Elevated peripheral blood levels of CXCL10 are associated with the presence of diabetic polyneuropathy in subjects with type 2 diabetes

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Abstract

Background: Diabetic peripheral neuropathy (DPN) has high morbidity and mortality. Major risk factors of DPN include metabolic changes, duration of diabetes, nerve ischemia, and derangements in regeneration and nerve repair programs. The implication of chemokines in the pathogenesis of various neuropathies and neuropathic pain processes has been studied previously. This pilot study aimed to evaluate the association between plasma levels of chemokines CXCL9, CXCL10, and CXCL11 with the presence of DPN in a cohort of type 2 diabetes (T2D) patients.

Methods: We have studied a total of 73 T2D patients (36 patients with DPN, and 37 without DPN). DPN was established through the Semmes-Weinstein test (SW). Plasma levels of circulating chemokines CXCL9, CXCL10, and CXCL11 were determined using Duoset ELISA kits (Abingdon, UK).

Results: In T2D patients with similar gender distribution, duration of the disease, peripheral macroangiopathy, and metabolic control, we detected significantly higher serum levels of chemokine CXCL10 among patients with DPN than among patients without DPN (57.6 ± 38.3 vs. 38.1 ± 33.4 pg/mL, respectively; p = 0.034). Serum levels of chemokine CXCL9 were also higher among patients with DPN (188.1 ± 72.7 and 150.4 ± 83.6 pg/mL, respectively, p = 0.06).

Conclusions: DPN in T2D patients was associated with a significant increase in CXCL10 circulating levels, suggesting a role for this chemokine in the DPN. Novel inflammatory markers that allow for early detection and therapeutic strategies in order to reverse and prevent the DPN should be investigated.

Background

Diabetic peripheral neuropathy (DPN), as defined by the American Diabetes Association, is a diagnosis of exclusion involving the presence of signs or symptoms of peripheral nerve dysfunction in individuals with diabetes [1]. DPN is probably the most prevalent, and largely underdiagnosed, chronic complication of diabetes and has high morbidity and mortality [1, 2]. Major risk factors of DPN include metabolic changes associated with chronic hyperglycemia and duration of diabetes, nerve ischemia, and derangements in regeneration and nerve repair programs [3].

Chronic hyperglycemia and increased levels of HbA1c lead to peripheral nerve injury through an array
of complex mechanisms that include the overactivation of the polyol pathway and accumulation of sorbitol [4], alterations of myoinositol [5], protein synthesis deficiency (myelin among others) [6], formation of advanced glycation end products, and oxidative stress [6, 7]. These interlinked metabolic pathways converge, activating the transcription factor NF-kB, which induces the expression of genes involved in inflammation, triggering pro-inflammatory and immune responses [3, 8-10]. Also, under hyperglycaemic conditions, microvascular alterations result and contribute to the development of DPN via tissue hypoxia and nerve ischemia [10-12].

Chemokines are a family of low molecular weight “chemotactic cytokines” (8-10 kDa) that, after binding to their specific G-protein coupled receptor, regulate immune cell migration. By inducing immune cell migration, these chemokines play a pivotal role not only in the innate immunity but also in the adaptive immune response, as well as in the maintenance of chronic inflammation [13]. While some chemokines have been described as pro-inflammatory mediators and are related to infection and inflammation, others are homeostatic and participate in the control of cell migration during tissue development and maintenance processes [14, 15].

Chemokines are classified into four main subfamilies according to the position of the cysteine residues on their N-terminal region: CXC, CC, C, and CX3C [14, 15]. The CXC subfamily is the largest of these and is subdivided into CXC chemokines that contain a glutamic acid-leucine-arginine motif (ELR) in the vicinity of the CXC residues and CXC chemokines that do not [14, 16]. Chemokines belonging to the latter subgroup include CXCL9, CXCL10, and CXCL11. These chemokines are produced by lymphocytes, monocytes, and endothelial cells from the small vessels. These chemokines act through the CXCR3 receptor and have inflammatory functions and antiangiogenic effects [17, 18], thus playing an important role in the control of immunity, inflammation, and angiogenesis [18].

