A Systemic Inflammatory Signature Reflecting Cross Talk Between Innate and Adaptive Immunity Is Associated With Incident Polyneuropathy: KORA F4/FF4 Study

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Prospective analyses of biomarkers of inflammation and distal sensorimotor polyneuropathy (DSPN) are scarce and limited to innate immunity. We therefore aimed to assess associations between biomarkers reflecting multiple aspects of immune activation and DSPN. The study was based on 127 case subjects with incident DSPN and 386 noncase subjects from the population-based Cooperative Health Research in the Region of Augsburg (KORA) F4/FF4 cohort (follow-up 6.5 years). Proximity extension assay technology was used to measure serum levels of biomarkers of inflammation. Of 71 biomarkers assessed, 26 were associated with incident DSPN. After adjustment for multiple testing, higher levels of six biomarkers remained related to incident DSPN. Three of these proteins (MCP-3/CCL7, MIG/CXCL9, IP-10/CXCL10) were chemokines, and the other three (DNER, CD40, TNFRSF9) were soluble forms of transmembrane receptors. The chemokines had neurotoxic effects on neuroblastoma cells in vitro. Addition of all six biomarkers improved the C statistic of a clinical risk model from 0.748 to 0.783 (P = 0.011). Pathway analyses indicated that multiple cell types from innate and adaptive immunity are involved in the development of DSPN. We thus identified novel associations between biomarkers of inflammation and incident DSPN pointing to a complex cross talk between innate and adaptive immunity in the pathogenesis of the disease.

Several lines of evidence have linked inflammatory processes to the development of distal sensorimotor polyneuropathy (DSPN). An activation of the immune system contributes to diabetic neuropathy, and inhibiting inflammatory pathways ameliorates the condition in different rodent models (1–5). Preclinical findings relating to an activation of the innate immunity are corroborated by cross-sectional studies in humans that have reported higher systemic levels of acute-phase proteins, proinflammatory cytokines, and soluble adhesion molecules in individuals with DSPN or neuropathic symptoms and deficits compared with individuals without DSPN (6–14).

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However, to characterize biomarkers of subclinical inflammation as risk factors of DSPN, prospective studies are required due to the potential of reverse causality (i.e., DSPN affecting inflammatory processes rather than vice versa), as recently highlighted for cardiovascular epidemiological research (15). The Cooperative Health Research in the Region of Augsburg (KORA) F4/FF4 cohort is the only cohort in which the relationship between biomarkers of inflammation and incident DSPN has been assessed so far (16). These analyses were based on eight biomarkers showing that cross-sectional and prospective associations between biomarkers of inflammation and DSPN overlap only partially (10,16,17). Importantly, we identified the proinflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α as novel biomarkers for incident DSPN and suggested that both cytokines may also improve the prediction of DSPN beyond established risk factors (16).

A crucial limitation of the aforementioned human studies—in addition to the mostly cross-sectional design—is the selection of biomarkers such as IL-6 and TNF-α reflecting only the activation state of innate immunity. Sural nerve biopsy specimens demonstrated a higher content of T cells in patients with peripheral neuropathy compared with healthy control subjects and thus suggested an involvement of the adaptive immunity (18,19). Supporting data from larger epidemiological studies are not available but would be relevant before inflammation can be addressed as a potential therapeutic target for DSPN (5,20,21).

Therefore, the aims of this study were *1*) to use a novel protein-based multimarker approach (22–25) for a detailed assessment of different aspects of immune activation in peripheral blood and to characterize inflammatory signatures that are associated with incident DSPN in a large sample of the general older population, *2*) to investigate the neurotoxic potential of biomarkers related to incident DSPN in an in vitro assay using human neuroblastoma cells, *3*) to assess their predictive value for incident DSPN, and *4*) to identify pathways and upstream regulators of differentially regulated biomarkers to gain insight into the mechanisms underlying DSPN.

RESEARCH DESIGN AND METHODS

Study Design and Participants

The study design has been described before (16,26,27). Briefly, this study is based on the KORA F4 (2006–2008) and the KORA FF4 studies (2013–2014), both follow-up examinations of the population-based KORA S4 study (1999–2001) conducted in Augsburg (Germany) and two adjacent counties. The assessment of anthropometric and metabolic variables, lifestyle factors, and glucose tolerance status using standard 75-g oral glucose tolerance tests was performed as previously reported (26,27).

These three examinations were performed in accordance with the Declaration of Helsinki, including written informed consent from all participants. The Bavarian Chamber of Physicians Ethics Board (Munich, Germany) approved the study.

Supplementary Fig. 1 describes the study sample for the current study. From 1,161 KORA F4 study participants aged 62–81 years, 1,048 individuals represented the baseline sample, which was used for correlation analysis between biomarkers of inflammation. Exclusions for the prospective study resulted in an analysis sample of 513 individuals without DSPN at baseline, including 127 incident case subjects and 386 noncase subjects. The mean follow-up time (±SD) was 6.46 ± 0.23 years. A drop-out analysis comparing participants and nonparticipants in KORA FF4 was published before (16).

