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

Short running title: Inflammatory Signature for DSPN

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**Prospective analyses on biomarkers of inflammation and distal sensorimotor polyneuropathy (DSPN) are scarce and limited to innate immunity. Therefore, we aimed to assess associations between biomarkers reflecting multiple aspects of immune activation and DSPN. The study was based on 127 cases with incident DSPN and 386 non-cases from the population-based Cooperative Health Research in the Region of Augsburg (KORA) F4/FF4 cohort (follow-up 6.5 years). Serum levels of biomarkers of inflammation were measured using proximity extension assay technology. Twenty-six out of 71 biomarkers 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, while 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. Thus, we 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, which reported higher systemic levels of acute-phase proteins, proinflammatory cytokines and soluble adhesion molecules in individuals with DSPN or neuropathic symptoms and deficits compared to individuals without DSPN (6-14).

However, to characterise 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 KORA F4/FF4 cohort was 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 tumour 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 TNFα reflecting only the activation state of innate immunity. Sural nerve biopsies demonstrated a higher content of T cells in patients with peripheral neuropathy compared to healthy controls 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 potential therapeutic target for DSPN (5, 20, 21).

Therefore, the aims of this study were (i) 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 characterise inflammatory signatures that are associated with incident DSPN in a large sample of the general older population. We also aimed (ii) to investigate the neurotoxic potential of biomarkers related to incident DSPN in an *in vitro* assay using human neuroblastoma cells, (iii) to assess their predictive value for incident DSPN and (iv) 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 Cooperative Health Research in the Region of Augsburg (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 (OGTT) was performed as reported (26, 27).

All three examinations were carried out in accordance with the Declaration of Helsinki, including written informed consent from all participants. The study was approved by the ethics board of the Bavarian Chamber of Physicians (Munich, Germany).

Supplementary Fig. 1 describes the study sample for the present study. From 1161 KORA F4 study participants aged 62-81 years, 1048 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 cases and 386 non-cases. The mean follow-up time (±SD) was 6.46±0.23 years. A drop-out analysis comparing participants and non-participants in KORA FF4 was published before (16).

**Assessment of DSPN**

The assessment of DSPN using the 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 (VPT) at the great toes. Age-dependent limits of normal VPT 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, which was used as continuous variable for the cross-sectional analysis. Incident DSPN was defined using a cut-off 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 labelled antibodies, a proximity-dependent DNA polymerisation event and a real-time quantitative polymerase chain reaction (qPCR) 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 normalised protein expression (NPX) values (22, 25), which are comparable in their distribution to $\log_2$-transformed protein concentrations. The normalisation procedure is required to convert Ct values from the qPCR to relative protein concentrations as described (22).

Supplementary Table 1 gives the full list of the 92 analytes including assay ID, abbreviated and full names, UniProt numbers, gene names, intra-assay coefficient of variation (CV), inter-assay CV, limit of detection (LOD) and percentage of samples with values below the LOD. The calculation of intra- and inter-assay 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. Furthermore, we excluded one biomarker because of an inter-assay CV >20%. The CVs for the 71 biomarkers in the final dataset were as follows: intra-assay CV mean±SD 3.6±1.5%, median ($25^{th}$; $75^{th}$ percentiles) 3.1 (2.7; 4.3)%, range 2.1-10.0%; inter-assay CV: 8.4±2.2%, median ($25^{th}$; $75^{th}$ percentiles) 8.4 (6.9; 9.6)%, range 4.6-16.6%.

Three of these 71 biomarkers (IL-6, IL-18, TNFα) had been measured before using ELISAs (16). $\text{Log}_2$-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. S2), and also the correlation between both biomarkers was similar when assessed by ELISA ($r$=0.15, $P$<0.0001) and PEA ($r$=0.17, $P$<0.0001). TNFα levels could not be compared because they were not measurable in the majority (95.9%) of serum samples using PEA.

***In vitro* neurotoxicity assay**

SH-SY5Y cells are a human neuroblastoma cell line commonly used as model to assess neurotoxic effects (30, 31). The cells (purchased from DSZM, Braunschweig, Germany) were seeded onto 96-well plates at $10^4$ cells/well (6 wells per experimental condition) in high glucose Dulbecco's Modified Eagle Medium (DMEM) GlutaMax supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, 1 mM pyruvate, 1,000 U/mL penicillin, 1,000 µg/mL streptomycin, and non-essential amino acids (all from Invitrogen/Life Technologies, Darmstadt, Germany), differentiated in the presence of 10 µM retinoic acid (Sigma-Aldrich, Taufkirchen, Germany) for 6 days and then incubated for 24 h without or with 200 ng/ml of 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, Darmstadt, Germany) by measuring fluorescence according to the manufacturer's protocol.

**Statistical Analysis**

Correlations between biomarkers of subclinical inflammation were estimated using Pearson's correlation coefficients (*r*) and corresponding *P* values. Additionally, we 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 package "GeneNet" (Version 1.2.13).

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 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 impact 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 described (16). Briefly, we calculated the C-statistic and computed the 95% CI using 2000 stratified bootstrap replicates. Differences in C-statistics were tested using the bootstrap test implemented in R's pROC package. We also calculated the category-free net reclassification improvement (NRI) and the integrated discrimination improvement (IDI) as reported before (16).