The implication of chemokines in the pathogenesis of various neuropathies and neuropathic pain processes has been studied previously [16, 19]. More specifically, in diabetic neuropathy, the roles of CXCL 9, 10, and 11 have been investigated in an animal model. Streptozotocin-induced diabetic mice presented raised spinal levels of these CXC chemokines following intrathecal administration and
increased neuropathic pain [20]. The authors suggested that these chemokines might participate in the development of diabetic neuropathy, which they described as a neuroinflammatory disorder, and that further research is warranted to complete the understanding of the underlying mechanisms [20]. As mentioned, CXCL9, CXCL10, and CXCL11 have been related to the regulation of angiogenesis and local vascular inflammation, suggesting a pivotal role in the development of microvascular lesions that could affect myelin and axons in DPN. Therefore, this pilot study aimed to evaluate the association between plasma levels of CXCL9, CXCL10, and CXCL11 with the presence of DPN in a cohort of type 2 diabetes (T2D) patients.

Methods

Study population

All participants were patients with T2D aged between 40 and 70 years old who were recruited from the Diabetes Unit of the Hospital Clínico Universitario of Valencia (HCUV) in Spain, by simple random sampling using random sampling tables. Patients with type 1 diabetes mellitus, MODY (Maturity Onset Diabetes of the Young), or secondary diabetes were excluded. Other exclusion criteria were presence of heart failure defined as New York Heart Association (NYHA) class II or above, kidney disease (estimated glomerular filtration rate < 30 ml/min/1.72 m²), liver cirrhosis or serum GPT levels at least twice the upper reference limit, hypothyroidism (TSH values > 5 µU/L), neuropathies or a family history of neuropathies different from diabetic neuropathy, neoplastic diseases or any inflammatory disease. Alcohol consumption greater than 20 g per day in men or 15 g per day in women and Charcot arthropathy or having undergone any previous amputation were also exclusion criteria. A total of 73 T2D patients were included: 36 patients with DPN and 37 patients without DPN. All participants included in the study continued with their usual antidiabetic treatment and their medications for other associated risk factors. The ethical committee of the HCUV approved the study, and all the included participants provided written informed consent.

Clinical Data And Anthropometric Parameters

A comprehensive medical history was taken, including age and sex, smoking habit, alcohol consumption, time of onset of diabetes, history of known cardiovascular disease, associated cardiovascular risk factors, such as hypertension or dyslipidemia, and current medications.
A physical examination, including vital tests, such as patient’s blood pressure and anthropometrical data, such as body mass index and abdominal circumference, was performed using standardized methods [21]. Ankle-brachial index (ABI) was obtained using a protocolized method calculating the ratio between the ankle systolic pressure and the brachial systolic pressure measured with a mercury sphygmomanometer and a Doppler ultrasound (Bi-directional Smartdrop 20) after 5–10 min of rest. Trained health care staff from the Research Unit at the HCUV performed all the procedures according to the research protocol.

**Diagnosis Of Dpn**

The criteria used to make the diagnosis of DPN has been described previously [21]. DPN was established through the Semmes-Weinstein test (SW) by applying 10 g of force with a 5.07-gauge monofilament [22]. A total of six plantar sites were explored, avoiding hyperkeratotic areas, three on each foot: the hallux and the first and the fifth metatarsals. A score of 1 was given when the patient perceived the force applied by the monofilament at each one of the sites and in at least two of three attempts. A score of 0 was given when the patient had no perception. Considering all six sites, a total score between 0 and 4 was considered pathological, whereas a score of 5 or 6 was reported as non-pathological.

The Neuropathy Symptom Score (NSS) and the Neurological Disability Score (NDS) were also performed and used to assess the severity and clinical criteria for symptomatic DPN [23].

**Biochemical Examinations**

Biochemical data were obtained from a blood test following a 10 h overnight fasting period and performed in the Functional Testing Unit within the Endocrinology Department at the HCUV in optimal conditions. Blood samples were analyzed in the Central Laboratory at the HCUV and in the Research Institute INCLIVA according to previously reported protocols [24]. Laboratory examinations were measured using standard procedures and included: full blood count, standard coagulation, liver enzymes, creatinine, glomerular filtration, fasting glucose, high sensitive C-reactive protein, and lipid profile (total cholesterol, LDL cholesterol, and triglycerides). High-density lipoprotein cholesterol was determined following a polyanion precipitation method, and ApoB and ApoA1 were measured by
immunoturbidimetric tests. Glycosylated hemoglobin (HbA1c) was measured using an NGSP-certified high-performance liquid chromatography (HPLC) assay [25].

