 0.008) and hsCRP (r = 0.35; p <0.001), and was also associated with trunk fat (r = 0.27; p = 0.016). In a multivariate linear regression excluding the advanced DKD group, serum PGRN remained associated with BMI (beta = 0.30; p = 0.004), and was also associated with T2DM (beta = 0.21; p = 0.040), independently of age, gender and hsCRP (Table 3).

Urinary PGRN was positively associated with albuminuria (r = 0.25; p = 0.010) and proteinuria (r = 0.38; p <0.001) (Table 2). In multivariate linear regression analysis, albuminuria was

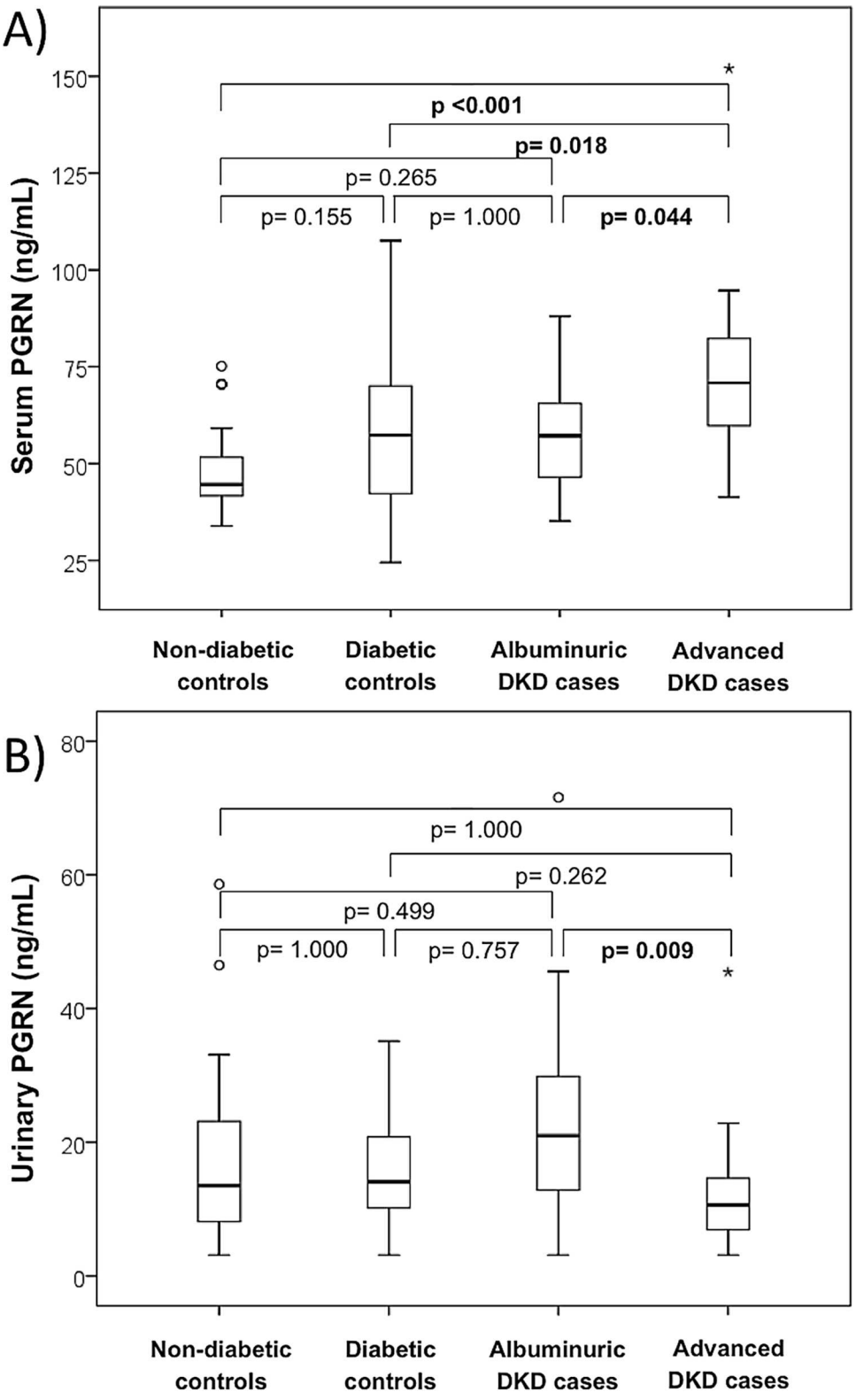

Fig 1. Boxplots showing serum (A) and urinary (B) levels of PGRN (ng/mL) according to study groups. PGRN: progranulin; DKD: diabetic kidney disease.

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Table 2. Correlations between serum / urinary PGRN and other parameters.

All patients	Serum PGRN r (P) (n = 114)	Urinary PGRN r (P) (n = 107)
Diabetes mellitus duration (years)	0.06 (0.555)	-0.07 (0.551)
Systolic blood pressure (mmHg)	0.01 (0.900)	-0.02 (0.863)
Diastolic blood pressure (mmHg)	0.04 (0.655)	0.17 (0.096)
Body mass index (kg/m^2)	0.27 (0.004)	0.12 (0.219)
Waist circumference (cm)	0.25 (0.007)	0.16 (0.091)
Body fat %	0.20 (0.042)	-0.01 (0.901)
Trunk fat (kg)	0.16 (0.117)	0.10 (0.300)
Fasting plasma glucose (mg/dL)	0.13 (0.158)	0.06 (0.551)
HbA1c (%, mmol/mol)	0.16 (0.095)	0.05 (0.611)
Total cholesterol (mg/dL)	0.03 (0.743)	0.01 (0.966)
LDL-cholesterol (mg/dL)	-0.08 (0.424)	-0.01 (0.951)
HDL-cholesterol (mg/dL)	-0.05 (0.622)	-0.14 (0.165)
Triglycerides (mg/dL)	0.15 (0.120)	0.08 (0.407)
hsCRP (mg/dL)	0.35 (<0.001)	0.18 (0.071)
IL-6 (pg/mL)	0.37 (<0.001)	-0.06 (0.553)
eGFR (mL/min/1.73m^2)	-0.22 (0.023)	0.16 (0.101)
Albuminuria (mg/L)	0.25 (0.008)	0.25 (0.010)
Proteinuria (mg/L)	0.24 (0.010)	0.38 (<0.001)

PGRN: progranulin; hsCRP: high-sensitivity C reactive protein; IL-6: interleukin-6; eGFR: estimated glomerular filtration rate.