The aforementioned statistical analyses were conducted with R version 3.3.3 (https://www.R-project.org/), SAS version 9.4 (SAS Institute Inc., Cary, NC) and GraphPad Prism version 7.01 (GraphPad Software, La Jolla, CA). A $P$ value <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 $P_{B\text{-}H}<0.05$ as significance threshold. $P_{B\text{-}H}$ can be interpreted as the probability of association of the differentially regulated biomarkers from this dataset with the

respective pathway by random chance alone. Additionally, 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 the fact that the differential regulation of biomarkers in our dataset is skewed towards positive associations with DSPN.

## RESULTS

### Study Population

Supplementary Table 2 presents the data for demographic, anthropometric, metabolic and lifestyle factors of the study population (*n*=127 cases, *n*=386 non-cases). The study sample is slightly smaller than the sample described in a previous study (16). Briefly, cases differed from non-cases at baseline by higher age, BMI, waist circumference, height, HbA1c and MNSI and by their smoking and physical activity behaviours, 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 high-sensitivity C-reactive protein.

As published before (16), excluded study participants (Supplementary Fig. 1) were older and overall less healthy, e.g. they had higher BMI, HbA1c, MNSI score and cytokine levels as well as lower eGFR 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. Thirty-five out of 71 biomarkers showed higher serum baseline levels

in incident cases (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. S4).

**Biomarkers of Subclinical Inflammation and Incident DSPN**

In model 1, 35 out 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, 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 6 in model 2 (Table 1, Supplementary Table S4). In model 2, the ORs (95% CI) for monocyte chemotactic protein 3 (MCP-3, also known as (a.k.a.) CC-chemokine ligand 7/CCL7), monokine induced by gamma interferon (MIG, a.k.a. C-X-C motif chemokine 9/CXCL9), interferon-gamma-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 tumour 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 ($\beta$ (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. S5).

**Neurotoxic Effects of Chemokines Associated with Incident DSPN**

MCP-3/CCL7, MIG/CXCL9 and IP-10/CXCL10 are secreted extracellular proteins. In order to test if these three chemokines also had direct neurotoxic effects, we tested their impact 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 6 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 (95% CI 0.034 (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, $P$=2x10$^{-5}$), 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**

Ingenuity pathway analysis 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 towards 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 TNFα (z=2.505, $P$=9x10$^{-12}$), IL-1β (z=2.167, $P$=2x10$^{-16}$) and IFNγ (z=1.975, $P$=2x10$^{-12}$) as potential positive upstream regulators that could explain the biomarker associations with incident DSPN in this study. In addition, IL-10 was identified as negative upstream regulator (z=-2.866, $P$=2x10$^{-10}$). Out of these four cytokines, data for TNFα and IL-10 were available in the KORA F4/FF4 cohort. TNFα showed positive correlations ($r$ between 0.063 and 0.238, age and sex-adjusted $P$<0.05) with 37 out 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 out 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 (16). With the present study, the number of biomarkers showing significant associations with incident DSPN increased from 2 (IL-6, TNFα) to 28. We used the correction for multiple testing to narrow down 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 TNFα to a clinical risk model improved model fit and reclassification, but not the predictive value assessed by C-statistic (16). Not surprisingly, the panel of 6 novel biomarkers from this study, i.e. MCP-3/CCL7, MIG/CXCL9, IP-10/CXCL10, DNER, CD40 and TNFRSF9, resulted in higher gains in NRI and IDI, i.e. improved reclassification, but also 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 over and above a clinical DSPN risk model. Our findings implicate that the incremental predictive value of biomarkers of inflammation cannot be estimated by hsCRP, IL-6 and a handful of other inflammation-related factors alone as commonly measured in epidemiological studies. Furthermore, we hypothesise 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 crosstalk 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 timepoint precluded the modelling 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, 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). Additionally, increased expression of chemokines was found in nerve biopsies from humans and rodents with peripheral neuropathies of different aetiologies (1, 34, 35). CXCL9 and CXCL10 are induced by the T-cell cytokine IFNγ, and IFNγ 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 NK cells), whereas CCL7 acts on C-C motif chemokine receptor 2 (CCR2)-expressing cells (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 towards stressed neuronal cells with

neurotoxic consequences. Indeed, immunohistochemical studies demonstrated the presence of macrophages and T-cells in sural nerve biopsies from patients with peripheral neuropathies (18, 19).

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

DNER is expressed in neurons (37), acts as Notch ligand (38) and has been identified as susceptibility gene for type 2 diabetes (39). Given the crucial role of Notch signalling 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. In addition, 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 TNFα 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 crosstalk 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). 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 IFNγ as potential positive and IL-10 as negative upstream regulators. The fact that we previously showed that TNFα levels measured using a high-sensitivity ELISA are indeed related to incident DSPN supports our *in silico* approach. Interestingly enough, TNFα, IL-1β and IL-10 are mainly monocyte/macrophage-derived, whereas IFNγ 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 randomised clinical trial (40), and it remains to be seen if IL-1β inhibition may also have beneficial effects in the prevention and/or therapy of DSPN.

**Strengths and Limitations**

This is the most comprehensive study 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 towards causal associations.

As to possible limitations, we used a clinical definition of DSPN which 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 biopsies, 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 IFNγ, 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 spill-over of these proteins into the circulation. The age of our study population at baseline was relatively high, resulting in a higher loss to follow-up than to be expected when studying younger samples. Additionally, 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 non-adult 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 (TNFα, IL-1β, IFNγ and IL-10) may explain the immune activation preceding DSPN as potential upstream regulators.

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**Duality of Interest.** The authors declare that there is no duality of interest associated with this manuscript.