Assessment of DSPN

The assessment of DSPN using the Michigan Neuropathy Screening Instrument (MNSI) has also been described before (16). The examination part of the MNSI included items for the appearance of feet, foot ulceration, ankle reflexes, and vibration perception threshold at the great toes. Age-dependent limits of normal vibration perception threshold were considered (28). The neuropathy assessment was extended by a bilateral examination of sensory perception using a 10-g monofilament (Neuropen) (16). This resulted in a total MNSI score ranging from 0 (all aspects normal) to a maximum of 10 points. Incident DSPN was defined using a cutoff at >3 points for the follow-up assessment in KORA FF4 in accordance with our previous study (16), thus satisfying the diagnostic criteria for possible DSPN according to the Toronto Diabetic Neuropathy Expert Group (29). Other potential causes of peripheral neuropathy, such as HIV infection and heavy alcohol consumption, were excluded, whereas data on hypothyroidism, vitamin deficiencies, and chronic inflammatory demyelinating polyneuropathy were not available.

Measurement of Biomarkers of Subclinical Inflammation

Biomarkers of subclinical inflammation were measured in fasting serum using the OLINK Inflammation multiplex immunoassay (OLINK Proteomics, Uppsala, Sweden). The OLINK Inflammation panel covers 92 protein biomarkers, including pro- and anti-inflammatory cytokines, chemokines, growth factors, and factors involved in acute inflammatory and immune responses, angiogenesis, fibrosis, and endothelial activation (Supplementary Table 1).

This immunoassay is based on the proximity extension assay (PEA) technology, which combines a detection step using oligonucleotide-labeled antibodies, a proximity-dependent DNA polymerization event, and a real-time quantitative PCR amplification. It has been used before to identify novel associations between biomarkers and cardiometabolic risk factors (22–25). The assay allows the relative quantification of analyte concentrations given as normalized protein expression (NPX) values (22,25), which are comparable in their distribution to $\log_2$-transformed protein concentrations. The normalization procedure is

required to convert threshold cycle values from the quantitative PCR to relative protein concentrations as previously described (22).

Supplementary Table 1 gives the full list of the 92 analytes, including assay ID, abbreviated, and full names, UniProt numbers, gene names, intraassay coefficient of variation (CV), interassay CV, limit of detection (LOD), and percentage of samples with values below the LOD. The calculation of intra- and interassay CVs was based on three control sera measured in duplicates on each plate (n = 16). We excluded 20 biomarkers that gave values below the LOD in ≥25% of all samples. For the remaining analytes, values below the LOD were substituted with the respective LOD. We excluded one biomarker because of an interassay CV >20%. The CVs for the 71 biomarkers in the final data set were intraassay CV: mean ± SD 3.6 ± 1.5%, median (25th; 75th percentiles) 3.1% (2.7; 4.3), range 2.1–10.0%; interassay CV: 8.4 ± 2.2%, median (25th; 75th percentiles) 8.4% (6.9; 9.6), range 4.6–16.6%.

Three of these 71 biomarkers (IL-6, IL-18, and TNF-α) had been measured before using ELISAs (16). Log_2-transformed absolute protein concentrations (ELISA) and NPX (PEA) were highly correlated for IL-6 (r = 0.85, $P <$ 0.001) and IL-18 (r = 0.88, $P < 0.001$) (Supplementary Fig. 2), and the correlation between both biomarkers was also similar when assessed by ELISA (r = 0.15, $P < 0.0001$) and PEA (r = 0.17, $P < 0.0001$). TNF-α levels could not be compared because they were not measurable in 95.9% of the serum samples using PEA.

In Vitro Neurotoxicity Assay

SH-SY5Y cells are a human neuroblastoma cell line commonly used as a model to assess neurotoxic effects (30,31). The cells (purchased from DSMZ, Braunschweig, Germany) were seeded onto 96-well plates at 10^4 cells/well (6 wells per experimental condition) in high glucose DMEM GlutaMax supplemented with 10% FBS, 25 mmol/L HEPES, 1 mmol/L pyruvate, 1,000 units/mL penicillin, 1,000 μg/mL streptomycin, and nonessential amino acids (all from Invitrogen/Life Technologies, Darmstadt, Germany), differentiated in the presence of 10 μmol/L retinoic acid (Sigma-Aldrich, Taufkirchen, Germany) for 6 days and then incubated for 24 h without or with 200 ng/mL recombinant CCL7, CXCL9, or CXCL10 (all from R&D Systems/BioTechne, Wiesbaden, Germany). Cell viability was assessed using the alamarBlue Cell Viability Assay (Invitrogen/Life Technologies) by measuring fluorescence according to the manufacturer's protocol.