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associated with urinary PGRN (beta = 0.28; p = 0.013) independently of eGFR, age, gender and T2DM (Table 3). Serum and urinary PGRN correlations with UAE were maintained. There was no correlation between serum and urinary PGRN (r = 0.08; p = 0.400).

Discussion

In the present study, we were able to better characterize serum and urinary PGRN levels in patients with T2DM according to renal function. Patients with diabetes and eGFR <60 mL/min/1.73m^2 presented elevated serum and reduced urinary levels of PGRN. Moreover, this adipokine was higher in serum of T2DM patients when compared to non-diabetic subjects, independently of renal function. Correlations with inflammatory, adiposity and renal function markers were also described.

Previous data regarding PGRN and kidney disease are reported by few studies. Xu et al. [18] found elevated serum PGRN concentrations in patients with T2DM and macroalbuminuria (UAE rate >300 mg/24h). However, that subset of patients also presented reduced eGFR [18]. In a recent study evaluating 532 patients with CKD stages 1–5, Richter et al. [20] observed that PGRN serum levels significantly increased with deterioration of renal function. Besides, in our sample, the elevated serum PGRN concentration among subjects with advanced DKD was accompanied with a low PGRN in urine. Despite we did not find a correlation between serum and urinary levels of PGRN, in agreement with Richter et al., who also did not find it in a subgroup of their study population [20, 28], it is supposed that renal filtration is an important route of PGRN elimination [28].

There is evidence that PGRN expression in the kidney is reduced in mice models of acute kidney injury [29] and DKD [30]; however, circulating PGRN is increased [29, 30]. In humans, higher levels of serum PGRN are observed in end-stage CKD [20] and also after nephrectomy,

Table 3. Multivariate linear regression analysis models.

Variable		Beta	P value
Dependent variable: Serum PGRN			
	All sample (n = 106)		
	Age (years)	-0.14	0.139
	Male gender	0.12	0.060
	Body mass index (kg/m^2)	0.05	0.595
	hsCRP (mg/dL)	0.12	0.244
	IL-6 (pg/mL)	0.09	0.364
	eGFR (mL/min/1.73m^2)	-0.28	0.006
	Type 2 diabetes mellitus	0.16	0.095
	Individuals with eGFR $\geq$ 60 mL/min/1.73m^2 (n = 90)		
	Age (years)	-0.07	0.462
	Male gender	0.08	0.422
	Body mass index (kg/m^2)	0.30	0.004
	hsCRP (mg/dL)	0.15	0.139
	Type 2 diabetes mellitus	0.21	0.040
Dependent variable: Urinary PGRN			
	All sample (n = 107)		
	Age (years)	-0.02	0.813
	Male gender	-0.07	0.465
	Albuminuria (mg/L)	0.28	0.013
	eGFR (mL/min/1.73m^2)	0.34	0.004
	Type 2 diabetes mellitus	0.19	0.848

PGRN: progranulin; hsCRP: high-sensitivity C reactive protein; IL-6: interleukin-6; eGFR: estimated glomerular filtration rate.

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a model of acute kidney insufficiency [28]. Moreover, a negative correlation between serum PGRN and eGFR has been previously reported in the literature [18, 20], even as we observed in the present study. Therefore, the relationship of PGRN with kidney disease appears to be its accumulation due to the eGFR decrease, suggesting renal clearance as a route of PGRN elimination. It corroborates our findings of higher PGRN in serum and lower in urine of advanced DKD cases.

On the other hand, increased circulating PGRN might be a compensatory mechanism to reduce renal deterioration, since it was demonstrated that PGRN could attenuate inflammation in an acute condition [29]. In a mouse model of renal ischemia-reperfusion injury, Zhou et al. [29] observed that PGRN deficiency was associated with higher elevation of serum creatinine and blood urea nitrogen, more severe morphological injury and higher inflammatory response. The administration of recombinant PGRN in vitro attenuated inflammation, exerting a protective role in acute kidney injury [29]. However, it is speculated that PGRN could play different functions in different metabolic conditions [19] and further studies are needed to understand whether increased serum PGRN in advanced DKD in T2DM patients could play an anti-inflammatory role or whether its accumulation in serum is only due to the decreased eGFR.

To our knowledge, this is the first study evaluating the association of urinary PGRN and DKD in T2DM. Previously, Schlatzer et al. [21] investigated PGRN in urine of 74 patients with type 1 diabetes mellitus and concluded that a panel of three proteins (Tamms-Horsfall glycoprotein, clusterin and human α-1 acid glycoprotein) plus PGRN could be used to predict early signs of DKD [21]. The present study design does not allow us to identify urinary PGRN as an

early marker of DKD. However, our findings show a positive correlation of urinary PGRN with albuminuria. Albumin is a protein of 67 kDa [31], a very similar size to PGRN (66–88 kDa) [1]. Possibly, their similar molecular size affects their renal clearance, but when eGFR decreases at <60 mL/min/1.73m^2, PGRN may accumulate in serum (advanced DKD patients presented higher PGRN in serum and lower in urine). However, the renal mechanisms possibly involved are still unknown.