**Author Contributions**. C.He. designed the study, contributed data, drafted the analysis plan, interpreted data and wrote the manuscript. J.M.K. drafted the analysis plan, performed the statistical analysis and contributed to data interpretation. M.C.-K. contributed data and contributed to the statistical analysis. A.S. and G.J.B. contributed data and contributed to data interpretation. W.R. and C.Hu. contributed and interpreted data. W.K., M.H. and C.M.

contributed data. J.K. contributed to the statistical analysis. A.P. and M.R. contributed data and contributed to data interpretation. B.T. and D.Z. designed the study, contributed to the analysis plan, contributed and 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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**Table 1 – Fully adjusted associations between biomarker levels and incident DSPN.**

| Biomarker | Full name | OR (95% CI) | *P* | $P_{B-H}$ |
|---|---|---|---|---|
| MCP-3 | Monocyte chemotactic protein 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 | Interleukin-15 receptor subunit alpha | **3.06 (1.29; 7.23)** | **0.011** | 0.078 |
| IL-10RB | Interleukin-10 receptor subunit beta | **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 | Interleukin-12 subunit beta | **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-1alpha (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 | Tumor necrosis factor 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 |
| TNFβ | Tumor necrosis factor-beta (lymphotoxin-alpha/LT-alpha) | **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 |

The table shows all biomarkers with significant associations in the prospective analysis. The full list of biomarkers and results is given in Supplementary Table 5.

OR (95% CI), corresponding *P* values (*P*) and Benjamini-Hochberg corrected *P* values ($P_{B-H}$) for incident DSPN are given for a 1-unit increase in normalised protein expression 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), HbA1c (%),

alcohol intake (none/moderate/high), smoking (never/ex/current), physical activity (active/inactive), use of lipid-lowering drugs (yes/no), use of non-steroidal anti-inflammatory drugs (NSAIDs) (yes/no), estimated glomerular filtration rate (eGFR; ml/min per $1.73m^2$), prevalent myocardial infarction and/or stroke (yes/no) and neurological conditions that might cause nerve damage (yes/no).

**Table 2 – Improvement of a clinical risk model for DSPN by 6 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) |
| **Δ C-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 | $2x10^{-5}$ |

* The clinical risk model includes age, sex, waist circumference, height, hypertension, total cholesterol, HbA1c, alcohol intake, smoking, physical activity, use of lipid-lowering drugs, use of non-steroidal 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).

**Table 3 – Ingenuity Pathway Analysis: canonical pathways enriched for biomarkers of incident DSPN**

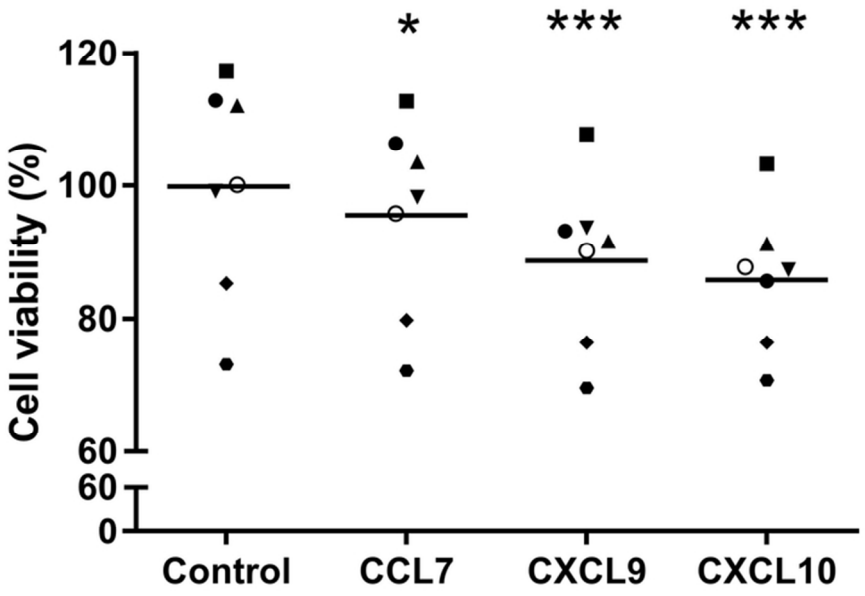
| # | Pathway | $P_{B\text{-}H}$ | Biomarkers |
|---|---|---|---|
| 1 | Granulocyte Adhesion and Diapedesis | 1.45E-07 | CXCL10, CCL20, CCL3, CXCL9, CCL19, CCL7, TNFRSF11B |
| 2 | Altered T Cell and B Cell Signaling in Rheumatoid Arthritis | 3.02E-06 | SLAMF1, CD40, IL12B, CSF1, LTA |
| 3 | Agranulocyte 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 | Crosstalk 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.

**Figure legend**

**Figure 1 - Neurotoxic effects of chemokines asociated with incident DSPN.**

Cell viability of SH-SY5Y cells was assessed for untreated cells (control) and cells incubated for 24 hours with 200 ng/ml of either 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 normalised 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 versus control (repeated-measures ANOVA with correction for multiple testing by controlling the false discovery rate).

