Brazilian Journal of
HEALTH AND PHARMACY

CCL20 is associated with type 2 diabetes mellitus regardless of age, physical activity, waist circumference and smoking in postmenopausal women

CCL20 está associado ao diabetes mellitus tipo 2 independentemente da idade, atividade física, circunferência da cintura e tabagismo em mulheres na pós-menopausa

Ana Luíza Quevedo[1]; Marilia Esteves Alves de Souza[1]; João Esteves Alves De Souza[1]; Fabio Lera Orsatti[2]; Claudio Lera Orsatti[1,2]*.

1 Biochemistry – Immunology Research Group, Department Health Science, Oeste Paulista University - UNOESTE, Jaú, São Paulo, Brazil.
2Applied Physiology, Nutrition and Exercise Research Group (PhyNEr), Institute of Health Sciences, Federal University of Triangulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil.

***Autor correspondente:** Claudio Lera Orsatti (ORCID: 0000-0001-9161-5737)
Email: orsatti@unoeste.br
Data de Submissão: 28/11/2023; Data do Aceite: 15/07/2024.

Citar: QUEVEDO, A. L.; SOUZA, M. E.A.; SOUZA, J.E.A.; ORSATTI, F.L.; ORSATTI, C.L. CCL20 is associated with type 2 diabetes mellitus regardless of age, physical activity, waist circumference and smoking in postmenopausal women. **Brazilian Journal of Health and Pharmacy**, v. 6, n. 3, p. 12 - 23, 2024.

ABSTRACT

CCL20 is a chemokine primarily expressed in lymphatic tissue and the liver, produced by endothelial cells, neutrophils, natural killer (NK) cells, Th17 cells, B cells, among others**.** CCL20, known for its role in recruiting inflammatory cells, emerges as a possible mediator of inflammation in T2DM**.** The objective was to evaluate serum CCL20 as a potential biomarker for T2DM in postmenopausal women. Participated in this cross-sectional study 160 postmenopausal women, aged over 45 years, with no personal history of cardiovascular disease, alcohol or drug use. The diabetic group consisted of 28 women. Serum CCL20 concentration was quantified using the ELISA technique, and confounding variables included age, physical activity, smoking, abdominal circumference, body fat percentage, PTH, insulin and glucose. The mean age was 70 years, menopause time of 23 ±8 years, waist circumference of 99 ±15 cm, body fat percentage of 43 ±5.7%, physical activity practiced by 17.9% and smoking in 2.1%. PTH = 58.7 ±23.8 pg/mL, insulin = 16.2 ±10.7 µU/mL, glucose = 105.4 ±8.0 mg/dL and CCL20 = 37.7 ±14.9 pg/mL. The logistic regression model showed that there was a significant association between high levels of CCL20 and T2DM (OR = 1.017, 95%CI = 1.001-1.033, P = 0.040), regardless of age, physical activity, abdominal circumference and smoking. For each one-unit rise in CCL20 concentration, the risk of diabetes increases by 1.7% (95%CI: 1-3.3%). CCL20 may be a promising biomarker to identify postmenopausal women with increased risk of developing T2DM.

Keywords: CCL20; Insulin resistance; T2DM; Menopause.

RESUMO

CCL20 é uma quimiocina expressa principalmente no tecido linfático e no fígado, produzida por células endoteliais, neutrófilos, célula natural killer (NK), células Th17, células B, entre outras. A CCL20, conhecida pelo seu papel no recrutamento de células inflamatórias, surge como um possível mediador da inflamação no T2DM. O objetivo foi

