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Deficits in systemic biomarkers of neuroinflammation and growth factors promoting nerve regeneration in patients with type 2 diabetes and polyneuropathy

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ABSTRACT

Introduction The determinants and mechanisms contributing to diabetic sensorimotor polyneuropathy (DSPN) remain unclear. Since neuroinflammation and altered nerve regeneration have been implicated in the pathogenesis of both DSPN and neuropathic pain, we hypothesized that the corresponding biomarkers could be associated with DSPN in general and could have the potential to discriminate between the painful and painless DSPN entities.

Methods In a cross-sectional study using multimarker proximity extension assay technology we assessed 71 serum biomarkers including cytokines, chemokines, growth factors, receptors, and others in patients with type 2 diabetes with DSPN (DSPN+) (n=304) or without DSPN (DSPN−) (n=158) and persons with normal glucose tolerance (NGT) without polyneuropathy (n=354).

Results After adjustment for multiple testing and sex, age, body mass index, HbA1c, and smoking, the serum levels of 17 biomarkers (four cytokines, five chemokines, four growth factors, two receptors, two miscellaneous) were lower in DSPN+ than in DSPN− and NGT. In DSPN+, six of these biomarkers were associated with peripheral nerve function. The concentrations of 15 other biomarkers differed between NGT and both DSPN+ and DSPN−, but not between DSPN+ and DSPN−. No differences in biomarker levels were found between patients with painful (n=164) and painless DSPN (n=140).

Conclusions Deficits in systemic cytokines, chemokines, and growth factors promoting nerve regeneration in patients with type 2 diabetes are linked to polyneuropathy in general but not specifically to the painful or painless entity.

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Significance of this study

What is already known about this subject?

- Inflammation and altered nerve regeneration have been implicated in the pathogenesis of both diabetic polyneuropathy and neuropathic pain, but it remains unclear whether serum markers of inflammation and growth factors are associated with diabetic polyneuropathy in general and also more specifically with the painful or painless entity.

What are the new findings?

- Deficits in systemic cytokines, chemokines, and growth factors promoting nerve regeneration in patients with type 2 diabetes are linked to polyneuropathy in general but not specifically to the painful or painless entity.

How might these results change the focus of research or clinical practice?

- When designing or implementing anti-inflammatory therapies for nerve injury involving myelinating cells, the various aspects of immune environment beneficial to myelin repair and potential differences between human and rodent immune cells should be considered.
- Given the importance of growth factors in normal nervous system development and maintenance, their therapeutic potential in regeneration of lost or damaged peripheral neurons should be further explored.

INTRODUCTION

Diabetic sensorimotor polyneuropathy (DSPN) is encountered in approximately 30% of patients with diabetes and accounts for considerable morbidity and an increased risk of mortality.[1] DSPN may present as a painful entity, the main feature of which is neuropathic pain and a painless variant that predisposes to foot ulceration. The distinctive aspects and patterns characterizing painful DSPN compared with painless DSPN have been addressed in a number of previous studies which indicate that painful DSPN is associated with female sex, obesity, and higher neuropathy severity when compared with the painless entity.[2] However, the question why one proportion of patients with DSPN develops

neuropathic pain, while the other remains painless has not yet been answered. Recent years have witnessed increasing evidence suggesting a role for inflammation in the causation of diabetic neuropathy in general[2 3] and specifically in the induction and maintenance of neuropathic pain.[2 4 5] Neuroinflammation is a well-controlled physiological process that serves to promote regeneration and healing, but chronic pain may emerge as a maladaptive mechanism if the resolution of neuroinflammation is disturbed.[6] Both in the peripheral nervous system (PNS) and central nervous system (CNS), mediators released by immune cells, such as cytokines, sensitize nociceptive signaling. Experimental data point to an immune pathogenesis of neuropathic pain, but clinical evidence of a central role of the immune system is less clear.[4] Likewise, experimental studies suggest that a crosstalk between oxidative stress and neuroinflammation culminating in the production of proinflammatory cytokines may be responsible for nerve tissue damage in neuropathies.[3]

We recently reported that proinflammatory cytokines predict the incidence and progression of polyneuropathy in the older general population.[7] Using a multimarker approach and both pathway and mediation analyses we suggested that multiple cell types from innate and adaptive immunity are involved in the development of polyneuropathy[8] and that inflammatory markers may also mediate the association between obesity and polyneuropathy in the older general population.[9] However, the specific role of inflammation in painful DSPN as opposed to the painless variant remains unclear. In fact, one study reported that among 18 inflammatory markers or growth factors only two showed increased systemic levels in patients with painful compared with those with painless DSPN.[10] Consequently, there is insufficient evidence to affirm that increased inflammation can be considered a discriminant between painful and painless DSPN.[2]