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69x46mm (300 x 300 DPI)

**Supplementary Table 1 – Biomarkers in the OLINK Inflammation panel and assay characteristics**

| Assay ID | Biomarker | Full name | UniProt No | Gene symbol | Intra-assay CV (%) | Inter-assay CV (%) | LOD (NPX) | Percentage of samples below LOD |
|---|---|---|---|---|---|---|---|---|
| 101_IL-8 | IL-8 | Interleukin-8 | P10145 | *CXCL8* | 3.0 | 8.9 | 1.69 | 0.0 |
| 102_VEGF-A | VEGF-A | Vascular endothelial growth factor A | P15692 | *VEGFA* | 2.9 | 7.6 | 2.22 | 0.0 |
| 103_BDNF* | BDNF | Brain-derived neurotrophic factor | P23560 | *BDNF* | 2.8 | 8.1 | 0.09 | 26.9 |
| 105_MCP-3 | MCP-3 | Monocyte chemotactic protein 3 (CCL7) | P80098 | *CCL7* | 6.5 | 10.3 | 0.84 | 3.0 |
| 106_GDNF | GDNF | Glial cell line-derived neurotrophic factor | P39905 | *GDNF* | 8.5 | 8.4 | 0.70 | 9.5 |
| 107_CDCP1 | CDCP1 | CUB domain-containing protein 1 | Q9H5V8 | *CDCP1* | 3.9 | 9.6 | 0.11 | 0.0 |
| 108_CD244 | CD244 | Natural killer cell receptor 2B4 | Q9BZW8 | *CD244* | 2.2 | 8.9 | 0.66 | 0.0 |
| 109_IL-7 | IL-7 | Interleukin-7 | P13232 | *IL7* | 2.7 | 6.0 | 1.04 | 0.1 |
| 110_OPG | OPG | Osteoprotegerin | O00300 | *TNFRSF11B* | 2.3 | 5.0 | 0.85 | 0.0 |
| 111_LAP TGF-beta-1 | LAP TGFβ1 | Latency-associated peptide transforming growth factor beta-1 | P01137 | *TGFB1* | 10.0 | 16.6 | 0.61 | 0.1 |
| 112_uPA | uPA | Urokinase-type plasminogen activator | P00749 | *PLAU* | 2.5 | 4.6 | 0.81 | 0.0 |
| 113_IL-6 | IL-6 | Interleukin-6 | P05231 | *IL6* | 4.4 | 10.7 | 1.15 | 2.1 |
| 114_IL-17C | IL-17C | Interleukin-17C | Q9P0M4 | *IL17C* | 6.2 | 6.9 | 0.95 | 22.3 |
| 115_MCP-1 | MCP-1 | Monocyte chemotactic protein 1 (CCL2) | P13500 | *CCL2* | 2.9 | 6.0 | 1.25 | 0.0 |
| 116_IL-17A* | IL-17A | Interleukin-17A | Q16552 | *IL17A* | 4.9 | 9.3 | 0.74 | 33.0 |
| 117_CXCL11 | CXCL11 | C-X-C motif chemokine 11 | O14625 | *CXCL11* | 2.5 | 5.8 | 1.02 | 0.0 |
| 118_AXIN1 | Axin-1 | Axin-1 | O15169 | *AXIN1* | 4.5 | 8.7 | 0.95 | 10.1 |
| 120_TRAIL | TRAIL | TNF-related apoptosis-inducing ligand (TNFSF10) | P50591 | *TNFSF10* | 2.7 | 7.2 | 0.59 | 0.0 |
| 121_IL-20RA* | IL-20RA | Interleukin-20 receptor subunit alpha | Q9UHF4 | *IL20RA* | n/a | n/a | 0.61 | 93.0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 122_CXCL9 | CXCL9 | C-X-C motif chemokine 9 | Q07325 | *CXCL9* | 3.2 | 5.4 | 0.70 | 0.0 |
| 123_CST5 | CST5 | Cystatin D | P28325 | *CST5* | 2.4 | 6.6 | 0.97 | 0.0 |
| 124_IL-2RB* | IL-2RB | Interleukin-2 receptor subunit beta | P14784 | *IL2RB* | n/a | n/a | 1.09 | 95.4 |
| 125_IL-1 alpha* | IL-1α | Interleukin-1 alpha | P01583 | *IL1A* | n/a | n/a | 1.77 | 96.9 |
| 126_OSM | OSM | Oncostatin-M | P13725 | *OSM* | 2.3 | 6.9 | 1.55 | 0.4 |
| 127_IL-2* | IL-2 | Interleukin-2 | P60568 | *IL2* | n/a | n/a | 0.68 | 99.9 |
| 128_CXCL1 | CXCL1 | C-X-C motif chemokine 1 | P09341 | *CXCL1* | 2.5 | 5.8 | 1.73 | 0.0 |
| 129_TSLP* | TSLP | Thymic stromal lymphopoietin | Q969D9 | *TSLP* | n/a | n/a | 1.41 | 99.2 |
| 130_CCL4 | CCL4 | C-C motif chemokine 4 | P13236 | *CCL4* | 2.8 | 5.8 | 1.38 | 0.0 |
| 131_CD6 | CD6 | T cell surface glycoprotein CD6 isoform | Q8WWJ7 | *CD6* | 3.8 | 12.9 | 1.55 | 0.0 |