avaliar a CCL20 no soro como um potencial biomarcador para o T2DM em mulheres pós-menopausa. Participaram deste estudo transversal 160 mulheres na pós-menopausa, com idade superior a 45 anos, sem histórico pessoal de doença cardiovascular, uso de álcool ou drogas. O grupo diabético foi composto por 28 mulheres. A concentração sérica de CCL20 foi quantificada pela técnica ELISA e as variáveis de confusão incluíram: idade, atividade física, tabagismo, circunferência abdominal, percentual de gordura corporal, PTH, insulina e glicose. A idade média foi de 70 anos, tempo de menopausa de 23±8 anos, circunferência da cintura de 99±15 cm, percentual de gordura corporal de 43±5,7%, atividade física praticada por 17,9% e tabagismo em 2,1%. PTH = 58,7 ±23,8 pg/mL, insulina = 16,2 ±10,7 µU/mL, glicose = 105,4 ±8,0 mg/dL e CCL20 = 37,7 ±14,9 pg/mL. O modelo de regressão logística mostrou que houve associação significativa entre níveis elevados de CCL20 e T2DM (OR = 1,017, IC95% = 1,001-1,033, P = 0,040), independentemente da idade, atividade física, circunferência abdominal e tabagismo. Para cada aumento de uma unidade na concentração de CCL20, o risco de diabetes aumenta em 1,7% (IC 95%: 1-3,3%). O CCL20 pode ser um biomarcador promissor para identificar mulheres na pós-menopausa com risco aumentado de desenvolver T2DM.

Palavras-chave: Quimiocina CCL20; Resistência à insulina; T2DM; Menopausa.

INTRODUCTION

Type 2 diabetes (T2DM) is an endocrine disorder associated with inflammation in various tissues, primarily pancreatic islets (BURKE et al., 2015; J BURKE, 2014), closely linked to the obesity epidemic (FAHED et al., 2022), becoming a global health concern. Individuals with T2DM face a high risk of micro and macrovascular complications due to hyperglycemia and individual components of insulin resistance (IR) and metabolic syndrome (MetS) (DEFRONZO et al., 2015). IR and impaired insulin secretion remain central deficits in T2DM; however, it is still unclear how phenotypic factors such as obesity, unhealthy diet, physical inactivity, and smoking (MOLINA-MOLINA et al., 2022) and genetic factors (KOCHETOVA et al., 2019) contribute to the pathophysiological disturbances leading to glucose homeostasis impairment in T2DM (DEFRONZO et al., 2015).

Postmenopausal women (PMW), in particular, may exhibit hyperinsulinemia, IR, increased body weight or obesity, and circulating markers of inflammation, which may contribute to the development and progression of T2DM (SOFTIĆ; MEŠALIĆ, 2023; XING; KIRBY; ALMAN, 2022). This suggests that the menopausal state is a potential risk factor for developing cardiovascular diseases (CVD) (DA SILVA et al., 2020; SOFTIĆ; MEŠALIĆ, 2023), metabolic disorders (ORSATTI CL et al., 2022), and cancer (BUTTROS et al., 2019; LAUDISIO et al., 2018). However, the impact of changes in endogenous hormonal environments on PMW with T2DM is controversial (CLAYTON et al., 2022; KIM, 2012).

Several serum biomarkers of inflammatory activity have been investigated in PMW, including polymorphic targets, interleukins, PCR, and heat shock protein (JOHNSON et al., 2017; ARREDONDO et al., 2021; LAU et al., 2019; ORSATTI et al., 2014, 2018; ORSATTI et al., 2022). Detecting and monitoring disease progression in PMW, especially with T2DM, is challenging. If factors associated with T2DM progression can be identified, it may be possible to develop more effective and personalized ways to screen or identify prognostic biomarkers for T2DM in PMW.

Chemokines belong to the family of secreted soluble proteins that coordinate the recruitment and activation of immune cells at sites of inflammation (CHANG; CHEN, 2020; MOSER et al., 2004). CCL20 is a chemokine primarily expressed in lymphatic tissue and the liver, produced by endothelial cells, neutrophils, natural killer (NK) cells, Th17 cells, B cells, among others (COMERFORD et al., 2010; GERARD; ROLLINS, 2001; LEE et al., 2013). It is well-established that CCL20 contributes to the recruitment of inflammatory cells (CAUX et al., 2002). This chemokine is signaled through its receptor CCR6 (C-C chemokine receptor type 6), which is expressed by immature dendritic cells (iDCs), B cells, T cells (pro-inflammatory Th17, regulatory Treg cells), NKT cells, and neutrophils (LEE et al., 2013; MARTÍNEZ-CHACÓN et al., 2021). The subsequent increase in chemokine receptor signaling following the response to these chemokines is associated with pathological inflammation (BURKE et al., 2015; CITRO et al., 2012; RANASINGHE; ERI, 2018), although this information is controversial in T2DM (SAFA et al., 2016; SHINJO et al., 2016; VAN DYKE et al., 2017; XING et al., 2022).

