Universidade Federal do Triângulo Mineiro
Programa de Pós-Graduação em Ciências da Saúde

Liliane Silvano Araújo

AVALIAÇÃO DE CITOCINAS E QUIMIOCINAS NO SORO E EM BIÓPSIAS RENAIS DE PACIENTES DIABÉTICOS COM E SEM ALTERAÇÃO RENAL

Uberaba - MG
2019

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde, área de concentração Patologia Investigativa, da Universidade Federal do Triângulo Mineiro, como requisito parcial para obtenção do título de Doutor.
Orientadora: Profa. Dra. Juliana Reis Machado e Silva
Coorientadora: Profa. Dra. Marlene Antônia dos Reis

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2019

Catalogação na fonte: Biblioteca da Universidade Federal do Triângulo Mineiro

Araújo, Liliane Silvano
A69a Avaliação de citocinas e quimiocinas no soro e em biópsias renais de pacientes diabéticos com e sem alteração renal / Liliane Silvano Araújo. -- 2019.
116 f. : il., graf., tab.

Tese (Doutorado em Ciências da Saúde) -- Universidade Federal do Triângulo Mineiro, Uberaba, MG, 2019
Orientadora: Profa. Dra. Juliana Reis Machado e Silva
Coorientadora: Profa. Dra. Marlene Antônia dos Reis

1. Diabetes mellitus tipo 2. 2. Nefropatias diabéticas. 3. Taxa de filtração glomerular. 4. Mediadores da inflamação. I. Silva, Juliana Reis Machado e. II. Universidade Federal do Triângulo Mineiro. III. Título.

CDU 616.379-008.64

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Uberaba, 13 de dezembro de 2019.

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Dedico este trabalho às minhas filhas queridas Isabella Silvano Vieira Alves e Sophia Silvano Borges Araújo, ao meu marido, Moacir Augusto de Araújo e aos meus pais Carlos Francisco Silvano e Vera Lúcia Pereira Silvano, amores da minha vida!

"Pra você guardei o amor
Que nunca soube dar
O amor que tive e vi sem me deixar
Sentir sem conseguir provar
Sem entregar
E repartir"

(Pra você guardei o amor – Nando Reis)

AGRADECIMENTOS

À minha orientadora Profa. Dra. Juliana Reis Machado e Silva, a quem tenho imensa admiração e carinho! Obrigada por todos ensinamentos, apoio e formação construída. Pela oportunidade de fazer o doutorado, por me conduzir no caminho da pesquisa e por confiar no meu potencial. Agradeço pelo crescimento e conhecimentos adquiridos ao longo desta jornada. Por me ensinar muito além da pesquisa e pela dedicação e compromisso com o trabalho. Agradeço pela paciência com minhas inseguranças, pelos incentivos e, sobretudo, a amizade! Me sinto honrada e abençoada por ter sido orientada por você e por me proporcionar o mérito de fazer parte de um grupo de pesquisa tão consolidado. Serei eternamente grata!

À minha coorientadora Profa. Dra. Marlene Antônia dos Reis, a quem também serei eternamente grata! Agradeço por ter depositado em mim confiança. Pelo conhecimento adquirido e incentivo a buscar o crescimento pessoal e profissional. Pelos valiosos conselhos, paciência e amizade construída ao longo desses anos. Deixo aqui minha sincera admiração e respeito pela sua pessoa e a certeza de que sou muito grata e honrada de fazer parte da sua equipe de trabalho.

AGRADECIMENTOS

À Deus por me conceder a vida e saúde para concretizar mais esta conquista.

À minha família amada, pelo apoio, compreensão, incentivo, paciência e amor dedicados a mim. Peço desculpas pelos momentos de ausência. Sem vocês minha vida não haveria de ter sentido... Amo incondicionalmente!

À minha amiga Crislaine Aparecida Silva, pela amizade construída e por todos os momentos compartilhados durante estes anos. Agradeço por estar ao meu lado sempre me incentivando e motivando minha autoconfiança. Obrigada pela prontidão em me ajudar, pelos conselhos e pela sua personalidade forte e decidida que sempre me inspira. Obrigada por tudo!

Aos professores e amigos da disciplina de Patologia Geral: Aline Silva, Aloísio Costa, Laura Penna, Lenaldo Branco, Lívia Alves, Lourimar Morais, Mara Ferraz, Maria Helena Soares, Roberta Borges, Rosana Corrêa e Vicente Antunes pelo convívio e amizade.

Em especial, meus sinceros agradecimentos aos amigos do Laboratório de Histopatologia da disciplina de Patologia Geral: Alberto Borba, Edson Santos e Vandair Gonçalves, por todo apoio, compreensão e colaboração. Sem o apoio de vocês tudo seria mais difícil. Muita gratidão pelo que fizeram por mim!

Aos amigos da pós graduação: Ana Luísa Martins, Bianca Torquato, Carlos Donizete, Laura Aguiar, Lívia Helena Pereira, Maria Luíza Monteiro e Mariana Oliveira pela amizade e conhecimentos compartilhados.

Às alunas de iniciação científica, Alexia Borges e Mikaela Martins, pelo aprendizado e oportunidade de coorientação.

Aos colaboradores deste trabalho, em especial, ao professor Marcos Vinícius da Silva pelo conhecimento e desenvolvimento de novas técnicas.

Às professoras Maria de Fátima Borges e Heloísa Marcelina da Cunha Palhares pela parceria e apoio no ambulatório com os pacientes.

Ao professor Virmondes Rodrigues Júnior pela parceria e pelos kits e anticorpos colaborados.

Aos pacientes que aceitaram participar do estudo, disponibilizando seu tempo e amostra de sangue.

Aos membros da banca examinadora por ter aceitado o convite, pela disponibilidade, pelo tempo dedicado à leitura deste trabalho e pelas considerações sugeridas.

Ao Programa de Pós-Graduação em Ciências da Saúde da UFTM e aos secretários, André e Tuânia, pelos esclarecimentos e disponibilidade.

Aos professores da Pós-Graduação, pela oportunidade de conhecimento durante as aulas cursadas.

Aos alunos da graduação pelo interesse e respeito durante o treinamento didático.

À Universidade Federal do Triângulo Mineiro e disciplina de Patologia Geral da UFTM, pela acolhida e estrutura que permitiu o desenvolvimento desta pesquisa.

Ao Serviço de Nefropatologia da UFTM e o Serviço de Patologia da USP/Ribeirão Preto - SP pelas amostras de tecido analisadas no estudo.

Aos órgãos de fomento: Conselho Nacional de Pesquisa (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de aperfeiçoamento de pessoal de nível superior (CAPES), Fundação de Ensino e Pesquisa de Uberaba (FUNEPU) e Empresa Brasileira de Serviços Hospitalares - EBSERH-HC/UFTM.

"Aprender é igual a mudar, e entender é apenas conhecer. A diferença entre os dois é o que diferencia os que fazem dos que apenas pensam em fazer."

(Paulo Vieira)

RESUMO

Introdução: O Diabetes Mellitus tipo 2 (DMT2) é o subtipo mais prevalente do Diabetes Mellitus, e a Nefropatia Diabética (ND) a complicação microvascular crônica mais frequente no DMT2, considerada a principal causa de insuficiência renal terminal no mundo. A inflamação crônica de baixo grau tem sido apontada como um dos fatores desencadeadores da lesão renal nos pacientes com DMT2. Porém, estudos ainda são necessários para avaliar a influência do processo inflamatório na promoção da diminuição da função renal nos pacientes DMT2 e na progressão da ND. Assim, o objetivo deste estudo foi avaliar as concentrações séricas de mediadores inflamatórios em pacientes DMT2 com ou sem alteração renal (AR), como também a expressão de citocinas e quimiocinas inflamatórias (IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, TNFR1 e Eotaxina) em biópsias renais de rim nativo de pacientes diabéticos com ND, bem como a relação desses mediadores com a diminuição da função renal. Material e métodos: Foram selecionadas amostras de soro de 76 pacientes com DMT2 e 24 indivíduos saudáveis. Os pacientes com DMT2 foram divididos em dois grupos de acordo com a taxa de filtração glomerular estimada (TFGe): Grupo DMT2 sem AR (n=56, com TFGe>60 mL/min/1.73m^2) e grupo DMT2 com AR (n=20, com TFGe<60 mL/min/1.73m^2). Níveis séricos de citocinas, quimiocinas e adipocinas foram avaliados pelo imunoensaio Multiplex e ELISA. Para análise *in situ*, 44 biópsias de rim nativo de pacientes com ND (grupo ND) e 23 casos controles (grupo controle) foram selecionados. Expressões *in situ* de eotaxina, MIP-1α, IL-8, IL-4, IL-10, TNF-α, TNFR1, IL-1β e IL-6 foram avaliadas pela técnica de imuno-histoquímica. Resultados: Níveis séricos de TNFR1 e leptina foram maiores no grupo DMT2 com AR do que no grupo DMT2 sem AR e grupo controle. Todos os pacientes com DMT2 apresentaram aumento dos níveis séricos de resistina, IL-8 e MIP-1α em comparação ao grupo controle. Níveis séricos de adiponectina foram maiores e de IL-4 diminuídos no grupo DMT2 com AR em comparação ao grupo controle. A TFGe correlacionou-se positivamente com IL-4 e negativamente com TNFR1, TNFR2 e leptina em pacientes com DMT2. No grupo DMT2 com AR, a TFGe apresentou correlação negativa com TNFR1 e resistina. O TNFR1 foi correlacionado positivamente com resistina e leptina, bem como resistina com IL-8 e leptina. Na análise *in situ* foi observado que o grupo ND apresentou aumento da expressão de IL-6, IL-1β, IL-4 e eotaxina, e diminuição da expressão de TNFR1 e IL-8 em comparação ao grupo controle. No entanto, as expressões de IL-10, TNF-α e MIP-1α não apresentaram diferença significativa entre os grupos. Em relação a inflamação intersticial, houve aumento das expressões de IL-6 nos escores 0 e 1 em relação ao escore 2, de IL-10 no escore 2 em relação

ao escore 0 e de eotaxina no escore 2 em comparação aos escores 0 e 1, enquanto que IL-8 e MIP-1α não apresentaram diferença significativa. Além disso, foi observado que a eotaxina apresentou uma tendência a ter correlação negativa com a TFGe. Conclusões: O aumento dos níveis séricos de TNFR1, adipocinas, quimiocinas e diminuição da IL-4 desempenham papel importante no processo inflamatório desenvolvido no DMT2 e diminuição da função renal. Além disso, sugerimos que o TNFR1 sérico é um forte preditor de disfunção renal em pacientes com DMT2. Na avaliação da expressão *in situ*, pacientes com ND apresentaram aumento das expressões de IL-6, IL-1β, IL-4 e eotaxina. Sendo observado que a expressão de eotaxina pode estar desempenhando um importante papel na progressão da inflamação intersticial na ND, como também pode estar relacionada com a diminuição da TFGe nestes pacientes.
Palavras-chave: Diabetes mellitus tipo 2. Nefropatia Diabética. Mediadores inflamatórios. Taxa de filtração glomerular estimada. Alteração renal. Biópsia renal. Inflamação intersticial.

ABSTRACT

Introduction: Type 2 Diabetes Mellitus (T2DM) is the most prevalent subtype of Diabetes Mellitus, and Diabetic Nephropathy (DN) is the most common chronic microvascular complication in T2DM, considered the leading cause of end-stage renal failure in the world. Low-grade chronic inflammation has been identified as one of the triggering factors for kidney injury in T2DM patients. However, studies are still needed to evaluate the influence of the inflammatory process on the promotion of decreased renal function in T2DM patients and on the progression of DN. Thus, the aim of this study was to evaluate the serum concentrations of inflammatory mediators in T2DM patients with or without renal alteration (RA), as well as the expression of inflammatory cytokines and chemokines (IL-1β, IL-4, IL-6, IL- 8, IL-10, TNF-α, TNFR1 and Eotaxin) in renal kidney biopsies native to diabetic patients with DN, as well as the relationship of these mediators with decreased renal function. Material and methods: Serum samples from 76 patients with T2DM and 24 healthy individuals were selected. Patients with T2DM were divided into two groups according to the estimated glomerular filtration rate (eGFR): T2DM group without RA (n= 56, with eGFR> 60 mL / min / 1.73m2) and T2DM group with RA (n= 20, with eGFR <60 mL / min / 1.73m2). Serum levels of cytokines, chemokines and adipokines were evaluated by Multiplex and ELISA immunoassay. For *in situ* analysis, 44 native kidney biopsies from patients with DN (DN group) and 23 control cases (control group) were selected. *In situ* expressions of eotaxin, MIP-1α, IL-8, IL-4, IL-10, TNF-α, TNFR1, IL-1β and IL-6 were evaluated by immunohistochemistry. Results: Serum levels of TNFR1 and leptin were higher in the T2DM group with RA than in the T2DM group without RA and control group. All patients with T2DM had increased serum resistin, IL-8 and MIP-1α levels compared to the control group. Serum adiponectin levels were higher and IL-4 decreased in the T2DM group with RA compared to the control group. eGFR positively correlated with IL-4 and negatively with TNFR1, TNFR2 and leptin in patients with T2DM. In the T2DM group with RA, eGFR was negatively correlated with TNFR1 and resistin. TNFR1 was positively correlated with resistin and leptin, as well as resistin with IL-8 and leptin. In the *in situ* analysis, it was observed that the DN group showed increased expression of IL-6, IL-1β, IL-4 and eotaxin, and decreased expression of TNFR1 and IL-8 compared to the control group. However, the expressions of IL-10, TNF-α and MIP-1α showed no significant difference between groups. Regarding interstitial inflammation, there was an increase in IL-6 expressions in scores 0 and 1 compared to score 2, IL-10 in score 2 compared to score 0, and eotaxin in score 2 compared to scores 0 and 1, while IL-8 and MIP-1α showed no significant difference.

In addition, it was observed that eotaxin tended to have a negative correlation with eGFR. Conclusions: Increased serum levels of TNFR1, adipokines, chemokines and decreased IL-4 play important role in the inflammatory process developed in T2DM and decreased renal function. In addition, we suggest that serum TNFR1 is a strong predictor of renal dysfunction in patients with T2DM. In the evaluation of *in situ* expression, patients with DN presented increased expression of IL-6, IL-1β, IL-4 and eotaxin. It was observed that eotaxin expression may be playing an important role in the progression of interstitial inflammation in DN, as well as being related to the decrease of eGFR in these patients.
Keywords: Type 2 diabetes mellitus. Diabetic Nephropathy. Inflammatory mediators. Estimated glomerular filtration rate. Renal alteration. Renal biopsy. Interstitial inflammation.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AGEs: Produtos finais de glicação avançada

CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration

CTGF: Fator de crescimento tecido conectivo

DRC: Doença renal crônica

DMT1: Diabetes Mellitus tipo 1

DMT2: Diabetes Mellitus tipo 2

ERO: Espécies reativas de oxigênio

EUA: Excreção urinária de albumina

FIAT: Fibrose intersticial e atrofia tubular

HE: Hematoxilina e eosina

ICAM-1: molécula de adesão intercelular-1

Ig: Imunoglobulina

IL: Interleucina

IL-1RA: Receptor antagonista da interleucina 1

IFN-γ: Interferon-γ

MBG: Membrana basal glomerular

MEC: Matriz extracelular

MET: Microscopia eletrônica de transmissão

MIP-1α: Proteína inflamatória de macrófagos-1α

MIP-1β: Proteína inflamatória de macrófagos-1β

ND: Nefropatia Diabética

NK: Natural killer

NF-kB: Falor nuclear kappa B

PAMS: Prata metenamina

PDGF: Fator de crescimento derivado de plaquetas

PKC: Proteína quinase C

RAGES: Receptor de produtos finais de glicação avançada

SOCS: Proteínas supressoras de sinalização de citocinas

TFG: Taxa de filtração glomerular

TFGe: Taxa de filtração glomerular estimada

TGF-β1: Fator transformador de crescimento beta 1

TMA: Tricômico de Masson Azul

TNF-α: Fator de necrose tumoral alfa

TNFR1: Receptor 1 do fator de necrose tumoral

TNFR2: Receptor 2 do fator de necrose tumoral

VCAM-1: Molécula de adesão celular vascular-1

VEGF: Fator de crescimento endotelial vascular

SUMÁRIO

Introdução

1 INTRODUÇÃO

1.1 DIABETES MELLITUS TIPO 2 E INFLAMAÇÃO

O Diabetes Mellitus tipo 2 (DMT2) é um dos subtipos mais prevalentes do Diabetes Mellitus, correspondendo 90 a 95% dos casos. Caracterizado como uma desordem metabólica resultante da deficiência relativa da produção de insulina e/ou de sua ação, que culminará no aumento dos níveis séricos de glicose. Possui etiologia complexa e multifatorial, envolvendo componentes genético e ambiental. Geralmente, acomete indivíduos a partir da quarta década de vida, e atualmente é considerado a principal causa de doença renal crônica (GREGG; LI; WANG; BURROWS *et al.*, 2014; MARATHE; GAO; CLOSE, 2017). De acordo com os dados da International Diabetes Federation, estima-se que o número de diabéticos na população com idade superior a 18 anos poderá alcançar 693 milhões até 2045 em todo o mundo (CHO; SHAW; KARURANGA; HUANG *et al.*, 2018).

O processo de hiperglicemia desenvolvido no DMT2 está fortemente associado ao desenvolvimento de complicações macrovasculares e microvasculares, podendo acometer vários órgãos, sendo o rim um dos mais acometidos (CHAWLA; CHAWLA; JAGGI, 2016).

A inflamação crônica de baixo grau, caracterizada pela produção de citocinas, quimiocinas e adipocinas, está envolvida nos processos patogênicos causadores de DMT2 e suas complicações, sendo a Nefropatia Diabética (ND) uma das complicações microvasculares mais prevalente (ARAÚJO; SILVA; SILVA; MONTEIRO *et al.*, 2016; CEBECI; CAKAN; GURSU; UZUN *et al.*, 2019; GUPTA; MARATHA; SIEDNIENKO; NATARAJAN *et al.*, 2017; NAVARRO; MORA, 2006; NAVARRO-GONZÁLEZ; MORA-FERNÁNDEZ, 2008). Evidências apontam que a ativação do sistema imune inato e do desenvolvimento de inflamação crônica sistêmica de baixo grau estão intimamente envolvidos na patogênese do DMT2 (CROOK, 2004; PICKUP, 2004).

A hiperglicemia está associada ao aumento da produção de citocinas mediada por mecanismos oxidativos. O excesso de produção de espécies reativas de oxigênio (ERO), associada à interação dos produtos finais de glicação avançada (AGEs) com seus receptores (RAGEs) no endotélio, leva à produção de citocinas pelas células endoteliais. O aumento expressivo da produção dessas citocinas está associado à disfunção endotelial (ESPOSITO; NAPPO; MARFELLA; GIUGLIANO *et al.*, 2002).

Várias citocinas secretadas tanto pelas células imunes quanto pelos adipócitos, encontram-se envolvidas no processo inflamatório do DMT2. Geralmente, níveis séricos de proteínas de fase aguda, como o fator de necrose tumoral-α (TNF-α), interleucina-6 (IL-6), IL-1β, proteína amiloide A, ácido siálico e proteína C reativa encontram-se elevados no DMT2. No entanto, outras citocinas, quimiocinas e adipocinas também podem estar desenvolvendo papéis importantes nesse processo inflamatório (ARAÚJO; SILVA; SILVA; MONTEIRO *et al.*, 2016; CROOK, 2004; PICKUP, 2004). Acredita-se que além da elevação de citocinas pró-inflamatórias, o DMT2 pode estar associado a uma resposta anti-inflamatória menos eficaz (GUEST; PARK; JOHNSON; FREUND, 2008). Curiosamente, algumas citocinas anti-inflamatórias, incluindo IL-4 e IL-10, compartilham os principais componentes de sinalização com o receptor de insulina e, por isso são suscetíveis a mecanismos de resistência semelhantes ao processo de resistência à insulina (GUEST; PARK; JOHNSON; FREUND, 2008; MORINO; PETERSEN; SHULMAN, 2006).

A IL-4 é uma citocina de perfil Th2, produzida por linfócitos $TCD4^+$, mastócitos, eosinófilos e basófilos. Tem ação na redução da secreção de citocinas pró-inflamatórias, incluindo o TNF-α, por macrófagos ativados e estimula a produção de uma série de moléculas anti-inflamatórias como, IL-1RA (receptor antagonista da IL-1) (VANNIER; MILLER; DINARELLO, 1992), IL-1R2 (POUSSET; CREMONA; DANTZER; KELLEY *et al.*, 2001) e receptores de TNF solúveis (MANNA; AGGARWAL, 1998), além de estimular a proliferação de células B e produção de IgG e IgE, importantes nas respostas alérgicas e anti-helmínticas (NELMS; KEEGAN; ZAMORANO; RYAN *et al.*, 1999). A IL-4 é pouco estudada no DMT2, no entanto já foi demonstrado que possui um papel regulador positivo na tolerância à glicose e sensibilidade à insulina, que pode ser devido a sua ação anti-inflamatória, inibindo a produção de citocinas indutoras de resistência à insulina, como TNF-α e IL-6 (CHANG; HO; LU; HUANG *et al.*, 2012). Um distúrbio da resposta anti-inflamatória pode ser um componente crítico da inflamação crônica encontrada no DMT2 (O'CONNOR; SHERRY; GUEST; FREUND, 2007). Elevados níveis séricos de IL-4 foi observado em pacientes DMT2 e obesos (BINISOR; MOLDOVAN; MOLDOVAN; ANDREI *et al.*, 2016) que pode ser devido à resistência a IL-4 em pacientes DMT2, resultado de um aumento adaptativo dos seus níveis (O'CONNOR; SHERRY; GUEST; FREUND, 2007).