Statistical Analysis

Correlations between biomarkers of subclinical inflammation were estimated using Pearson correlation coefficients (r) and corresponding P values. We also used a Gaussian graphical model (GGM) to illustrate the conditional dependence structure between all biomarkers of inflammation (32). A GGM is an undirected graph in which each edge represents the partial correlation between two variables. These partial correlations quantify the associations between two variables corrected for all remaining variables. To estimate the GGM, we used the R 1.2.13 package "GeneNet."

Associations between biomarkers of subclinical inflammation and incident DSPN were assessed using logistic regression models of increasing complexity (separate models for each biomarker) adjusting for the same set of confounders as in our previous analysis on inflammation and incident DSPN (16). Differences in the associations between individuals with normal glucose tolerance and prediabetes or diabetes were assessed using an interaction term to estimate potential effect modification by prediabetes/diabetes status. As a sensitivity analysis, associations between biomarkers of inflammation and increases in MNSI (dependent variable: MNSI at follow-up, model adjusted for baseline MNSI) were assessed using linear regression analysis.

The effect of biomarkers on cell viability was assessed using repeated-measures ANOVA and correction for multiple comparisons.

The improvement of prediction models for incident DSPN by biomarkers was estimated by comparing the previously described risk model containing all covariates from the fully adjusted logistic regression model with the same risk model additionally including the biomarkers of inflammation that were significantly associated with the outcome after correction for multiple testing, as previously described (16). Briefly, we calculated the C statistic and computed the 95% CI using 2,000 stratified bootstrap replicates. Differences in C statistics were tested using the bootstrap test implemented in the R pROC package. We also calculated the category-free net reclassification improvement (NRI) and the integrated discrimination improvement (IDI), as reported before (16).

The statistical analyses were conducted with R 3.3.3 (https://www.R-project.org/), SAS 9.4 (SAS Institute, Cary, NC), and GraphPad Prism 7.01 (GraphPad Software, La Jolla, CA) software. A P value of <0.05 was considered to indicate nominal statistical significance. We adjusted for multiple testing using the Benjamini-Hochberg procedure.

Ingenuity Pathway Analysis (IPA) software (QIAGEN, Hilden, Germany) was used to identify biological pathways that are enriched for the biomarkers of inflammation found associated with incident DSPN in the fully adjusted model. The significance of canonical pathways was assessed using P values adjusted for multiple testing using the Benjamini-Hochberg procedure with $P_{B\text{-}H}$ of <0.05 as the significance threshold. $P_{B\text{-}H}$ can be interpreted as the probability of association of the differentially regulated biomarkers from this data set with the respective pathway by random chance alone. In addition, the IPA Upstream Regulator analysis was performed to identify upstream transcriptional regulators that could explain the biomarker pattern found associated with incident DSPN. This analysis uses activation z scores to infer activation states of predicted regulators and overlap P values to estimate significant overlaps between differentially regulated genes

and genes regulated by a transcriptional regulator of interest. In this study, *z* scores were bias-corrected to take into account that the differential regulation of biomarkers in our data set is skewed toward positive associations with DSPN.

RESULTS

Study Population

Supplementary Table 2 presents the data for demographic, anthropometric, metabolic, and lifestyle factors of the 127 case subjects and 386 noncase subjects in the study population. The study sample is slightly smaller than the sample described in a previous study (16). Briefly, case subjects differed from noncase subjects at baseline by higher age, BMI, waist circumference, height, HbA_{1c}, and MNSI, and by their smoking and physical activity behaviors, whereas no differences were observed for sex, glucose tolerance status, hypertension, serum lipids, kidney function, alcohol intake, history of myocardial infarction, other neurological diseases, and hs-CRP.

As published before (16), excluded study participants (Supplementary Fig. 1) were older and overall less healthy; for example, they had higher BMI, HbA_{1c}, MNSI score, and cytokine levels as well as a lower estimated glomerular filtration rate than participants in both KORA F4 and FF4 with complete data for this analysis.

Serum levels of biomarkers of inflammation measured by PEA are presented in Supplementary Table 3. Of 71 biomarkers assessed, 35 showed higher serum baseline levels in incident case subjects (age and sex-adjusted $P < 0.05$), whereas no biomarker was significantly downregulated. Supplementary Fig. 3 gives a matrix of all pairwise correlations among biomarkers, which were almost all positive and mainly in the range of r between 0.1 and 0.4 (Supplementary Fig. 4).

Biomarkers of Subclinical Inflammation and Incident DSPN

In model 1, 35 of 71 biomarkers were associated with incident DSPN at $P < 0.05$ (Supplementary Table 4). Associations were slightly attenuated after adjustment, resulting in 26 nominally significant associations in model 2 (Table 1 and Supplementary Table 5). We found no evidence for differences in these associations between individuals with normal glucose tolerance and prediabetes/diabetes based on *P* values for interaction by prediabetes/diabetes status (data not shown).