Nevertheless, CCL20, known for its role in recruiting inflammatory cells, emerges as a possible mediator of inflammation in T2DM (MARTÍNEZ-CHACÓN et al., 2021; SAFA et al., 2016). However, the exact role of serum CCL20 in T2DM is not well-established, and this information is limited in PMW. Thus, we hypothesize that serum CCL20 plays a significant role in inflammation associated with T2DM in PMW and that they have an increased risk of developing cardiometabolic complications. Therefore, the aim of this study was to investigate the serological profile of CCL20 in the pathogenesis of T2DM in postmenopausal women, identifying the possibility of prognostication for T2DM in PMW, with a focus on detecting and monitoring disease progression.

METHODS

Participants and design study

This is a clinical, analytical, and cross-sectional study. The study population consisted of 160 PMW seen at the Climacteric and Menopause Outpatient Clinic. Postmenopause was defined according to DAVIES, SARRI and LUMSDEN (2017) and REES et al. (2022), and the NICE and EMAS guidelines recommend diagnosis to be made without laboratory tests in healthy women over 45 years old with symptoms of menopause: perimenopause based on vasomotor symptoms and irregular periods; menopause or climacteric in women who have not menstruated for at least 12 months (who do not use hormonal contraception); menopause based on symptoms in women who have undergone hysterectomy. Thus, it was defined that participants based on the date of the last menstruation at least 12 months ago, age > 45 years (DAVIES; SARRI; LUMSDEN, 2017; REES et al., 2022). These women were selected based on the date of their last menstruation being at least 12 months ago, age > 45 years, without a personal history of cardiovascular disease, and no alcohol or drug consumption. One group included 28 PMW previously diagnosed with T2DM (ADA, 2022). To ensure result consistency, exclusion criteria were established. PMW with current or past coronary artery disease, cerebrovascular disease, aneurysmal disease, peripheral artery disease, chronic kidney disease, insulin-dependent diabetes, liver disease, autoimmune diseases, alcoholics or substance-dependent individuals, as well as those using hormonal therapy, were excluded. Before participating in the study, all participants were adequately informed about the research objectives, procedures involved, and data confidentiality. They agreed to participate voluntarily and signed the Informed Consent Form, as required by Resolution No. 466/2012 of the National Health Council. The conduct of this study strictly adhered to ethical principles and regulations. It was approved

by the Research Ethics Committee of the Faculty of Medicine of Botucatu (No. 2709692), São Paulo State University, and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice principles.

Experimental Approach to the Problem

On the day of the consultation, data related to current age, age at menopause, duration of menopause, current smoking status, hormone therapy use, history of chronic diseases (hypertension, diabetes, cardiovascular diseases), medication use, physical activity, blood pressure, and waist circumference were collected through interviews. Patients with a daily smoking habit were defined as smokers, regardless of the number of cigarettes smoked. Women who engaged in moderate-intensity aerobic exercise for at least 30 minutes five times a week (150 minutes/week) or resistance exercises three days a week were considered active ("WHO guidelines on physical activity and sedentary behaviour", [s.d.]). All the information was gathered during the actual appointment, conducted by the physician.

Anthropometry and fat percentage

For anthropometric assessment, the following were verified: waist circumference and fat percentage. For waist measurement, the circumference between the last rib and the anterosuperior iliac crest was considered, with the reading being taken at the time of expiration (NCPE, 2001). Fat percentage (body fat) was assessed by dual X-ray absorptiometry [(DXA) Hologic; QDR-200, Waltham, MA, USA]. The DXA measurements were performed at the same time of day (at pre- and post-intervention) between 08h00 am and 10h00 am, after 8-10 hours of fasting. To standardize the level of hydration, the volunteers were instructed to consume 2-L of water during the day prior to the DXA assessments. The volunteers dressed in light and comfortable clothes with no metal fastenings. All DXA measurements were performed by the same experienced examiner.