O TNF-α é uma citocina pleiotrópica de perfil Th1, conhecida por suas propriedades pró-inflamatórias, que desempenha papel essencial na mediação dos processos inflamatórios. É

uma proteína homotrimérica transmembrana produzida por muitas células, incluindo células adiposas, endoteliais e leucócitos. O TNF-α exerce sua função via dois receptores, o receptor 1 do TNF (TNFR1) e o receptor 2 do TNF (TNFR2). Membros da superfamília dos receptores de TNF, o TNFR1 é expresso em quase todas as células (NIEWCZAS; GOHDA; SKUPIEN; SMILES *et al.*, 2012), enquanto que o TNFR2 é restrito ao linfócito T e outras poucas células (YANG; WANG; BRAND; ZHENG, 2018). Os receptores, TNFR1 e TNFR2, são proteínas transmembranas. Formas solúveis podem ser geradas através da clivagem enzimática do receptor por metaloproteinases inflamatórias ou pela inclusão do receptor nos exossomos secretores (HAWARI; ROUHANI; CUI; YU *et al.*, 2004; LAINEZ; FERNANDEZ-REAL; ROMERO; ESPLUGUES *et al.*, 2004).

Além de suas propriedades pró-inflamatórias, o TNF-α também exerce efeitos no metabolismo da glicose e dos lipídios (HOTAMISLIGIL; SHARGILL; SPIEGELMAN, 1993). Em baixas concentrações, o TNF-α atua localmente como um regulador da resposta inflamatória imunológica (efeitos autócrinos e parácrinos). Já em altas concentrações, o TNF-α entra na circulação, e de forma sistêmica atua como um fator endócrino, associado à resistência à insulina (FASSHAUER; PASCHKE, 2003). O TNF-α causa um aumento na liberação de ácidos graxos pelos adipócitos, resultando em níveis aumentados de ácidos graxos livres, que podem deteriorar a sinalização da insulina (RUAN; MILES; LADD; ROSS *et al.*, 2002). Além disso, o TNF-α pode agir inibindo a transdução do sinal de insulina e também diminuindo sua secreção (HOTAMISLIGIL, 1999). Já foi demonstrado que pacientes DMT2 apresentaram elevados níveis séricos de TNF-α, que foi confirmado pela correlação positiva e significativa entre TNF-α e glicose (CHEN; WANG; LIU; MA *et al.*, 2013). Estudos com indivíduos DMT2 tem associado os elevados níveis séricos dos receptores 1 e 2 do TNF-α ao risco de doença renal terminal, como também têm demonstrado uma associação do aumento dos níveis séricos do TNFR1 com a diminuição da taxa de filtração glomerular estimada (TFGe) (DOODY; JACKSON; ELLIOTT; CANAVAN *et al.*, 2018; NIEWCZAS; GOHDA; SKUPIEN; SMILES *et al.*, 2012).

O interferon-γ (IFN-γ) é uma citocina produzida principalmente por células T, B e *natural killer* (NK). Age na inibição da proliferação de células que sintetizam IL-4, IL-5, IL-6, IL-10 e IL-13 e na diminuição da produção de algumas imunoglobulinas. O IFN-γ tem sido implicado na patogênese de várias doenças autoimunes, em processos inflamatórios crônicos (FARRAR; SCHREIBER, 1993) e na estimulação da expressão de receptores de quimiocinas

em células mesangiais humanas (UCIECHOWSKI; SCHWARZ; GESSNER; SCHMIDT *et al.*, 1998). Foi demonstrado que tanto indivíduos com DMT2 quanto com ND apresentaram elevados níveis séricos de IFN-γ quando comparado ao grupo controle, com tendência a permanecerem mais elevados na ND (ANAND; VASANTHAKUMAR; MOHAN; BABU *et al.*, 2014). Por outro lado, recentemente, um estudo experimental demonstrou que o INF-γ pode apresentar efeito protetor no rim de animais DMT2 ao exercer supressão da expressão de TGF-β1 e colágeno IV e atenuar o acúmulo excessivo de matriz mesangial (DU; DONG; LI; LI *et al.*, 2018).

As quimiocinas são uma grande família de pequenas citocinas de baixo peso molecular que varia de 7 a 15KDa e possuem capacidades de controle da adesão, quimiotaxia e ativação leucocitária. Juntamente aos seus receptores, as quimiocinas controlam a migração e a residência das células imunes. Algumas quimiocinas apresentam perfil pró-inflamatório e podem ser induzidas durante a resposta imune no sítio de infecção, enquanto outras são consideradas homeostáticas e estão envolvidas no controle da migração celular durante o desenvolvimento ou a manutenção dos tecidos. Existem duas grandes subfamílias de quimiocinas baseadas na posição dos resíduos de cisteínas: a família das quimiocinas CXC e CC. De modo geral, as quimiocinas CXC, também conhecidas como as alfa-quimiocinas, estimulam a quimiotaxia de neutrófilos, enquanto que as quimiocinas CC, também conhecidas como beta-quimiocinas, estimulam principalmente a quimiotaxia de monócitos, mas também de basófilos, eosinófilos, linfócitos T e as células NK (PALOMINO; MARTI, 2015).

A IL-8 (CXCL8) foi a primeira quimiocina a ser descoberta e exerce predominantemente efeito quimioatrativo para neutrófilos (GERARD; ROLLINS, 2001), estimula a liberação de seus grânulos, aumenta a expressão de moléculas de adesão pelas células endoteliais e antagoniza a produção IgE estimulada pela IL-4 (ZWAHLEN; WALZ; ROT, 1993). Produzida principalmente por monócitos/macrófagos e em menor quantidade por fibroblastos, células endoteliais, queratinócitos, hepatócitos, melanócitos e condrócitos. IL-1, TNF-α e IFN-γ são seus principais estimuladores (BAGGIOLINI; DEWALD; MOSER, 1994). Aumento dos níveis urinários de IL-8 já foram relatados em pacientes com DMT2 e com ND, assim como uma associação do aumento de IL-8 com o declínio da taxa de filtração glomerular (VERHAVE; BOUCHARD; GOUPIL; PICHETTE *et al.*, 2013).

As quimiocinas, proteína inflamatória de macrófagos-1α (MIP-1α /CCL3) e a proteína inflamatória de macrófagos-1β (MIP-1β /CCL4) pertencem a subfamília CC das quimiocinas e

induzem a expressão de moléculas de adesão e moléculas co-estimuladoras na superfície das células T, células NK, macrófagos e monócitos. Essas quimiocinas além de mediarem a quimiotaxia dessas células, também promovem a secreção de citocinas pró-inflamatórias, como a IL-1, IL-6 e TNF-α a partir de fibroblastos e macrófagos (LUSTER, 1998). Em estudos com pacientes DMT2 foi observado aumento dos níveis séricos de MIP-1α e MIP-1β em associação com o declínio da taxa de filtração glomerular (KONENKOV; KLIMONTOV; MYAKINA; TYAN *et al.*, 2015; PERLMAN; CHEVALIER; WILKINSON; LIU *et al.*, 2015).

Outra quimiocina da subfamília CC é a eotaxina (CCL11), que tem ação quimiotática principalmente de eosinófilos. A eotaxina é secretada por células endoteliais, macrófagos, fibroblastos e células musculares lisas (GARCIA-ZEPEDA; ROTHENBERG; OWNBEY; CELESTIN *et al.*, 1996). Já foi demonstrado que o processo de hiperglicemia prolongado aumenta a excreção urinária de eotaxina (CHERNEY; SCHOLEY; SOCHETT; BRADLEY *et al.*, 2011) e o aumento tanto dos níveis urinários, quanto dos séricos de eotaxina pode estar associado às complicações do DMT2 (ADELA; REDDY; GHOSH; AGGARWAL *et al.*, 2019; LIU; ZHAO; WILLCOX; XU *et al.*, 2010).

Além das citocinas e quimiocinas, as adipocinas, como a adiponectina, a resistina e a leptina também encontram-se associadas à inflamação e ao diabetes. A adiponectina é uma adipocina secretada exclusivamente por adipócitos humanos (KADOWAKI; YAMAUCHI, 2005) e exerce seu efeito biológico ao se ligar a receptores específicos encontrados em várias células, principalmente miócitos e células hepáticas (BRUUN; LIHN; VERDICH; PEDERSEN *et al.*, 2003). Níveis plasmáticos de adiponectina são inversamente correlacionados com os níveis de insulina, apresenta efeitos benéficos na resistência à insulina e propriedades anti-inflamatórias (TURER; SCHERER, 2012) e anti-oxidativas (MATSUDA; SHIMOMURA, 2014). Sugere-se que a ação anti-inflamatória exibida pela adiponectina seja pela inibição da produção de citocinas pró-inflamatórias, como IL-6 e TNF-α nos macrófagos e/ou por reduzir sua ação fagocitária (GARCIA; FEVE; FERRÉ; HALIMI *et al.*, 2010).

A leptina é uma adipocina secretada principalmente por adipócitos, mas que também pode ser produzida por outras células, como os macrófagos. Produto proteico do gene da obesidade (*ob*), tem sido relacionada a vários fatores metabólicos e inflamatórios, e a expressão desse gene é regulada por diferentes fatores, incluindo níveis de insulina. Os níveis séricos de leptina são diretamente proporcionais à massa total de gordura e em condições inflamatórias, há o aumento de sua produção. A leptina também pode modular as respostas imunes inata e

adaptativa, incluindo as respostas de células T, ativação de monócitos e macrófagos e indução de mediadores pró-inflamatórios (LONTCHI-YIMAGOU; SOBNGWI; MATSHA; KENGNE, 2013). Encontra-se envolvida no controle da ingestão alimentar, levando à supressão do apetite, no entanto pacientes obesos apresentam hiperleptinemia devido ao desenvolvimento de resistência à leptina, possivelmente pelo mecanismo de interrupção da sinalização da cascata do receptor de leptina pelas proteínas supressoras de sinalização de citocinas (SOCS) (KATSIKI; MIKHAILIDIS; BANACH, 2018; O'CONNOR; JOHNSON; FREUND, 2006). Elevados níveis de leptina estão associados com resistência à insulina e desenvolvimento de DMT2 (ANDRADE-OLIVEIRA; CÂMARA; MORAES-VIEIRA, 2015). Porém, já foi demonstrado que tanto a diminuição, quanto o aumento da concentração de leptina são fatores de risco para o declínio da função renal em pacientes DMT2 (HANAI; BABAZONO; MUGISHIMA; YOSHIDA *et al.*, 2011).

A resistina é uma proteína secretada principalmente em macrófagos e monócitos nos seres humanos e possui efeitos pró-inflamatórios (BOKAREWA; NAGAEV; DAHLBERG; SMITH *et al.*, 2005; STEPPAN; BAILEY; BHAT; BROWN *et al.*, 2001). O aumento dos níveis séricos de citocinas pró-inflamatórias foi associado ao aumento dos níveis séricos de resistina nos pacientes com DMT2 (BOKAREWA; NAGAEV; DAHLBERG; SMITH *et al.*, 2005; MCTERNAN; FISHER; VALSAMAKIS; CHETTY *et al.*, 2003). Também foi demonstrado recentemente, que pacientes DMT2 microalbuminúricos e com diminuição da função renal apresentaram aumento significativo dos níveis séricos de resistina em comparação aos pacientes DMT2 com função renal normal (CEBECI; CAKAN; GURSU; UZUN *et al.*, 2019).

O desequilíbrio entre mediadores é considerado o gatilho causador ou exacerbador das complicações do DMT2. A ativação do sistema imune inato, por si só, induz a hiperglicemia e a resistência à insulina. Dessa maneira, diabetes e a inflamação encontram-se simultaneamente envolvidas, desempenhando papéis de fonte de alimentação recíproca (GUEST; PARK; JOHNSON; FREUND, 2008).

1.2 NEFROPATIA DIABÉTICA

A ND é uma complicação microvascular crônica resultante tanto do DMT1 quanto do DMT2 (BATLLE, 2003). Ocorre em 20% a 30% dos pacientes com DMT2 e é considerada a principal causa de insuficiência renal terminal com necessidade de terapia renal substitutiva (CHO; SHAW; KARURANGA; HUANG *et al.*, 2018). Clinicamente é caracterizada pelo aumento da excreção urinária de albumina (maior que 300 mg/24h), declínio na taxa filtração glomerular e aumento da pressão arterial (GROSS; NEHME, 1999; PARVING; SMIDT; FRIISBERG; BONNEVIE-NIELSEN *et al.*, 1981).

Em 1936, Kimmelstiel e Wilson descreveram lesões intercapilares glomerulares encontradas em pacientes com DMT2 que, posteriormente vieram a ser denominadas "nódulos de Kimmelstiel e Wilson" ou expansão mesangial. Estas lesões são observadas em 40 a 50% dos pacientes diabéticos em fase de proteinúria (KIMMELSTIEL; WILSON, 1936).

A patogênese da ND vem sendo investigada em vários aspectos. A hiperglicemia desempenha um papel central e representa o ponto de partida na ativação de várias vias. Os mecanismos patogênicos subjacentes a esta doença, envolve a geração de espécies reativas de oxigênio (ERO), acúmulo de AGEs e ativação de vias como a da proteína quinase C (PKC), do poliol e das hexosaminas (CHAWLA; CHAWLA; JAGGI, 2016). A ativação destes mecanismos levam a várias respostas celulares, à expressão de fatores de secreção e acúmulo de matriz extracelular (MEC), que em última instância vão resultar em alterações da barreira de filtração glomerular com expansão mesangial, esclerose glomerular nodular e fibrose túbulo-intersticial , que irão resultar em albuminúria progressiva, diminuição da taxa de filtração glomerular e elevação da pressão arterial (ARORA; SINGH, 2013; EL MESALLAMY; AHMED; BASSYOUNI; AHMED, 2012; GARUD; KULKARNI, 2014).

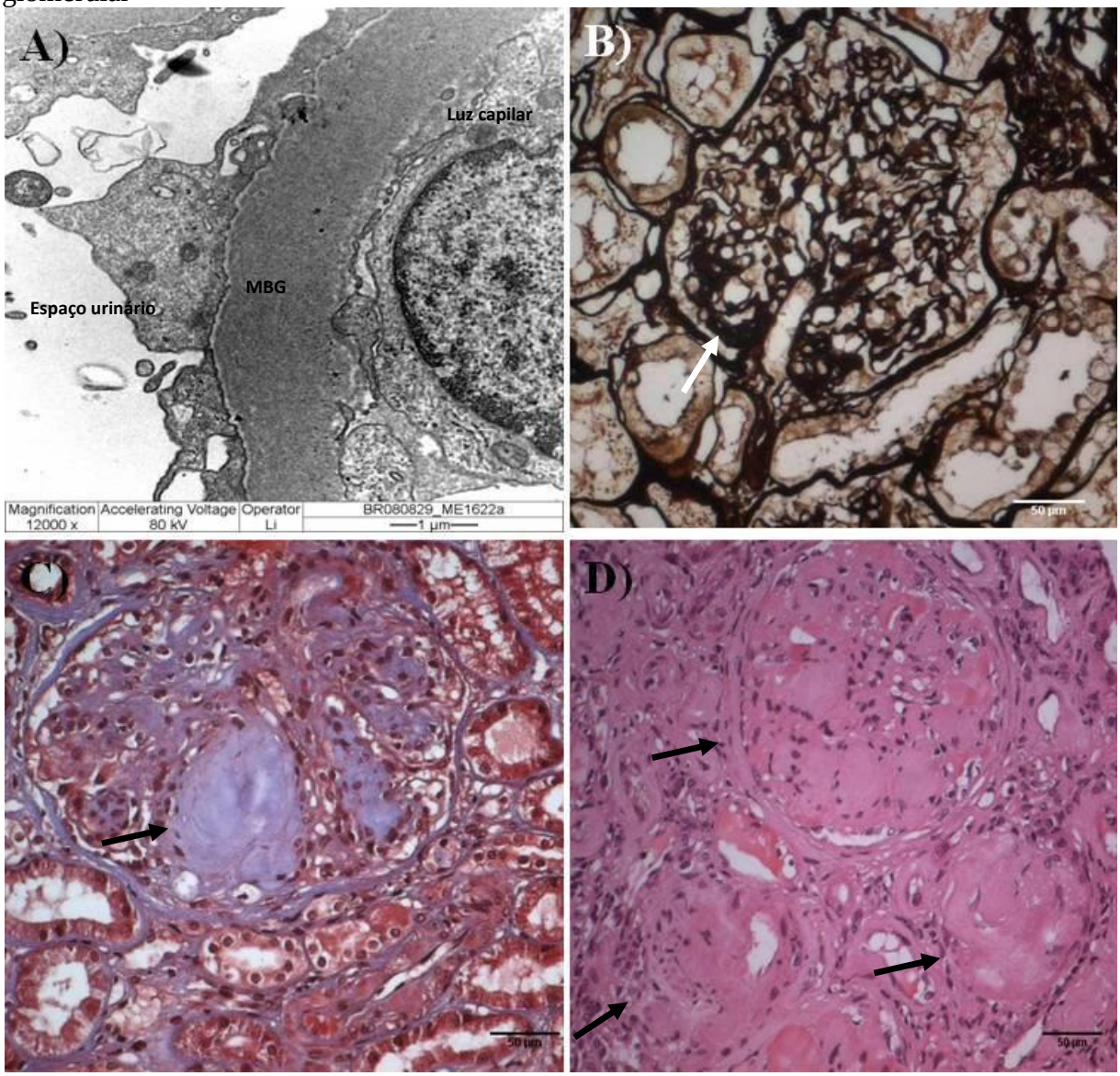
Em condições de hiperglicemia crônica, a geração de ERO que ativa fatores de transcrição (NF-kB – fator nuclear kappa B) e o acúmulo de AGEs, além de contribuírem com o aumento da MEC (GARUD; KULKARNI, 2014), também contribuem para liberação de citocinas pró-inflamatórias e expressão de fatores de crescimento e moléculas de adesão, tais como fator de crescimento transformador β1 (TGF-β1), fator de crescimento endotelial vascular (VEGF), fator de crescimento tecido conectivo (CTGF), fator de crescimento derivado de plaquetas (PDGF), TNF-α e IL-6 que parecem estar envolvidos na patogênese da ND (BONVENTRE, 2012). Estudos experimentais com diabetes relatam que o estresse oxidativo

aumenta com o acúmulo de AGEs (HA; SAUL; TAWFIK; ZORRILLA *et al.*, 2012) e que a inibição experimental de AGEs diminui a expressão de fatores de crescimento (VAN BUREN; TOTO, 2013).

Morfologicamente, as alterações da ND apresentam-se de forma semelhante tanto no paciente com DMT1 como no paciente com DMT2 (HAYASHI; KARASAWA; INN; SAITOU *et al.*, 1992) e todas as células do rim, tais como, endotélio glomerular, célula mesangial, podócitos e o epitélio tubular podem ser alvos da injúria hiperglicêmica (KANWAR; WADA; SUN; XIE *et al.*, 2008). Dentre as principais alterações morfológicas que ocorrem neste ambiente estão o acúmulo de MEC que vai evoluir para glomeruloesclerose difusa e nodular (nódulos de Kimmelstiel e Wilson), espessamento da membrana basal glomerular (MBG) e dos túbulos, hipertrofia glomerular, expansão de células mesangiais, apagamento dos pedicelos e fibrose túbulo-intersticial (DALLA VESTRA; SALLER; BORTOLOSO; MAUER *et al.*, 2000; MASON; WAHAB, 2003).

As alterações morfológicas glomerulares da ND, decorrentes tanto do DMT1 ou DMT2, são discriminadas em quatro tipos de classes de acordo com a gravidade das lesões. Classe I se refere ao espessamento da MBG visualizado pela microscopia eletrônica transmissão (MET), sem presença de alterações que se enquadram nos critérios das classes seguintes. Classe II está relacionada à expansão mesangial leve (IIa) ou acentuada (IIb) sem esclerose nodular (lesão de Kimmelstiel-Wilson). Classe III, definida pela esclerose nodular observada em pelo menos um dos glomérulos da amostra. E Classe IV, definida como glomeruloesclerose diabética avançada, na qual mais de 50% dos glomérulos apresentam esclerose global (Figura 1). A avaliação do grau de comprometimento intersticial e vascular é expressa em scores de acordo com a intensidade e extensão da lesão (TERVAERT; MOOYAART; AMANN; COHEN *et al.*, 2010).

Figura 1 – Características morfológicas das Classes da Nefropatia Diabética no compartimento glomerular



Fonte: PATGE, 2019.