After adjustment for multiple testing ($P_{B\text{-}H} < 0.05$), the numbers of biomarkers for incident DSPN were reduced to 26 in model 1 and to 6 in model 2 (Table 1 and Supplementary Table 4). In model 2, the odds ratios (95% CI) for monocyte chemotactic protein 3 (MCP-3, also known as [a.k.a.] CC-chemokine ligand 7 [CCL7]), monokine induced by γ-interferon (MIG, a.k.a. C-X-C motif chemokine 9 [CXCL9]), interferon-γ-induced protein 10 (IP-10, a.k.a. C-X-C motif chemokine 10 [CXCL10]), Delta and Notch-like epidermal growth factor-related receptor (DNER), CD40L receptor (CD40), and tumor necrosis factor receptor superfamily member 9 (TNFRSF9) ranged from 1.47 (1.17; 1.86) to 3.88 (1.56; 9.66) at $P_{B\text{-}H}$ between <0.001 and 0.047 (Table 1).

In a sensitivity analysis, five of these six biomarkers were also positively associated with an increase in the MNSI score, when used as a continuous variable in model 2 (β [95% CI]) ranging from 0.17 (0.04; 0.30) to 0.56 (0.29; 0.82) and $P_{B\text{-}H}$ between <0.001 and 0.047 for MCP-3/CCL7, MIG/CXCL9, IP-10/CXCL10, CD40, and TNFRSF9 ($P_{B\text{-}H}$ = 0.254 for DNER).

An analysis of the conditional dependence structure of all 71 biomarkers showed that MIG/CXCL9 and IP-10/CXCL10 were highly correlated, whereas MCP-3/CCL7, DNER, CD40, and TNFRSF9 were independent of each other (Supplementary Fig. 5).

Neurotoxic Effects of Chemokines Associated With Incident DSPN

MCP-3/CCL7, MIG/CXCL9, and IP-10/CXCL10 are secreted extracellular proteins. We determined whether these three chemokines also had direct neurotoxic effects by testing their effect on the viability of human neuroblastoma cells. Treatment with MCP-3/CCL7, MIG/CXCL9, and IP-10/CXCL10 reduced cell viability by 4.4% ($P < 0.05$), 11.3% ($P < 0.001$), and 14.2% ($P < 0.001$), respectively (Fig. 1).

Prediction of Incident DSPN by Biomarkers of Subclinical Inflammation

The addition of the six biomarkers associated with incident DSPN at $P_{B\text{-}H} < 0.05$ to a clinical risk model increased the *C* statistic from 0.748 to 0.783 (difference 0.034 [95% CI 0.008; 0.060], $P = 0.011$) (Table 2). The measures of category-free NRI and IDI were 0.352 (95% CI 0.154; 0.551, $P < 0.001$) and 0.049 (95% CI 0.027; 0.071, $P = 2 \times 10^{-5}$), respectively, thus indicating an improvement of reclassification and discrimination by the novel biomarkers (Table 2).

Canonical Pathways Enriched for Biomarkers of Incident DSPN and Potential Upstream Regulators

IPA revealed an enrichment of the 26 biomarkers that were associated with incident DSPN in the fully adjusted model in 14 canonical pathways ($P_{B\text{-}H} < 0.001$) (Table 3). As discussed below, these pathways point toward an involvement and cross talk between multiple cell types from innate immunity (#1, #7, #8, #13) and adaptive immunity (#2, #3, #4, #8, #10, #12, #14). This cross talk involves processes such as antigen presentation (#2, #5, #7, #10, #12, #13) and chemotaxis (#1, #3) and may also implicate autoimmune reactivities (#2, #4) and a hepatic component (#6, #11).

Pathway analysis identified TNF-α ($z = 2.505$, $P = 9 \times 10^{-12}$), IL-1β ($z = 2.167$, $P = 2 \times 10^{-16}$), and interferon (IFN)-γ ($z = 1.975$, $P = 2 \times 10^{-12}$) as potential positive upstream regulators that could explain the biomarker associations with incident DSPN in this study. In addition,