Studies regarding PGRN in diabetes should consider the eGFR, since kidney disease is a common complication that could influence the result. Previous data comparing patients with T2DM to non-diabetic subjects have reported elevated serum PGRN associated with the disease [10–12]. The studies of Tönjes et al. [12] and Youn et al. [11] observed increased serum PGRN in patients with T2DM; however they did not consider eGFR in their analysis. On the other hand, Qu et al. [10] excluded patients with renal disease from their study and, even so, observed elevated serum PGRN in patients with T2DM, independently of obesity. Lastly, Xu et al. [18] did not find a significant difference in serum PGRN when comparing non-diabetic subjects with normoalbuminuric T2DM patients. In the present study, serum PGRN differences among albuminuric DKD cases, diabetic controls and non-diabetic groups did not reach statistical significance. But when diabetic controls and albuminuric DKD patients were grouped and compared to subjects without diabetes, we observed that serum PGRN are higher in patients with diabetes, independently of kidney disease.

The association of PGRN in diabetes is in accordance with its putative role in insulin resistance [14, 19]. Experimental studies reported that PGRN promotes IL-6 expression, impacting on insulin signaling [14]. The adipokine PGRN has been previously associated with inflammatory markers, as hsCRP [11, 15] and IL-6 [10, 15, 18]. In our study, we also observed a positive correlation between serum PGRN, hsCRP and IL-6, corroborating the pro-inflammatory effects previously suggested for PGRN [3, 10]. There is also some evidence supporting a correlation of PGRN with HbA1C and fasting plasma glucose [10, 11, 32]; however, these associations were not observed by Xu et al. [18], even as we did not observe them in the present study.

PGRN is secreted by adipocytes [3] and acts as a chemoattractant molecule which bring monocytes into adipose tissue, favoring chronic inflammation, obesity and its consequences [11]. Previous studies report the association of PGRN with obesity, with higher serum levels in obese subjects, independently of diabetes [10, 11]. In our sample, serum PGRN was associated with BMI and also with other measurements of adiposity such as waist circumference and BF %, corroborating previous data [10–12, 32].

This study provides a comprehensive and integrated evaluation of a new adipokine in DKD in T2DM. However, there are some limitations. First, the cross-sectional design does not allow an investigation of a causative role between DKD development and changes in PGRN levels. Second, the sample size is relatively small, but we had power to conduct the study, based on previous sample size calculation. Indeed, secondary analyses must be carefully interpreted. Further studies with a longitudinal design are necessary to investigate the association of urinary PGRN levels and DKD in T2DM.

In conclusion, our results suggest that serum PGRN depends on eGFR. Serum PGRN is elevated among patients with low eGFR and urinary PGRN correlates with albuminuria. Furthermore, PGRN correlates with adiposity and inflammation markers, and is associated with T2DM. Prospective studies are needed to find out whether the PGRN might be related to the prognosis of DKD.

Author Contributions

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Formal analysis: BBN LHC.

Funding acquisition: LHC.

Investigation: BBN TCK DC.

Methodology: BBN LHC.

Project administration: BBN LHC.

Resources: LHC.

Supervision: LHC.

Visualization: BBN DC LHC.

Writing – original draft: BBN LHC.

Writing – review & editing: BBN DC LHC.

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6 ARTIGO ORIGINAL II

Título

Progranulin serum levels in human kidney transplant recipients: A longitudinal study

Autores

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Progranulin serum levels in human kidney transplant recipients: A longitudinal study

Short title: Progranulin in kidney transplantation

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Abstract

Background: The adipokine progranulin has metabolic proprieties, playing a role in obesity and insulin resistance. Its levels seems to be dependent of renal function, since higher progranulin concentration is observed in patients with end-stage kidney disease. However, the effect of kidney transplantation on progranulin remains unknown. **Objective:** To assess the serum progranulin levels in kidney transplant recipients before and after kidney transplantation. **Methods:** Forty-six prospective kidney transplant recipients were included in this longitudinal study. They were evaluated before transplantation and at three and twelve months after transplantation. Clinical, anthropometric and laboratorial measurements were assessed. Progranulin was determined with enzyme-linked immunosorbent assays. **Results:** Serum progranulin significantly decreased in the early period after transplantation (from 72.78 ± 2.86 ng/mL before transplantation to 40.65 ± 1.49 ng/mL at three months; $p<0.001$) and increased at one year (53.15 ± 2.55 ng/mL; $p<0.001$ vs. three months), remaining significantly lower than before transplantation ($p<0.001$) ($p_{over\ time}<0.001$). At one year after transplantation, there was a significant increase in body mass index, trunk fat and waist circumference compared to immediate period after transplantation. **Conclusion:** Progranulin serum levels are increased before transplantation and a reduction is observed in the early period after transplantation, possibly attributed to an improvement in renal function. At one year after transplantation, increment in progranulin levels is probably associated to increased body mass index, waist circumference and trunk fat.

Introduction

Progranulin (PGRN), also known as proepithelin, granulin/epithelin precursor, or PC cell–derived growth factor, has emerged as a protein with growth factor-like properties, being involved in tissue remodeling, tumorigenesis and neurodegenerative diseases (1-4). More recently, it has been associated with obesity and insulin resistance (5, 6). PGRN is expressed in epithelial cells, immune cells, neurons and also in adipocytes (7), emerging as a novel adipokine with a function in glucose and insulin metabolisms (5, 6). PGRN plays a role in adipose tissue, recruiting monocytes (8) and promoting interleukin-6 (IL-6) expression (5), which favors inflammation and insulin resistance. Previous studies have demonstrate that serum PGRN is increased in obesity and type 2 diabetes mellitus (T2DM) (8-10).

Elevated serum PGRN could also been observed in chronic kidney disease (CKD) (11, 12), and patients at stage 5 of CKD have increased PGRN levels (12). Moreover, patients with diabetic kidney disease, with estimated glomerular filtration rate (eGFR) <60 mL/min/1.73m², also presented higher serum PGRN than diabetic and non-diabetic patients with preserved renal function (11). A negative correlation between serum PGRN and eGFR has been described (11-13).