A) Alça capilar com espessamento da MBG visualizado pela MET caracterizando a Classe I da ND (12000X). B) Glomérulo com expansão mesangial (seta) caracterizando a Classe II da ND (PAMS – 820X). C) Glomérulo com esclerose nodular ou "nódulo de Kimmelstiel-Wilson" representado pela região arredondada com aumento de matriz corado em azul, com os núcleos dispostos na periferia do nódulo, permanecendo os capilares abertos ao redor da esclerose nodular (seta) caracterizando a Classe III da ND (TMA – 820X). D) Glomérulos com esclerose global (setas) caracterizando a Classe IV da ND (HE – 820X).

1.3 NEFROPATIA DIABÉTICA E INFLAMAÇÃO

Evidências indicam que mecanismos imunológicos e inflamatórios desempenham papéis significantes no desenvolvimento e progressão da ND, sendo então considerada uma doença inflamatória (MORA; NAVARRO, 2006; TUTTLE, 2005). Diversas células, como monócitos e macrófagos, e outras moléculas como quimiocinas, citocinas, fatores de crescimento, moléculas de adesão e fatores nucleares encontram-se implicados no processo relacionado a ND (WADA; MAKINO, 2013).

A fibrose é a característica mais importante e fundamental da ND e a inflamação parece ser o gatilho central no aparecimento e progressão da fibrose renal se não controlada (WADA; MAKINO, 2013). Em estudos clínicos, as concentrações circulantes de marcadores inflamatórios têm sido encontradas em pacientes com DMT1 e DMT2, e estes marcadores parecem prever o início e progressão das complicações diabéticas (HARJUTSALO; GROOP, 2014; PICKUP; CHUSNEY; THOMAS; BURT, 2000). Foi demonstrado que as concentrações de citocinas pró-inflamatórias aumentam com a progressão da ND (BRUNO; MERLETTI; BIGGERI; BARGERO *et al.*, 2003; FESTA; D'AGOSTINO; HOWARD; MYKKÄNEN *et al.*, 2000) e que são independentemente relacionadas com a excreção urinária de albumina apresentando uma associação direta com marcadores clínicos de lesão glomerular e túbulo-intersticial (NAVARRO; MORA; MACA; GARCA, 2003). Estudos experimentais com modelos diabéticos demonstraram que a inibição do recrutamento de células inflamatórias para o rim representa um fator protetor na ND (AWAD; KINSEY; KHUTSISHVILI; GAO *et al.*, 2011; CHOW; NIKOLIC-PATERSON; OZOLS; ATKINS *et al.*, 2005).

A participação de citocinas inflamatórias na patogênese da ND foi sugerida pela primeira vez em 1991 por Hasegawa e colaboradores (HASEGAWA; NAKANO; SAWADA; UNO *et al.*, 1991). Neste trabalho, os autores demonstraram que os macrófagos peritoneais cultivados com membranas basais glomerulares de animais diabéticos produziram quantidades significativamente maiores de citocinas inflamatórias, tais como TNF-α e IL-1, quando comparados com aqueles cultivados com membranas basais glomerulares de animais normais.

Concentrações plasmáticas de algumas citocinas inflamatórias estão elevadas em pacientes diabéticos sendo fortes preditores do desenvolvimento desta doença (PRADHAN; MANSON; RIFAI; BURING *et al.*, 2001; SCHMIDT; DUNCAN; SHARRETT; LINDBERG *et al.*, 1999; SPRANGER; KROKE; MÖHLIG; HOFFMANN *et al.*, 2003). O DMT2 por si só

está associado com a inflamação, mas a inflamação também contribui significativamente para o desenvolvimento de ND (DALLA VESTRA; MUSSAP; GALLINA; BRUSEGHIN *et al.*, 2005).

Todas as células renais (endoteliais, epiteliais, mesangiais e tubulares) são também capazes de sintetizar citocinas pró-inflamatórias, tais como TNF-α, IL-1 e IL-6, e, por conseguinte, estas citocinas, atuando de forma parácrina ou autócrina e podem induzir uma variedade de efeitos em diferentes estruturas renais desenvolvendo um papel importante no desenvolvimento e progressão de várias doenças renais incluindo a ND (NORONHA; NIEMIR; STEIN; WALDHERR, 1995; OSTENDORF; BURG; FLOEGE, 1996). Hoje se sabe que entre as citocinas inflamatórias, a IL-1β, IL-6, IL-18 e TNF- α são relevantes para o desenvolvimento da ND, desempenhando diversas ações potencialmente envolvidas no desenvolvimento de suas complicações (NAVARRO-GONZÁLEZ; MORA-FERNÁNDEZ, 2008).

A IL-1β é produzida por uma variedade de tecidos e tipos de células, incluindo macrófagos, neurônios, células β do pâncreas e tecido adiposo (BLUTHÉ; DANTZER; KELLEY, 1997). Conhecida por induzir a secreção de outras citocinas, as funções da IL-1β são contra reguladas em parte pela inibição competitiva da IL-1RA e IL-1R2 (BLUTHÉ; DANTZER; KELLEY, 1992). Em modelos experimentais de ND foi observado um aumento da expressão renal de IL-1β, que está relacionada com a expressão de fatores quimiotáticos e moléculas de adesão (NAVARRO; MILENA; MORA; LEÓN *et al.*, 2006; SASSY-PRIGENT; HEUDES; MANDET; BÉLAIR *et al.*, 2000). A IL-lβ aumenta a síntese de molécula de adesão intercelular 1 (ICAM-1) e molécula de adesão celular vascular-1 (VCAM-1) por células endoteliais glomerulares e induz a síntese *de novo* e expressão de ICAM-1 pelas células mesangiais glomerulares e epitélios tubulares renais. Além disso, esta citocina induz expressão transitória de E-selectina por células endoteliais (BRADY, 1994; PARK; KIM; LEE; KIM *et al.*, 2000) como também, encontra-se envolvida com o desenvolvimento de alterações hemodinâmicas intraglomerulares relacionadas à síntese de prostaglandina pelas células mesangiais (PFEILSCHIFTER; PIGNAT; VOSBECK; MÄRKI, 1989). Encontra-se diretamente relacionada com o aumento da permeabilidade das células endoteliais vasculares (ROYALL; BERKOW; BECKMAN; CUNNINGHAM *et al.*, 1989) e envolvida no processo de proliferação das células mesangiais e síntese de matriz (MELCION; LACHMAN; KILLEN; MOREL-MAROGER *et al.*, 1982; NAVARRO; MORA, 2005).

A IL-6 é produzida principalmente por células do sistema imunológico, músculo esquelético e fígado, porém outros tipos de células também podem produzir IL-6 como, as células da glia e células endoteliais (KISHIMOTO, 2005). É uma citocina pró-inflamatória e um importante mediador da proliferação de células mesangiais, do aumento da permeabilidade das células endoteliais e aumento da produção de matriz extracelular (FORBES; COOPER, 2013). Níveis de IL-6 são mais elevados em pacientes com ND em comparação com pacientes com diabetes, porém sem nefropatia (MAHADEVAN; LARKINS; FRASER; FOSANG *et al.*, 1995). Além disso, a hibridização *in situ* realizada em biópsias renais humanas demonstrou um aumento da expressão de RNAm que codifica a IL-6 em células infiltradas no mesângio, interstício e túbulos que também apresentaram uma relação positiva com a gravidade da expansão mesangial (SUZUKI; MIYAZAKI; NAKA; KOJI *et al.*, 1995). A IL-6 também tem sido relacionada com o aumento da expressão de fibronectina (COLEMAN; RUEF, 1992) e aumento da espessura da MBG (DALLA VESTRA; MUSSAP; GALLINA; BRUSEGHIN *et al.*, 2005). Pacientes com DMT2 apresentaram aumento na produção de IL-6 associado não somente com a ND, mas também com o espessamento da MBG, lesão crucial e mais precoce da ND sendo considerado um forte marcador de declínio da função renal (DALLA VESTRA; MUSSAP; GALLINA; BRUSEGHIN *et al.*, 2005).

O TNF-α é produzido principalmente pelos monócitos, macrófagos e células T, mas também intrinsecamente pelas células renais. Apresenta efeitos citotóxicos para as células glomerulares, mesangiais e epiteliais e pode induzir dano renal de forma direta (DONG; SWAMINATHAN; BACHMAN; CROATT *et al.*, 2007). No glomérulo, o TNF-α estimula a produção de prostaglandinas pelas células mesangiais e pode ser responsável pela alteração da microcirculação glomerular. Essa citocina também induz atividade pro coagulante do endotélio e aumenta sua permeabilidade (FERNÁNDEZ-REAL; VENDRELL; GARCÍA; RICART *et al.*, 2012). Muitos estudos de pacientes com ND têm encontrado elevados níveis séricos e urinários de TNF-α quando comparados com pacientes não diabéticos. E estas concentrações aumentam com a progressão da ND. Estes achados mostram que existe uma forte relação entre os elevados níveis desta citocina inflamatória e o desenvolvimento e progressão da lesão renal no DMT2 (NAVARRO; MORA; MACA; GARCA, 2003; NAVARRO; MORA; MUROS; GARCÍA, 2006). Outros estudos têm demonstrado que o TNF-α e seus receptores (TNFR1 e TNFR2) estão associados com a redução da função renal nos pacientes DMT2, como também têm associado os elevados níveis séricos de TNFR1 com a ND (NAVARRO-GONZÁLEZ;

MORA-FERNÁNDEZ; MUROS DE FUENTES; GARCÍA-PÉREZ, 2011; NIEWCZAS; GOHDA; SKUPIEN; SMILES *et al.*, 2012). Além disso, já foi demonstrado que ambos receptores, TNFR1 e TNFR2, desempenham papel importante no desenvolvimento da fibrose tubulointersticial (GUO; MORRISSEY; MCCRACKEN; TOLLEY *et al.*, 1999).

A IL-8 (CXCL8) tem sido sugerida como um marcador para avaliar o grau de lesão renal no estágio inicial da ND (NIEMIR; STEIN; CIECHANOWICZ; OLEJNICZAK *et al.*, 2004). No rim, as principais fontes de IL8 são representadas pelos podócitos e células endoteliais dos vasos intersticiais, e uma pequena quantidade é expressa pelas células epiteliais tubulares. O aumento de IL-8 nas células endoteliais, próximas ao sítio inflamatório, facilita a travessia dos leucócitos ativados pelo endotélio por meio da alteração da expressão de moléculas de adesão (NIEMIR; STEIN; CIECHANOWICZ; OLEJNICZAK *et al.*, 2004). A elevada produção de citocinas no rim e o aumento da permeabilidade da barreira de filtração glomerular, podem contribuir para o aumento dos níveis urinários de citocinas em pacientes microalbuminúricos (LIU; ZHAO; WILLCOX; XU *et al.*, 2010). Elevadas concentrações urinárias de IL-8 já foram detectadas nas fases iniciais da ND (TASHIRO; KOYANAGI; SAITOH; SHIMIZU *et al.*, 2002). Além disso, já foi demonstrado que a IL-8 apresentou associação negativa com a taxa de filtração glomerular estimada e positiva com o índice de massa corporal (VIANNA; SOARES; SILVEIRA; ELMIRO *et al.*, 2013).

Outras quimiocinas, além da IL-8, também se encontram envolvidas na patogênese da ND. Estudos de pacientes com DMT2 e ND revelaram aumentos dos níveis séricos de MIP-1α e MIP-1β até mesmo nos estágios precoces da ND (KONENKOV; KLIMONTOV; MYAKINA; TYAN *et al.*, 2015; PERLMAN; CHEVALIER; WILKINSON; LIU *et al.*, 2015). Como também foi observado que pacientes DMT2 microalbuminúricos apresentaram elevados níveis urinários de eotaxina quando comparados com pacientes normoalbuminúricos e controle (LIU; ZHAO; WILLCOX; XU *et al.*, 2010).

Entre as citocinas de perfil anti-inflamatório, a IL-10 é uma citocina sintetizada em várias células imunes, principalmente células CD8+ ativadas. Sua produção é prejudicada por muitas citocinas, como IL-4, IL-13 e IFN-γ, e também pela sua autorregulação. Tem ação de inibir citocinas pró-inflamatórias, principalmente TNF-α, IL-1 e IL-6 produzidas por macrófagos e monócitos ativados, e estimula a produção de citocinas anti-inflamatórias (ZHANG; AN, 2007). Alguns estudos relataram que pacientes DMT2 com ND apresentaram níveis séricos elevados de IL-10 e correlação positiva entre IL-10 e albuminúria

(MYŚLIWSKA; ZORENA; SEMETKOWSKA-JURKIEWICZ; RACHOŃ *et al.*, 2005; WONG; HO; TONG; YEUNG *et al.*, 2007). No entanto, já foi descrito que a baixa capacidade de produção de IL-10 está associada à síndrome metabólica e ao DMT2 (VAN EXEL; GUSSEKLOO; DE CRAEN; FRÖLICH *et al.*, 2002). Outra citocina de perfil Th2 que também exerce efeitos anti-inflamatórios é a IL-4. Apesar de ser considerada uma importante citocina nas doenças renais, por estimular a síntese de MEC pelas células mesangiais e epiteliais glomerulares (FURUSU; MIYAZAKI; KOJI; ABE *et al.*, 1997), pacientes com ND apresentaram baixos níveis séricos de IL-4 (PERLMAN; CHEVALIER; WILKINSON; LIU *et al.*, 2015). Também já foi demonstrado que não houve alteração significativa nos níveis séricos de IL-4 nos pacientes com ND quando comparado aos pacientes sem ND (WU; CHEN; LU; CHEN *et al.*, 2010).

1.4 DOENÇA RENAL CRÔNICA E DIABETES MELLITUS TIPO 2

A doença renal crônica (DRC) é um grave problema de saúde pública, cuja prevalência tem aumentado de forma epidêmica em todo o mundo nos últimos anos. Apresenta caráter progressivo e está associada à elevada morbidade e mortalidade. Estima-se que aproximadamente 35% dos pacientes com DMT2 desenvolvem DRC (KIM; PARK; CHO; KIM, 2018). Vários fatores estão associados à instalação e progressão da DRC, incluindo, obesidade, hipertensão arterial sistêmica e diabetes mellitus. Além desses, evidências apontam a inflamação na fisiopatologia da DRC e a ativação do sistema imune em estágios precoces e tardios da DRC. Acredita-se que a modulação da resposta imunoinflamatória pode se tornar alvo para tratamento da DRC (LI; TANG; YI, 2019; VIANNA; SOARES; TAVARES; TEIXEIRA *et al.*, 2011).

A DRC é definida como a diminuição do ritmo de filtração glomerular abaixo de 60 mL/min /1,73 m^2 ou presença de anormalidades na estrutura ou na função renal, considerada uma complicação frequente nos pacientes diabéticos. Os critérios diagnósticos para DRC consistem na presença de um ou mais marcadores de lesão do parênquima renal, como a albuminúria e/ou a taxa de filtração glomerular (TFG) menor que 60 mL/min /1,73 m^2 persistentes por mais de três meses (INKER; ASTOR; FOX; ISAKOVA *et al.*, 2014).

O desfecho da DRC é caracterizado pela progressiva fibrose glomerular e/ou túbulo-intersticial, lesão dos capilares peritubulares por hipóxia e perda de funcionamento dos néfrons

por esclerose glomerular e atrofia tubular (EDDY, 2005). Nesse contexto fisiopatológico de progressão da lesão renal tem sido cada vez mais relacionada a participação de mecanismos inflamatórios (MEZA LETELIER; SAN MARTÍN OJEDA; RUIZ PROVOSTE; FRUGONE ZAROR, 2017; RUIZ-ORTEGA; LORENZO; SUZUKI; RUPÉREZ *et al.*, 2001).

O rastreamento da DRC deve ser iniciado logo após o diagnóstico de diabetes nos pacientes DMT2. A identificação precoce do risco de perda progressiva da função renal nestes pacientes pode protelar as complicações decorrentes do diabetes. Mediante impossibilidade de realizar-se a medida da TFG ou da excreção urinária de albumina (EUA), na prática clínica tem-se utilizado a TFGe, calculada a partir da fórmula CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) (LEVEY; STEVENS; SCHMID; ZHANG *et al.*, 2009), como parâmetro recomendado para avaliar a função renal dos pacientes DMT2. A constatação de uma TFGe< 60 mL/min/1.73m^2, por si só, pode caracterizar a diminuição da função renal (LEVEY; DE JONG; CORESH; EL NAHAS *et al.*, 2011; PENA; HEINZEL; HEINZE; ALKHALAF *et al.*, 2015), podendo esse evento anteceder ao aparecimento de microalbuminúria (KROLEWSKI; NIEWCZAS; SKUPIEN; GOHDA *et al.*, 2014). A DRC é classificada em cinco estágios de acordo com a TFG: Estágio 1 (TFG normal ou elevada: ≥ 90 mL/min/1.73m^2), Estágio 2 (TFG levemente reduzida: 60-89 mL/min/1.73m^2), Estágio 3a (moderada redução da TFG: 45-59 mL/min/1.73m^2), Estágio 3b (redução marcada da TFG: 30-44 mL/min/1.73m^2), Estágio 4 (redução grave da TFG: 15-29 mL/min/1.73m^2) e Estágio 5 (insuficiência renal: TFG < 15 mL/min/1.73m^2) (LEVEY; DE JONG; CORESH; EL NAHAS *et al.*, 2011).

Mediadores inflamatórios têm sido envolvidos na instalação e progressão da DRC no DMT2 (LIU; DENG; SUN; YE *et al.*, 2016; LIU; ZHAO; WILLCOX; XU *et al.*, 2010; VERHAVE; BOUCHARD; GOUPIL; PICHETTE *et al.*, 2013). Recentemente, foi demonstrado que marcadores plasmáticos e urinários indicam que o declínio renal progressivo precoce, no contexto do DMT2, tem múltiplos determinantes (NOWAK; SKUPIEN; SMILES; YAMANOUCHI *et al.*, 2018). Dessa forma é incontestável o papel da inflamação na DRC, sendo fundamental o entendimento dos efeitos dos mediadores inflamatórios na instalação e progressão da lesão renal, tendo em vista a possibilidade de definir novos marcadores prognósticos e, talvez até, alvos terapêuticos alternativos e mais eficientes.

Atualmente, não existem medidas específicas para prevenir o desenvolvimento da ND. As principais estratégias terapêuticas são baseadas no rigoroso controle dos riscos modificáveis como o controle da hipertensão, dos níveis séricos de glicose e da dislipidemia, mas que nem

sempre impedem a progressão da ND. Portanto, um melhor conhecimento do papel de mediadores inflamatórios na patogênese da ND poderia facilitar a identificação dos pacientes de alto risco de progressão da doença, assim como, do desenvolvimento e progressão da disfunção renal (WILLIAMS; TUTTLE, 2005).

Assim, este estudo tem o objetivo de avaliar os seguintes mediadores inflamatórios séricos e *in situ*: interleucina-1β (IL-1β), IL-4, IL-6, IL-10, fator de necrose tumoral-α (TNF-α), os receptores do fator de necrose tumoral-1 (TNFR1) e 2 (TNFR2), interferon-γ (IFN-γ), as quimiocinas: interleucina-8 (IL-8/CXCL8), proteína inflamatória de macrófagos-1α (MIP-1α/CCL3), proteína inflamatória de macrófagos-1β (MIP-1β/CCL4), eotaxina (CCL11) e as adipocinas: adiponectina, leptina e resistina; com intuito de contribuir com a identificação de possíveis fatores que possam estar implicados no processo inflamatório crônico desenvolvido no DMT2, e nas complicações que afetam a função renal desses pacientes.

Frente a uma situação desafiadora devido à ausência ou silenciamento de sintomas, a utilização de métodos alternativos para a identificação precoce do declínio da função renal é fundamental para implementar estratégias preventivas e proporcionar um melhor manejo e acompanhamento aos pacientes diabéticos.

Justificativa

2 JUSTIFICATIVA

O Diabetes Mellitus tipo 2 (DMT2) é um dos subtipos mais prevalentes do Diabetes Mellitus, considerada mundialmente a principal causa de doença renal crônica. Uma de suas complicações microvasculares mais frequentes é a nefropatia diabética (ND), chegando a acometer cerca de 20 a 30% dos pacientes com DMT2 (CHO; SHAW; KARURANGA; HUANG *et al.*, 2018; GREGG; LI; WANG; BURROWS *et al.*, 2014).

Cada vez mais evidências apontam que a inflamação de baixo grau, caracterizada pela produção de citocinas, quimiocinas e adipocinas, está envolvida nos processos patogênicos causadores do DMT2 e suas complicações (ARAÚJO; SILVA; SILVA; MONTEIRO *et al.*, 2016; CEBECI; CAKAN; GURSU; UZUN *et al.*, 2019; GUPTA; MARATHA; SIEDNIENKO; NATARAJAN *et al.*, 2017; NAVARRO; MORA, 2006). O desequilíbrio entre esses mediadores inflamatórios pode ser considerado o gatilho causador ou exacerbador das complicações do DMT2, estabelecendo assim uma relação de envolvimento simultâneo entre diabetes e inflamação (GUEST; PARK; JOHNSON; FREUND, 2008).