Blood samples

In the routine assessment of PMW at the Climacteric and Menopause Outpatient Clinic of the Botucatu Medical School-UNESP and Blood samples were collected from each participant, after 12h of fasting. Fasting glucose levels were measured using an automatic biochemical analyzer (RAXT, Technician, USA). This was done through colorimetric quantification with specific commercial reagents (Bayer, USA). Optimal values were defined as blood glucose levels <100 mg/dL (FAHED et al., 2022). Additionally, measurements of parathyroid hormone (PTH) and insulin were conducted using a chemiluminescence assay with an automated Immulite 2000® immunoassay system (Diagnostic Products Corporation, Los Angeles, CA, USA). Normal reference ranges considered were PTH of 11 to 65 pg/mL and insulin of 6.0–27.0 µIU/mL (NAHAS-NETO et al., 2018).

Blood samples (20 ml) were collected from the PMW directly into dry tubes with serum separating gel and centrifuged at 3,000 rpm for 10 minutes. The resulting serum was separated into 500 µL aliquots and stored at -80ºC. For the serological determination of the chemokine CCL20, an immunoassay was prepared for analysis in a 96-well plate using a custom cytokine Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel (Millipore Corp., Billerica, MA) following the kit-specific protocols provided by Millipore. Analytes were quantified using a Magpix analytical test instrument employing xMAP Technology (Luminex Corp., Austin, TX) and xPONENT 4.2 software (Luminex). xMAP technology utilizes fluorescent-coded magnetic microspheres coated with analyte-specific capture antibodies to simultaneously measure multiple analytes in a specimen. Once analytes were captured, a biotinylated detection antibody bound to the complex, followed by the attachment of streptavidin PE as a reporter molecule. Magnetic

beads were held in a monolayer by a magnet within the instrument, where two LEDs excited the internal microsphere dye and the reporter molecule's dye, respectively. A CCD camera captured these images, which were subsequently analyzed by Milliplex Analyst software. CCL20 concentrations (pg/mL) were determined based on the fit of a standard curve for mean fluorescence intensity versus pg/mL. Each assay included two quality controls (control 1, low level; control 2, high level). The inter-assay precision for CCL20 was 11.6% CV.

Statistical analyzes

All analyses were performed using JAMOVI (version 2.3). Results were expressed as total numbers, means, standard deviations and percentages. The nonparametric Mann-Whitney test was used to compare the control group with the TDM2 group. Bivariate analysis, using a linear regression model adjusted for age, physical activity (yes or no), smoking (yes or no), and waist circumference, was used to evaluate the influence of CCL20 on PMW with T2DM. All values (unadjusted values) are described as means and standard deviations. The significant level was set at $p<0.05$.

RESULT AND DISCUSSION

The clinical and laboratory characteristics of the 160 PMW assessed within the T2DM (n=28) and control (n=132) groups are presented in Table 1. Physical inactivity was reported by 71.2%, while only 28.8% of study participants reported regular light walking at least five times a week. Current smoking was represented by 23.75% of the study population. Biochemical analysis revealed serum PTH levels of 58.6±23.8 pg/ml in the T2DM group and 60.8±21.2 pg/ml in the control group. The T2DM group showed serum insulin levels of 16.2±10.7 µu/ml and glucose levels of 105.4±8 mg/dl, while the control group exhibited parameters of 9.7±5.8 µu/ml (p=0.001) and 89.6±9.8 mg/dl (p=0.001), respectively. CCL20 exhibited serum levels of 37.70±78.7 pg/ml in the T2DM group and 10.84±14.5 pg/ml in the control group (p=0.011).