Kidney transplantation is often followed by complications such as weight gain, increased body fat, dyslipidemia, metabolic syndrome and new onset diabetes after transplantation. These complications are possibly related to immunosuppressive therapy and changes in the metabolism (14-16). Many adipokines have been studied in this context (17). After kidney transplantation, their levels decrease, perhaps due to an improvement in eGFR (14, 18); however, in the later period after transplantation, adipokine serum levels tend to increase (14).

Considering that the effect of kidney transplantation on PGRN serum levels has not been studied, and that this adipokine is related to renal function, we aimed to assess the effect of kidney transplant on serum PGRN concentration and its association with metabolic indexes.

Materials and methods

Design and patients

Forty-six patients who underwent kidney transplantation at the Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil) between December 2014 and August 2015 were included in this longitudinal study. Kidney recipients were evaluated before transplantation and at three and twelve months after transplant. Data and blood samples at pre-transplant period were collected two days before surgery for the living donor recipients and immediately before surgery for the deceased donor organ recipients. A control group of 40 outpatients attending at the same hospital was included in the study. Controls were selected based on their renal function (eGFR between 30 and 90 mL/min/1.73m^2), in order to match then to kidney recipients at twelve months (considering that eGFR at this moment would be comparable to controls). Moreover, groups were paired by age, gender and body mass index (BMI). Exclusion criteria were age below 18 years old, multiorgan transplantation, re-transplants, cancer, acute infections, Cushing's disease, systemic lupus erythematosus disease, pregnancy, alcohol or drug abuse and kidney recipients who did not reach three months of transplantation with a functioning graft.

This study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre and all subjects received adequate information about the study and gave their written informed consent.

Clinical, anthropometric and laboratorial assessment

Demographic and clinical data were assessed using a standard questionnaire and review of medical registry, including the following variables: age, gender, ethnicity, primary kidney disease, dialysis modality and duration, donor type, immunosuppressive agents used, cumulative prednisone dose, arterial hypertension and previous or development of diabetes mellitus. Hypertension was defined by blood pressure ≥140/90 mmHg or antihypertensive medication use; while new onset diabetes after transplantation was defined by American Diabetes Association criteria (19, 20).

Anthropometric assessment consisted of weight, height, and waist circumference. Body weight (kg) and height (m) were evaluated in order to calculate BMI (kg/m^2) (21). Waist circumference was measured at the midpoint between the lowest rib and the iliac crest, using a flexible, inelastic measuring tape (22). Body fat percentage (BF%) was assessed by two methods: 1) Tetrapolar bioelectric impedance device (Biodynamics 450; Biodynamics Corp Seattle, Washington, USA), using current of 800 microA and frequency of 50HkHz was performed at all study moments and groups; and 2) Dual-energy X-ray absorptiometry (DEXA) using a Lunar iDXA Densitometer and enCORE software (version 13,60,033; GE Healthcare, Madison, USA) was applied in control patients and in kidney recipients at three and twelve months after transplantation, since the pre-transplant logistics did not allow this

analysis. Values obtained by two methods were positively correlated in the present study (BF% at 3 months $r=0.88$, $p<0.001$ and 12 months $r=0.71$, $p<0.001$). The measurements were performed with the patient fasting, without shoes, wearing light clothing, in a stable condition (23).

Blood samples were drawn after 12-hour overnight fasting and sera obtained by centrifugation were stored in duplicates at -80ºC. PGRN, adiponectin (ADPN) and IL-6 were determined with enzyme-linked immunosorbent assays (all R&D Systems, Minneapolis, MN, USA). Of all samples, 36.6%, 31.3% and 28.3% were performed in duplicates for PGRN, ADPN and IL-6, respectively. The assay sensitivity and assay range was 0.54 ng/mL and 1.56-100 ng/mL for PGRN, 0.891 ng/mL and 3.9-250 ng/mL for ADPN and 0.7 pg/mL and 3.12-300 pg/mL for IL-6. For PGRN and ADPN, the inter-assay coefficient was less than 8.5%, while for IL-6 it was less than 4%.

Serum creatinine, high-sensitivity C reactive protein (hsCRP), insulin, fasting plasma glucose (FPG), total cholesterol, HDL-cholesterol and triglycerides were determined using standard local laboratory techniques. LDL-cholesterol was calculated using the Friedewald formula when triglyceride levels were lower than 400 mg/dL. The Homeostasis Model Assessment (HOMA) index was used to calculate the insulin resistance: HOMA=plasma insulin (μUI/mL) x fasting glucose (mmol/L) / 22.5. The eGFR was assessed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (24).

Statistical analyses

The sample size calculation was based on a previous study that reported PGRN serum levels according to the 5 stages of CKD (12). The PGRN concentration observed in patients at stage 5 was considered to be equivalent to pre-transplant period, while PGRN levels observed at stage 3 were considered to be similar to post-transplant period and control group, since an equivalent eGFR was expected. A difference of 25 ng/mL between moments and groups was admitted. Considering $\alpha=0.05$ and $\beta=0.10$ errors and 20% losses after kidney transplantation, the estimated sample size was 35 individuals in control group and 45 kidney transplant patients.

Data were analyzed using the Statistical Package for Social Sciences version 20.0 program (SPSS, Chicago, IL). Normality of continuous variables was assessed by the Shapiro Wilk test. Data with normal distribution are presented as mean ± standard error, whereas data with asymmetric distribution are presented as median (interquartile range). Generalized estimating equations with linear model and Bonferroni correction were used to assess changes over time in kidney transplantation group when variables presented normal distribution. For nonparametric variables, Friedman test was used. Body fat and trunk fat assessed by DEXA were compared at three and twelve months in kidney transplant recipients by paired t test. For comparisons between control group an kidney recipients at twelve months after transplantation, Student's t test or Mann Whitney test were used, as appropriate. Categorical variables were compared among groups by Chi-square test and they are reported as absolute numbers and percentages. Correlations were tested by Pearson's or Spearman's correlation coefficient, according to variable

158 distribution. Only valid cases were included in each analysis. The level of statistical
159 significance was established at lower than 5%.