Table 1—Fully adjusted associations between biomarker levels and incident DSPN

Biomarker	Full name	OR (95% CI)	*P*	$P_{B\text{-}H}$
MCP-3	MCP 3 (CCL7)	1.84 (1.24; 2.73)	0.003	0.043
CDCP1	CUB domain-containing protein 1	1.50 (1.06; 2.12)	0.022	0.105
CD244	Natural killer cell receptor 2B4	2.01 (1.07; 3.80)	0.031	0.105
OPG	Osteoprotegerin	2.69 (1.26; 5.73)	0.010	0.078
uPA	Urokinase-type plasminogen activator	2.45 (1.21; 4.95)	0.013	0.084
CXCL9	C-X-C motif chemokine 9	1.69 (1.26; 2.28)	<0.001	<0.001
CD6	T cell surface glycoprotein CD6 isoform	1.86 (1.17; 2.97)	0.009	0.078
SLAMF1	Signaling lymphocytic activation molecule (SLAM)	1.79 (1.09; 2.94)	0.022	0.105
LIF-R	Leukemia inhibitory factor receptor	2.26 (1.04; 4.91)	0.039	0.107
CCL19	C-C motif chemokine 19	1.32 (1.03; 1.69)	0.029	0.105
IL-15RA	IL-15 receptor subunit α	3.06 (1.29; 7.23)	0.011	0.078
IL-10RB	IL-10 receptor subunit β	2.53 (1.14; 5.61)	0.023	0.105
HGF	Hepatocyte growth factor	1.99 (1.06; 3.75)	0.033	0.105
IL-12B	IL-12 subunit β	1.51 (1.03; 2.21)	0.035	0.105
CD5	T-cell surface glycoprotein CD5	1.95 (1.07; 3.56)	0.028	0.105
MIP-1α	Macrophage inflammatory protein-1 alpha (C-C motif chemokine 3 [CCL3])	1.57 (1.03; 2.04)	0.037	0.105
CXCL10	C-X-C motif chemokine 10 (IP-10)	1.47 (1.17; 1.86)	0.001	0.018
EIF4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1	1.62 (1.03; 2.54)	0.036	0.105
DNER	Delta and Notch-like epidermal growth factor-related receptor	3.88 (1.56; 9.66)	0.004	0.047
EN-RAGE	Protein S100-A12 (EN-RAGE)	1.43 (1.06; 1.92)	0.018	0.105
CD40	CD40L receptor	3.39 (1.64; 7.04)	0.001	0.018
FGF-19	Fibroblast growth factor 19	1.36 (1.03; 1.78)	0.029	0.105
TNFRSF9	TNF receptor superfamily member 9	2.46 (1.47; 4.13)	0.001	0.018
CCL20	C-C motif chemokine 20	1.34 (1.09; 1.65)	0.006	0.061
TNF-β	TNF-β (lymphotoxin-α/LT-α)	1.72 (1.03; 2.88)	0.037	0.105
CSF-1	Macrophage colony-stimulating factor 1	3.19 (1.13; 8.99)	0.028	0.105

All biomarkers with significant associations in the prospective analysis are shown. The full list of biomarkers and results is given in Supplementary Table 5. OR (95% CI), corresponding *P* values and Benjamini-Hochberg corrected *P* values ($P_{B\text{-}H}$) for incident DSPN are given for a 1-unit increase in NPX levels. Results are from the fully adjusted model 2: adjusted for baseline age (years), sex, waist circumference (cm), height (cm), hypertension (yes/no), total cholesterol (mg/dL), HbA_{1c} (%), alcohol intake (none/moderate/high), smoking (never/former/current), physical activity (active/inactive), use of lipid-lowering drugs (yes/no), use of nonsteroidal anti-inflammatory drugs (yes/no), estimated glomerular filtration rate (mL/min per 1.73 m^2), prevalent myocardial infarction and/or stroke (yes/no), and neurological conditions that might cause nerve damage (yes/no).

IL-10 was identified as negative upstream regulator ($z = -2.866$, $P = 2 \times 10^{-10}$). Of these four cytokines, data for TNF-α and IL-10 were available in the KORA F4/FF4 cohort. TNF-α showed positive correlations (r between 0.063 and 0.238, age and sex-adjusted $P < 0.05$) with 37 of 71 biomarkers and significant negative correlations (r between −0.136 and −0.070, age and sex-adjusted $P < 0.05$) with another 3 biomarkers, whereas IL-10 was positively correlated with 70 of 71 biomarkers (r between 0.073 and 0.437, age and sex-adjusted $P < 0.05$) (data not shown).

DISCUSSION

This study identified multiple biomarkers of subclinical inflammation that are independently associated with incident DSPN. Six biomarkers of inflammation remained associated with incident DSPN after correction for multiple testing and improved the predictive value of a risk model for DSPN comprising established risk factors. Three of these biomarkers, which are secreted chemokines, were shown to have direct neurotoxic effects. Pathway analyses suggested a complex cross talk between innate and adaptive immunity contributing to DSPN.