Mesmo diante dos tratamentos disponíveis baseados no rigoroso controle dos riscos modificáveis, como o controle da hipertensão, dos níveis séricos de glicose e da dislipidemia, nem sempre são capazes de impedir a progressão da ND ou protegerem contra a diminuição da função renal. Portanto, um melhor conhecimento do papel das citocinas, quimiocinas e adipocinas inflamatórias na patogênese da ND se faz necessário para auxiliar pesquisas futuras voltadas para intervenções terapêuticas mais avançadas e eficazes.

A quantificação de mediadores inflamatórios *in situ* e séricos pode ser uma ferramenta útil para avaliação da influência do processo inflamatório na promoção da diminuição da função renal nos pacientes com DMT2 e na progressão da ND. Dessa maneira, seria possível identificar possíveis fatores que exercem contribuição efetiva no desenvolvimento e manutenção do processo inflamatório crônico de baixo grau e suas complicações repercutidas na função renal dos pacientes com DMT2 e naqueles com ND.

Dessa maneira, estudos voltados para um melhor conhecimento dos mecanismos imunológicos envolvidos na patogênese do DMT2 e suas complicações podem contribuir para que, no futuro, sejam estabelecidas novas e melhores estratégias de rastreio que contribuem para detecção precoce de indivíduos com DMT2 em risco de comprometimento mais rápido da função renal, com vista a proporcionar melhor acompanhamento a esses pacientes.

Objetivos

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar os níveis séricos de mediadores inflamatórios em pacientes com DMT2 com ou sem alteração renal, como também a expressão de citocinas e quimiocinas inflamatórias (IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, TNFR1 e Eotaxina) em biópsias renais de rim nativo de pacientes diabéticos com Nefropatia Diabética, bem como descrever a relação desses mediadores com a diminuição da função renal.

3.2 OBJETIVOS ESPECÍFICOS

1. Descrever a caracterização clínico-epidemiológica dos pacientes DMT2 com ou sem alteração renal atendidos no Ambulatório de Endocrinologia da Universidade Federal do Triângulo Mineiro, e dos pacientes com diagnóstico de ND que realizaram biópsia renal no Serviço de Nefropatologia da Universidade Federal do Triângulo Mineiro entre os anos de 1996 a 2018;
2. Quantificar as citocinas e quimiocinas (IL-4, IFN-γ, TNF-α, IL-8, Eotaxina, MIP-1α e MIP-1β) no soro de pacientes DMT2 com ou sem alteração renal e pacientes controle;
3. Quantificar as adipocinas (Adiponectina, Resistina e Leptina) e as citocinas (TNFR1 e TNFR2) no soro de pacientes DMT2 com ou sem alteração renal e pacientes controle;
4. Quantificar a expressão de citocinas e quimiocinas inflamatórias (IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, TNFR1, MIP-1α e Eotaxina) nas biópsias renais de pacientes com ND e controle;
5. Relacionar as expressões *in situ* e os níveis séricos das citocinas, quimiocinas e adipocinas com os dados clínico-laboratoriais e morfológicos;
6. Relacionar as expressões *in situ* e os níveis séricos das citocinas, quimiocinas e adipocinas com a função renal, determinada pela taxa de filtração glomerular estimada (TFGe);
7. Correlacionar as alterações morfológicas com as expressões *in situ* dos mediadores inflamatórios nas biópsias renais de pacientes com ND.

Resultados

4 RESULTADOS

ARTIGO 1

TÍTULO: **Influence of inflammatory mediators on type 2 diabetes mellitus and decreased renal function**

SITUAÇÃO:

- PLOS ONE Decision: Revision required [PONE-D-19-24297] - [EMID:800861c31b2d900a] em 22/10/19.
- Processo de resposta aos revisores.

Artigo 1

Influence of inflammatory mediators on type 2 diabetes mellitus and decreased renal function

Inflammation in type 2 diabetes mellitus and renal dysfunction

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Abstract

Aim: To evaluate the serum concentrations of inflammatory mediators in patients with type 2 diabetes mellitus (T2DM) with or without renal alteration (RA) function. Methods: Serum samples from 76 patients with T2DM and 24 healthy individuals were selected. Patients with T2DM were divided into two groups according to eGFR (> or < 60mL/min/1.73m^2). Cytokines, chemokines and adipokines levels were evaluated using the Multiplex immunoassay and ELISA. Results: TNFR1 and leptin were higher in the T2DM group with RA than in the T2DM group without RA and control group. All patients with T2DM showed increased resistin, IL-8, and MIP-1α compared to the control group. Adiponectin were higher and IL-4 decreased in the T2DM group with RA compared to the control group. eGFR positively correlated with IL-4 and negatively with TNFR1, TNFR2, and leptin in patients with T2DM. In the T2DM group with RA, eGFR was negatively correlated with TNFR1 and resistin. TNFR1 was positively correlated with resistin and leptin, as well as resistin with IL-8 and leptin. Conclusion: Increased levels of TNFR1, adipokines, chemokines and decrease of IL-4 play important role in the inflammatory process developed in T2DM and decreased renal function. We also suggest that TNFR1 is a strong predictor of renal dysfunction in patients with T2DM.

Key words: type 2 diabetes mellitus, inflammatory mediators, estimated glomerular filtration rate, renal alteration

Introduction

Type 2 diabetes mellitus (T2DM) is one of the most prevalent subtypes of diabetes mellitus (DM). It is a metabolic disorder resulting from the relative deficiency of insulin production and/or its action, which leads to increased serum glucose levels, which is considered the main cause of chronic kidney disease (CKD) [1, 2]. Hyperglycemia in T2DM is strongly associated with the development of macrovascular and microvascular complications, which may result in decreased renal function [3]. Studies suggest that low-grade inflammation, characterized by the production of cytokines, chemokines, and adipokines, is involved in the pathogenic processes that cause T2DM and its complications [4-7].

The imbalance between mediators triggers or enhances T2DM complications. Activation of the innate immune system alone induces hyperglycemia and insulin resistance. Thus, diabetes and inflammation are simultaneously involved, feeding a positive feedback loop [8].

Early identification of the risk of progressive loss of renal function in patients with T2DM might delay diabetes complications in these patients. Due to the lack of feasibility in measuring glomerular filtration rate, in clinical practice, the estimated glomerular filtration rate (eGFR) and albuminuria have been used as parameters to evaluate the renal function of patients with T2DM. An eGFR <60 mL/min/1.73 m^2 might characterize decreased renal function [9, 10]. Plasma and urinary markers have recently shown that that early progressive renal decline, in the context of T2DM, has multiple causes [11].

Given the need to identify possible factors that contribute to low-grade inflammation and its complications, as reflected in the renal function of patients with T2DM, this study aimed to evaluate the serum concentrations of inflammatory mediators in patients with T2DM with or

without renal alteration (RA), determined by the eGFR, and verify the relationship of these mediators to decreased renal function.

Patients and methods

Patients

Type 2 DM patients were recruited in the Endocrinology Outpatient Clinic of the Federal University of Triangulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil, between December of 2016 and May of 2017. Healthy volunteers were recruited from the facilities of the Federal University of Triangulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil. Patients included in the study had T2DM diagnosis, age over 18 years-old and were in medical follow up in Endocrinology Outpatient Clinic of the UFTM. Healthy people aged above 18 years old and with normal renal function were also included for comparison. Pre-diabetic patients, T2DM patients aged under 18 years old and healthy people without sufficient data for eGFR calculation (age, race, serum creatinine and gender) were excluded from the study.

A total of 100 adult patients were recruited for this study, 76 of whom had T2DM (28 men and 48 women) and 24 were healthy volunteers (10 men and 14 women). The patients with T2DM were divided into two groups according to the eGFR (mL/min/1.73 m^2) using the equation proposed by the Chronic Kidney Disease Epidemiology Collaboration study (CKD-EPI) [12]. These were the T2DM group without RA (n=56, patients with T2DM with eGFR>60 mL/min/1.73 m^2), with median age of 59.5 (18–84) years, and the T2DM group with RA (n=20, patients with T2DM with eGFR <60 mL/min/1.73 m^2), with median age of 75 (37–94) years. The control group consisted of 24 healthy patients without DM and with eGFR >60 mL/min/1.73 m^2, with median age of 34 (22–58) years.

Clinical and laboratory data of the patients in the study were obtained from the information in the follow-up medical records of the patients with T2DM and the results of routine blood tests previously acquired from the volunteers.

The study was conducted in the laboratories of General Pathology Department and Immunology Department of the Federal University of Triangulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil. This study was approved by the Research Ethics Committee of the Federal University of Triângulo Mineiro (number 3,001,006).

Methods

T2DM patients were individually addressed during the routine clinical consultation, in doctor´s office. Healthy people were referred to a reserved room in the General Pathology Department of UFTM. They were instructed about the research and those who agreed to participate, signed the consent form and had the biological sample collected. Then, they were referred to a reserved and appropriate blood collection room, where general data of the participants were also recorded.

The sample was collected in a sterile tube, containing a separating gel, and centrifuged, after 30 min of rest, at 3,000 rpm, at 4°C, for 15 min to obtain the serum. The serum sample was stored at -80°C until analysis.

The serum cytokines were quantified using the Multiplex immunoassay - MAGPIX™ System (Lot #5028196) following the manufacturer's instructions, in which the following mediators were detected: interleukin-4 (IL-4), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-8 (CXCL8), eotaxin, macrophage inflammatory protein-1α (MIP-1α), and MIP-1β.

The adipokines (adiponectin, resistin, and leptin), tumor necrosis factor receptor-1 (TNFR1), and TNFR2 were measured by the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) method using R&D Systems® antibody pairs, following the manufacturer's instructions: Human Adiponectin (Catalog DY1065), Human Resistin (Catalog DY1359), Human Leptin (Catalog DY398), Human TNF RI (Catalog DY225), and Human TNF RII (Catalog DY726).

Statistical analysis

In the statistical analysis, an electronic spreadsheet (Microsoft Excel) was elaborated, and the data were analyzed using the GraphPad Prism software, version 7.0 (GraphPad Software, USA). The variables were tested for normality using the Kolmogorov-Smirnov test. For a non-normal distribution, we used the Mann-Whitney test (U) in the comparison between the two groups and the Kruskal-Wallis test (H), followed by the Dunn's post hoc test, among three or more groups. The proportions were compared using the chi-square test (χ^2) or Fisher's exact test. We used the Pearson's test (r) to correlate parametric variables and the Spearman's test (rS) for nonparametric variables. Differences were considered statistically significant when $p < 0.05$.

Results

Clinical and laboratory characteristics of the participants

A total of 100 patients were selected for the study and classified into three groups: the control group with 24 patients (24%), T2DM group without RA with 56 (56%) patients, and T2DM group with RA with 20 (20%) patients. According to the general characteristics of the groups, there was a predominance of women in the three groups, with 14 (58.3%) in the control

675 group, 36 (64.3%) in the T2DM group without RA, and 12 (60%) in the T2DM group with RA.
676 The patients were mainly Caucasian, with 20 (83.3%) in the control group, 45 (80.4%) in the
677 T2DM group without RA, and 16 (80%) in the T2DM group with RA. Most patients with
678 T2DM had hypertension, with 36 (64.3%) in the T2DM group without RA and 18 (90%) in the
679 T2DM group with RA, whereas there was no patient with hypertension in the control group.
680 The body mass index (BMI) was higher in the T2DM groups than in the control group. Patients
681 with T2DM with RA had longer DM duration compared to patients with T2DM without RA.
682 Moreover, most patients in both groups reported the use of insulin to control diabetes, with 38
683 (67.8%) in the T2DM group without RA and 17 (85%) in the T2DM group with RA.

684 Regarding the laboratory data, as expected, fasting serum glucose and glycated
685 hemoglobin levels were higher in patients with T2DM. Serum urea and creatinine levels were
686 higher in the T2DM group with RA than in other groups. Serum total cholesterol, high-density
687 lipoprotein cholesterol, and triglyceride levels were similar among groups. However, serum
688 low-density lipoprotein cholesterol levels were higher in the control group than in other groups.
689 Regarding the habit of drinking alcohol, most 14 (58.3%) patients in the control group reported
690 social use, while 30 (53.6%) patients in T2DM group without RA and 12 (60%) patients in
691 T2DM group with RA reported not to consume alcohol. Most patients in all groups were non-
692 smokers. Regarding physical activities, half of the patients both in control and T2DM with RA
693 groups did not practice physical activities as well as 35 (62.5%) patients of T2DM group
694 without RA. The clinical and laboratory characteristics of the patients are detailed in Table 1.

695 **Table 1. Clinical and laboratory data of diabetic and control groups.**

	Control (n=24)	**T2DM without RA (n=56)**	**T2DM with RA (n=20)**
Age (years)			
Median (Min-Max)	34 (22-58)	59.5 (18-84)	74 (37-94)
Gender n (%)			
Male	10 (41.7%)	20 (35.7%)	08 (40%)
Female	14 (58.3%)	36 (64.3%)	12 (60%)

Color n (%)			
White	20 (83.3%)	45 (80.4%)	16 (80%)
Not white	04 (16.7%)	11 (19.7%)	04 (20%)
SAH n (%)			
Yes		36 (64.3%)	18 (90%)
No	24 (100%)	20 (35.7%)	02 (10%)
BMI (kg/m^2)			
Median (Min-Max)	24.34 (19.6-30.4)	27.19 (18.17-42.1)	26.5 (18.4-36.8)
Course DM (years)			
Median (Min-Max)		11.5 (0.4-30)	17.5 (0.5-40)
Insulin n (%)			
Yes		38 (67.8%)	17 (85%)
No		18 (32.2%)	03 (15%)
eGFR (mL/min/1.73m^2)			
Mean ± SD	93.81 ± 18.14	89.55 ± 21.17	40.72 ± 16.95
Fasting glucose (mg/dL)			
Median (Min-Max)	86.9 (69-101)	169.9 (94.9-596.9)	143.3 (53.7-366.7)
HgA1c (%)			
Median (Min-Max)	4.8 (4.5-5.7)	8.6 (5.9-15.1)	7.6 (5.3-10.9)
Urea (mg/dL)			
Median (Min-Max)	32 (23.3-40)	29 (18.4-61.7)	56.5 (34.1-191.9)
Creatinine (mg/dL)			
Median (Min-Max)	0.87 (0.6-1.4)	0.82 (0.41-1.3)	1.27 (0.95-10.61)
TC (mg/dL)			
Median (Min-Max)	183.1 (148-282)	162.9 (93.3-275.3)	168(104.3-265.3)
HDL (mg/dL)			
Median (Min-Max)	53 (27-76)	52 (26-97)	50 (32-101)
LDL (mg/dL)			
Median (Min-Max)	98 (74-138)	67.6 (24-192.3)	78.8 (48.7-186.2)
TG (mg/dL)			
Median (Min-Max)	97 (46-235)	125.5 (28-558)	151 (32-323)
Alcohol drinking habit n (%)			
Yes (socially)	14 (58.3%)	9 (16.1%)	1 (5%)
No	9 (37.5%)	30 (53.6%)	12 (60%)
Past		5 (8.9%)	
NI	1 (4.2%)	12 (21.4%)	7 (35%)
Smoking n (%)			
Yes	1 (4.2%)	6 (10.7%)	
No	22 (91.6%)	32 (57.1%)	12 (60%)
Past		9 (16.1%)	2 (10%)
NI	1 (4.2%)	9 (16.1%)	6 (30%)
Practicing exercise n (%)			
Yes	11 (45.8%)	21 (37.5%)	10 (50%)
No	12 (50%)	35 (62.5%)	10 (50%)
NI	1 (4.2%)		

DM: Diabetes Mellitus. RA: Renal alteration. SAH: Systemic arterial hypertension. BMI: Body mass index. MG: Minas Gerais. eGFR: Estimated glomerular filtration rate. HgA1c: Glycated hemoglobin. TC: Total cholesterol. HDL: High density lipoprotein. LDL: Low density lipoprotein. TG: Triglycerides. NI: Not informed. SD: Standard deviation.

Imbalance in serum cytokine production in patients with T2DM with RA

Inflammatory cytokines were analyzed in patients with T2DM to evaluate their production in the context of renal function. Patients with T2DM showed a significant decrease in serum IL-4 and TNFR2 levels ($p=0.0165$, $U=617$, and $p=0.0024$, $U=541.5$, respectively) and a significant increase in TNFR1 level compared to controls ($p=0.0035$; $U=554.5$). However, there was no significant difference in TNF-α level between the groups ($p=0.7919$; $U=879$), and there was only a tendency of increased IFN-γ level in patients with T2DM ($p=0.0724$; $U=690$). Comparing the groups based on RA, the T2DM group with RA had decreased IL-4 levels compared to the control group ($p=0.0090$; $H=9.413$, Dunn's post hoc test) and increased TNFR1 levels when compared to the T2DM group without RA and control group ($p<0.0001$; $H=20.58$, Dunn's post hoc test). Patients without RA showed a decrease in TNFR2 level compared to the control group ($p=0.0017$; $H=12.76$, Dunn's post hoc test), and there was no significant difference in TNF-α ($p=0.9606$; $H=0.0803$, Dunn's post hoc test) and IFN-γ levels ($p=0.1986$; $H=3.233$ Dunn's post hoc test) between the groups (Fig 1).

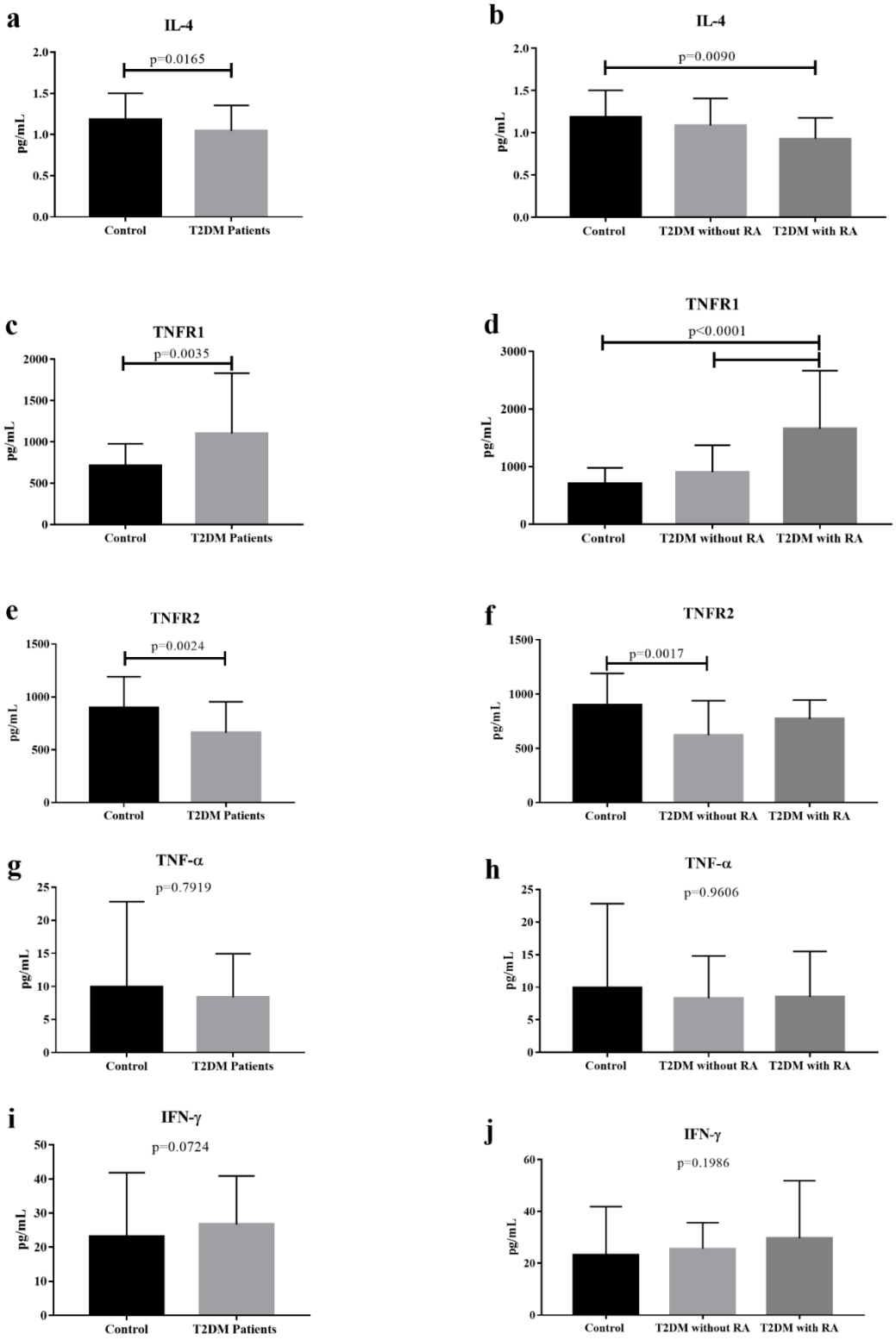


Fig 1. Serum cytokine concentrations in the T2DM group without and with RA and control group. (a) Serum IL-4 level in T2DM patients and controls and (b) T2DM patients without and with RA vs. controls. (c) Serum TNFR1 level in T2DM patients and controls and

(d) T2DM patients without and with RA vs. controls. (e) Serum TNFR2 level in T2DM patients and controls and (f) T2DM patients without and with RA vs. controls. (g) Serum TNF-α in T2DM patients and controls and (h) T2DM patients without and with RA vs. controls. (i) Serum INF-γ level in T2DM patients and controls and (j) T2DM patients without and with RA vs. controls. The results were expressed as mean ± standard deviation. RA, renal alteration.