Diabetes results in multiple processes of degeneration, remodeling, and regeneration in axons, glia cells, and the axon-surrounding microenvironment, ultimately culminating in impaired peripheral nerve function.[1] The growth and survival of the nervous system are regulated by neurotrophins which provide trophic support by promoting survival and/or growth of neurons and tropic support by directing the movement of extending neurites. Thus, neurotrophins are vital for nervous system development and function, and impaired neurotrophin signaling in development or due to injury leads to devastating effects.[11] Despite the ample evidence from experimental studies supporting the role of neurotrophins in the pathogenesis of both diabetic neuropathy[12] and neuropathic pain,[13] studies focusing on neurotrophins as biomarkers in human DSPN are scarce. Two relatively small studies reported higher systemic levels of nerve growth factor[14] and transforming growth factor beta 1 (TGF-ß1)[15] in patients with type 2 diabetes with DSPN than in those without DSPN. Apart from these reports, no study has hitherto systematically assessed the role of these factors neither in larger cohorts of patients with type 2 diabetes with DSPN, nor specifically in those with the painful and painless entity.

Since inflammation and altered nerve regeneration have been implicated in the pathogenesis of both DSPN and neuropathic pain, we aimed to determine whether serum markers of inflammation and growth factors are associated with DSPN in general and also more specifically with the painful or painless entity using a novel comprehensive protein-based multimarker approach.

MATERIALS AND METHODS

Study design and participants

This cross-sectional study included 304 patients with type 2 diabetes and DSPN (DSPN+) participating in the Probing the Role of Sodium Channels in Painful Neuropathy Study (PROPANE) as well as 354 persons with normal glucose tolerance (NGT) without polyneuropathy and 158 individuals with type 2 diabetes without DSPN (DSPN–) from the Cooperative Health Research in the Region of Augsburg (KORA) Survey F4. The inclusion criteria and study design were described previously.[16] In brief, the inclusion criteria were age ≥18 years, type 2 diabetes according to the American Diabetes Association criteria,[17] and presence of DSPN.[18] Exclusion criteria were other causes of neuropathy and concomitant diseases that might interfere with the participant's ability to fill in questionnaires.[16] The study design has been described before.[6] In brief, this study included individuals from KORA F4 (2006–2008), a follow-up examination of the population-based KORA S4 study (1999–2001) conducted in Augsburg (Germany) and two adjacent counties. Anthropometric and metabolic parameters, lifestyle factors, and glucose tolerance status using standard 75 g oral glucose tolerance tests were assessed as reported previously.[7] NGT was classified according to the American Diabetes Association criteria using fasting glucose and 2-hour glucose values.[17]

Neurological assessment

In the PROPANE study, neurological examination was performed using the Neuropathy Disability Score.[19] Neurological symptoms were assessed by Neuropathy Symptom Score (NSS)[19] and neuropathic pain by the 11-point Numerical Rating Scale (NRS) scoring average and maximum pain over 24 hours. Electrophysiological testing, quantitative sensory testing (QST), skin biopsies, and neuropathy score surveys were performed as previously described.[16] Sensory nerve conduction velocity (SNCV) and sensory nerve action potential (SNAP) were determined in the median, ulnar, and sural nerves, while motor nerve conduction velocity (MNCV) was measured in the peroneal and tibial nerves, all at a skin temperature of 33°C–34°C using surface electrodes (Nicolet VikingQuest; Natus Medical San Carlos, CA). Vibration perception thresholds (VPT) were measured at the second metacarpal bone and medial malleolus using the method of limits (Vibrameter; SBMEDIC Electronics, Solna, Sweden). Thermal detection thresholds (TDT) were measured using method of limits for warm and cold stimuli

at the thenar eminence and the dorsum of the foot (TSA-II NeuroSensory Analyzer; Medoc, Ramat Yishai, Israel). Sensory, sensorimotor, and/or small fiber DSPN was diagnosed as possible, probable or confirmed according to the Toronto Consensus criteria.[18] Patients were subdivided into two groups based on the diagnosis of painful DSPN (n=164) or painless DSPN (n=140). The presence of pain in the distal lower limbs lasting ≥1 year with a pain intensity ≥4 (24 hours average or maximum) on the NRS in the absence of analgesic treatment, or according to the medical history (recall and/or records) prior to analgesic treatment, was used to define painful DSPN.[16] Patients with painless DSPN reported a pain intensity on the 24 hours average NRS of 0, except for 11 who had an NRS between 0.5 and 1.5 without analgesic treatment.

In the KORA F4 study, the examination part of the Michigan Neuropathy Screening Instrument (MNSI)[20] was used to exclude clinical DSPN as previously described.[7] In brief, items for the appearance of feet, foot ulceration, ankle reflexes and VPT at the great toes were included in the examination part of the MNSI. For normal VPT age-dependent limits were considered.[21] The neuropathy assessment was extended by a bilateral examination of touch/pressure sensation (TPS) using a 10 g monofilament (Neuropen).[7] The total range of the MNSI score was from 0 (all aspects normal) to a maximum of 10 points. Clinical distal sensorimotor polyneuropathy was excluded if the MNSI score was ≤3 points. Average pain in the feet over 24 hours was assessed using the 11-point NRS.