Plasma levels of circulating chemokines CXCL9, CXCL10, and CXCL11 were determined using Duoset ELISA kits (Abingdon, UK). Further details have been explained elsewhere [18].

Statistical analysis

The normal distribution of the variables was evaluated with the Kolmogorov-Smirnov test. Means for quantitative variables were compared between the two groups with the Student’s t-test for unpaired data for variables with normal distribution and, for non-normal distribution variables, the Kolmogorov-Smirnov test was used as well as the Wilcoxon test for independent samples. The Variance test and the one-way ANOVA test were used to analyze three variables. Results are expressed as the mean ± the standard deviation (SD). P-values < 0.05 were considered significant. Statistical analysis was performed using the Statistical Package for Social Sciences (SPPS) software v.12.1.3 (SPSS Chicago, IL).

Results

We included a total of 73 patients with T2D (47 men and 26 women). The patient characteristics, according to gender, are shown in Table 1. All included patients were divided into two groups according to the presence or absence of DPN, diagnosed by the SW monofilament test: 36 patients with DPN (22 men and 14 women) and 37 without DPN (25 men and 12 women).

| Male (n = 47) | Female (n = 26) | p  |
|--------------|----------------|----|
| Age (years)  | 63.4 ± 11.3    | 64.9 ± 10.4 | NS |
| Evolution of diabetes (years) | 12.5 ± 10.7 | 10.7 ± 9.1 | NS |
| BMI (kg/m2)  | 33.3 ± 5.6     | 30.3 ± 4.9  | 0.034 |
| Waist circumference (cm) | 108.4 ± 10.9 | 106.6 ± 11.1 | NS |
| SBP (mmHg)   | 149.6 ± 16.9   | 147.0 ± 25.3 | NS |
| DBP (mmHg)   | 83.3 ± 13.5    | 82.4 ± 11.7  | NS |
| Fasting glucose (mg/dL) | 160.3 ± 49.7 | 160.9 ± 50.9 | NS |
| HbA1c (%)    | 7.6 ± 1.6      | 7.9 ± 1.7   | NS |
| eGFR (mL/min/1.73²) | 105.2 ± 47.1 | 94.7 ± 50.9 | NS |

Comparing patients’ characteristics between the two groups, no statistically significant differences were found, except for age (Table 2). Patients in the DPN group were older than patients in the group without DPN (68.5 ± 7.9 and 59.4 ± 11.6, respectively; p < 0.001). Patients in the group without DPN
used oral hypoglycemic agents more often than patients in the group with DPN.

Table 2
Clinical and anthropometric parameters in the studied groups of type 2 diabetic patients divided according to the presence of diabetic polyneuropathy.

|                      | DPN + (n = 36) | DPN - (n = 37) | p     |
|----------------------|----------------|----------------|-------|
| Male (n)             | 22             | 25             | NS    |
| Female (n)           | 14             | 12             | NS    |
| Age (years)          | 68.5 ± 7.9     | 59.4 ± 11.6    | <0.001|
| Evolution of diabetes (years) | 13.6 ± 10.8 | 9.8 ± 9.4 | NS    |
| BMI (kg/m²)          | 30.6 ± 4.6     | 31.9 ± 5.8     | NS    |
| Waist circumference (cm) | 106.7 ± 9.6   | 108.7 ± 12.7   | NS    |
| SBP (mm Hg)          | 151.7 ± 20.5   | 145.7 ± 19.7   | NS    |
| DBP (mm Hg)          | 80.7 ± 12.7    | 85.2 ± 12.6    | NS    |
| Treatment for hypertension, n (%) | 26 (75) | 23 (65) | NS    |
| Hypoglycemic treatment Diet (n) | 2 | 3 | 0.021 |
| Oral hypoglycemic agents (n) | 15 | 27 | |
| Insulin (n)          | 9              | 2              |      |
| Insulin + OHA (n)    | 10             | 5              |      |
| Documented myocardial infarction, n (%) | 18 (50) | 12 (32) | NS    |
| ABI                  | 0.84 ± 0.17    | 0.88 ± 0.15    | NS    |
| Statins, n           | 22             | 25             | NS    |
| NDS n, (%) 0 1 2 3   | 10 (27.8) 11 (30.6) 12 (33.3) 3 (8.3) | 29 (78.4) 7 (18.9) 1 (2.7) 0 (0.0) | <0.001 |
| NSS n, (%) 0 1 2 3   | 19 (52.8) 6 (16.7) 3 (8.3) 8 (22.2) | 32 (86.5) 3 (8.1) 2 (5.4) 0 (0.0) | < 0.001 |

Abbreviations: DPN: diabetic polyneuropathy, BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, ABI: ankle-brachial index, NDS: Neurological Disability Score, NSS: Neuropathy Symptom Score.