160

161 Results

162 Clinical characteristics

163 Sixty-four patients were assessed for eligibility. The exclusions were: eight re-
164 transplantation, five kidney-pancreas transplantation and two transplants in
165 systemic lupus erythematosus recipients. Forty-nine kidney transplant recipients were
166 initially enrolled in the study. Three patients did not reach three months of
167 transplantation with functioning graft and were not included in the analyses. Of the
168 forty-six patients included, two lost their kidney graft and two died before the first year
169 of transplantation.

170 Kidney transplant recipients and controls had similar age, gender and ethnicity
171 distribution (Table 1). Hypertension had high prevalence in both groups, and diabetes
172 mellitus was present in 19.6% in kidney patients before transplantation and in 28.2%
173 of control patients (p=0.444). After transplantation, five patients (10.9%) developed
174 new-onset diabetes after transplant and the prevalence of diabetes in the study group
175 at the first year of transplantation was 30.5% (p>0.999 vs. control group) (Table 1).

176 Among the study group, most patients had unknown primary kidney disease
177 (32.6%), followed by hypertension (21.7%), diabetes mellitus (17.4%),
178 glomerulonephritis (10.9%), adult polycystic kidney disease (10.9%), and other
179 conditions (6.5%) (Table 1). Before transplantation, most patients were on

hemodialysis (91.3%) and the median dialysis duration was 24 (14.5 – 52.5) months. Most patients (78.3%) received their kidney from deceased donors (Table 1).

Table 1. Basal characteristics of the study groups.

	TRANSPLANTATION GROUP (n=46)	CONTROL GROUP (n=40)	P value
Age, years	49.2 ± 2.1	51.8 ± 2.0	0.402
Male gender, n (%)	27 (58.7)	23 (57.5)	>0.999
White ethnicity, n (%)	30 (65.2)	30 (75.0)	0.356
Hypertension, n (%)	41 (89.1)	35 (87.5)	>0.999
Diabetes mellitus, n (%)			
Basal	9 (19.6)	11 (28.2)	0.444
Post-transplant	14 (30.5)		>0.999
Primary kidney disease, n (%)			
Unknown	15 (32.6)		
Hypertension	10 (21.7)		
Diabetes mellitus	8 (17.4)		
Glomerulonephritis	5 (10.9)		
Polycystic kidney disease	5 (10.9)		
Others	3 (6.5)		
Renal replacement therapy, n (%)			
Hemodialysis	42 (91.3)		
Hemodialysis and peritoneal dialysis	2 (4.3)		
Preemptive transplantation	2 (4.3)		
Dialysis duration before transplantation, months	24 (14.5 – 52.5)		
Donor type, deceased, n (%)	36 (78.3)		

Immunosuppression was achieved with prednisone, tacrolimus and mycophenolate for 97.8% of the transplant recipients. One patient (2.2%) received cyclosporine instead of tacrolimus. Induction therapy was employed in 82.6% of patients, basiliximab was used for 10 patients (21.7%) and antithymocyte globulin (ATG) for 28 (60.9%). The cumulative per patient prednisone dose at the end of the first year after transplantation was 3.35 (3.29 – 3.46) g.

Laboratorial, anthropometric and body composition characteristics

Laboratory tests, anthropometric measurements and body composition variables are presented in Table 2. As expected, renal function improved after transplantation at 12 months and was similar to control group at this time. Except for IL-6, that was higher in the kidney recipients, no other studied variable presented statistically significant differences between groups.

In the transplant group, changes in hsCRP, FPG, total cholesterol, HDL-cholesterol, BMI and waist circumference were observed over time (Table 2). In the early period after transplantation, there was a significant reduction in the hsCRP and increment in the FPG, total cholesterol and HDL-cholesterol levels. At one year after transplantation, there was a significant increase in BMI and waist circumference compared to immediate period after transplantation. There was also a gain in trunk fat during this period (Table 2).

207 **Table 2. Laboratorial, anthropometric and body composition characteristics of kidney transplant recipients and control group.**

	TRANSPLANTATION GROUP				CONTROL GROUP (n=40)	P value* (between groups)
	Before transplant (n=46)	3 months (n=46)	12 months (n=42)	P value (over time)		
eGFR (mL/min/1.73m²)	7.87 ± 0.42 [a]	49.3 ± 2.9 [b]	56.7 ± 3.7 [c]	**<0.001**	58.6 ± 2.9	0.772
IL-6 (pg/mL)	4.46 (3.12 – 6.92)	3.12 (3.12 – 5.87)	4.41 (3.12 – 7.54)	0.317	3.12 (3.12 – 4.03)	**<0.001**
hsCRP (mg/dL)	4.43 (2.14 – 10.31) [a]	2.20 (0.92 – 5.05) [b]	3.19 (1.33 – 6.37) [ab]	**0.022**	4.63 (1.60 – 9.48)	0.425
FPG (mg/dL)	91.0 (83.0 – 98.3) [a]	101.0 (86.8 – 115.0) [b]	97.0 (86.0 – 108.5) [ab]	**0.007**	93.0 (83.5 – 110.3)	0.568
Insulin (mg/dL)	7.60 (4.75 – 14.30)	9.85 (6.93 – 13.03)	8.90 (6.40 – 13.73)	0.065	9.85 (6.58 – 13.70)	0.498
HOMA	1.73 (0.98 – 3.31)	2.53 (1.63 – 3.55)	2.29 (1.34 – 3.83)	0.103	2.63 (1.52 – 3.77)	0.699
Total cholesterol (mg/dL)	165.6 ± 5.7 [a]	188.9 ± 6.8 [b]	179.9 ± 6.0 [ab]	**0.001**	184.1 ± 8.3	0.695
HDL-cholesterol (mg/dL)	35.3 ± 2.0 [a]	46.3 ± 2.1 [b]	46.3 ± 2.3 [b]	**<0.001**	44.2 ± 1.8	0.467
LDL-cholesterol (mg/dL)	92.9 ± 4.2	102.0 ± 5.1	96.8 ± 4.5	0.226	109.4 ± 7.1	0.120
Triglycerides (mg/dL)	158.0 (108.0 – 226.8)	183.0 (126.8 – 232.3)	148.5 (111.5 – 202.0)	0.262	136.5 (88.5 – 213.3)	0.224
Body mass index (kg/m²)	27.3 ± 0.7 [ab]	26.7 ± 0.7 [a]	27.9 ± 0.8 [b]	**<0.001**	29.5 ± 0.80	0.215
Waist circumference (cm)	93.3 ± 2.7 [ab]	93.8 ± 1.7 [a]	96.3 ± 2.0 [b]	**0.006**	99.9 ± 2.3	0.257
Bioimpedance body fat (%)	26.5 ± 1.5	27.5 ± 1.2	28.0 ± 1.3	0.404	27.9 ± 7.4	0.910
DEXA body fat (%)	-	33.2 ± 1.4	34.3 ± 1.4	0.064	35.2 ± 1.3	0.665
DEXA trunk fat (kg)	-	13.8 ± 0.9	14.8 ± 1.0	**0.018**	16.7 ± 1.1	0.186