Multimarker assessment (OLINK inflammation panel)

Biomarkers of subclinical inflammation were measured in fasting serum using the OLINK Inflammation multiplex immunoassay (OLINK Proteomics, Uppsala, Sweden) as described before.[8] In brief, the OLINK inflammation panel consists of 92 protein biomarkers including cytokines and chemokines as well as growth, acute inflammatory/immune response, angiogenesis, fibrosis, and endothelial activation factors. The OLINK Proteomics immunoassay is based on the proximity extension assay technology combining a detection step using oligonucleotide-labeled antibodies, a proximity-dependent DNA polymerization event, and a real-time quantitative PCR amplification. The assay measures the relative concentration of the analytes as normalized protein expression values which are comparable in their distribution to log2-transformed protein concentrations. We excluded 20 biomarkers that gave values below the limit of detection (LOD) in ≥25% of all samples. For the remaining analytes, values below the LOD were substituted with the respective LOD. Moreover, we excluded one biomarker because of an interassay coefficient of variation>20%.

Statistical analysis

Categorical data were expressed as percentages of participants while continuous data were expressed as mean±SD. Categorical variables were compared using the χ^2 test. Continuous data were assessed using non-parametric Kruskal-Wallis test (for all three groups) or Mann-Whitney test (for two groups). The analyses were adjusted for multiple testing (three groups and 71 biomarkers) using the Bonferroni correction: $\alpha=0.05/3\times71=0.000235$. All group comparisons were adjusted for sex, age, body mass index (BMI), HbA1c, and smoking using multiple linear regression analyses. Correlations between biomarkers of subclinical inflammation and neurophysiological parameters were estimated using Spearman rank correlation (r). Associations between biomarkers and neurophysiological parameters were assessed using multiple linear regression analyses with adjustments for sex, age, BMI, HbA1c, and smoking. All statistical tests were performed two sided. The level of significance was set at $\alpha=0.05$. All analyses were performed using SPSS V.22.0 software.

RESULTS

The demographic and clinical data of the three groups are listed in table 1. When compared with patients without DSPN (DSPN−), those with DSPN (DSPN+) were younger and more frequently male and current smokers and had lower diastolic blood pressure, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol and higher systolic blood pressure, diabetes duration, and HbA1c ($p<0.05$). Compared with the NGT group, DSPN+ patients were more frequently male and current smokers and had lower diastolic blood pressure, cholesterol, and LDL cholesterol and higher systolic blood pressure and HbA1c ($p<0.05$). When compared with the NGT group, DSPN− patients were older and more frequently male and current smokers and had lower cholesterol, LDL cholesterol, and HDL cholesterol as well as higher systolic blood pressure and HbA1c ($p<0.05$). No other differences between the groups were noted, except for the higher rates of abnormal VPT, TPS, and ankle reflexes in the DSPN+ group.

Table 2 lists the 18 biomarkers for which differences in serum levels were found between the DSPN+ and DSPN− groups as well as the DSPN+ and NGT groups. The levels of 17 biomarkers were lower, while the CCL20 (MIP-3α) concentration was higher in DSPN+ patients than in the DSPN− and NGT groups ($p<0.05$). There were no differences between the DSPN− and NGT groups with respect to all biomarkers listed, except for CCL20 (MIP-3α) the level of which was higher in DSPN− patients than NGT persons ($p<0.05$). No other differences between the groups were found. Moreover, levels of the 71 biomarkers were tested for differences between patients with painful (n=161) and painless DSPN (n=141). After Bonferroni correction for multiple testing and adjustment for sex, age, BMI, HbA1c, and smoking, no differences in any of these biomarkers between the groups could be detected (data not shown).

Table 3 shows the 15 biomarkers for which differences in serum levels were noted between NGT persons and either DSPN− or DSPN+ patients or both. Compared with the NGT group, after full adjustment using model 3, DSPN+ patients showed higher serum concentrations of CCL19 (MIP-3β), CCL23 (MIP-3), CCL25 (TECK), FGF21, and SIRT2 and lower levels of FGF19, ST1A1, and uPA ($p<0.05$). After

Table 1 Demographic and clinical characteristics of the three groups studied.