| 132_SCF | SCF | Stem cell factor (c-Kit-ligand) | P21583 | *KITLG* | 2.1 | 5.8 | 1.08 | 0.0 |
| 133_IL-18 | IL-18 | Interleukin-18 | Q14116 | *IL18* | 3.0 | 6.4 | 1.36 | 0.0 |
| 134_SLAMF1 | SLAMF1 | Signaling lymphocytic activation molecule (SLAM) | Q13291 | *SLAMF1* | 6.4 | 10.7 | 1.08 | 0.0 |
| 135_TGF-alpha | TGFα | Transforming growth factor alpha | P01135 | *TGFA* | 3.1 | 9.5 | -0.19 | 0.0 |
| 136_MCP-4 | MCP-4 | Monocyte chemotactic protein 4 (CCL13) | Q99616 | *CCL13* | 3.0 | 9.5 | 0.29 | 0.0 |
| 137_CCL11 | Eotaxin | Eotaxin (CCL11) | P51671 | *CCL11* | 2.8 | 6.9 | 1.43 | 0.0 |
| 138_TNFSF14 | TNFSF14 | Tumor necrosis factor ligand superfamily member 14 (LIGHT) | O43557 | *TNFSF14* | 2.8 | 7.8 | 1.30 | 0.0 |
| 139_FGF-23 | FGF-23 | Fibroblast growth factor 23 | Q9GZV9 | *FGF23* | 5.0 | 7.6 | 0.31 | 0.2 |
| 140_IL-10RA* | IL-10RA | Interleukin-10 receptor subunit alpha | Q13651 | *IL10RA* | 2.6 | 9.2 | 0.82 | 26.5 |
| 141_FGF-5 | FGF-5 | Fibroblast growth factor 5 | Q8NF90 | *FGF5* | 4.3 | 8.7 | 0.61 | 2.5 |
| 142_MMP-1 | MMP-1 | Matrix metalloproteinase-1 | P03956 | *MMP1* | 2.3 | 5.4 | 2.12 | 0.0 |
| 143_LIF-R | LIF-R | Leukemia inhibitory factor receptor | P42702 | *LIFR* | 3.4 | 10.1 | 1.20 | 0.0 |
| 144_FGF-21 | FGF-21 | Fibroblast growth factor 21 | Q9NSA1 | *FGF21* | 3.1 | 7.3 | 0.84 | 0.0 |
| 145_CCL19 | CCL19 | C-C motif chemokine 19 | Q99731 | *CCL19* | 2.9 | 7.0 | 0.90 | 0.0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 148_IL-15RA | IL-15RA | Interleukin-15 receptor subunit alpha | Q13261 | *IL15RA* | 5.6 | 10.5 | 0.15 | 2.4 |
| 149_IL-10RB | IL-10RB | Interleukin-10 receptor subunit beta | Q08334 | *IL10RB* | 3.1 | 10.4 | 1.17 | 0.0 |
| 150_IL-22 RA1* | IL-22RA1 | Interleukin-22 receptor subunit alpha-1 | Q8N6P7 | *IL22RA1* | n/a | n/a | 1.55 | 99.8 |
| 151_IL-18R1 | IL-18R1 | Interleukin-18 receptor 1 | Q13478 | *IL18R1* | 3.0 | 7.6 | 0.85 | 0.0 |
| 152_PD-L1 | PD-L1 | Programmed cell death 1 ligand 1 | Q9NZQ7 | *CD274* | 4.8 | 9.6 | 1.87 | 0.0 |
| 153_Beta-NGF | Beta-NGF | Beta-nerve growth factor | P01138 | *NGF* | 3.6 | 7.6 | 1.02 | 0.0 |
| 154_CXCL5 | CXCL5 | C-X-C motif chemokine 5 | P42830 | *CXCL5* | 2.7 | 5.8 | 1.57 | 0.0 |
| 155_TRANCE | TRANCE | TNF-related activation-induced cytokine (TRANCE, TNFSF11, RANKL, OPGL) | O14788 | *TNFSF11* | 4.6 | 8.8 | 1.32 | 0.0 |
| 156_HGF | HGF | Hepatocyte growth factor | P14210 | *HGF* | 2.5 | 7.5 | 1.11 | 0.0 |
| 157_IL-12B | IL-12B | Interleukin-12 subunit beta | P29460 | *IL12B* | 3.2 | 6.9 | 0.69 | 0.0 |
| 158_IL-24* | IL-24 | Interleukin-24 | Q13007 | *IL24* | n/a | n/a | 1.36 | 93.5 |
| 159_IL-13* | IL-13 | Interleukin-13 | P35225 | *IL13* | n/a | n/a | 1.14 | 94.9 |
| 160_ARTN* | Artemin | Artemin | Q5T4W7 | *ARTN* | n/a | n/a | 0.72 | 95.3 |
| 161_MMP-10 | MMP-10 | Matrix metalloproteinase-10 (SL-2) | P09238 | *MMP10* | 2.7 | 8.8 | 1.13 | 0.0 |
| 162_IL-10 | IL-10 | Interleukin-10 | P22301 | *IL10* | 5.3 | 10.4 | 1.04 | 0.0 |
| 163_TNF* | TNFα | Tumor necrosis factor-alpha | P01375 | *TNF* | n/a | n/a | 1.08 | 95.9 |
| 164_CCL23 | CCL23 | C-C motif chemokine 23 | P55773 | *CCL23* | 2.9 | 6.1 | 0.90 | 0.0 |
| 165_CD5 | CD5 | T-cell surface glycoprotein CD5 | P06127 | *CD5* | 3.1 | 9.3 | 1.22 | 0.0 |