Table 1. Clinical, anthropometric and laboratory analyses of PMW (n = 160)

VARIABLES	GROUP	N	MEAN ±SD
Age, *y*	Control	132	67 ±6.5
	T2DM	28	70 ±7
Menopause time, *y*	Control	132	20 ±8.5
	T2DM	28	23 ±8
Menopause age, *y*	Control	132	47 ±6
	T2DM	28	47 ±5.5
WC, *cm*	Control	132	92.5 ±10
	T2DM	28	99 ±15
Body fat,%	Control	132	43.1 ±7
	T2DM	28	43 ±5.7
PTH, *pg/ml*	Control	132	60.8 ±21.2
	T2DM	28	58.6 ±23.8
Insulin, *µu/ml*	Control	132	9.7 ±5.8
	T2DM	28	16.2 ±10.7
Glucose, *mg/dl*	Control	132	89.6 ±9.8
	T2DM	28	105.4 ±8
CCL20, *pg/ml*	Control	132	10.8 ±14.5
	T2DM	28	37.7 ±78.8

Legenda: WC: waist circumference; PTH: Parathyroid hormone; CCL20: Chemokine (C-C motif) ligand 20; SD = standard deviation.

The association between CCL20 and risk indicators for T2DM is presented in Table 2. Elevated levels of CCL20 were significantly associated with the presence of T2DM (OR = 1.017, 95% CI = 1.001-1.033, p = 0.040), independently of age (OR = 1.058, 95% CI = 0.991-1.129, P = 0.091), physical activity (OR = 0.571, 95% CI = 0.188-1.734, p = 0.323), abdominal circumference (OR = 1.048, 95% CI = 1.005-1.092, p = 0.029), and smoking (OR = 0.380, 95% CI = 0.079-1.827, p = 0.227) in postmenopausal women.

Table 2. Logistic regression to assess the relationship between predictive factors and T2DM

COEFFICIENT MODEL – T2DM				
Variables	**95% CI**			
	OR	**Lower**	**Upper**	***p***
CCL20 pg/ml	1.017	1,001 –	1,033	0,040[a]
Age	1.058	0,991 –	1,129	0,091
WC	1.048	1,005 –	1,092	0,029
Physical Activity:				
1 – 0	0.571	0,188 –	1,734	0,323
Smoke:				
1 – 0	0.380	0,079 –	1,827	0,227

Legenda: CCL20, Chemokine (C-C motif) ligand 20; WC, waist circumference; OR, odds ratio; CI, confidence interval. 1, performs (physical activity or smoking). 0, does not perform (physical activity or smoking).

[a]Significantly different if $p < 0.05$ (logistic regression)

This study assessed 160 PMW in groups of T2DM and controls. Both groups exhibited approximately 43% body fat and central fat distribution. The majority were physically inactive, with only 28.8% reporting regular walking. Smoking was present in 23.75% of the population. Our analyses revealed significantly higher serum levels of CCL20 in the T2DM group. The association between CCL20 and T2DM risk was confirmed, even after adjustments for age, physical activity, abdominal circumference, and smoking in PMW.

PMW from urban societies present risk factors for T2DM due to an increased proportion of women over 50 years with obesity, physical inactivity, inadequate diet, as well as metabolic risk factors such as hyperglycemia (TARDIVO et al., 2010).

CC chemokine receptors play a pivotal role in chronic inflammatory responses and may be promising therapeutic targets for the treatment of chronic inflammatory diseases, including T2DM, atherosclerosis, and metabolic syndrome (WHITE et al., 2013). The role of chemokines in attracting immune cells to inflammatory sites, selectively blocking CC chemokine receptors could be an effective strategy to reduce chronic inflammation, prevent tissue architecture and function loss, and thus improve the quality of life of patients. This therapeutic approach may be

particularly relevant given the persistence of chronic inflammatory diseases and their significant impact on public health and the economy (LAU et al., 2019).

In studies with animal models, elevated levels of circulating CCL20 were associated with obesity, and the regulation of CCL20 appears to depend on pancreatic beta cells, which are differentially affected through the NF-κB pathway, resulting in the recruitment of inflammatory cells and contributing to tissue inflammation and damage (BURKE et al., 2015; CHANG; CHEN, 2020). Despite studies with animal models, these deserve attention and such observations underscore the importance of the interaction between CCL20, inflammation, and the development of T2DM (GERARD; ROLLINS, 2001). A significant connection was observed between dietary energy density and the development of T2DM in PMW (HINGLE et al., 2017). We understand that a high dietary energy density compromises metabolic homeostasis by stimulating inflammation mediators (ULLAH et al., 2021), playing a crucial role in the pathogenesis of T2DM (CHANG; CHEN, 2020), and one of the main players in this process is CCL20 (HUANG; POLLOCK; CHEN, 2014).