	NGT (KORA F4)	DSPN– (KORA F4)	DSPN+ (PROPANE)
n	354	158	304
Male (%)	41	59*	76*†
Age (years)	68.9±5.3	71.0±5.5*	68.0±9.3†
BMI (kg/m^2)	26.9±3.7	30.8±4.4*	30.8±5.3*
Current smokers (%)	10	10	17*†
Systolic BP (mm Hg)‡	124±20	133±20*	139±20*†
Diastolic BP (mm Hg)‡	74±10	74±10	72±9*†
Cholesterol (mg/dL)‡	228±40	212±40*	192±46*†
LDL cholesterol (mg/dL)‡	145±36	132±34*	118±37*†
HDL cholesterol (mg/dL)‡	60±14	49±11*	52±16†
Creatinine (mg/dL)‡	0.91±0.27	1.02±0.50	1.02±0.42
Diabetes duration (years)	–	7.6±5.8	13.5±9.6†
HbA1c (%) HbA1c (mol/mmol)	5.49±0.28 36.5±3.0	6.55±0.97* 48.1±10.7*	7.35±1.30*† 56.8±14.2*†
NRS 24 hours average pain (feet)	0.43±1.51	0.41±1.39	0.14±0.77§ 4.16±2.80¶
Abnormal VPT right (%)	10	11	50
Abnormal VPT left (%)	7	9	57
Abnormal TPS right (%)	4	8	31
Abnormal TPS left (%)	3	7	27
Absent ankle reflex right (%)	2	1	48
Absent ankle reflex left (%)	1	3	45

Data are expressed as percentages or mean±SD.
*P<0.05 versus NGT.
†P<0.05 versus DSPN–.
‡After adjustment for sex, age, BMI, HbA1c, and smoking.
§Painless DSPN (n=140).
¶Painful DSPN (n=164).
BMI, body mass index; BP, blood pressure; DSPN, diabetic sensorimotor polyneuropathy; HDL, high-density lipoprotein; KORA, Cooperative Health Research in the Region of Augsburg; LDL, low-density lipoprotein; NGT, normal glucose tolerance; NRS, Numerical Rating Scale; PROPANE, Probing the Role of Sodium Channels in Painful Neuropathy Study; TPS, touch/pressure sensation; VPT, vibration perception threshold.

adjustment using model 2, DSPN+ patients had higher levels of interleukin 6 (IL6), IL18R1, MMP10 (SL-2), and SLAMF1 (CD150) than the NGT group (p<0.05). After full adjustment using model 3, DSPN– patients had higher serum concentrations of IL6, FGF21, and IL18R1 than persons with NGT (p<0.05). Compared with the NGT group, after adjustment using model 2, DSPN– patients showed higher concentrations of LIF-R and SLAMF1 (CD150) and after adjustment using model 1 higher levels of IL18, CCL19 (MIP-3β), and 4E-BP1 (p<0.05). No other differences between the groups were observed.

The mean values with statistical tests of all 71 analyzable biomarkers assessed from the OLINK inflammation panel are given in online supplementary table 1.

Table 4 shows the associations between the biomarkers listed in table 2 and neurophysiological tests in the DSPN+ group. Among the cytokines, both TNFSF12 (TWEAK) and TNFSF14 (LIGHT) were positively associated with peroneal motor MNCV, sural SNCV, sural SNAP, and cold TDT on the hand, while TNFSF12 (TWEAK) was associated with tibial MNCV and TNFSF14 (LIGHT) was associated with cold TDT on the foot (p<0.05). Among the chemokines, positive associations of CCL20 (MIP-3α) with metacarpal VPT and warm TDT on the hand were found, while CXCL1 (MGSA-α) was associated with cold TDT on the hand (p<0.05). Among the growth factors, DNER was positively associated with tibial MNCV, peroneal MNCV, and ulnar SNAP (p<0.05). Finally, MMP1 was positively associated with sural SNCV and SNAP (p<0.05).

DISCUSSION

The results of this study using a multimarker approach demonstrate that reduced serum levels of multiple biomarkers of neuroinflammation and growth factors promoting nerve regeneration are linked to polyneuropathy in patients with type 2 diabetes. Among 71 biomarkers studied (see online supplementary table 1), after stringent adjustment for important potential confounders and multiple testing, among the 18 markers associated with DSPN, remarkably, all but one were lower in patients with DSPN compared with those without DSPN and NGT subjects. Surprisingly, no differences in biomarker levels were noted between patients with painful and painless DSPN. Collectively, these data show that DSPN in type 2 diabetes is associated primarily with reduced rather than enhanced neuroinflammation, independent of the presence of neuropathic pain. Thus, these findings do not support the hypothesis of a major role for enhanced inflammation in the pathogenesis of both DSPN in general and painful DSPN in particular.

Apart from our two recent reports using the identical multimarker approach in the elderly general population,[8 9] there are no published studies with which to directly compare the present results. Doupis *et al*[10] reported that while the levels of 13 out of 18 inflammatory markers or growth factors were increased or decreased, respectively, in patients with DSPN compared with those without DSPN, only two of these (C-reactive protein and soluble intercellular adhesion molecule 1) were higher in patients with painful than in those with painless DSPN. Oddly, the authors interpreted these results as evidence of increased inflammation in painful DSPN.[10] Moreover, that study had several drawbacks. Both persons with type 1 and type 2 diabetes were included. The groups with painful and painless DSPN were classified based on the NSS≥4 and NSS<4 points, respectively, but this score includes both painful and painless symptoms without an option to discriminate between them. Finally, no correction for multiple testing was undertaken. Thus, in the light of the present data, increased systemic inflammation cannot be added to the factors contributing to painful DSPN relative to painless DSPN such as female sex, obesity, and higher neuropathy severity.[2] By contrast, the levels of multiple inflammatory markers were increased