| 166_MIP-1 alpha | MIP-1α | Macrophage inflammatory protein-1alpha (C-C motif chemokine 3/CCL3) | P10147 | *CCL3* | 3.3 | 9.0 | 1.62 | 0.0 |
| 167_Flt3L | Flt3L | Fms-related tyrosine kinase 3 ligand | P49771 | *FLT3LG* | 2.7 | 8.1 | 1.28 | 0.0 |
| 168_CXCL6 | CXCL6 | C-X-C motif chemokine 6 | P80162 | *CXCL6* | 2.4 | 8.9 | 1.26 | 0.0 |
| 169_CXCL10 | CXCL10 | C-X-C motif chemokine 10 (IP-10) | P02778 | *CXCL10* | 3.1 | 6.0 | 1.53 | 0.0 |
| 170_4E-BP1 | EIF4EBP1 | Eukaryotic translation initiation factor 4E-binding protein 1 | Q13541 | *EIF4EBP1* | 3.8 | 8.6 | 0.66 | 0.0 |
| 171_IL-20* | IL-20 | Interleukin-20 | Q9NYY1 | *IL20* | n/a | n/a | 0.96 | 95.4 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 172_SIRT2 | SIRT2 | SIR2-like protein 2 | Q8IXJ6 | *SIRT2* | 5.4 | 9.7 | 0.87 | 0.0 |
| 173_CCL28 | CCL28 | C-C motif chemokine 28 | Q9NRJ3 | *CCL28* | 4.1 | 14.6 | 0.73 | 1.0 |
| 174_DNER | DNER | Delta and Notch-like epidermal growth factor-related receptor | Q8NFT8 | *DNER* | 2.3 | 8.0 | 0.71 | 0.0 |
| 175_EN-RAGE | EN-RAGE | Protein S100-A12 (EN-RAGE) | P80511 | *S100A12* | 4.9 | 11.0 | 0.78 | 0.0 |
| 176_CD40 | CD40 | CD40L receptor | P25942 | *CD40* | 2.5 | 7.8 | 0.81 | 0.0 |
| 177_IL-33* | IL-33 | Interleukin-33 | O95760 | *IL33* | n/a | n/a | 0.84 | 98.0 |
| 178_IFN-gamma* | IFNγ | Interferon-gamma | P01579 | *IFNG* | n/a | n/a | 0.94 | 98.5 |
| 179_FGF-19 | FGF-19 | Fibroblast growth factor 19 | O95750 | *FGF19* | 2.9 | 8.4 | 0.68 | 0.0 |
| 180_IL-4* | IL-4 | Interleukin-4 | P05112 | *IL4* | n/a | n/a | 0.81 | 91.9 |
| 181_LIF* | LIF | Leukemia inhibitory factor | P15018 | *LIF* | n/a | n/a | 1.28 | 95.0 |
| 182_NRTN* | Neurturin | Neurturin | Q99748 | *NRTN* | n/a | n/a | 1.04 | 97.2 |
| 183_MCP-2 | MCP-2 | Monocyte chemotactic protein 2 (MCP-2, CCL8) | P80075 | *CCL8* | 2.7 | 8.0 | 1.27 | 0.0 |
| 184_CASP-8** | Caspase-8 | Caspase-8 | Q14790 | *CASP8* | 7.1 | 35.9 | 1.46 | 0.7 |
| 185_CCL25 | CCL25 | C-C motif chemokine 25 | O15444 | *CCL25* | 3.2 | 7.6 | 0.70 | 0.0 |
| 186_CX3CL1 | CX3CL1 | Fractalkine | P78423 | *CX3CL1* | 3.7 | 10.8 | 1.31 | 0.0 |
| 187_TNFRSF9 | TNFRSF9 | Tumor necrosis factor receptor superfamily member 9 | Q07011 | *TNFRSF9* | 3.0 | 9.7 | 1.31 | 0.0 |
| 188_NT-3 | NT-3 | Neurotrophin-3 | P20783 | *NTF3* | 4.8 | 10.5 | 0.33 | 0.7 |
| 189_TWEAK | TWEAK | Tumor necrosis factor (Ligand) superfamily, member 12 (TWEAK) | O43508 | *TNFSF12* | 2.3 | 6.2 | 1.07 | 0.0 |
| 190_CCL20 | CCL20 | C-C motif chemokine 20 | P78556 | *CCL20* | 3.2 | 7.1 | 0.89 | 0.0 |
| 191_ST1A1 | ST1A1 | Sulfotransferase 1A1 | P50225 | *SULT1A1* | 6.4 | 14.0 | 0.69 | 4.5 |
| 192_STAMPB | STAMBP | STAM-binding protein | O95630 | *STAMBP* | 3.6 | 9.2 | 1.16 | 0.0 |
| 193_IL-5* | IL-5 | Interleukin-5 | P05113 | *IL5* | n/a | n/a | 1.21 | 72.6 |
| 194_ADA | ADA | Adenosine deaminase | P00813 | *ADA* | 3.7 | 8.8 | 0.99 | 0.0 |
| 195_TNFB | TNFβ | Tumor necrosis factor-beta (lymphotoxin- | P01374 | *LTA* | 4.4 | 8.8 | 1.08 | 0.0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | alpha/LT-alpha) | | | | | | |
| 196_CSF-1 | CSF-1 | Macrophage colony-stimulating factor 1 | P09603 | *CSF1* | 2.2 | 8.8 | 0.93 | 0.0 |