208 eGFR: estimated glomerular filtration rate; IL-6: interleukin-6; hsCRP: high sensitivity C reactive protein; FPG: fasting plasma glucose; HOMA: Homeostasis
209 Model Assessment; DEXA: Dual-energy X-ray absorptiometry. * Control group was compared to transplantation group at 12 months.

Serum PGRN significantly decreased in the early period after transplantation (from 72.78 ± 2.86 ng/mL before transplantation to 40.65 ± 1.49 ng/mL at three months; $p<0.001$) and increased at one year (53.15 ± 2.55 ng/mL; $p<0.001$ vs. three months), remaining significantly lower than before transplantation ($p<0.001$) ($p_{over\ time}<0.001$). At 12 months, PGRN value was similar to controls (53.31 ± 2.11 ng/mL; $p=0.972$) (Figure 1).

Serum ADPN had a not-significant drop at three months [from 11.64 (7.74-22.55) µg/mL before transplantation to 9.08 (7.16-17.63) µg/mL at three months; $p=0.380$] and further dropped at twelve months [8.10 (6.06-11.82) µg/mL; $p<0.001$ vs. pre-transplant and $p=0.036$ vs. three months; $p_{over\ time}<0.001$]. At one year after transplantation, ADPN levels were significantly higher in kidney transplant recipients as compared to controls [3.71 (2.55-6.04) µg/mL; $p<0.001$) (Figure 2).

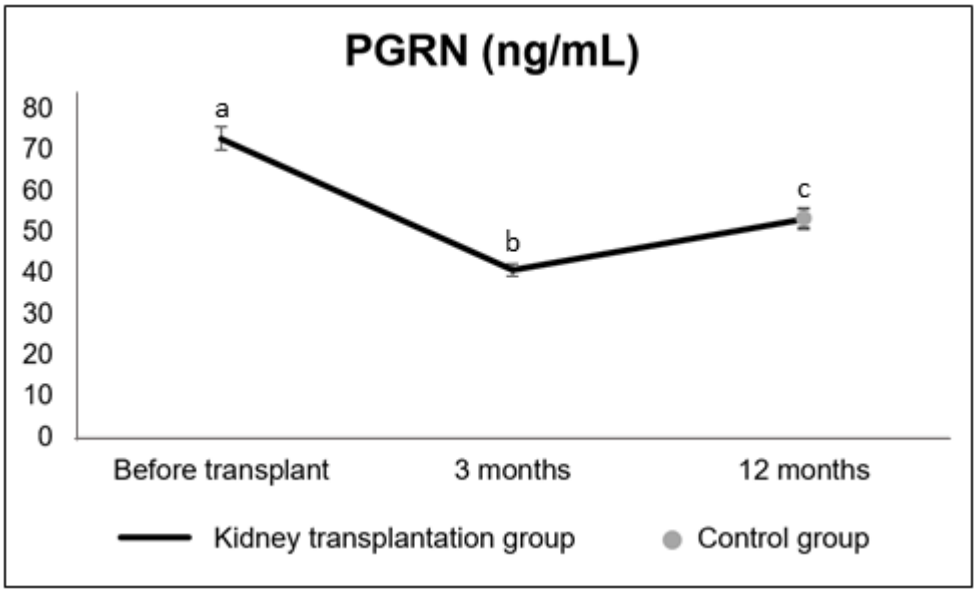

Figure 1. Serum PGRN up to 12 months after transplantation and in control group (mean ± standard error). PGRN: progranulin. Significant differences in the kidney transplant group are presented by letters (a, b, c).