The biological parameters of the two groups are described in Table 3. No statistically significant differences were observed between the two groups when comparing fasting glucose and glycosylated hemoglobin. However, the estimated glomerular filtration rate was significantly lower among patients with DPN than among patients without DPN (87.5 ± 35.4 and 115.4 ± 55.7, respectively; p = 0.013).

No differences were found in the lipid profile between the two groups. Considering the classification of patients with DPN, no significant differences were observed between the classification performed using the monofilament test results and the ones carried out with the NSS and NDS scales (Table 2).
Table 3
Biological parameters in the studied groups of type 2 diabetic patients divided according to the presence of diabetic polyneuropathy.

| Parameter                | DPN + (n = 36)       | DPN - (n = 37)       | p       |
|--------------------------|----------------------|----------------------|---------|
| Fasting glucose (mg/dL)  | 162.5 ± 48.6         | 158.6 ± 51.6         | NS      |
| HbA1c (%)                | 7.9 ± 1.6            | 7.5 ± 1.6            | NS      |
| eGFR (mL/min/1.73²)      | 87.5 ± 35.4          | 115.4 ± 55.7         | 0.013   |
| TC (mg/dL)               | 191.1 ± 37.0         | 185.5 ± 46.4         | NS      |
| TG (mg/dL)               | 151.4 ± 71.3         | 144.1 ± 89.3         | NS      |
| cHDL (mg/dL)             | 49.6 ± 13.3          | 47.6 ± 11.3          | NS      |
| cLDL (mg/dL)             | 112.1 ± 28.6         | 109.4 ± 42.5         | NS      |
| hsCRP (mg/l)             | 10.1 ± 2.7           | 3.9 ± 3.3            | 0.01    |
| CXCL10 (pg/mL)           | 57.6 ± 38.3          | 38.1 ± 33.4          | 0.034   |
| CXCL9 (pg/mL)            | 188.1 ± 72.7         | 150.4 ± 83.6         | 0.06    |
| CXCL11 (pg/mL)           | 59.0 ± 50.8          | 43.0 ± 36.5          | NS      |

Abbreviations: DPN: diabetic polyneuropathy, TC: total cholesterol, TG: triglycerides, HDLc. High-density lipoprotein cholesterol, LDLc: low-density lipoprotein cholesterol, eGFR: estimated glomerular filtration rate, hsCRP: high sensitivity C-reactive protein.

The analysis of serum concentrations of chemokines is shown in Table 3. Serum levels of chemokine CXCL10 were significantly higher among patients with DPN than among patients without DPN (57.6 ± 38.3 and 38.1 ± 33.4 pg/mL, p = 0.034). Serum levels of chemokine CXCL9 were also higher among patients with DPN (188.1 ± 72.7 and 150.4 ± 83.6 pg/mL), but this did not reach statistical significance (p = 0.06). No significant differences were found between the two groups regarding CXCL11 serum levels. Furthermore, patients with DPN presented significantly higher plasma levels of high sensitivity C reactive protein compared to patients without DPN.

Discussion
This preliminary study investigating the relationship between CXCL9, 10, and 11 chemokines and DPN has some important findings. When comparing patients with T2D with and without DPN classified using the monofilament test, we found that the serum concentrations of chemokine CXCL10 were significantly higher among patients with DPN. We also found that serum CXCL 9 and CXCL 11 levels were raised in the DPN group. However, no statistical significance was reached. To the best of our knowledge, this is an original work showing the direct link between elevated circulating CXCL10 levels and the presence of DPN.