Table 2 Biomarkers for which differences in mean levels were found between patients with diabetic sensorimotor polyneuropathy (DSPN+, n=304; PROPANE study) and participants from the KORA F4 study without DSPN (DSPN–, n=158) and those with normal glucose tolerance (NGT, n=354)

Biomarkers	NGT	DSPN–	DSPN+	P value*
Cytokines				
Oncostatin M	4.83±0.62	5.06±0.63	4.35±0.75†‡	4.9×10^{-25}
TNFSF10 (TRAIL)	8.12±0.30	8.08±0.33	7.90±0.41†‡	8.6×10^{-16}
TNFSF12 (TWEAK)	9.45±0.29	9.30±0.32	9.06±0.35†‡	2.7×10^{-42}
TNFSF14 (LIGHT)	5.61±0.53	5.65±0.55	4.90±0.77†‡	2.2×10^{-40}
Chemokines				
CCL4 (MIP-1β)	8.18±0.57	8.36±0.64	7.93±0.68†‡	8.4×10^{-14}
CCL8 (MCP-2)	9.50±0.77	9.47±0.72	9.21±0.77†‡	3.0×10^{-8}
CCL20 (MIP-3α)	4.91±1.18	5.24±1.11	5.79±1.23†‡	5.0×10^{-25}
CCL28 (MEC)	1.56±0.38	1.57±0.44	1.29±0.34†‡	2.4×10^{-21}
CXCL1 (MGSA-α)	9.42±0.48	9.49±0.47	9.27±0.65†‡	3.3×10^{-5}
CXCL11 (I-TAC)	7.89±0.78	7.99±0.61	7.46±0.78†‡	6.4×10^{-18}
Growth factors				
HGF	8.52±0.36	8.79±0.40†	8.42±0.41†‡	2.3×10^{-18}
TGF-α	4.51±0.51	4.64±0.56	3.91±0.51†‡	3.6×10^{-49}
LAP-TGFβ1	7.89±0.53	7.99±0.33	7.46±0.40†‡	2.6×10^{-52}
Neurotrophin-3	1.03±0.34	1.00±0.33	0.87 ± 0.40†‡	6.4×10^{-13}
Receptors				
TNFRSF5 (CD40)	10.25±0.33	10.30±0.43	10.15±0.46†‡	5.1×10^{-7}
DNER	8.33±0.23	8.28±0.26	8.14±0.30†‡	1.5×10^{-17}
Miscellaneous				
AXIN1	1.55±0.47	1.61±0.54	1.34±0.48†‡	5.7×10^{-12}
MMP1	14.43±0.75	14.44±0.74	14.06±0.92†‡	2.7×10^{-8}

Data are expressed as mean±SD (the unit is normalized protein expression (NPX) which is comparable in its distribution to log2-transformed protein concentration).
*Kruskal-Wallis test with Bonferroni correction ($p<2.35\times10^{-4}$).
†P<0.05 versus NGT group.
‡P<0.05 versus DSPN– group after adjustment for sex, age, body mass index (BMI), HbA1c, and smoking (NGT vs DSPN groups) or sex, age, BMI, HbA1c, smoking, and diabetes duration (DSPN– vs DSPN+).
CCL, chemokine (C-C motif) ligand; CD, cluster of differentiation; CXCL, chemokine (C-X-C motif) ligand; DNER, delta and Notch-like epidermal growth factor-related receptor; HGF, hepatocyte growth factor; I-TAC, interferon-inducible T-cell alpha chemoattractant; KORA, Cooperative Health Research in the Region of Augsburg; LAP-TGFβ1, latency-associated peptide transforming growth factor beta 1; LIGHT, homologous to **l**ymphotoxin, exhibits **i**nducible expression and competes with HSV **g**lycoprotein D for binding to **h**erpesvirus entry mediator, a receptor expressed on **T** lymphocytes; MCP-2, monocyte chemoattractant protein 2; MEC, mucosae-associated epithelial chemokine; MGSA-α, melanoma growth-stimulating activity alpha; MIP, macrophage inflammatory protein; MMP1, matrix metalloproteinase-1; PROPANE, Probing the Role of Sodium Channels in Painful Neuropathy Study; TGF-α, transforming growth factor alpha; TNFRSF5, tumor necrosis factor receptor superfamily 5; TNFSF12, tumor necrosis factor ligand superfamily member 12; TNFSF14, tumor necrosis factor ligand superfamily member 14; TRAIL, TNF-related apoptosis-inducing ligand; TWEAK, TNF-related weak inducer of apoptosis.

both in the groups with and without DSPN when compared with the NGT group, but without differences between the DSPN groups, suggesting that these markers may play a particular role in the context of diabetes per se rather than DSPN. This notion is supported by applying different adjustment models to the data. For example, the differences in IL18, CCL19, and 4E-BP1 levels between patients without DSPN and NGT persons obtained with model 1 were abrogated when entering BMI into the model. Likewise, after adding HbA1c to the model, the differences between the groups with and/or without DSPN and NGT individuals were abolished for IL6, IL18R1, LIF-R, MMP10, and SLAMF1 (table 3).