Inflammatory Signature for DSPN

Our study identified 26 biomarkers of inflammation that are associated with incident DSPN after adjustment for multiple confounders. The vast majority of these biomarkers have not been investigated in the context of DSPN before. In particular, prospective associations between biomarkers of inflammation and DSPN have previously been assessed only in the KORA F4/FF4 cohort

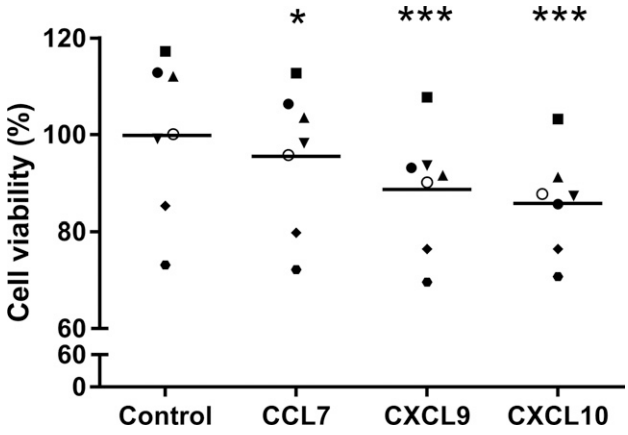

Figure 1—Neurotoxic effects of chemokines associated with incident DSPN. Cell viability of SH-SY5Y cells was assessed for untreated cells (control) and cells incubated for 24 h with 200 ng/mL CCL7, CXCL9, or CXCL10. The mean value of the alamarBlue Viability Assay (fluorescence units) from all control experiments was set to 100%, and values from experiments with chemokine treatment were normalized to this mean control value. Data are expressed as mean values from seven independent experiments ($n = 6$ replicates per treatment for each experiment), with black lines indicating the mean values for each treatment. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. control (repeated-measures ANOVA with correction for multiple testing by controlling the false discovery rate).

(16). With the current study, the number of biomarkers showing significant associations with incident DSPN increased from 2 (IL-6, TNF-α) to 28. We used the correction for multiple testing to narrow the number of biomarkers that could serve to improve the prediction of DSPN. In our previous study, we provided evidence that the addition of IL-6 and TNF-α to a clinical risk model improved model fit and reclassification but not the predictive value assessed by the *C* statistic (16). Not surprisingly, the panel of six novel biomarkers from this study—MCP-3/CCL7, MIG/CXCL9, IP-10/CXCL10, DNER, CD40, and TNFRSF9—resulted in higher gains in NRI and IDI (i.e., improved reclassification) and in a notable increase in C statistics by 0.034. To put this finding into perspective, the addition of the novel and clearly relevant cardiovascular risk marker troponin I improved a prognostic model for cardiovascular death in 10 population-based cohorts only by an increase of 0.007 in C statistics (33).

We are not aware of other cohorts that have reported an increase in C statistics by novel biomarkers beyond a clinical DSPN risk model. Our findings implicate that the incremental predictive value of biomarkers of inflammation cannot be estimated by hs-CRP, IL-6, and a handful of other inflammation-related factors alone as commonly measured in epidemiological studies. Furthermore, we hypothesize that the combination of biomarkers of inflammation with biomarkers reflecting other mechanisms implicated in the pathogenesis of DSPN (e.g., oxidative stress, lipid metabolism, glycation) could have the potential to lead to a clinically relevant test to identify individuals at high risk for DSPN in the general older population.

Novel Insights Into the Pathogenesis of DSPN

The list of biomarkers associated with incident DSPN and the subsequent identification of DSPN-related pathways and upstream regulators indicate that the role of the immune system in the pathogenesis of DSPN is obviously complex and based on a cross talk between most of its components. Our study had the strength that the prospective design allowed the identification of changes in biomarker levels before the onset of DSPN, but the limitation to one time point precluded the modeling of biomarker trajectories preceding the onset of clinical DSPN and a more precise assessment of systemic inflammation.

Starting with MCP-3/CCL7, MIG/CXCL9, IP-10/CXCL10, DNER, CD40, and TNFRSF9 that showed the strongest associations with DSPN, it is striking that the first three biomarkers are secreted proteins with chemotactic activities (chemokines) and that three further chemokines (CCL19, CCL20, and MIP-1α/CCL3) showed nominally significant associations with incident DSPN. In contrast, the latter three are soluble forms of transmembrane proteins mediating contact-based cell-to-cell communication.

Our in vitro experiments revealed direct neurotoxic effects of MCP-3/CCL7, MIG/CXCL9, and IP-10/CXCL10. These data extend previous studies using mouse models that suggested that chemokines, including CXCL9 and CXCL10, may contribute to diabetic neuropathic pain (2,4). In addition, increased expression of chemokines was found in nerve biopsy samples from humans and rodents with peripheral neuropathies of different etiologies (1,34,35). CXCL9 and CXCL10 are induced by the T-cell cytokine IFN-γ, and IFN-γ deficiency has been shown to completely prevent autoimmune peripheral neuropathy in NOD mice with partial loss of autoimmune regulator (Aire) function (36). Collectively, our data and preclinical studies implicate chemokines in different manifestations of peripheral neuropathies. CXCL9 and CXCL10 attract cells expressing C-X-C motif chemokine receptor 3 (CXCR3; predominantly T cells, but also B and natural killer cells), whereas CCL7 acts on cells expressing C-C motif chemokine receptor 2 (CCR2; mainly monocytes). This may serve as first indication that different cell types of both innate and adaptive immunity may contribute to the development of DSPN. In addition to direct neurotoxic effects of chemokines, it is also possible that nerve-derived chemokines mediate the chemoattraction of immune cells toward stressed neuronal cells with neurotoxic consequences. Indeed, immunohistochemical studies demonstrated the presence of macrophages and T cells in sural nerve biopsy samples from patients with peripheral neuropathies (18,19).