Increased serum adipokine production in patients with T2DM with RA

Observing the imbalance in serum cytokine production in patients with T2DM with RA and considering T2DM as a low-grade chronic inflammatory process, we analyzed adipokine production in these patients. There was a significant increase in serum adiponectin and resistin levels in patients with T2DM compared to the control group (p=0.0230, U=631.5, and p=0.0003, U=478.5, respectively). Moreover, there was a tendency for increased leptin levels in patients with T2DM compared to that in the control group (p=0.0844; U=698). Comparing the groups based on RA, there was an increase in adiponectin levels in the patients with T2DM with RA compared to the control group (p=0.0422; H=6.329, Dunn's post hoc test). Regardless of RA, patients with T2DM showed an increase in resistin levels compared to the control group (p=0.0014; H=13.12, Dunn's post hoc test). However, the serum leptin levels were significantly higher in the T2DM group with RA compared to those in the T2DM group without RA and control group (p=0.0076; H=9.759, Dunn's post hoc test) (Fig 2).

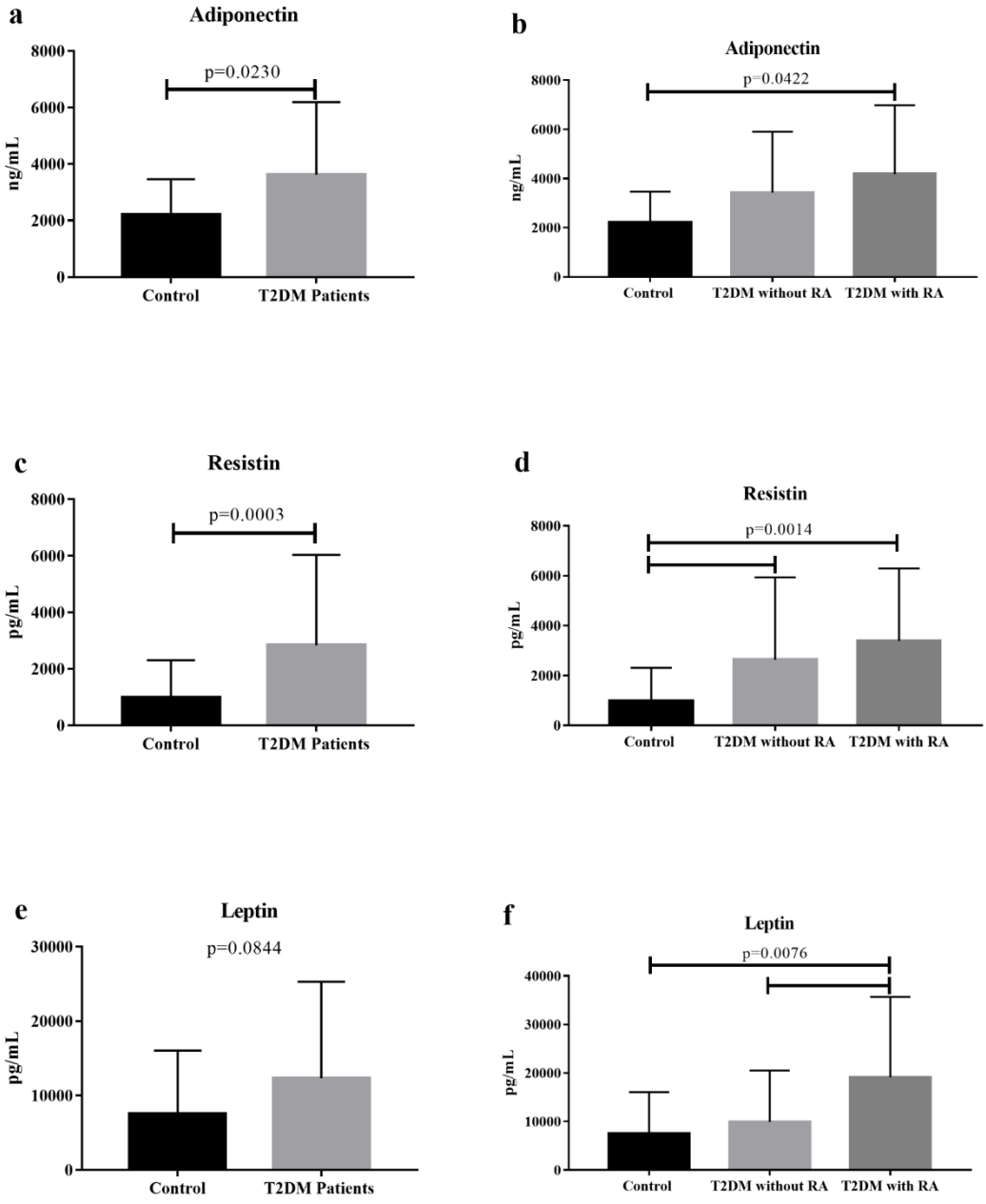
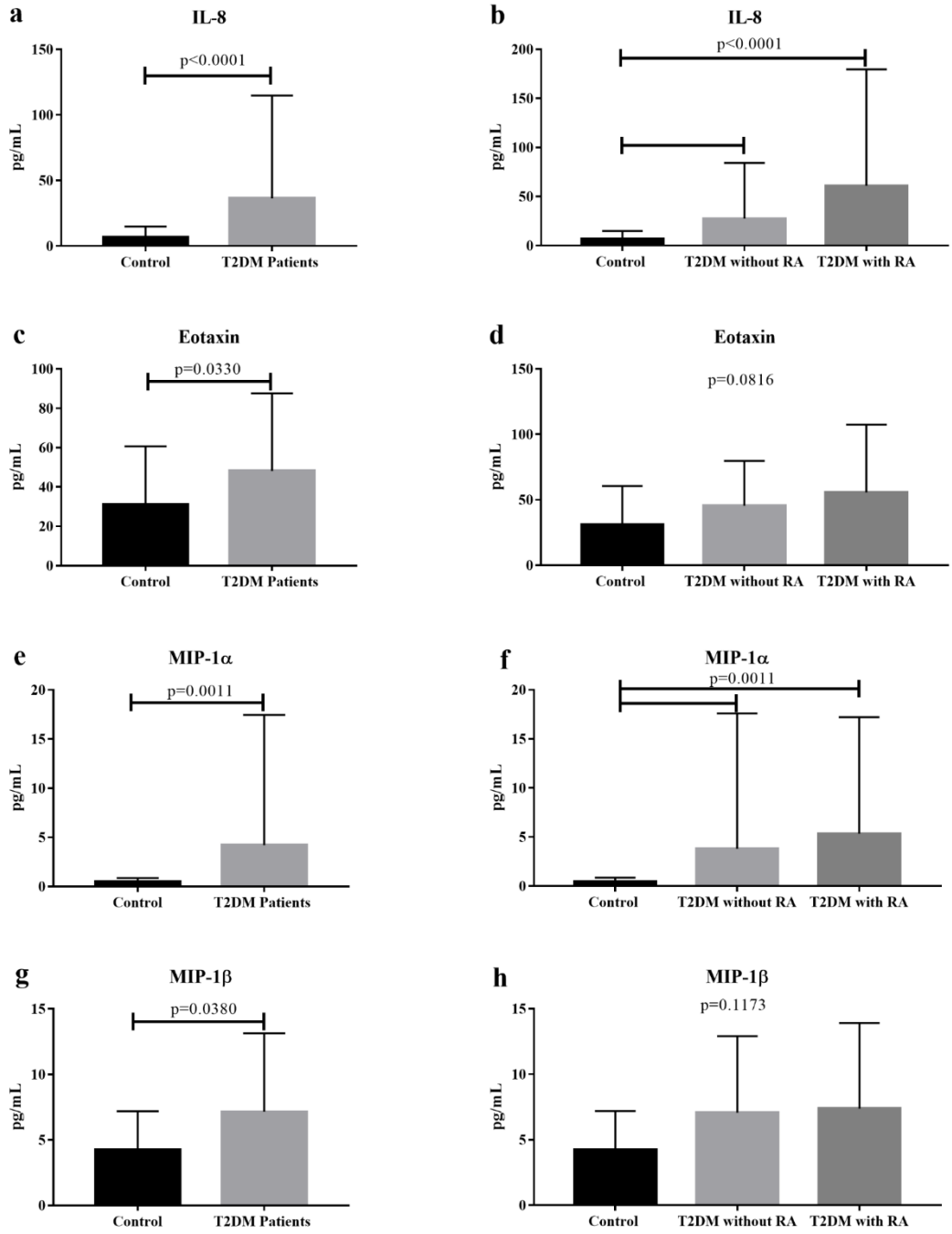


Fig 2. Serum adipokine levels in T2DM group without and with RA and control group. (a) Serum adiponectin levels in T2DM patients and controls and (b) T2DM patients without and with RA vs. controls. (c) Serum resistin level in T2DM patients and controls and (d) T2DM patients without and with RA vs. controls. (e) Serum leptin level in T2DM patients and controls and (f) T2DM patients without and with RA vs. controls. The results were expressed as mean ± standard deviation. RA, renal alteration.

Increased serum chemokine production in patients with T2DM with RA

Observing the increase in adipokine production associated with the imbalance in cytokine production in patients with T2DM with RA, we analyzed the chemokine production in these patients. There was a significant increase in serum IL-8 ($p<0.0001$; $U=322$), eotaxin ($p=0.0330$; $U=648.5$), MIP-1α ($p=0.0011$; $U=541$), and MIP-1β ($p=0.0380$; $U=655.5$) levels in T2DM patients compared to those in the control group.

Comparing the groups based on RA, it was found that, regardless of RA, serum IL-8 levels remain significantly elevated in the T2DM group without RA and T2DM group with RA compared to that in the control group ($p<0.0001$; $H=22.8$, Dunn's post hoc test). The same mechanism was observed with regard to the MIP-1α level ($p=0.0011$; $H=13.61$, Dunn's post hoc test). There was no significant difference in eotaxin ($p=0.0816$; $H=5.011$, Dunn's post hoc test) and MIP-1β levels ($p=0.1173$; $H=4.286$, Dunn's post hoc test) between the groups. However, it is possible to observe that both eotaxin and MIP-1β tend to behave similarly to IL-8 and MIP-1α (Fig 3).



766
767 **Fig 3. Serum chemokine levels in the T2DM group without and with RA and control**
768 **group.** (a) Serum IL-8 level in T2DM patients and controls and (b) T2DM patients without and
769 with RA vs. controls. (c) Serum eotaxin level in T2DM patients and controls and (d) T2DM
770 patients without and with RA vs. controls. (e) Serum MIP-1α level in T2DM patients and
771 controls and (f) T2DM patients without and with RA vs. controls. (g) Serum MIP-1β level in

T2DM patients and controls and (h) T2DM patients without and with RA vs. controls. The results were expressed as mean ± standard deviation. RA, renal alteration.

Correlation between eGFR and cytokines/ chemokines/ adipokines in patients with T2DM with RA

To evaluate the relationship of mediators in patients with T2DM with RA, correlations between eGFR and cytokines/chemokines/adipokines were analyzed. Patients with T2DM had a positive and significant correlation between eGFR and IL-4 ($p=0.0346$; $rS=0.2428$) and a negative and significant correlation between eGFR and TNFR1 ($p<0.0001$; $rS=-0.4559$), TNFR2 ($p=0.0096$; $rS=-0.2956$), and leptin ($p=0.0178$; $rS=-0.2712$). In the T2DM group with RA, eGFR was negatively and significantly correlated with TNFR1 ($p<0.0001$; $r=-0.9101$) and resistin ($p=0.0141$; $r=-0.5396$, Fig 4).

Patients with T2DM with RA showed a positive and significant correlation between TNFR1 and resistin ($p=0.0002$; $rS=0.7349$) and leptin ($p=0.0420$; $rS=0.4586$). A positive and significant correlation was also observed between resistin and leptin ($p=0.0192$; $r=0.5185$) and between resistin and IL-8 ($p=0.0087$; $rS=0.57$, Fig 5).

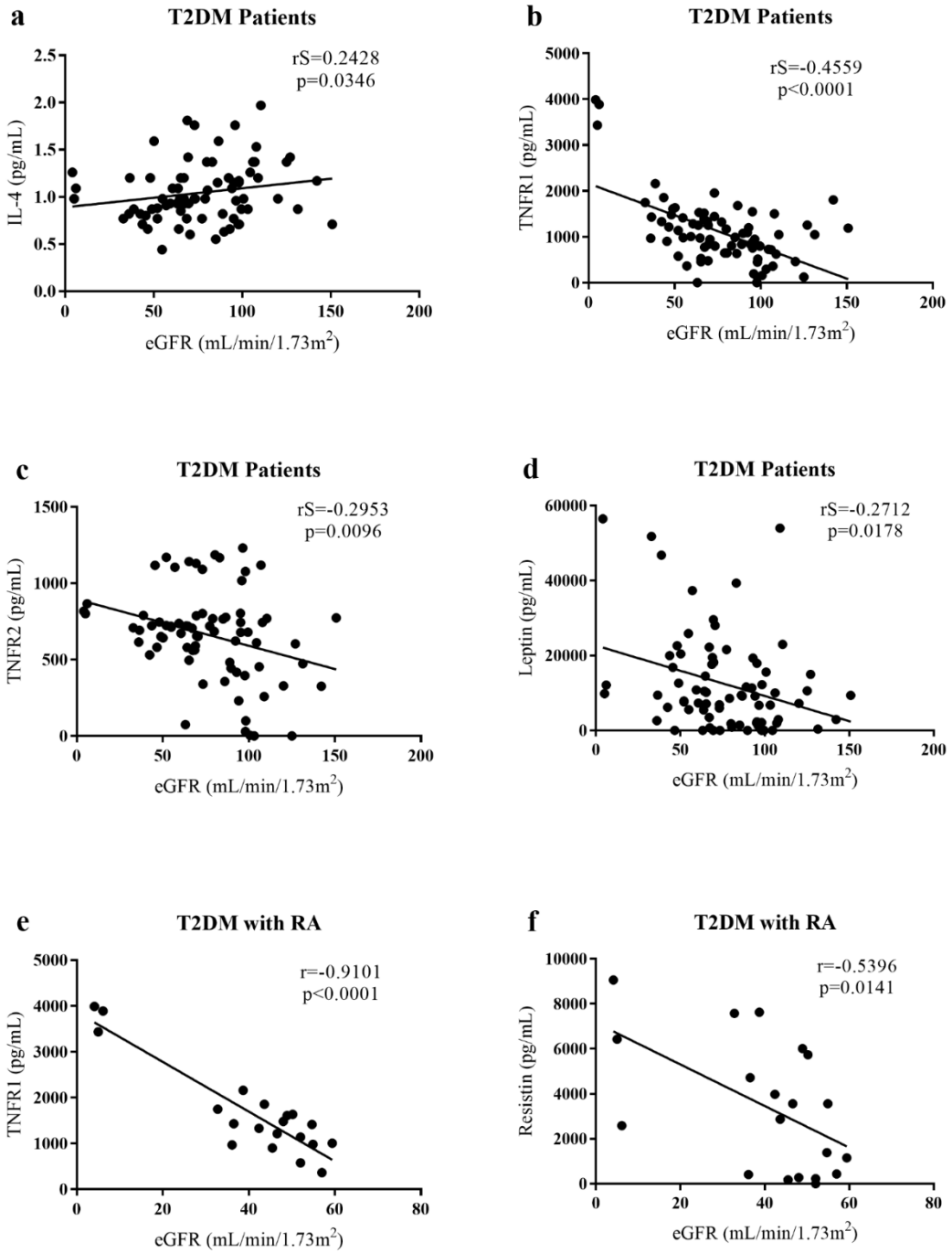


Fig 4. Correlations between cytokine serum levels and estimated glomerular filtration rate (eGFR) in T2DM patients and T2DM with RA. (a) Positive and significant correlation between IL-4 level and eGFR in T2DM patients. (b) Negative and significant correlation, in T2DM patients, between TNFR1 level and eGFR, (c) between TNFR2 level and eGFR and (d) between leptin level and eGFR. (e) Negative and significant correlation, in T2DM with RA

group, between TNFR1 level and eGFR and (f) between resistin level and eGFR. RA, renal alteration.

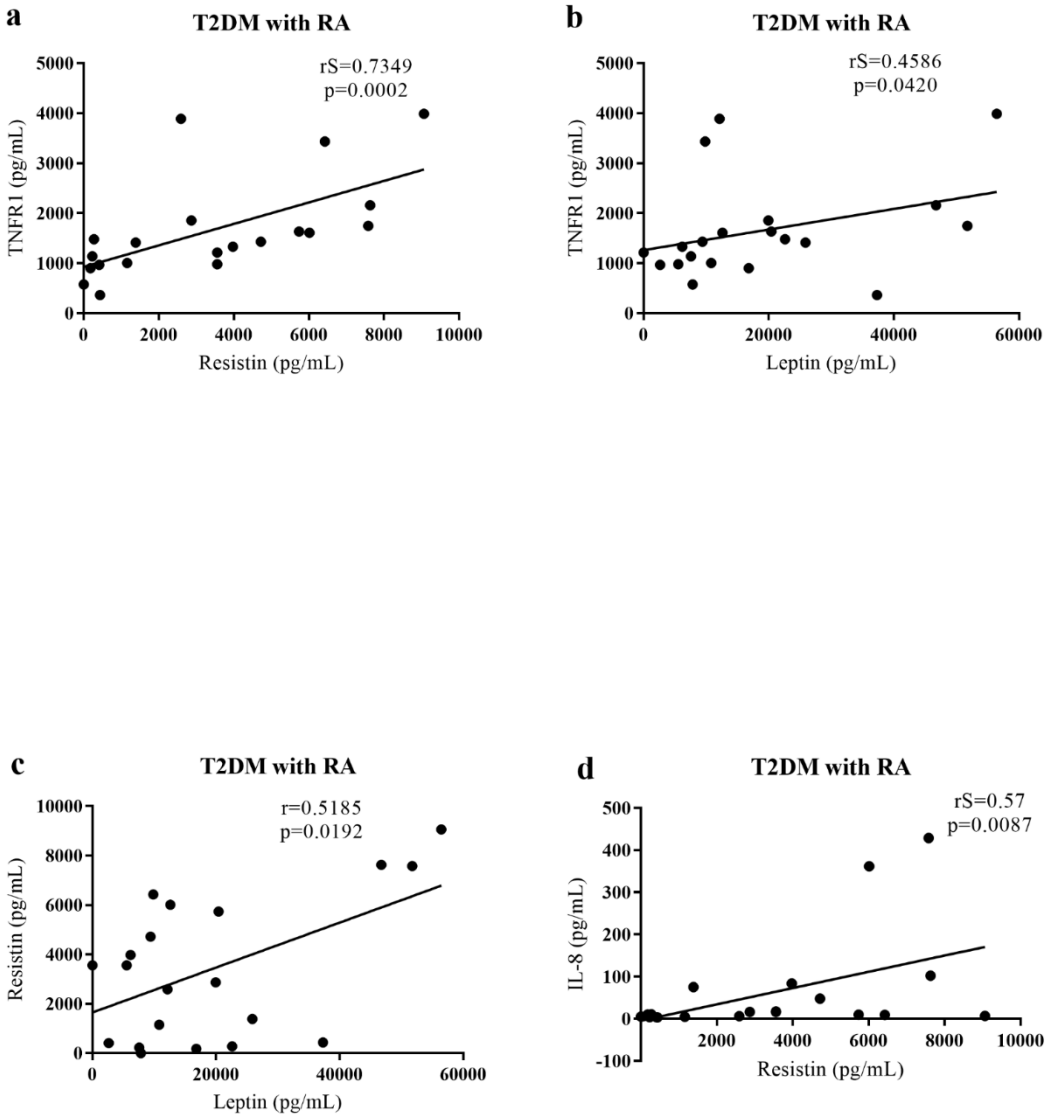


Fig 5. Correlations between serum cytokine and adipokine levels in patients with T2DM with RA. (a) Positive and significant correlation between TNFR1 and resistin levels, (b) between TNFR1 and leptin levels, (c) between resistin and leptin levels, (d) between resistin and IL-8 levels in patients with T2DM with RA. RA, renal alteration.

Discussion

Although hyperglycemia is considered the main triggering factor of Diabetic Nephropathy (DN), low-grade chronic inflammation is one of the triggering factors of kidney injury in patients with T2DM [13-15]. Several studies are being conducted to determine the actual role of inflammatory cytokines in the development and progression of diabetic kidney disease [16-18]. In this context, this study analyzed the serum cytokine/chemokine/adipokine levels in patients with T2DM with or without RA as determined by eGFR in relation to those in healthy patients, to investigate the association of these inflammatory mediators with decreased renal function.