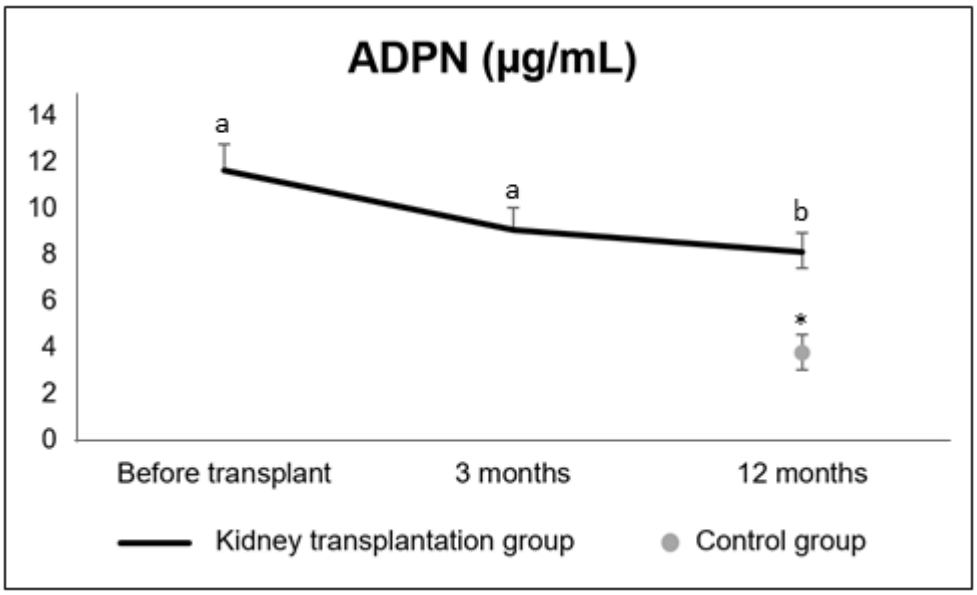

Figure 2. Serum ADPN up to 12 months after transplantation and control group (median ± standard error). ADPN: adiponectin. Significant differences in the kidney transplant group are presented by letters (a, b, c). * Significant difference between groups.

Correlations

Correlations between serum PGRN levels and eGFR, anthropometric and laboratory variables in kidney transplant recipients are presented in Table 3. A positive and significant correlation was observed between serum PGRN and IL-6 before kidney transplantation (r=0.413; p=0.004) and at three months after transplant (r=0.328; p=0.026) (Table 3). No other correlations were observed.

Table 3. Correlations between serum PGRN and laboratory and anthropometric variables in kidney transplant recipients.

	PGRN		
	Before transplant r (p)	**3 months r (p)**	**12 months r (p)**
eGFR	-0.244 (0.102)	-0.215 (0.152)	-0.297 (0.056)
Adiponectin	0.215 (0.151)	-0.028 (0.856)	0.007 (0.967)
Interleukin-6	**0.413 (0.004)**	**0.328 (0.026)**	0.282 (0.071)
High sensitivity C reactive protein	0.191 (0.215)	0.192 (0.202)	0.200 (0.205)
Fasting plasma glucose	-0.142 (0.348)	0.190 (0.205)	0.180 (0.260)
HOMA	-0.154 (0.319)	0.223 (0.137)	0.160 (0.318)
Body mass index	-0.006 (0.968)	0.066 (0.664)	-0.023 (0.886)
Waist circumference	0.116 (0.444)	0.225 (0.133)	0.203 (0.203)
Bioimpedance body fat	-0.229 (0.144)	0.056 (0.713)	-0.056 (0.728)
DEXA body fat	-	-0.029 (0.846)	0.074 (0.642)
DEXA trunk fat	-	0.072 (0.633)	0.066 (0.678)

eGFR: estimated glomerular filtration rate; HOMA: Homeostasis Model Assessment; DEXA: Dual-energy X-ray absorptiometry.

Discussion

In this longitudinal study, it was possible to demonstrate changes in PGRN serum concentration overtime after kidney transplantation. In patients with end-stage renal disease, PGRN is elevated, and it decreases upon eGFR improvement in the early period after kidney transplantation. At one year, an increment in PGRN is observed, seems to be independent of eGFR, and remained significantly lower than before transplantation.

The reduction in serum PGRN observed immediately after transplantation could be attributed to improvement in kidney function. To our knowledge, this is the first study evaluating the effect of kidney transplantation (and consequently greater eGFR) in PGRN levels. It seems that renal clearance is an important route of PGRN elimination. Some studies have demonstrate that patients with impaired renal function have increased PGRN circulating levels (11-13). Richter et al. (12) evaluated 532 patients with stages 1-5 of CKD and identified that PGRN serum levels are different among groups, being higher at stage 5. In that subset of patients, eGFR or CKD stage were independently associated with PGRN (12). Recently similar results were reported in patients with advanced diabetic kidney disease, who presented higher PGRN serum levels and lower urinary excretion (11). Also, Xu et al. (13) observed increased PGRN levels in macroalbuminuric T2DM patients, with reduced eGFR. In support, in a mice model of CKD, higher circulating PGRN levels were reported (25). These findings are consistent with the PGRN kinetics reported in the present study.

Weight gain is common following kidney transplantation. It occurs mainly due to increased appetite and freedom of dietary restrictions needed during the dialysis period (14, 26, 27). In this study, kidney transplant recipients had increased BMI,

waist circumference and trunk fat at one year after transplantation compared to three months. It was previously reported that these conditions are associated with higher serum PGRN (8-10). Qu et al. (10) compared serum PGRN in obese and non-obese patients without kidney disease and found higher levels in obese individuals. Youn et al. (8) investigated the relationship between PGRN and visceral adiposity. They found that patients with a predominantly visceral fat distribution had significantly higher PGRN serum levels (8). In agreement, we found increased serum PGRN levels at one year after transplantation, when patients presented altered adiposity markers. Previous studies show that PGRN has a positive correlation with BMI (8, 10, 11, 28), BF% and waist circumference (8, 9, 11, 28, 29). Such findings are notably absent in our study population due the limited sample size, short-term follow-up and small variability of the parameters.

We also observed a significant correlation between serum PGRN and IL-6 before and in the early period after transplantation. That comes in support to previous studies (10, 30) and suggests an important relationship between these markers pointing to PGRN as a pro-inflammatory molecule. Matsubara et al. (5) demonstrate that PGRN promotes IL-6 expression in adipose cells, which impairs insulin signaling. This adipokine has also chemotactic activity, recruiting monocytes into adipose tissue (8). Moreover, PGRN binds to the tumor necrosis factor receptor 1 (TNFR-1) (28, 31) and experimentally induces adipose insulin resistance (32). In humans, increased PGRN serum levels have been found in individuals with insulin resistance (33) and T2DM (6, 8-10).