DNER, CD40, and TNFRSF9, in contrast to the chemokines, are transmembrane proteins with soluble forms. These results are less straightforward to interpret, because whether higher circulating levels of these isoforms are caused by an upregulation of their expression or increased proteolytic shedding from cell membranes is not known.

DNER is expressed in neurons (37), acts as a Notch ligand (38), and has been identified as a susceptibility gene

Table 2—Improvement of a clinical risk model for DSPN by six biomarkers of subclinical inflammation associated with incident DSPN

	Clinical risk model*	Clinical risk model + MCP-3/CCL7, MIG/CXCL9, IP-10/CXCL10, DNER, CD40, and TNFRSF9
C statistic (95% CI)	0.748 (0.698; 0.799)	0.783 (0.735; 0.830)
ΔC statistic (95% CI)	Reference	0.034 (0.008; 0.060)
P	N/A	0.011
Category-free net reclassification index (95% CI)	Reference	0.352 (0.154; 0.551)
P	N/A	<0.001
Integrated discrimination index (95% CI)	Reference	0.049 (0.027; 0.071)
P	N/A	2×10^{-5}

N/A, not applicable. *The clinical risk model includes age, sex, waist circumference, height, hypertension, total cholesterol, HbA_{1c}, alcohol intake, smoking, physical activity, use of lipid-lowering drugs, use of nonsteroidal anti-inflammatory drugs, estimated glomerular filtration rate, prevalent myocardial infarction, and neurological conditions that might cause nerve damage (i.e., all covariates from the fully adjusted model, see Table 1). The same clinical risk model was used in a previous KORA F4/FF4 analysis that was based on a slightly larger study sample (16).

for type 2 diabetes (39). Given the crucial role of Notch signaling in neurogenesis, an association between DNER and DSPN appears biologically plausible.

CD40 (a.k.a. TNFRSF5) and TNFRSF9 are members of the TNF receptor superfamily expressed by cells of innate and adaptive immunity, and CD40 expression has also been shown for neurons. The interaction between both proteins and their ligands provides costimulatory signals to T cells and, therefore, also implicates activated T cells in the development of DSPN.

The simultaneous consideration of all upregulated biomarkers of inflammation using bioinformatic tools broadens the scope of single-biomarker analyses. Previous epidemiological studies implicated biomarkers derived from innate immune cells such as IL-6 and TNF-α in the development of DSPN (7,8,10,16), and this study identified multiple pathways with contributions from innate immunity (mainly monocytes and granulocytes). An important novel finding of this study is that half of the DSPN-related pathways involve cells from adaptive immunity, predominantly T cells. This is also underlined by an enrichment of pathways involving antigen presentation and chemotaxis, which reflect cross talk between cells from innate and adaptive immunity based on cell-to-cell contact and humoral factors, respectively, and with pathways involved in autoimmune diseases (rheumatoid arthritis, multiple sclerosis).

Table 3—IPA: canonical pathways enriched for biomarkers of incident DSPN

No.	Pathway	$P_{B\text{-}H}$	Biomarkers
1	Granulocyte adhesion and diapedesis	1.45E-07	CXCL10, CCL20, CCL3, CXCL9, CCL19, CCL7, TNFRSF11B
2	Altered T cell and B cell signaling in rheumatoid arthritis	3.02E-06	SLAMF1, CD40, IL12B, CSF1, LTA
3	Agranulocyte adhesion and diapedesis	3.02E-06	CXCL10, CCL20, CCL3, CXCL9, CCL19, CCL7
4	Pathogenesis of multiple sclerosis	3.24E-06	CXCL10, CCL3, CXCL9
5	T helper cell differentiation	3.47E-05	CD40, IL12B, IL10RB, TNFRSF11B
6	Hepatic fibrosis/hepatic stellate cell activation	3.98E-05	CD40, CSF1, HGF, CXCL9, TNFRSF11B
7	Cross talk between dendritic cells and natural killer cells	5.50E-05	IL15RA, CD40, IL12B, LTA
8	Communication between innate and adaptive immune cells	5.89E-05	CXCL10, CD40, IL12B, CCL3
9	Role of hypercytokinemia/hyperchemokinemia in the pathogenesis of influenza	2.00E-04	CXCL10, IL12B, CCL3
10	Th1 pathway	2.00E-04	CD40, IL12B, LTA, IL10RB
11	Hepatic cholestasis	3.55E-04	IL12B, LTA, FGF19, TNFRSF11B
12	Th1 and Th2 activation pathway	5.62E-04	CD40, IL12B, LTA, IL10RB
13	Dendritic cell maturation	6.03E-04	CD40, IL12B, LTA, TNFRSF11B
14	TREM1 signaling	7.08E-04	CD40, CCL3, CCL7