In our study, patients with T2DM had decreased serum IL-4 levels compared to the control group. A decreased IL-4 level was also found in patients with T2DM with RA compared to that in the control group, indicating that not only diabetes but also RA characterized by eGFR <60 mL/min/1.73 m^2 is associated with decreased IL-4 level. IL-4 is a Th2 profile anti-inflammatory cytokine that acts to reduce the secretion of proinflammatory cytokines by activated macrophages and stimulates the production of a number of anti-inflammatory molecules, such as IL-1ra [19], IL-1R2 [20], and soluble TNF receptors [21]. Patients with DN [22], as well as those with T2DM [23], show decreased serum IL-4 levels. Results of experimental studies with db/db mice suggested that suppression of the inflammatory process by anti-inflammatory cytokines is impaired in T2DM [24, 25]. The decrease in serum IL-4 levels compromises its action in reducing the effects of IL-1 and IL-8 [26, 27], determining a factor of worse evolution of T2DM. Thus, the decrease in IL-4 level may be associated with the development of inflammatory process complications. This culminates in impairing the renal function of these patients, since serum IL-4 level decreases as eGFR decreases, as shown by the positive and significant correlation between IL-4 level and eGFR. Possibly, the decrease in

serum IL-4 levels in the patients of this study could be due to the increase in cytokine, adipokine, and chemokine levels.

In this study, the results of patients with T2DM were different with respect to serum TNFR levels as TNFR1 level increased and TNFR2 level decreased compared to those in the control group. One of the main findings of our study was that, exclusively, the increase in TNFR1 level distinguished the patients with T2DM with RA from those with T2DM without RA and healthy volunteers. This fact was confirmed by the negative and significant correlation between eGFR and TNFR1 in patients with T2DM and even stronger correlation in patients with T2DM with RA. Thus, this result shows that TNFR1 can predict a decrease in renal function in patients with T2DM. Although TNFR2 is decreased in patients with T2DM without RA compared to those in the control group, it was noted that its serum level might increase in patients with T2DM with RA, which was strengthened by the fact that eGFR correlates negatively with TNFR2 level. Nevertheless, despite the relevance of the results found with their receptors, we did not observe any significant difference between the groups in relation to TNF-α.

TNF-α is a pleiotropic cytokine that plays an important role in the mediation of inflammatory processes. It is a transmembrane homotrimeric protein, which is produced by many cells, including fat, endothelial cells, and leukocytes. In plasma, TNF-α appears free or bound to the circulating TNFR1 and TNFR2 [28]. In a 12-year follow-up study conducted in patients with T2DM, it was observed that, of all markers analyzed, only TNFR1 and TNFR2 were associated with the risk of end-stage renal disease. A stronger association was found with TNFR1, suggesting that high serum levels of this receptor can predict the progression of T2DM to CKD [29]. Other studies have shown that elevated plasma TNFR1 levels are associated with decreased eGFR in patients with T2DM [30, 31], which corroborates our findings. In contrast, a recent study showed that patients with T2DM had increased plasma levels of not only TNFR1

but also TNF-α and TNFR2 compared to the control group, which differs from our findings. However, similar to our results, TNFR1 and TNFR2 were strongly associated with kidney injury [32]. It is still unclear why serum TNFR levels are more closely associated with eGFR. One possible explanation is that, because TNFR levels are at least 100 times greater than TNF-α levels, circulating TNFRs play an important role in the progression of diabetic kidney disease, regardless of the TNF-α levels [33].

In association with the cytokine findings, our study found an increase in serum adipokine levels in patients with T2DM. Patients with T2DM with RA showed a significant increase in adiponectin level. Regardless of RA, patients with T2DM also showed an increase in resistin level. However, serum resistin levels tend to increase as eGFR decreases, which was confirmed by the negative and significant correlation found between eGFR and resistin level. Additionally, another important finding of our study was in relation to the serum leptin levels, which established its importance in distinguishing patients with T2DM with RA, also showing a negative and significant correlation with eGFR. In light of these findings, our results demonstrate that an increase in adipokine levels is related to a decrease in renal function in patients with T2DM.

Adiponectin is an adipokine secreted exclusively by human adipocytes [34]. It has beneficial effects on insulin resistance and anti-inflammatory [35] and anti-oxidative properties [36]. It is suggested that the anti-inflammatory action of adiponectin is due to the inhibition of proinflammatory cytokine production, such as IL-6 and TNF-α, by macrophages and/or reduction of their phagocytic action [37]. We observed a significant increase in adiponectin level in our patients. Perhaps, this factor has contributed in the serum levels of proinflammatory cytokines, such as TNF-α and IFN-γ. Although some studies reported that patients with T2DM have lower circulating quantities of adiponectin than those without T2DM [38, 39], other studies have shown that, under various kidney disease conditions [40, 41] and in patients with

879 T2DM with CKD [42, 43] the serum adiponectin levels are increased, which corroborates our
880 findings. Another study evaluating more than 1,200 patients with T2DM showed an inverse
881 relationship between serum adiponectin levels and eGFR [44]. The relationship of adiponectin
882 and CKD is still controversial. It is suggested that, in individuals with kidney dysfunction,
883 increased adiponectin levels represent not only a decrease in renal excretion but also a
884 temporary homeostatic mechanism in an attempt to reduce renal damage through anti-
885 inflammatory and anti-oxidative mechanisms [45, 46].

886 Resistin is a protein secreted mainly by macrophages and monocytes in humans and has
887 proinflammatory effects [47, 48]. The association between serum resistin levels and CKD in
888 diabetes is also unclear. It was recently observed that patients with microalbuminuria and
889 T2DM with eGFR <60 mL/min/1.73 m^2 showed a significant increase in serum resistin levels
890 compared to patients with T2DM with normal renal function. Additionally, serum resistin levels
891 were correlated negatively with eGFR and positively with C-reactive protein level. Thus, the
892 main determinants of resistin levels in patients with T2DM are renal function level and
893 inflammation [7]. Axelsson et al. demonstrated that high resistin levels in patients with T2DM
894 with CKD were associated with decreased eGFR and inflammation [49]. A prospective cohort
895 study showed that high resistin and TNFR2 levels are related to a higher risk of decline in renal
896 function [50]. Moreover, an increase in resistin levels was observed in the early stages of CKD
897 [51]. This means that even in mild renal function, there is already an increase in resistin level,
898 which corroborates our findings. In agreement, other investigations suggest that resistin might
899 promote endothelial dysfunction by enhancing the oxidative stress, an effect that would
900 eventually culminate in glomerular dysfunction [52, 53] and that the adverse effects of resistin
901 could be attributed to its ability to stimulate proinflammatory cytokine production [47, 54].

902 Another adipokine with proinflammatory effects, which promotes the synthesis of other
903 inflammatory cytokines, is leptin. It is involved in the control of food intake, leading to appetite

suppression. Patients with obesity have hyperleptinemia due to the development of leptin resistance [55]. High leptin levels are associated with insulin resistance and development of T2DM [56]. It has been shown that an increase in serum leptin levels are related to a decline in eGFR, and this association has been described to be stronger in women [57] and patients with CKD [58]. Both the decrease and increase in leptin levels are risk factors for the decline in renal function in patients with T2DM [59]. Our results showed that patients with T2DM with decreased renal function had increased serum leptin levels. In addition to a decreased renal excretion due to renal dysfunction, unfavorable actions of leptin, such as the activation of the sympathetic nervous system, rather than causing beneficial effects, may affect the renal function decline in patients with hyperleptinemia. This can be then further compromised, due to the leptin resistance found in these patients [59].

Among the chemokines analyzed, we observed that patients with T2DM had a significant increase in serum IL-8, eotaxin, MIP-1α, and MIP-1β levels compared to the control group. One study evaluated urinary cytokine levels in patients with T2DM with normo- and microalbuminuria and found a significant increase in urinary IL-8, IP-10, MCP-1, G-CSF, eotaxin, RANTES, and TNF-α levels in patients with microalbuminuria compared to patients with normoalbuminuria. Patients with microalbuminuria had a significant increase in GM-CSF, MIP-1α, and MIP-1β levels compared to the control group. These results indicated that determination of the urine cytokine level might be useful in the diagnosis and early treatment of diabetic nephropathy [60].

Our results showed that the increase in serum IL-8 levels in patients with T2DM is independent of the presence of RA, although its increase, accompanied by decrease in eGFR, is noticeable. IL-8 (CXCL8) was the first chemokine to be discovered and has a predominantly chemoattractant effect on neutrophils [61, 62]. It enhances the expression of adhesion molecules by endothelial cells and antagonizes IgE production stimulated by IL-4 [63]. It is produced

mainly by monocytes/macrophages and, to a lesser extent, by fibroblasts, endothelial cells, keratinocytes, hepatocytes, melanocytes, and chondrocytes. IL-1, TNF-α, and IFN-γ are its main stimulators [64]. In the kidneys, podocytes and endothelial cells of interstitial vessels are the main sources of IL-8, while tubular epithelial cells express small amounts of this cytokine. In inflammatory kidney diseases, the IL-8 expression increases fivefold compared to those in normal structures. It increases the level of endothelial cells near the inflammatory site, facilitates the recruitment and crossing of leukocytes through the endothelium, and alters the expression of adhesion molecules [65]. Urinary IL-8 levels have been observed to be elevated in the early stages of diabetic nephropathy in patients with T2DM [66]. Another study that evaluated the association between urinary cytokine levels and decreased eGFR in patients with T2DM with DN found that increased urinary levels of IL-6, IL-8, TNF-α, and TFG-β were predictors of a faster decline in renal function, indicating the clinical utility of these levels in stratifying the risk of renal disease progression [67]. In patients with T2DM, IL-8 was negatively associated with eGFR and positively associated with BMI [68]. These studies reveal that there is an association between increased IL-8 level and decreased eGFR in patients with T2DM. Our results showed that the increase in serum IL-8 level anticipated a decrease in renal function in patients with T2DM. A possible explanation is that the hyperglycemic environment itself promotes increased serum levels of this chemoattractant cytokine. These contribute to the onset and progression of the inflammatory process, from recruitment, especially of neutrophils, to vascular changes, such as increased permeability that favors the arrival of new inflammatory cells to the inflammatory site, which results in renal function impairment [69].

Similar to that found in relation to serum IL-8 levels, in our study, the patients with T2DM also showed an increase in serum MIP-1α and MIP-1β levels compared to the control group. However, only MIP-1α level showed a significant difference in the evaluation of T2DM group without RA and T2DM group with RA in relation to the control group. Thus, similar to

the IL-8 level, the increase in serum MIP-1α levels in patients with T2DM is independent of the presence of RA. However, its increase also seems to accompany the decrease in eGFR. MIP-1α (CCL3) and MIP-1β (CCL4) belong to the CC subfamily of chemokines and induce the expression of adhesion and costimulatory molecules on the surface of T cells, NK cells, macrophages, and monocytes. These chemokines not only mediate the chemotaxis of these cells but also promote the secretion of proinflammatory cytokines [70]. One study evaluated the serum levels of inflammatory cytokines in 64 patients with T2DM with CKD, and it was observed that patients with eGFR of 30–59 mL/min/1.73 m^2 had increased serum MIP-1α levels. This was associated with the decline in eGFR and also correlated positively with urinary albumin excretion [71]. Patients with T2DM with diagnosis of DN showed an increase in serum MIP-1β levels in CKD stages 1–2 [72]. Thus, corroborating these studies, our results suggest that increased serum MIP-1α and MIP-1β levels may anticipate the decline in renal function of patients with T2DM.

In this study, patients with T2DM showed an increase in serum eotaxin levels compared to the control group. However, there was no difference between patients with T2DM with and those without RA. Although there was no such difference, a trend of increased serum eotaxin levels in these patients was noted. Eotaxin is a CC chemokine that acts on chemotaxis, mainly of eosinophils. It is secreted by endothelial cells, macrophages, fibroblasts, and smooth muscle cells [73]. In 2015, a study conducted in African American patients with type 1 diabetes was the first to report that increased plasma eotaxin levels are an independent predictor of renal failure [74]. A prolonged hyperglycemia process increases the excretion of urinary eotaxin and other inflammatory mediators [75]. Increased urinary eotaxin levels were found in patients with microalbuminuria and T2DM compared to patients with normoalbuminuria and controls [60]. In addition to angiogenic properties [76] and contributing to renal interstitial eosinophilia [77], studies have shown that an increase in serum eotaxin levels in patients with T2DM could play

an important role in the process of atherosclerosis that developed in patients with T2DM [78] and chronic renal disease [72]. Increased serum eotaxin levels were also observed in obese mice and humans [79]. Thus, it is possible to associate the increase in serum and urinary eotaxin levels with the development of T2DM complications and renal function impairment, as probably related to obesity that may affect patients with T2DM and favor the low-grade chronic inflammatory process [72, 79].

Low-grade chronic inflammation promoted by T2DM is associated with macrophage infiltration in the kidney. Monocytes/macrophages and neutrophils are considered primordial cells that drive inflammation and concomitant production of proinflammatory cytokines in vivo [80, 81]. Increased infiltration of activated monocytes, macrophages, and T lymphocytes are described in the kidneys of patients with T2DM with DN [82, 83] reinforcing the hypothesis that T2DM is a disease of the innate immune system [84, 85]. Thus, our study demonstrates that patients with T2DM with RA show increased stimulation for the recruitment of innate immune cells, through an increase in the serum levels of pro-inflammatory chemokines, stimulated by the increase in adipokines and TNFR1, with consequent decrease in IL-4, favoring the inflammatory process. Hence, this immune mechanism could be associated with and plays an important role in promoting the decline in renal function in patients with T2DM. Therefore, our study highlights the importance of this screening for increased serum TNFR1, adipokine, and chemokine levels and decreased serum IL-4 level in patients with T2DM to identify individuals at risk of progressive loss of renal function.

We demonstrated that TNFR1 correlated positively with resistin and leptin in patients with T2DM with RA, showing its contribution to the increase of these adipokines in conditions of decreased renal function. Furthermore, under this same condition, resistin was shown to be positively related to leptin and IL-8. Thus, adipokines, especially resistin and leptin, TNFR1,

and IL-8, exert similar behaviors in patients with T2DM with decreased renal function in their inflammatory process.

Conclusions

Our study showed that serum TNFR1, IL-4, adipokines, and chemokines play an important role in the inflammatory process in T2DM and decreased renal function. Moreover, our data indicate that TNFR1 is a strong predictor of renal dysfunction in patients with T2DM.

Acknowledgements

The authors appreciate the financial support of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Fundação de Ensino e Pesquisa de Uberaba (FUNEPU).

The authors thank Federal University of Triângulo Mineiro, General Pathology Discipline and the following employees of the Nephropathology service: Alberto Borba, Edson Santos, João Noberto, Laura Penna, Lívia Alves and Vandair Gonçalves.

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Artigo 2

ARTIGO 2

TÍTULO: **Renal expression of cytokines and chemokines in diabetic nephropathy**

SITUAÇÃO:

- Artigo formatado de acordo com as normas da revista BMC Nephrology.

Renal expression of cytokines and chemokines in diabetic nephropathy

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ABSTRACT

Introduction: Diabetic nephropathy (DN) is the leading cause of end-stage renal disease worldwide. Inflammatory mediators have been implicated in the pathogenesis of DN, which is thus considered an inflammatory disease. However, further studies are required to assess the renal damage caused by the action of these molecules. Therefore, the objective of this study was to analyze the expression of cytokines and chemokines in renal biopsies from DN patients and to correlate it with interstitial inflammation and decreased renal function. Methods: Forty-four native renal biopsies from DN patients and 23 control cases were selected. The *in situ* expression of eotaxin, MIP-1α (macrophage inflammatory protein-1α), IL-8 (interleukin-8), IL-4, IL-10, TNF-α (tumor necrosis factor-α), TNFR1 (tumor necrosis factor receptor-1), IL-1β, and IL-6 was analyzed by immunohistochemistry. Results: The DN group showed a significant increase in IL-6 ($p<0.0001$), IL-1β ($p<0.0001$), IL-4 ($p<0.0001$), and eotaxin ($p=0.0012$) expression, and a decrease in TNFR1 ($p=0.0107$) and IL-8 ($p=0.0262$) expression compared to the control group. However, there were no significant differences in IL-10 ($p=0.4951$), TNF-α ($p=0.7534$), and MIP-1α ($p=0.3816$) expression among the groups. Regarding the interstitial inflammation, there was a significant increase in IL-6 in scores 0 and 1 compared to that in score 2 ($p=0.0035$), in IL-10 in score 2 compared to that in score 0 ($p=0.0479$), and in eotaxin in score 2 compared to that in scores 0 and 1 ($p<0.0001$), whereas IL-8 ($p=0.0513$) and MIP-1α ($p=0.1801$) showed no significant differences. There was a tended for negative correlation between eotaxin and the estimated glomerular filtration rate (eGFR) ($p=0.0566$). Conclusion: Our results indicated an increased *in situ* production of cytokines and chemokines in DN, including IL-6, IL-1β, IL-4, and eotaxin. Moreover, it was observed that eotaxin plays an important role in the progression of interstitial inflammation in DN and may be associated with decreases in the eGFR in these patients.

Keywords: diabetic nephropathy, cytokines, chemokines, renal biopsy, interstitial inflammation

INTRODUCTION

Diabetic nephropathy (DN) is a chronic microvascular complication that affects about 20 to 30% of patients with type 2 diabetes mellitus (T2DM). It is considered the leading cause of end-stage renal failure requiring renal replacement therapy (1, 2), although its pathogenesis has not yet been fully elucidated. Immune and inflammatory mechanisms play important roles in the development and progression of DN, which is considered a chronic inflammatory disease (3, 4). Several cells, such as monocytes, macrophages, and lymphocytes, as well as chemokines and cytokines, have been implicated in this process (5, 6). Among them, it is known that IL-1β, IL-6, TNF-α (tumor necrosis factor-α), IL-8, MIP-1α (macrophage inflammatory protein-1α) are relevant for the development of DN, as they play actions potentially involved in the onset of its complications (7-9).

Patients with DN have a predominance of increased levels of inflammatory mediators in the serum and urine, even in the early stages of the disease, and in the decreased renal function (9-13). However, the extent to which renal damage is caused by the action of immune cell-derived cytokines and chemokines and the importance of such inflammatory mechanisms on the development and progression of DN requires further investigation (14, 15).

Renal biopsies are considered the gold standard for the diagnosis of glomerulopathies; however, diabetic patients are only subjected to renal biopsies in cases of atypical clinical courses of DN. Atypical presentations include microalbuminuria without diabetic retinopathy, a rapid decline in glomerular filtration rate, rapidly increasing proteinuria, a sudden onset of nephrotic syndrome, hematuria, a period of less than five years since the diagnosis of diabetes, or signs and symptoms of systemic diseases (16, 17).

However, further studies using this type of samples to investigate the mechanisms associated with the expression of inflammatory mediators implicated in the pathogenesis of DN are required, as the level of inflammatory mediators is reflective of the direct action of the molecules in the organs, as well as the relationship with DN. Therefore, the objective of this study was to analyze the expression of cytokines and chemokines, such as, IL-1β, IL-6, IL-4, IL-10, TNF-α, TNFR1 (tumor necrosis fator receptor-1), IL-8, MIP-1α e eotaxin, in renal biopsies from DN patients and determine its correlation with interstitial inflammation and decreased renal function.

MATERIAL AND METHODS

Patients

Forty-four cases of native renal biopsies from adult patients diagnosed with DN were selected from the Renal Pathology Service database of the Federal University of Triângulo Mineiro (UFTM), Uberaba-MG, Brazil, in the period from 1996 to 2018. All cases of DN without overlap of other kidney disease of patients over 18 years and with satisfactory samples for analysis were included in the study. The control group (n=23) consisted of kidneys obtained from the autopsies of patients >18 years old, with no evidence of infection or previous renal changes. Cases with autolysis, acute tubular necrosis, and congestion with moderate to severe changes were excluded from the control group. These samples were obtained from the Pathology Service of the University of São Paulo/Ribeirão Preto. This study was approved by the Ethics and Research Committee of the Federal University of Triângulo Mineiro (no. 3.001.006).

Renal histopathology

The diagnosis of DN was performed based on the evaluation of native kidney fragments, with three samples used for light microscopy (LM), direct immunofluorescence (IF), and transmission electron microscopy (TEM) analyses according to the standard procedures (18).

For LM, 2-µm paraffin sections were stained with hematoxylin and eosin (H&E), picrosirius, methenamine silver, and Masson's trichrome. LM was used to analyze morphological changes and interstitial inflammation. The interstitial inflammation in DN was evaluated using scores, including score 0 (absence of interstitial inflammation), score 1 [interstitial inflammation related to interstitial fibrosis and tubular atrophy (IFTA)] and score 2 (interstitial inflammation in areas other than IFTA). The DN classes were defined according to the pathologic classification of DN (19).