A study examined the role of chemokines, specifically CCL2, in postmenopausal women with osteoporosis and found that higher levels of CCL2 were associated with greater severity of the condition (YANG et al., 2016). Although the focus of this study is on CCL2, it is relevant to mention that another study conducted by Hogling et al. (2016) identified a relationship between CCL18 and insulin resistance, as well as metabolic risk in women (HOGLING et al., 2016). However, controversy remains regarding the utility of chemokine mediators as markers of chronic diseases, and few studies have specifically examined this association in postmenopausal women (SZMUILOWICZ; STUENKEL; SEELY, 2009). Another study showed that the CCR5Δ32 genetic variant in the CC chemokine receptor 5 is known to lead to CCR5 deficiency and is associated with mortality in T2DM (MUNTINGHE et al., 2009).

A recent study emphasized the association between CCL20 and abdominal aortic aneurysm (AAA), suggesting a possible link between inflammation and AAA pathogenesis mediated by CCL20 (SOTO et al., 2017). The presence of elevated CCL20 levels as a sensitive biomarker may represent a valuable tool for the diagnosis and risk assessment of AAA in patients (SOTO et al., 2017) and other chronic diseases (LEE et al., 2022; MARTÍNEZ-CHACÓN et al., 2021; RANASINGHE; ERI, 2018; SAFA et al., 2016; SOTO et al., 2017; VAN DYKE et al., 2017). Despite previous studies, this current study presents novel results by demonstrating that circulating levels of CCL20 in postmenopausal women with T2DM are directly related to the risk of developing the disease. We found that for every one-unit increase in CCL20 concentration, the risk of developing T2DM increased by 1.7% (95% CI: 1-3.3%). This is particularly relevant, considering that T2DM is one of the major risk factors for cardiovascular diseases in women (DONG et al., 2017), according to recent evidence that also points to a possible connection between T2DM and menopause (SLOPIEN et al., 2018).

It has been demonstrated that circulating pro-inflammatory molecules are potential biomarkers for cardiometabolic diseases, and therefore, the detection and monitoring of inflammation are important for early diagnosis (LIBBY et al., 2019). These research findings highlight the importance of understanding the relationship between inflammatory biomarkers, specifically chemokines like CCL20, and the development of chronic medical conditions. Inflammation plays a substantial role in various health conditions, and these biomarkers may become targets for future therapeutic interventions (POCKLEY et al., 2014). However, it is imperative to conduct further studies to fully elucidate the underlying mechanisms and therapeutic potential of these associations.

On the other hand, this study has some limitations,

notably in its methodology; the lack of causality since associations are identified; the inability to follow participants over time to assess longitudinal changes. The sample size of the T2DM group is limited, which restricts the ability to generalize results to a larger population and may affect the precision of estimates of associations between variables. Therefore, longitudinal studies with this specific population are needed to evaluate clinical outcomes of T2DM. Despite the limitations, this is one of the few studies that assessed associations of serum CCL20 levels as a parameter of inflammation mediation of T2DM, especially in PMW, bringing future perspectives in menopausal women's research. Given the importance of T2DM as a risk factor for cardiovascular diseases in women (CLAYTON et al., 2022; XING; KIRBY; ALMAN, 2022), the link between T2DM and menopause is a relevant topic that deserves further attention (SLOPIEN et al., 2018).

CONCLUSION

In conclusion, our study suggests that CCL20 may be a promising biomarker to identify PMW at increased risk of developing T2DM. Our findings also suggest that CCL20 may have significant future implications for the diagnosis of T2DM in PMW contributing to their overall well-being and healthy ageing.

FUNDING

FLO receives a research grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (National Council for Scientific and Technological Development – CNPq).

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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