The calculation of inter- and intra-assay CVs and of the percentage of samples below the LOD was based on all measurements within KORA F4, i.e. including the set of 1048 samples representing the study baseline.

LOD, limit of detection; n/a, not applicable (NPX of control measurements below LOD); NPX, normalised protein expression values.

*Excluded from analysis because missing data for >25%.

**Excluded from analysis because inter-assay CV >20%.

**Supplementary Table 2 - Baseline characteristics of the KORA F4 study population**

| Variable | No incident DSPN | Incident DSPN | *P* |
|---|---|---|---|
| *n* | 386 | 127 | |
| Age (years) | 67.9 ± 4.6 | 70.2 ± 5 | <0.001 |
| Sex (% male / female) | 49.2 / 50.8 | 55.9 / 44.1 | 0.169 |
| BMI (kg/m$^2$) | 27.6 ± 3.9 | 29.2 ± 4 | <0.001 |
| Waist circumference (cm) | 94.7 ± 11.3 | 100.1 ± 11.4 | <0.001 |
| Height (cm) | 166 ± 9 | 167 ± 9 | 0.016 |
| HbA1c (%) | 5.66 ± 0.48 | 5.83 ± 0.69 | 0.014 |
| HbA1c (mmol/mol) | 39 ± 6 | 40 ± 7 | 0.014 |
| Glucose tolerance status (NGT / IFG / IGT / IFG&IGT / ndT2D / kT2D) (%) | 45.9 / 21.2 / 9.3 / 9.6 / 5.4 / 8.5 | 38.6 / 20.5 / 9.4 / 11.0 / 4.7 / 15.7 | 0.490 |
| Hypertension (%) * | 56.5 | 65.4 | 0.452 |
| Total cholesterol (mmol/l) † | 6.06 ± 1.01 | 5.82 ± 1.12 | 0.094 |
| Fasting triglycerides (mmol/l) † | 1.27 (0.97; 1.77) | 1.29 (1.05; 1.67) | 0.745 |
| Use of lipid-lowering drugs (%) | 23.1 | 26.8 | 0.643 |
| eGFR (ml/min per 1.73m$^2$) ‡ | 80.2 ± 13.1 | 76.9 ± 14.3 | 0.498 |
| Smoking (never / former / current) (%) | 51.6 / 42.7 / 5.7 | 54.3 / 33.9 / 11.8 | 0.002 |
| Alcohol intake (none / moderate / high (%) § | 31.1 / 59.8 / 9.1 | 30.7 / 55.1 / 14.2 | 0.078 |
| Physically active (%) ‖ | 62.2 | 41.7 | <0.001 |
| Myocardial infarction (%) | 5.2 | 7.1 | 0.850 |
| Neurological conditions that might cause nerve damage (%) | 15.1 | 20.5 | 0.078 |
| Use of nonsteroidal anti-inflammatory drugs (%) ¶ | 1.0 | 2.4 | 0.375 |
| MNSI | 2.0 (0.5; 2.0) | 2.0 (2.0; 2.5) | <0.001 |
| hsCRP (mg/l) | 1.30 (0.68; 2.48) | 1.59 (0.74; 3.42) | 0.144 |

Data are given as mean ± SD, median (25$^{th}$; 75$^{th}$ percentiles) or percentages. The *P* values for the comparison of cases and non-cases were derived from logistic regression analysis (likelihood ratio tests comparing models with the respective variable, age and sex as independent variables to models with age and sex only). All analyses were adjusted for age

and sex except associations with age (sex-adjusted only) or sex (age-adjusted only). hsCRP was $log_2$-transformed prior to logistic regression.

BMI, body mass index; eGFR, estimated glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; kT2D, known type 2 diabetes; MNSI, Michigan Neuropathy Screening Instrument; ndT2D, newly-diagnosed type 2 diabetes; NGT, normal glucose tolerance.

* Blood pressure of 140/90 mmHg or higher, or antihypertensive medication given that the subjects were aware of being hypertensive.

† Individuals using lipid-lowering drugs excluded.

‡ The eGFR was calculated using the chronic kidney disease epidemiology (CKD-EPI) creatinine equation.

§ Classified as none (0 g/day), moderate (≥0 to <20 g/day for women, ≥0 to <40 g/day for men) or high (≥20 g/day for women, ≥40 g/day for men).

‖ Study participants were classified as physically active if they participated in sports in summer and in winter and if they reported ≥1 h sports/week in at least one of the seasons.

¶ Nonsteroidal anti-inflammatory drugs except acetylsalicylic acid used as platelet aggregation inhibitor.

The incidence study sample is a subgroup of a slightly larger sample (*n*=133 cases, *n*=397 non-cases) that was described in a previous KORA publication (1).

**Supplementary Table 3 – Normalised protein expression levels for biomarkers of subclinical inflammation at baseline for the KORA F4 study population**