The main novel finding of the present study is the reduction of both systemic markers of neuroinflammation and growth factors associated with DSPN per se rather than painful DSPN in particular. At first sight these results come as a surprise, since on the one hand the current perception suggests that an activation of the inflammatory cascade with proinflammatory cytokine upregulation plays a vital role in structural and functional damage of the peripheral nerves leading to DSPN.[3]

Table 3 Biomarkers for which differences between persons from the KORA F4 study with normal glucose tolerance (NGT, n=354) and either patients without diabetic sensorimotor polyneuropathy (DSPN–, n=158) or those with DSPN (DSPN+, n=304; PROPANE study) or both were noted

Biomarkers	NGT	DSPN–	DSPN+	P value*
Cytokines				
IL6	2.22±0.81	2.58±0.73†‡§	2.57±0.83†‡	1.4×10^{-13}
IL18	8.65±0.58	8.86±0.65†	8.82±0.60	7.0×10^{-6}
Chemokines				
CCL19 (MIP-3β)	9.80±0.92	10.06±0.87†	10.28±0.95†‡§	9.7×10^{-13}
CCL23 (MIP-3)	9.98±0.47	10.02±0.49	10.15±0.77†‡§	1.9×10^{-4}
CCL25 (TECK)	6.63±0.58	6.71±0.54	6.89±0.60†‡§	2.1×10^{-7}
Growth factors				
FGF19	7.87±0.88	7.60±0.93	7.58±0.97†‡§	5.4×10^{-5}
FGF21	5.69±1.06	6.41±1.11†‡§	6.25±1.40†‡§	2.4×10^{-13}
Receptors				
IL18R1	7.38±0.37	7.68±0.43†‡§	7.69±0.53†‡	1.2×10^{-19}
LIF-R	4.57±0.28	4.73±0.35†‡	4.72±0.38†‡	5.7×10^{-8}
Miscellaneous				
4E-BP1	6.81±0.56	7.10±0.64†	7.03±1.19	9.0×10^{-8}
MMP10 (SL-2)	6.41±0.58	6.51±0.66	6.61±0.62†‡	6.9×10^{-6}
SIRT2	2.28±0.48	2.37±0.53	2.54±0.69†‡§	4.5×10^{-7}
SLAMF1 (CD150)	2.41±0.50	2.73±0.60†‡	2.74±0.58†‡	1.2×10^{-17}
ST1A1	2.08±0.85	1.99±0.78	1.78±0.84†‡§	6.0×10^{-6}
uPA	9.89±0.27	9.86±0.33	9.80±0.36†‡§	8.9×10^{-5}

Data are expressed as mean±SD (the unit is normalized protein expression (NPX) which is comparable in its distribution to log2-transformed protein concentration).
*Kruskal-Wallis test with Bonferroni correction ($p<2.35\times10^{-4}$).
†P<0.05 versus NGT group after adjustment for model 1: sex, age, and smoking.
‡P<0.05 versus NGT group after adjustment for model 2: model 1+BMI.
§P<0.05 versus NGT group after adjustment for model 3: model 2+HbA1c.
BMI, body mass index; CCL, chemokine (C-C motif) ligand; CD, cluster of differentiation; FGF, fibroblast growth factor; IL6, interleukin 6; KORA, Cooperative Health Research in the Region of Augsburg; MIP, macrophage inflammatory protein; PROPANE, Probing the Role of Sodium Channels in Painful Neuropathy Study; SIRT2, SIR2-like protein; SL-2, stromelysin-2; ST1A1, sulfotransferase 1A1; TECK, thymus-expressed chemokine; uPA, urokinase-type plasminogen activator.

On the other hand, mediators released by immune cells, such as cytokines, which sensitize nociceptive signaling in the PNS and CNS may contribute to an immune pathogenesis of neuropathic pain.[4 5] Since inflammation is a common component of trauma and disease in the nervous system, an active inflammatory response is often considered deleterious to myelin health.[22] While inflammation can certainly damage myelin, inflammatory cascades can also augment myelin repair, including processes initiated by infiltrating immune cells and local Schwann cells in the peripheral nerves.[21] Supporting this notion is our finding that the levels of most biomarkers which were lower in patients with DSPN than in those without DSPN were actually numerically higher in the latter group than in the NGT group. It is tempting to speculate that this suggests a biphasic pattern of neuroinflammatory markers during the course of diabetes with a trend towards an increase before the development of DSPN and subsequent decrease after DSPN has developed. Against this background, below we provide the rationale from experimental and translational studies for the reduced systemic biomarker levels in association with DSPN (table 2, except for CCL20) by outlining their neuroprotective properties.