In the present study, the diagnosis of polyneuropathy was based on the response to the monofilament test as it has already been published by our group and reported previously in the literature [21, 26, 27]. Lee et al. [28] described a sensitivity of 93% and a specificity of 100% when performing a ten-
points monofilament examination and comparing it to nerve conduction studies. In their work, the
diagnostic threshold was defined as the inability to perceive at least four sites out of the ten
evaluated. Nevertheless, Baraz et al. [29] reported that in terms of sensitivity and specificity, there
were no significant differences between the monofilament testing in three to four points and the
monofilament testing in ten points. In the present study, we carried out the DPN classification using
the monofilament test in three points, and its results were also consistent with the NDS and NSS
scales. Therefore, we retained this method as a valid tool to screen for DPN.
Regarding CXCL10, we found that serum concentrations were higher among diabetic patients with
DPN. Chemokine CXCL10 is functionally classified as an inflammatory chemokine. The role of the
CXCL10/CXCR3 axis in the immunopathogenesis of type 1 diabetes has been extensively studied with
the blockade of the CXCL10/CXCR3 system considered as a promising therapeutic target [30, 31].
Chemokine CXCL10 has also been suggested to be a potential biomarker of inflammation and
angiogenesis in T2D patients. In fact, a previous study [32] involving 100 patients with T2D and 150
healthy controls found that serum values of CXCL10 and CXCL11 were increased among the T2D
patients when compared to the healthy controls.
In another study [33], hyperglycemia promoted the expression of CXCR3 in activated CD8 + T
lymphocytes and the expression of CXCL9, CXCL10, and CXCL11 on Schwann cells, leading to
Schwann cells apoptosis. This study suggests that CXCR3 and its ligands (CXCL19, CXCL10, and
CXCL11) participate in the development of DPN and that this axis could represent a novel therapeutic
target for preventing DPN.
However, fewer studies have explored the role of CXCL10 in the DPN in T2D patients. A recent study
based on a German prospective cohort [34], the population-based Cooperative Health Research in the
Region of Augsburg (KORA) involving 127 subjects with incipient DPN and 386 without, evaluated the
association between chronic inflammatory biomarkers and the development of DPN. Among all the
biomarkers analyzed, it was found that three chemokines – CCL7, CXCL9, and CXCL10 – were
independently associated with DPN after adjustment and correction for multiple testing. It was also
found that, when the investigators tested their effect in vitro on human neuroblastoma cells, those
chemokines had direct neurotoxic effects [34]. This study supports our findings suggesting further research evaluating the role of CXCL9 and CXCL10 in the pathogenesis of DPN should be undertaken. Our study has several limitations. Since it is a preliminary study, a limited number of patients were included. Additionally, conduction nerve studies were not conducted in the study participants to establish the diagnosis and severity of DPN. Moreover, due to the inherent nature of the study, no causal effect can be established. However, we are hopeful that our study will be complemented by further research.

Conclusions
In our work, DPN in T2D patients was associated with a significant increase in CXCL10 circulating levels, suggesting a role for this chemokine in the DPN. These preliminary results should motivate further research to gain a better understanding of the complex pathogenic pathways implicated in the development of DPN. DPN has a major clinical impact and often develops in predisposed subjects, despite well-controlled diabetes. Novel biological markers that allow for early detection and therapeutic strategies in order to reverse and prevent the disease should now be investigated.

Abbreviations
ABI: Ankle-brachial index; DPN: diabetic peripheral neuropathy; HCUV: Hospital Clínico Universitario of Valencia; HPLC: high-performance liquid chromatography; MODY: Maturity Onset Diabetes of the Young; NDS: Neurological Disability Score; NF-kB: Nuclear Factor kappa Beta; NSS: Neuropathy Symptom Score; SD: standard deviation; SPSS: Statistical Package for Social Sciences; SW: Semmes-Weinstein test; T2D: type 2 diabetes.

Declarations

Ethics approval and consent to participate
The present study was approved by the Ethical Committee of the Hospital Clínico Universitario of Valencia.
All the participants gave written informed consent.

Consent for publication
This manuscript does not contain any individual person’s data in any form (including individual details, images or videos).
Availability of data and materials

The datasets generated during and/or analyzed in the current study are available from the corresponding author on a reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

LP and JTR contributed to the conception and design of the study; PA, AP, SM-H and JFA contributed to the acquisition and analysis of data; MJS and LP performed the laboratory experiments; SM-H, MJS, JFA, LP and JTR contributed to interpretation of the results; PA, AP, SM-H, MJS, JFA, LP and JTR contributed to drafting and revision of the text. All authors read and approved the final manuscript.

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