For IF, IgG, IgM, and IgA immunoglobulins, kappa and lambda light chains, C3 and C1q complement fractions, and fibrinogen were detected in 2-µm frozen tissues using fluorescein isothiocyanate (FITC)-conjugated antibodies (Dako, Copenhagen, Denmark). IF was used to exclude or identify kidney disease overlapping ND. For TEM, the tissue was first fixed in 2.5% Karnovsky + 0.2% ruthenium red, then fixed in 2% osmium tetroxide. Next, the tissue was dehydrated using a graded series of alcohol and acetone solutions before embedding in Epon 812 resin. Ultra-thin sections of 60 nm were prepared and placed in nickel grids. The sections were then stained with uranyl acetate and examined under a transmission electron microscope (EM-900; Zeiss, Germany) (18). TEM was used to measure the thickness of the glomerular basement membrane (GBM) and to exclude or identify kidney disease overlapping ND. All cases of DN overlapping with other kidney diseases were excluded from the study.

Immunohistochemistry

Immunohistochemistry was performed manually on slides containing 2-µm paraffin-embedded tissue sections using the Novolink non-biotin polymer system (Novolink Polymer

Detection System Kit; BL, UK) according to the manufacturer's recommendations. Specifications of the antibodies used are summarized in table 1.

Table 1 - Immunohistochemistry specifications

Primary antibody	Supplier	Clone or code	Antigenic recovery	Concentration
Anti-eotaxin monoclonal antibody	Thermo Fisher Scientific	43911	Citrate pH 6.0	1:100
Anti-MIP-1α (macrophage inflammatory protein-1α) (CCL3) polyclonal antibody	Thermo Fisher Scientific	PA5-32496	Citrate pH 6.0	1:1600
Anti-IL-4 (interleukin-4) polyclonal antibody	Thermo Fisher Scientific	PA5-25165	Citrate pH 6.0	1:1300
Anti-IL-8 (CXCL8) polyclonal antibody	Thermo Fisher Scientific	PA5-79113	Citrate pH 6.0	1:1400
Anti-IL-10 polyclonal antibody	Thermo Fisher Scientific	PA5-79457	Citrate pH 6.0	1:1200
Anti-TNF-α (tumor necrosis factor-α) monoclonal antibody	Thermo Fisher Scientific	2C8	Citrate pH 6.0	1:1200
Anti-TNF Receptor I (tumor necrosis fator receptor-1) monoclonal antibody	abcam	H398	Citrate pH 6.0	1:300
Anti-IL-1β polyclonal antibody	Novus Biologicals	NBP1-19775	Citrate pH 6.0	1:40
Anti-IL-6 polyclonal antibody	abcam	ab6672	Citrate pH 6.0	1:300

Source: the author himself

Quantification of in situ immunostaining

All fields of renal biopsy samples and 40 fields of autopsy kidney fragments, which included glomerular and tubulointerstitial compartments, were analyzed. Immunostained cells that showed an intense brownish staining were marked by the observer using the interactive AxionCam ICc 5 (Zeiss, Germany) image analysis system with a 40× objective (final

magnification of 1,600×). The results were expressed as the percentage of marked area compared to the total area of the analyzed fields.

Statistical analysis

A spreadsheet (Microsoft Excel) was created for the statistical analysis. Data analysis was performed using GraphPad Prism version 7.0 (GraphPad Software, USA). Normality was tested using the Kolmogorov-Smirnov test. In cases of normal distribution and similar variances, the parametric ANOVA (F) test was used, followed by the *post-hoc* Tukey's test and the Student's *t*-test (t). In cases of a non-normal distribution, the Kruskal-Wallis (H) test was used, followed by the *post-hoc* Dunn's test and the Mann-Whitney (U) test. The proportions were compared by the Chi-square test (χ^2). The Pearson's test (r) was used to determine correlations with parametric variables and the Spearman's test (rS) for non-parametric variables. The differences were considered statistically significant when $p < 0.05$.

RESULTS

General characteristics of control and DN groups

A total of 44 cases of DN were selected. The subjects had a median age of 53 (23-75) years, where most were male (24; 54.55%) and white (34; 77.27%). The control group subjects (n=23) had a median age of 44 (19-80) years with a male predominance (12; 52.17%).

Most DN patients were hypertensive (26; 59.1%), had a mean time since diagnosis of diabetes mellitus of 13.66 ± 6.58 years, and a mean GBM thickness of 750.69 ± 184.03 nm. The laboratory data of these patients showed mean creatinine levels of 2.22 ± 1.47 mg/dL and mean urea levels of 81.9 ± 41.9 mg/dL. The estimated glomerular filtration rate (eGFR) was reduced, showing a mean of 48.46 ± 34.51 mL/min/1.73m^2. The mean proteinuria was within the nephrotic range with values of 5.02 ± 4.35 g/day. Regarding the DN classification, 27

(61.5%) biopsies were classified as class III, corresponding to the nodular sclerosis class (Kimmelstiel-Wilson nodules), 11 (25%) as class IV, and 6 as classes I, IIa, and IIb. There were 2 (4.5%) cases per class. The general characteristics of the patients are summarized in table 2.

Table 2 General characteristics of the Control and DN groups

	Control Group (n=23)	**DN Group (n=44)**
Age (years)		
Median (Min-Max)	44 (19-80)	53 (23-75)
Mean ± SD	47±16.57	50.3 ± 13.73[a]
Gender n (%)		
Male	12 (52.17%)	24 (54.55%)[b]
Female	11 (47.83%)	20 (45.55%)
Color n (%)		
White		34 (77.27%)
Not white		4 (9.09%)
NI		6 (13.64%)
SAH n (%)		
Yes		26 (59.1%)
No		7 (15.9%)
NI		11 (25%)
Course DM (years)		
Mean ± SD		13.66 ± 6.58
GBM thickness (nm)		
Mean ± SD		750.69 ± 184.03
Classes DN (n)		
Class I		2 (4.5%)
Class IIa		2 (4.5%)
Class IIb		2 (4.5%)
Class III		27 (61.5%)
Class IV		11 (25%)
Urea (mg/dL)		
Mean ± SD		81.9 ± 41.9
Creatinine (mg/dL)		
Mean ± SD		2.22 ± 1.47
eGFR (mL/min/1.73m^2)		
Mean ± SD		48.46 ± 34.51
Proteinuria (g/day)		
Mean ± SD		5.02 ± 4.35

DN: Diabetic Nephropathy. DM: Diabetes Mellitus. SAH: Systemic arterial hypertension. GBM: Glomerular basement membrane. eGFR: Estimated glomerular filtration rate. SD: Standard deviation.

[a]: t= 0.8607; p=0.3927

[b]: χ^2 = 0.03417; p=0.8533

Role of cytokines and chemokines in diabetic nephropathy

The expression of inflammatory cytokines and chemokines was analyzed in patients with DN to determine their expression profile in this disease. The DN group showed a significant increase in the cytokines IL-6 ($p<0.0001$; U=82, Figure 1A and 1B), IL-1β ($p<0.0001$; t=5.16, Figure 1C and 1D), and IL-4 ($p<0.0001$; U=182, Figure 1E and 1F) and a decrease in TNFR1 ($p=0.0107$; t=2.631, Figure 2A and 2B) compared to the control group. In contrast, there were no significant differences between the groups for the cytokines IL-10 ($p=0.4951$; t=0.6862, Figure 2C and 2D) and TNF-α ($p=0.7534$; t=0.3155, Figure 2E and 2F).

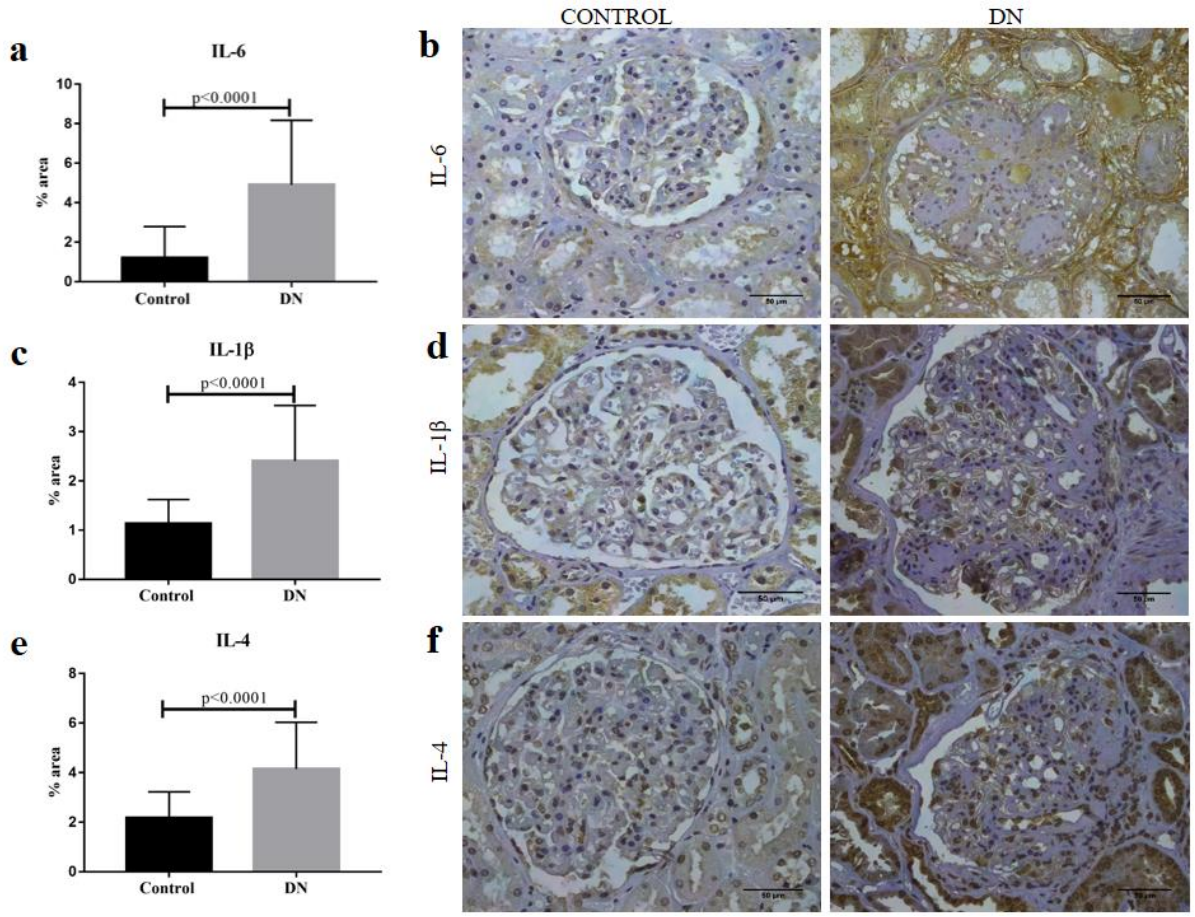

Figure 1- *In situ* expression of IL-6, IL-1β, and IL-4 in the glomerular and tubulointerstitial compartments in patients with diabetic nephropathy (DN) and the control group. (a) IL-6 expression in the control and DN groups. (b) IL-6 immunostaining in the control and DN groups. (c) IL-1β expression in the control and DN groups. (d) IL-1β immunostaining in the control and DN groups. (e) IL-4 expression in the control group and DN groups. (f) IL-4 immunostaining in the control and DN groups. The results are expressed as mean ± standard deviation. The bars represent the mean and the line above represents the standard deviation.

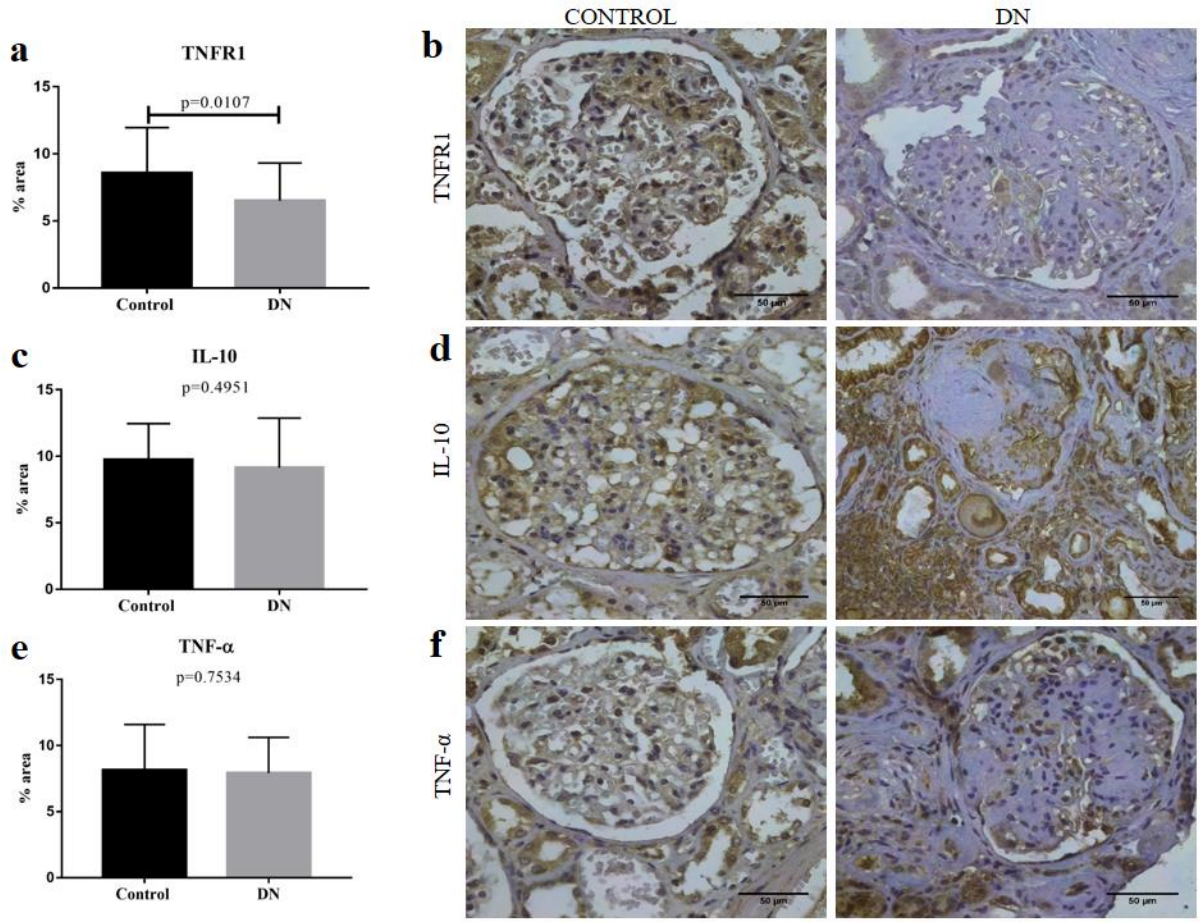

Figure 2- *In situ* expression of TNFR1, IL-10, and TNF-α in the glomerular and tubulointerstitial compartments in patients with diabetic nephropathy (DN) and the control group. (a) TNFR1 expression in the control and DN groups. (b) TNFR1 immunostaining in the control and DN groups. (c) IL-10 expression in the control and DN groups. (d) IL-10 immunostaining in the control and DN groups. (e) TNF-α expression in the control and DN groups. (f) TNF-α immunostaining in the control and DN groups. The results are expressed as mean ± standard deviation. The bars represent the mean and the line above represents the standard deviation.

The analysis of chemokine expression showed a significant increase in eotaxin (p=0.0012; U=265.5, Figure 3A and 3B) expression and a decrease in IL-8 (p=0.0262; t=2.275, Figure 3C and 3D) expression in the DN group compared to the control group. However, there were no significant differences between the groups for MIP-1α (p=0.3816; t=0.8811, Figure 3E and 3F) expression.

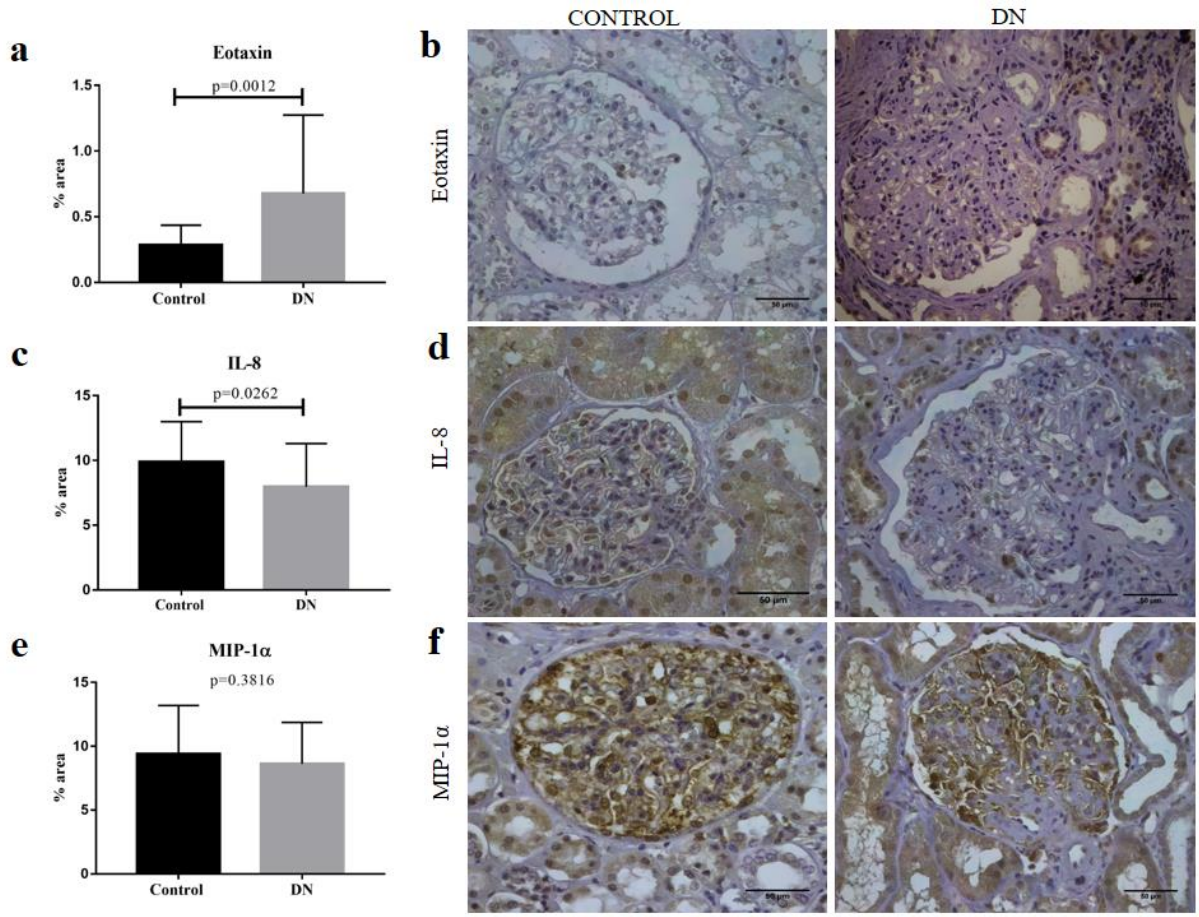

Figure 3. *In situ* expression of the chemokines eotaxin, IL-8, and MIP-1α in the glomerular and tubulointerstitial compartments in patients with diabetic nephropathy (DN) and the control group. (a) Eotaxin expression in the control and DN groups. (b) Eotaxin immunostaining in the control and DN groups. (c) IL-8 expression in the control and DN groups. (d) IL-8 immunostaining in the control and DN groups. (e) MIP-1α expression in the control and DN groups. (f) MIP-1α immunostaining in the control and DN groups. The results are expressed as the mean ± standard deviation. The bars represent the mean and the line above represents the standard deviation.

Relationship of cytokines and chemokines in the evaluation of interstitial inflammation in diabetic nephropathy

After determining the cytokine and chemokine expression profile in DN, we analyzed how these inflammatory mediators could be related to the interstitial inflammation of this disease.There was a significant increase in IL-6 in scores 0 and 1 compared to that in score 2 ($p=0.0035$; $F=6.592$, Figure 4A) and a significant increase in IL-10 in score 2 compared to that in score 0 ($p=0.0479$; $F=3.295$, Figure 4B). For the chemokines, there was a significant increase in eotaxin in score 2 compared to that in scores 0 and 1 ($p<0.0001$; $H=19.19$, Figure 4C),

whereas IL-8 (p=0.0513; F=3.208, Figure 4D) and MIP-1α (p=0.1801; F=5.203, Figure 4E) showed no significant differences between the groups.