Cytokines

Oncostatin M is a member of the IL6 family of cytokines which is regulated in most cells of the CNS. It is a powerful neuroprotective cytokine which prevents the expression of the N-methyl-D-aspartate receptor which plays a major role in the pathogenesis of neuropathic pain.[23] Oncostatin M also confers neuroprotection against ischemic stroke, and hence may represent a promising drug candidate for stroke treatment.[24]

TNFSF10 (TRAIL) has pleiotropic functions and protects against cardiovascular disease (CVD), insulin resistance, non-alcoholic steatohepatitis (NASH), and vascular inflammation. Low TRAIL plasma levels are predictors of CVD and are reduced in patients with NASH. It has been suggested that increasing TRAIL levels may be

Table 4 Associations of the biomarkers listed in table 2 with neurophysiological tests in the group with DSPN (n=304; PROPANE study). Only correlations achieving p<0.01 were considered

Biomarker	Nerve function tests	r	P value	β	P value
TNFSF12 (TWEAK)	Tibial MNCV	0.164	0.005	0.122	0.037
	Peroneal MNCV	0.207	0.0003	0.155	0.006
	Sural SNCV	0.244	<0.0001	0.208	0.0002
	Sural SNAP	0.259	<0.0001	0.242	<0.0001
	Cold TDT hand	0.226	<0.0001	0.195	0.001
TNFSF14 (LIGHT)	Peroneal MNCV	0.159	0.006	0.123	0.031
	Sural SNCV	0.210	0.0003	0.184	0.002
	Sural SNAP	0.205	0.0004	0.191	0.001
	Cold TDT hand	0.214	0.0002	0.131	0.029
	Cold TDT foot	0.182	0.002	0.126	0.035
CCL20 (MIP-3α)	Metacarpal VPT	0.182	0.002	0.183	0.002
	Warm TDT hand	0.165	0.004	0.182	0.002
CXCL1 (MGSA-α)	Cold TDT hand	0.168	0.004	0.124	0.035
DNER	Tibial MNCV	0.227	<0.0001	0.217	0.0002
	Peroneal MNCV	0.228	<0.0001	0.176	0.002
	Ulnar SNAP	0.166	0.005	0.194	0.001
MMP1	Sural SNCV	0.172	0.003	0.150	0.008
	Sural SNAP	0.177	0.002	0.168	0.003

Linear regression analyses were adjusted for sex, age, body mass index (BMI), HbA1c, smoking, and diabetes duration.
CCL, chemokine (C-C motif) ligand; CXCL1, chemokine (C-X-C motif) ligand 1; DNER, delta and Notch-like epidermal growth factor-related receptor; DSPN, diabetic sensorimotor polyneuropathy; LIGHT, homologous to **l**ymphotoxin, exhibits **i**nducible expression and competes with HSV **g**lycoprotein D for binding to **h**erpesvirus entry mediator, a receptor expressed on **T** lymphocytes; MGSA-α, melanoma growth-stimulating activity alpha; MIP, macrophage inflammatory protein; MMP1, matrix metalloproteinase-1; MNCV, motor nerve conduction velocity; PROPANE, Probing the Role of Sodium Channels in Painful Neuropathy Study; SNAP, sensory nerve action potential; SNCV, sensory nerve conduction velocity; TDT, thermal detection threshold; TNFSF, tumor necrosis factor ligand superfamily; TWEAK, TNF-related weak inducer of apoptosis; VPT, vibration perception threshold.

an attractive therapeutic strategy to reduce insulin resistance as well as liver and vascular inflammation/injury.[25]

TNFSF12 (TWEAK) exerts its biologic effects by binding to the Fn14 receptor (fibroblast growth factor-inducible 14 kDa protein) which is involved in axonal regrowth by injured neurons in the PNS. In cultured mouse neural progenitor cells, TWEAK exerted age-dependent effects on neurite extension.[26]

TNFSF14 (LIGHT) plays an important role in the development and progression of chronic experimental autoimmune encephalomyelitis—one of the models used for multiple sclerosis. The ablation of LIGHT leads to an acute aggravation of the clinical signs of the disease.[27]

Chemokines

CCL20 was the only biomarker showing higher levels in patients with DSPN than in those without DSPN. Consistent with this finding are experimental studies demonstrating that CCL20 is directly toxic to primary neurons and oligodendrocytes subjected to oxygen glucose deprivation. The temporal expression profile of CCL20, coupled with in vitro toxicity to primary cells, suggests that this chemokine exerts deleterious effects on cell viability following traumatic brain injury.[28]