PGRN metabolic functions are not fully understood. Some authors have reported that binding PGRN to TNFR-1 could impair tumor necrosis factor alfa (TNF-α) binding to its receptor, resulting in an anti-inflammatory effect (34, 35). This

hypothesis is also supported in a mice model of renal ischemia-reperfusion injury, where PGRN deficiency was associated with higher elevation of serum creatinine and blood urea nitrogen and its administration in vitro attenuated inflammation (36). Similarly, PGRN-deficient mice exposed to lipopolysaccharide (LPS) injection as an endotoxin-induced acute kidney injury model, presented increments of inflammatory markers, serum creatinine and blood urea nitrogen (37). Moreover, administration of recombinant PGRN before LPS treatment in wild-type mice was associated with reduced renal injury (37). Finally, in a mice model of hyperhomocysteinemia (a risk factor for kidney disease), PGRN-deficient mice also presented exacerbated renal injury, that could be ameliorated by pretreatment with recombinant human PGRN (38). In this context, PGRN could be a renal protective molecule in an inflammatory environment.

ADPN is a well-described anti-inflammatory adipokine, that is frequently reduced in obesity and insulin resistance conditions (39). In CKD, ADPN levels have been reported to be increased (18, 40, 41). This is in agreement with our findings before transplantation. It was previously described that the adipose tissue production of ADPN is increased in end-stage renal disease, contributing for its increased circulating levels (42). The beneficial effects of higher ADPN, however, are not effective in CKD, mainly due to ADPN resistance at the post-receptor level (43). In the present work, ADPN concentration decreased at three months and the decrement reached statistical significance at one year, findings that are corroborated by previous studies (17, 18, 41). This reduction could be associated with improved eGFR and other factors as increased in BMI, waist circumference and trunk fat (18, 39). We further observed that serum ADPN was increased in renal recipients compared to controls, which is also in accordance with other studies (17, 44, 45). No association

between PGRN and ADPN was observed. A finding corroborated by many (8, 30, 46), but not all (12) studies.

Other adipokines, mainly leptin, were previously evaluated in kidney transplant recipients (17). Serum leptin levels evaluated up to five years after renal transplantation (14) presented a similar behavior to PGRN: elevated levels pre-transplantation, decline in the early period after transplantation and later increment (14, 47). We believe that the reasons for such behaviors are similar: improvement in eGFR, followed by metabolic changes and weight gain occurring after kidney transplantation (14, 47).

This study provides relevant information regarding the effects of kidney transplantation on PGRN serum levels. However, there are some limitations. First, follow-up is relatively short and longer evaluation is needed to a better understanding of the relationships between PGRN and other parameters in kidney transplantation. Second, bioelectric impedance and DEXA methods have limitations in estimating body fat in kidney graft recipients (48); and parameters derived from these methods might be seeing cautiously.

In conclusion, PGRN serum levels are increased before transplantation and a reduction is observed in the early period after transplantation, possibly attributed to an improvement in the renal function. At later period of transplantation, increase in PGRN could be associate to metabolic changes, including higher BMI, waist circumference and trunk fat.

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7 CONCLUSÕES

Esta tese teve o objetivo de elucidar mecanismos de ação e funções da PGRN no metabolismo, além de investigar a relação dos níveis dessa adipocina na doença renal do diabetes e no transplante renal.

Através do estudo de revisão, foi possível identificar a PGRN como uma interessante molécula que parece desempenhar diferentes funções no organismo em distintas situações metabólicas. A ação inflamatória da PGRN associada à resistência insulínica e DM tipo 2 está bem descrita na literatura. Diversos mecanismos de ação têm sido apontados, como o aumento da expressão de IL-6, ligação com o TNFR-1, entre outros. Por outro lado, algumas funções anti-inflamatórias têm sido sugeridas para a PGRN, envolvendo esta molécula em doenças como psoríase e artrite. Quanto à função renal, está estabelecido que há uma relação com a redução da TFG, ocorrendo um aumento nos níveis séricos de PGRN. Acredita-se que a principal causa esteja associada à depuração renal, porém outras hipóteses têm sido sugeridas, relacionando a PGRN como uma molécula protetora que aumenta nessa condição.

No primeiro estudo original publicado, reforçamos evidências prévias de que os níveis de PGRN dependem da TFG e que pacientes em estágio avançado da DRD apresentam níveis elevados de PGRN sérica. Este foi o primeiro estudo avaliando a associação dos níveis urinários de PGRN na DRD no DM tipo 2. Observou-se que pacientes com DM tipo 2 e TFG < 60 mL/min/1,73m² apresentam maiores concentrações de PGRN no soro e menores na urina. Além disso, encontramos associações entre os níveis de PGRN e marcadores de obesidade e

inflamação, corroborando estudos anteriores. Ainda, foi possível estudar essas relações considerando a função renal. Neste contexto, identificou-se que o comprometimento da TFG é o principal fator que interfere nos níveis séricos de PGRN; e quando avaliam-se pacientes com função renal normal, parâmetros de obesidade e inflamação ganham influência (conforme demonstrado nas análises multivariadas).

No segundo estudo original, apresentamos os níveis séricos de PGRN em diferentes momentos do transplante renal. Como esperado, no pré-transplante renal, os níveis de PGRN estão aumentados, corroborando com achados anteriores. Após o transplante, os níveis reduzem no período imediato. Em um ano, aumentam significativamente em relação aos 3 meses, independente da função renal, mas mantém-se inferiores aos níveis observados no pré-transplante renal. Possivelmente a redução no primeiro momento esteja associada à melhora da TFG; enquanto o aumento no segundo período esteja relacionado ao ganho de peso em um ano pós-transplante. Porém, deve-se considerar ainda a hipótese de que a PGRN possa estar desempenhando o seu papel anti-inflamatório nesse contexto, atuando como mecanismo de proteção renal.

Apesar do conhecimento adicionado com esta tese, mais estudos são necessários para compreender se a elevação dos níveis de PGRN na doença renal pode ou não desempenhar um papel metabólico. Nesse sentido, é preciso elucidar se a ação da PGRN na DRC pode contribuir para um ambiente inflamatório e propenso à resistência insulínica ou, por lado, contribuir para um ambiente anti-inflamatório de proteção renal.