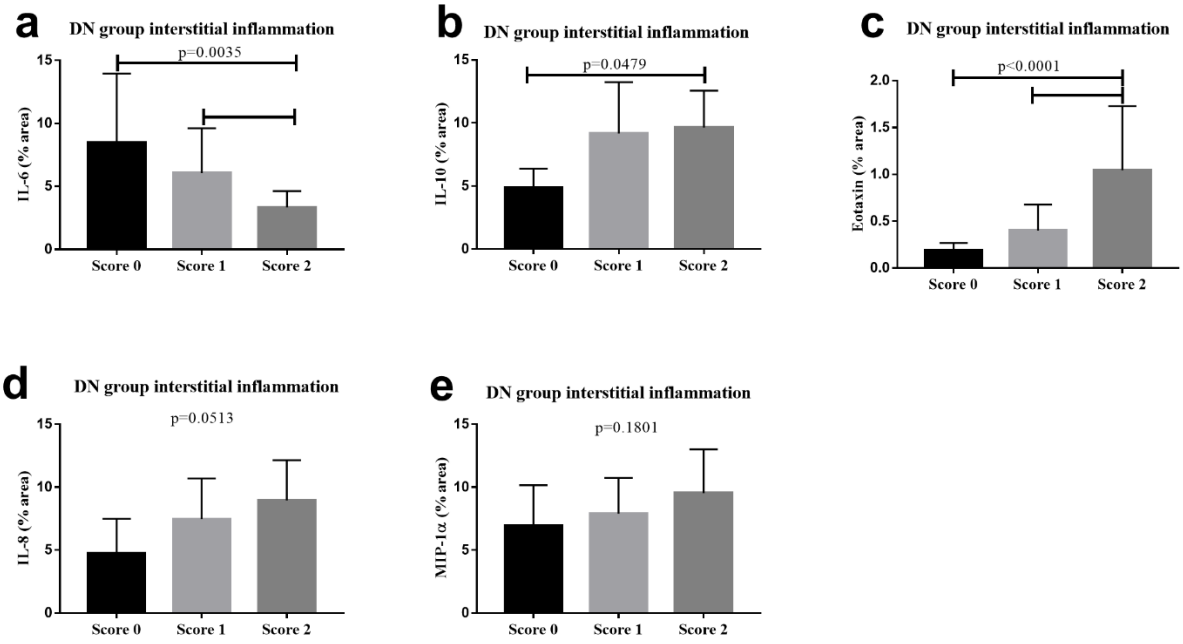

Figure 4. Comparison between *in situ* cytokine and chemokine expression and interstitial inflammation in patients with diabetic nephropathy (DN). (a) IL-6 expression in cases classified as scores 0, 1, and 2 for interstitial inflammation in the DN group. (b) IL-10 expression in cases classified as scores 0, 1, and 2 for interstitial inflammation in the DN group. (c) Eotaxin expression in cases classified as scores 0, 1, and 2 for interstitial inflammation in the DN group. (d) IL-8 expression in cases classified as scores 0, 1, and 2 for interstitial inflammation in the DN group. (e) MIP-1α expression in cases classified as scores 0, 1, and 2 for interstitial inflammation in the DN group. The results are expressed as mean ± standard deviation. The bars represent the mean and the line above represents the standard deviation.

Correlation between the estimated glomerular filtration rate (eGFR) and chemokine expression in diabetic nephropathy

Since there was a predominant increase in chemokine expression as interstitial inflammation progressed, the potential correlation between chemokine expression and decreased eGFR was analyzed in the DN patients. It was observed that only eotaxin tended to have a negative correlation with eGFR (p=0.0566; rS=–0.3253, Figure 5A), while MIP-1α (p=0.3167; r=–0.1798, Figure 5B) and IL-8 (p=0.9297; r=0.01571, Figure 5C) showed no significant correlation.

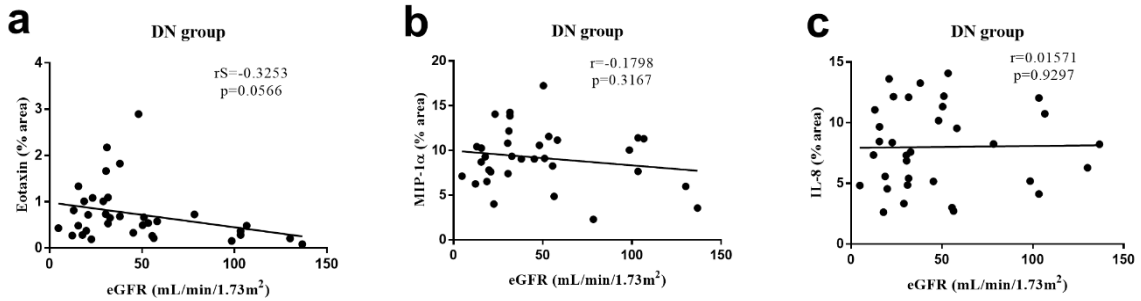

Figure 5. Correlation between estimated glomerular filtration rate (eGFR) and *in situ* chemokine expression in patients with diabetic nephropathy (DN). (a) Negative and significant correlation trend between eGFR and eotaxin in the DN group. (b) Non-significant correlation between eGFR and MIP-1α in the DN group. (c) Non-significant correlation between eGFR and IL-8 in the DN group.

DISCUSSION

This study analyzed the *in situ* expression of cytokines and chemokines in the renal biopsies of patients with DN and related this expression with interstitial inflammation and eGFR to improve our understanding of the immune and inflammatory mechanisms that may be acting directly on the kidney and decreasing renal function. Our results showed an increase expression of proinflammatory cytokines IL-6 and IL-1β, as well as of the Th2 cytokine, IL-4 and the chemokine eotaxin in DN patients. In contrast, TNFR1 and IL-8 expression was reduced in DN. These findings suggest that in DN there may be a simultaneous sharing of cytokine actions of the innate and acquired immune response, associated with the increase of a potent eosinophilic chemokine.

The analysis of interstitial inflammation showed that eotaxin expression may play an important role in interstitial inflammation in DN, since its expression was increased exclusively in score 2, in which interstitial inflammation is related to areas other than IFTA and represents a more severe condition. On the other hand, IL-6 expression was higher in scores 0 and 1 compared to that in score 2, whereas IL-10 expression was higher in score 2 compared to that in score 0. Possibly, increase IL-10 expression in score 2 may be affecting IL-6 expression through its pro-fibrotic and anti-inflammatory action.

Further supporting the role of eotaxin in DN, in addition to the relationship between its increased expression and interstitial inflammation in patients with DN, there is a correlation between eotaxin expression and decreased eGFR. Therefore, the action of eotaxin on DN may influence both interstitial inflammation and eGFR, indicating its possible role in the pathogenesis of DN.

Kidney cells (endothelial, mesangial, epithelial, and tubular) are able to synthesize cytokines and chemokines in a specific manner for cells and stimuli. Cytokines, chemokines, growth factors, adhesion molecules, nuclear factors, and immune cells, such as monocytes, lymphocytes, and macrophages, have been previously demonstrated to be implicated in the pathogenesis of DN (20-22).

IL-1β and IL-6 are among the cytokines that play an important role in the pathogenesis of DN, affecting renal and infiltrating cells. IL-1β induces the expression of the intercellular adhesion molecule 1 (ICAM-1) via mesangial and tubular cells and increases the vascular permeability and the expression of chemokines, resulting in the proliferation and synthesis of the extracellular matrix (ECM) in the glomerular mesangium (23). IL-6 acts on mesangial cell proliferation and promotes the synthesis of ECM and the thickening of the GBM, in addition to affecting vascular permeability and facilitating neutrophil infiltration into the tubulointerstitium, leading to DN progression (22, 24). Studies using experimental DN models have shown a correlation between the increased renal expression of IL-1β and the increased expression of chemotactic factors and adhesion molecules (25, 26). Previously, T2DM patients with DN were found to show an increased production of IL-6, which was associated with a thickening of the GBM and was considered a strong marker of renal function decline (27). Since the thickening of the GBM is the earliest morphological alteration in DN associated with increased IL-6 production, it is possible that increased *in situ* IL-6 expression occurs from the early stages of DN, whereby patients show increased expression even without interstitial

inflammation (score 0) or in score 1. Moreover, actions associated with increased IL-1β and IL-6 expression promote greater cell infiltration in the kidney, which may exacerbate the inflammatory process and lead to impaired renal function.

Studies have reported that the serum IL-10 levels are elevated in T2DM patients with DN and that there is a positive correlation between IL-10 and albuminuria (28-30). It has been shown that mononucleated cells are able to adopt an anti-inflammatory phenotype in the tissue repair process later in the course of inflammation, which is believed to occur after exposure to IL-10. These cells eliminate cellular and matrix debris and generally promote the resolution of renal inflammation, stimulating renal tubular cell proliferation and angiogenesis (31). The increased IL-10 production most probably represents a compensatory mechanism due to the increase in the expression of proinflammatory cytokines and is a negative regulator of inflammation, which corroborates our findings.

Although IL-4 stimulates the synthesis of ECM through glomerular mesangial and epithelial cells, DN patients were found to have low serum levels of IL-4 (9). Furthermore, no significant differences were found of this cytokine serum levels when comparing patients with and without DN (14). However, our results show that the *in situ* expression of IL-4 is increased in patients with DN. The main morphological alteration associated with DN is the progressive accumulation of ECM, which may account for the increased expression and suggests that the action of IL-4 is more effective *in situ*, in promoting the synthesis of ECM, than systemically.

Studies with T2DM patients have shown that only TNFR1 and TNFR2 receptors are associated with a risk of end-stage renal disease, wherein elevated serum levels of TNFR1 are associated with DN (32) and decreased renal function (33, 34). TNFR1 is mainly present in the glomerular cells and endothelial cells of the peritubular capillaries (35). High serum levels of TNFR1 have been associated with global sclerosis, increased ECM, decreased glomerular filtration, and foot process effacement in T2DM patients (36). Although *in vitro*-activated

TNFR1 induces tissue damage via proinflammatory signals and/or cell death (37), the mechanisms associating TNF receptors with DN remain unknown (36, 38). However, it has been shown that glomerular and tubular TNFR1 expression is not associated with a loss of renal function nor with any clinical parameters in DN patients (39). Our results showed decreased *in situ* expression of TNFR1 in DN, which suggests that elevated serum levels of TNFR1 may be mostly implicated in the progression of DN.

Eotaxin is a CC chemokine that is mainly chemotactic for eosinophils, from the activation of its CCR3 receptor, and is secreted by endothelial cells, macrophages, fibroblasts, and smooth muscle cells (40). Roy et al. showed that eotaxin could be used as an independent predictor of renal failure. However, the relationship between an increased eotaxin plasma concentration and the progression to renal failure in diabetic patients remains poorly understood (41). Elevated levels of urinary eotaxin are associated with prolonged hyperglycemia and microalbuminuria in T2DM patients (42). In the kidneys, eotaxin has been reported to contribute to renal interstitial eosinophilia; however, these results do not refer to DN (43).

The slow and continuous decline of renal function is associated with progressive tubulointerstitial damage and renal fibrosis, which is characterized by the accumulation of leukocytes, fibroblasts, EMC, and tubular atrophy (2, 44). The accumulation of macrophages and lymphocytes in the interstitium is critical for tubular and interstitial damage, since these cells are the main sources of proinflammatory and pro-fibrotic cytokines (45, 46). Eotaxin is a potent chemoattractant chemokine and/or activator of eosinophils but may also be involved in the regulation of other cells. In atherosclerosis, smooth muscle cells express eotaxin and macrophages and mast cells express the CCR3 receptor, suggesting that eotaxin and its receptor contribute to the recruitment and activation of inflammatory cells in the atheroma (47). It was also observed that Th2 lymphocytes, neutrophils, and bronchial endothelial cells also express

the CCR3 receptor, suggesting the potential role of eotaxin in the non-eosinophilic inflammatory process (48, 49).

Macrophages are the main inflammatory cells involved in renal damage, the accumulation of which is correlated with the severity of DN (5, 50, 51) and mesangial expansion (52). Mast cells also infiltrate the tubulointerstitial compartment and release inflammatory mediators and proteolytic enzymes. The intensity of macrophage infiltration and the extent of mast cell degranulation has been previously associated with the level of tubulointerstitial inflammation and with decreased eGFR in DN (53). Thus, we suggest that the increased *in situ* expression of eotaxin may be related to its contribution to the recruitment and activation of cells other than eosinophils, which strongly promotes further infiltration and accumulation of these cells in the kidney. This may also account for the decreased *in situ* expression of IL-8 found in the patients studied here. The action of eotaxin associated with the action of inflammatory cytokines may worsen the inflammatory process and impair renal function, which corroborates our findings and suggests that eotaxin exerts an *in situ* role in the pathogenesis of DN.

Therefore, our findings indicate that the *in situ* expression analysis of cytokines and chemokines, especially eotaxin, could be used to assist in the analysis of renal function impairment based on the analysis of interstitial inflammation developed in patients with DN.

CONCLUSION

Our results show that the *in situ* expression of cytokines and chemokines, including IL-6, IL-1β, IL-4, and eotaxin, is increased in patients with DN. In addition, eotaxin plays an important role in the progression of interstitial inflammation in DN patients and may be related to decreased eGFR in these patients.

1710 LIST OF ABBREVIATIONS

1711 DN: Diabetic nephropathy

1712 eGFR: Estimated glomerular filtration rate

1713 ECM: Extracellular matrix

1714 F: ANOVA test

1715 FITC: Fluorescein isothiocyanate

1716 GBM: Glomerular basement membrane

1717 H: Kruskal-Wallis test

1718 H&E: Hematoxylin and eosin

1719 ICAM-1: Intercellular adhesion molecule 1

1720 IF: Direct immunofluorescence

1721 IFTA: Interstitial fibrosis and tubular atrophy

1722 Ig: Immunoglobulin

1723 IL: Interleukin

1724 LM: Light Microscopy

1725 MIP-1α: Macrophage inflammatory protein-1α

1726 nm: Nanometers

1727 r: Pearson test

1728 rS: Spearman test

1729 SAH: Systemic arterial hypertension

1730 SD: Standard deviation

1731 t: Student's t test

1732 T2DM: Type 2 diabetes mellitus

TEM: Transmission electron microscopy

TNF-α: Tumor necrosis factor-α

TNFR1: Tumor necrosis fator receptor-1

TNFR2: Tumor necrosis fator receptor-2

U: Mann Whitney test

$\chi 2$: Chi square test

DECLARATIONS

Ethics approval and consent to participate

The patients involved in the study signed a consent form and this study was approved by the Ethics Committee of the Federal University of Triangulo Mineiro, Protocol 3.001.006.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

We declare not having conflict of interest related to this study.

Funding

Not applicable.

Authors' contributions

Conceptualization: JRM and LSA

Formal analysis: JRM and LSA

Performed the pathological diagnosis: MAR and MLGRM

Methodology: LSA, BGST, CAS, ALMSM and MVS

Supervised the manuscript: JRM

Writing – original draft: LSA

Wrinting – review and editing: LSA, BGST, CAS, ALMSM, MLGRM, MVS, MAR and JRM

All authors read and approved the final manuscript.

Acknowledgements

The authors appreciate the financial support of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Fundação de Ensino e Pesquisa de Uberaba (FUNEPU).

The authors thank Federal University of Triângulo Mineiro, General Pathology Discipline and the following employees of the Nephropathology Service: Alberto Borba, Edson Santos, João Nolberto, Laura Penna, Lívia Alves and Vandair Gonçalves.

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Considerações finais

CONSIDERAÇÕES FINAIS

ARTIGO I

I. Os indivíduos envolvidos no estudo apresentavam idade entre 18 a 94 anos, sendo a maioria do gênero feminino e da cor branca. A maioria dos pacientes DMT2 eram hipertensos, tinham mediana de IMC em torno de 27 (Kg/m^2) e relataram o uso de insulina no controle do diabetes associado a outros medicamentos hipoglicemiantes. O grupo DMT2 com AR apresentou maior curso da doença e maiores níveis séricos de ureia e creatinina em relação ao grupo DMT2 sem AR. Enquanto que os valores séricos de colesterol total, HDL-c e triglicérides foram similares entre os grupos.

II. Os pacientes DMT2 apresentaram diminuição dos níveis séricos de IL-4 e aumento dos níveis séricos de IL-8, eotaxina, MIP-1α e MIP-1β em comparação ao grupo controle. O grupo DMT2 com AR apresentou diminuição dos níveis séricos de IL-4 em comparação ao grupo controle e independente da alteração renal foi observado aumento de IL-8 e MIP-1α em comparação ao grupo controle.

III. Os pacientes DMT2 apresentaram aumento de TNFR1, adiponectina e resistina, e diminuição dos níveis séricos de TNFR2 em comparação ao grupo controle. O grupo DMT2 com AR apresentou aumento de TNFR1 e leptina em comparação aos grupos DM2 sem AR e controle, como também, de adiponectina em comparação ao grupo controle, sendo que os níveis elevados de resistina foram independentes da alteração renal em comparação ao grupo controle.

IV. A TFGe correlacionou-se de forma positiva com a IL-4, e negativa com TNFR1, TNFR2 e Leptina nos pacientes DMT2. Já no grupo DMT2 com AR, a TFGe correlacionou-se de forma negativa com TNFR1 e resistina. Ainda foi observado que neste grupo o TNFR1 correlacionou-se de forma positiva com a resistina e a leptina, da mesma forma, a resistina correlacionou-se com a leptina e IL-8.

ARTIGO II

V. Os casos de ND apresentaram mediana de idade de 53 (23-75) anos, dos quais a maioria eram do gênero masculino e brancos. No grupo controle a mediana de idade foi de 44 (19-80) anos com predomínio do gênero masculino. A maioria dos pacientes com ND eram hipertensos e apresentavam média do tempo de DM de 13,66 ± 6,58 anos e média da espessura da MBG de 750,69 ± 184,03 nm. A média da creatinina foi de 2,22 ±1,47 mg/dL e de ureia 81,9 ± 41,9 mg/dL. A TFGe apresentou-se diminuída e a média da

proteinúria se manteve em níveis nefróticos. A maioria dos casos de ND foram classificados em classe III.

VI. Aumento da expressão de IL-6, IL-1β, IL-4 e eotaxina, e diminuição da expressão de TNFR1 e IL-8 foram observados no grupo ND em comparação ao grupo controle.

VII. A expressão de eotaxina apresentou tendência de correlação negativa com a TFGe.

VIII. Na inflamação intersticial, aumento da expressão de IL-6 foi observado nos escores 0 e 1 em relação ao escore 2, e aumento da expressão de IL-10 no escore 2 em relação ao escore 0 nos casos de ND. Dentre as quimiocinas, a expressão de eotaxina foi aumentada no escore 2 em comparação aos escores 0 e 1.

Conclusões

CONCLUSÕES

Nosso estudo mostrou que de forma sistêmica os elevados níveis séricos de TNFR1, das adipocinas (adiponectina, resistina e leptina) e das quimiocinas (IL-8, MIP-1α, MIP-1β e eotaxina) e diminuição de IL-4, desempenham papel importante no processo inflamatório desenvolvido no DMT2 e na diminuição da função renal. Além disso, o TNFR1 sérico mostrou-se um forte preditor de disfunção renal nos pacientes com DMT2. No entanto, na avaliação da expressão *in situ*, os casos de pacientes com ND apresentaram aumento das expressões de IL-6, IL-1β, IL-4 e eotaxina. Sendo observado que a expressão de eotaxina pode estar desempenhando um importante papel na progressão da inflamação intersticial na ND, como também pode estar relacionada com a diminuição da TFGe nestes pacientes. Dessa maneira, podemos concluir que as análises de mediadores inflamatórios séricos e *in situ* são importantes e se complementam, no auxílio da avaliação do comprometimento da função renal a partir da análise do processo inflamatório desenvolvido no DMT2 e na ND.

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ANEXO A – Parecer consubstanciado do CEP

UFTM - UNIVERSIDADE FEDERAL DO TRIÂNGULO MINEIRO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação histopatológica e de citocinas séricas em pacientes diabéticos com ou sem alteração renal

Pesquisador: Juliana Reis Machado e Silva

Área Temática:

Versão: 4

CAAE: 88997018.6.0000.5154

Instituição Proponente: Universidade Federal do Triangulo Mineiro

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.001.006

Apresentação do Projeto:

Segundo os pesquisadores:

A Nefropatia Diabética é considerada a principal causa de insuficiência renal terminal com necessidade de terapia renal substitutiva. De acordo com os dados da International Diabetes Federation, o número de diabéticos na população adulta com idade superior a 20 anos poderá ultrapassar 430 milhões em 2030 (Atkins, 2005).

A patogênese da Nefropatia Diabética vem sendo intensamente investigada. Pouco se sabe sobre os mecanismos envolvidos na evolução e progressão desta entidade. Alterações nos podócitos e na espessura da membrana basal glomerular (MBG) são cruciais para o aparecimento gradual de proteinúria nestes pacientes (White, Bilous e Group, 2004).

O podócito é um dos componentes responsável pela integridade da barreira de filtração glomerular, alterações como achatamento ou redução no número de células podem levar ao desenvolvimento de proteinúria (Lemley et al., 2002). Estudos revelam que o apagamento dos processos podocitários é evidente nas fases de proteinúria maciça, porém, foi observado que os pacientes normoalbuminúricos ou microalbuminúricos também podem apresentar certo grau de apagamento e que a gravidade destas lesões é proporcional ao aumento da taxa de excreção de albumina (Perrin et al., 2006; Toyoda et al., 2007).

Foi observado que lesões podocitárias graves podem levar a perda de podócitos pela urina com consequente diminuição do número de podócitos no glomérulo. Pacientes com Diabetes Mellitus

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Continuação do Parecer: 3.001.006

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

UBERABA, 05 de Novembro de 2018

Assinado por:
Alessandra Cavalcanti de Albuquerque e Souza
(Coordenador(a))

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