CXCL1 is a major neutrophil chemoattractant that binds to the chemokine receptor CXCR2 on neutrophils and oligodendrocytes. Estrogen receptor β ligand treatment in a mouse model of multiple sclerosis induces an increase in peripheral and brain CXCL1 levels that correlate with an increase in axon remyelination. Potential neuroprotective benefits arising from the presence of CXCL1 could have implications for improved therapies against multiple sclerosis.[29] CXCL1 also plays an antinociceptive role in peripheral nerve injury-induced neuropathic pain, which is possibly mediated by infiltrating neutrophils.[30]

Growth factors

Hepatocyte growth factor (HGF) plays important roles in Schwann cell-mediated nerve repair, suggesting that HGF gene transfer may provide a useful tool for treating peripheral neuropathy. A clinical trial evaluating the safety and efficacy of a plasmid (VM202) containing two human HGF isoforms administered by two intramuscular injections in patients with painful DSPN showed improvement in pain intensity and quality of life after 3 months.[31]

TGF-α is known to play multiple roles in the CNS, including the provision of neurotropic properties that protect neurons against various neurotoxic insults,

suggesting that therapeutic modulation of TGF-α regulation could afford neuroprotection.[32]

TGF-ß1 is expressed by Schwann cells, which is both sufficient and necessary for mediating the synapse-promoting effects of Schwann cells at the developing neuromuscular junction, strengthening the concept that glial cells contribute to synaptogenesis in both the PNS and CNS.[33]

NT-3 belongs to the neurotrophin family of trophic factors, best known for their effects in promoting neuronal survival. In contrast to our data, one small study showed higher serum NT-3 levels in patients with type 2 diabetes than controls,[34] but neurological phenotyping was not performed.

Receptors

TNFRSF5 (CD40), a member of the TNF receptor superfamily, is a major regulator of dendrite growth and elaboration in the developing brain.[35]

DNER serves an important role in the developing CNS and also modulates the length, polarity, and synaptogenesis of spiral ganglion neurons in the inner ear via the Notch signaling pathway.[36]

Miscellaneous

AXIN1 is a scaffold protein that regulates neuronal differentiation and morphogenesis in vitro. Recent studies suggest an emerging role of AXIN1 in gene expression and cytoskeletal regulation during neurogenesis, neuronal polarization, and axon formation.[37]

MMPs are a family of secreted endopeptidases expressed by neurons and glia. Regulated MMP activity contributes to physiological synaptic plasticity, while dysregulated activity can stimulate injury. Overexpression of MMP-1 in vivo increases dendritic complexity and induces biochemical and behavioral endpoints.[38]

Collectively, these largely experimental studies are in line with the concept that impaired neuroprotection, axon myelination, and nerve regeneration may underlie the systemic depletion of multiple markers of neuroinflammation and growth factors observed herein. This was supported by concordant associations, that is, higher levels of TWEAK, LIGHT, CXCL1, DNER, and MMP1 were associated with better nerve conduction and QST parameters, whereas higher CCL20 levels were associated with QST worsening.

The strengths of this study are the relative large sample size, comprehensive multimarker assessment including representative cytokines, chemokines, growth factors, receptors, and multiple miscellaneous markers as well as stringent adjustment in particular for multiple testing using the Bonferroni correction method. Nonetheless, the present study has some limitations. First, the cross-sectional study design does not provide any insights into the predictive value of these markers on the progression of DSPN which will be determined in a prospective 5-year follow-up within the PROPANE study. Second, due to the different study designs the criteria to diagnose or exclude DSPN differed between the two study cohorts. While the Toronto criteria were used to diagnose DSPN in the PROPANE study,[18] the MNSI examination part[20] was used to exclude DSPN in the KORA F4 study. However, while the Toronto criteria are generally accepted, we are also confident that the widely used MNSI is an appropriate tool to exclude clinically relevant DSPN, although the presence of subclinical DSPN could not be ruled out in the KORA F4 cohort. It is likely that uniform diagnostic criteria would have contributed to a more precise phenotyping as to the presence and absence of DSPN. Thus, a bias toward a less sensitive detection of DSPN introduced by using only clinical criteria in the KORA F4 cohort cannot be excluded.

CONCLUSIONS

This study demonstrated that the systemic levels of multiple biomarkers of neuroinflammation and growth factors that promote nerve regeneration are associated with polyneuropathy in patients with type 2 diabetes. Collectively, these data show that DSPN in type 2 diabetes is associated primarily with reduced rather than enhanced neuroinflammation and DSPN per se rather than painful DSPN in particular. Thus, when designing or implementing anti-inflammatory therapies for nerve injury involving myelinating cells, the various aspects of immune environment beneficial to myelin repair should be considered.[22] Given the importance of growth factors in normal nervous system development and maintenance, their therapeutic potential in regeneration of lost or damaged peripheral neurons should be further explored.[11] Lastly, although therapeutic agents targeting novel mechanisms of neuroimmune interaction are also promising in the context of neuropathic pain, potential differences between human and rodent immune cells should be considered when designing clinical trials of such agents.[4]

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