The table shows all canonical pathways ($P_{B\text{-}H} < 10^{-3}$) based on significant associations between biomarkers of inflammation and incident DSPN in the fully adjusted model (see also Table 1) and the biomarkers belonging to the respective pathway.

Collectively, these findings raise the question whether endogenous and/or exogenous antigens may play a role in the induction and maintenance of neuronal damage leading to manifest DSPN.

Finally, this study identified TNFα, IL-1β, and IFN-γ as potential positive and IL-10 as negative upstream regulators. The fact that we previously showed that TNF-α levels measured using a high-sensitivity ELISA are indeed related to incident DSPN supports our in silico approach. Interestingly enough, TNF-α, IL-1β, and IL-10 are mainly monocyte/macrophage-derived, whereas IFN-γ is predominantly secreted by T cells, thus adding another line of evidence for a contribution of both innate and adaptive immunity to DSPN. So far, only IL-1β has been successfully targeted in the context of cardiovascular events in a large randomized clinical trial (40), and whether IL-1β inhibition may also have beneficial effects in the prevention and/or therapy of DSPN remains to be seen.

Strengths and Limitations

This is the most comprehensive study to date to assess associations between biomarkers of inflammation and incident DSPN. Major strengths are the prospective design, the population-based sample, the sample size, and the detailed immunophenotyping. The use of pathway-based analyses enabled us to gain deeper insight into the complexity of immune activation preceding DSPN. The results of the in vitro study corroborated the epidemiological findings and pointed toward causal associations.

Possible limitations include our use of a clinical definition of DSPN that was not confirmed by nerve conduction studies in our cohort. Hypothyroidism, vitamin deficiencies, chronic inflammatory demyelinating polyneuropathy, or rare autoimmune diseases as potential other causes of peripheral neuropathy could not be excluded. Moreover, this study assessed systemic levels of biomarkers of subclinical inflammation but not local levels (e.g., in skin or nerve biopsy specimens), which would provide complementary insight into mechanisms underlying DSPN. It is conceivable that biomarker measurements, particularly in peripheral nerves, would result in the identification of additional predictors of DSPN due to a presumably stronger correlation between biomarker levels and nerve damage and to more profound insights into the pathomechanisms of the disease, but such examinations were not feasible in the present epidemiological setting.

We also have no data for serum levels of IL-1β and IFN-γ, because these are below the level of detection in a large proportion of such population-based cohorts. However, this does not preclude that these cytokines play major roles in age- and diabetes-related comorbidities due to their expression patterns in various tissues without substantial spillover of these proteins into the circulation. The age of our study population at baseline was relatively old, resulting in a higher loss to follow-up than is expected when studying younger samples. In addition, our data cannot be extrapolated to young adults or other ethnic groups.

Finally, we used an established human cell line to assess neurotoxicity (30,31). Primary human peripheral neurons would have been the ideal model system, but these cells would require nerve biopsies in adults and are not available for in vitro culture. Alternatively, human neural progenitor cells or rodent dorsal root ganglion neurons could have been used, but these have their own limitations such as nonadult origin or species difference. We used a supraphysiological concentration of the three chemokines for our neurotoxicity assay, based on the assumption that local levels at neuronal cell membranes most likely exceed systemic levels by at least one or two orders of magnitude due to chemokine release by both neurons and infiltrating leukocytes.

Conclusions

We found that multiple biomarkers of subclinical inflammation were associated with incident DSPN. After extensive adjustment and correction for multiple testing, six biomarkers of inflammation (MCP-3/CCL7, MIG/CXCL9, IP-10/CXCL10, DNER, CD40, and TNFRSF9) emerged as novel risk factors of incident DSPN. Addition of these biomarkers improved a clinical risk model for DSPN. The three chemokines also showed direct neurotoxic effects in vitro. Pathway analyses corroborate that not only cells of the innate immunity but also T cells and other adaptive immune cells may be involved in the pathogenesis of DSPN. Cytokines reflecting both the innate and adaptive arms of the immune system (TNF-α, IL-1β, IFN-γ, and IL-10) may explain the immune activation preceding DSPN as potential upstream regulators.

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interpreted data. All authors reviewed and edited the manuscript and approved of its submission. C.He. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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