| Variable | No incident DSPN | Incident DSPN | *P* |
|---|---|---|---|
| *n* | 386 | 127 | |
| IL-8 | 6.60 ± 0.52 | 6.81 ± 0.62 | 0.002 |
| VEGF-A | 10.84 ± 0.50 | 10.9 ± 0.47 | 0.497 |
| MCP-3 | 1.83 ± 0.50 | 2.09 ± 0.64 | <0.001 |
| GDNF | 1.10 ± 0.31 | 1.10 ± 0.32 | 0.818 |
| CDCP1 | 3.21 ± 0.65 | 3.51 ± 0.62 | 0.002 |
| CD244 | 5.52 ± 0.33 | 5.61 ± 0.39 | 0.009 |
| IL-7 | 4.63 ± 0.49 | 4.68 ± 0.46 | 0.507 |
| OPG | 10.13 ± 0.31 | 10.24 ± 0.32 | 0.011 |
| LAP TGFβ1 | 7.91 ± 0.35 | 7.93 ± 0.34 | 0.633 |
| uPA | 9.87 ± 0.29 | 9.95 ± 0.41 | 0.022 |
| IL-6 | 2.21 ± 0.70 | 2.44 ± 0.72 | 0.012 |
| IL-17C | 1.36 ± 0.51 | 1.50 ± 0.61 | 0.066 |
| MCP-1 | 11.08 ± 0.43 | 11.19 ± 0.45 | 0.043 |
| CXCL11 | 7.82 ± 0.70 | 8.03 ± 0.82 | 0.023 |
| Axin-1 | 1.54 ± 0.47 | 1.56 ± 0.48 | 0.592 |
| TRAIL | 8.10 ± 0.28 | 8.12 ± 0.29 | 0.303 |
| CXCL9 | 7.27 ± 0.75 | 7.68 ± 0.89 | <0.001 |
| CST5 | 6.43 ± 0.46 | 6.41 ± 0.48 | 0.257 |
| OSM | 4.82 ± 0.64 | 5.01 ± 0.64 | 0.005 |
| CXCL1 | 9.40 ± 0.49 | 9.43 ± 0.45 | 0.342 |
| CCL4 | 8.23 ± 0.59 | 8.28 ± 0.61 | 0.353 |
| CD6 | 4.56 ± 0.46 | 4.70 ± 0.53 | 0.002 |
| SCF | 9.90 ± 0.36 | 9.90 ± 0.37 | 0.768 |
| IL-18 | 8.67 ± 0.61 | 8.76 ± 0.51 | 0.267 |
| SLAMF1 | 2.41 ± 0.45 | 2.58 ± 0.55 | 0.001 |
| TGFα | 4.48 ± 0.52 | 4.61 ± 0.53 | 0.016 |
| MCP-4 | 3.98 ± 0.55 | 4.13 ± 0.60 | 0.048 |
| Eotaxin | 8.67 ± 0.42 | 8.72 ± 0.43 | 0.527 |
| TNFSF14 | 5.56 ± 0.52 | 5.62 ± 0.60 | 0.156 |
| FGF-23 | 1.36 ± 0.48 | 1.48 ± 0.57 | 0.051 |
| FGF-5 | 1.24 ± 0.29 | 1.29 ± 0.29 | 0.235 |
| MMP-1 | 14.39 ± 0.80 | 14.45 ± 0.61 | 0.460 |
| LIF-R | 4.57 ± 0.28 | 4.65 ± 0.29 | 0.006 |

| Variable | No incident DSPN | Incident DSPN | *P* |
|---|---|---|---|
| FGF-21 | 5.76 ± 1.01 | 5.89 ± 1.00 | 0.189 |
| CCL19 | 9.76 ± 0.91 | 10.05 ± 0.88 | 0.003 |
| IL-15RA | 0.71 ± 0.26 | 0.81 ± 0.28 | 0.002 |
| IL-10RB | 7.17 ± 0.28 | 7.28 ± 0.33 | 0.003 |
| IL-18R1 | 7.40 ± 0.40 | 7.51 ± 0.40 | 0.007 |
| PD-L1 | 4.25 ± 0.48 | 4.32 ± 0.40 | 0.315 |
| Beta-NGF | 1.82 ± 0.31 | 1.91 ± 0.35 | 0.024 |
| CXCL5 | 11.52 ± 0.72 | 11.62 ± 0.75 | 0.051 |
| TRANCE | 4.62 ± 0.60 | 4.68 ± 0.59 | 0.207 |
| HGF | 8.51 ± 0.36 | 8.68 ± 0.40 | <0.001 |
| IL-12B | 5.34 ± 0.63 | 5.51 ± 0.65 | 0.011 |
| MMP-10 | 6.35 ± 0.58 | 6.51 ± 0.61 | 0.021 |
| IL-10 | 2.42 ± 0.42 | 2.48 ± 0.41 | 0.163 |
| CCL23 | 9.95 ± 0.46 | 10.02 ± 0.52 | 0.281 |
| CD5 | 5.29 ± 0.38 | 5.42 ± 0.39 | 0.003 |
| MIP-1α | 4.42 ± 0.49 | 4.62 ± 0.67 | 0.001 |
| Flt3L | 8.89 ± 0.40 | 8.98 ± 0.45 | 0.041 |
| CXCL6 | 8.65 ± 0.54 | 8.74 ± 0.51 | 0.074 |
| CXCL10 | 9.32 ± 0.84 | 9.74 ± 1.09 | <0.001 |
| EIF4EBP1 | 6.79 ± 0.52 | 7.01 ± 0.58 | 0.002 |
| SIRT2 | 2.25 ± 0.46 | 2.33 ± 0.52 | 0.094 |
| CCL28 | 1.52 ± 0.38 | 1.56 ± 0.39 | 0.463 |
| DNER | 8.32 ± 0.26 | 8.34 ± 0.26 | 0.084 |
| EN-RAGE | 4.89 ± 0.75 | 5.14 ± 0.79 | 0.002 |
| CD40 | 10.19 ± 0.32 | 10.31 ± 0.34 | 0.001 |
| FGF-19 | 7.74 ± 0.84 | 7.88 ± 0.87 | 0.074 |
| MCP-2 | 9.52 ± 0.74 | 9.61 ± 0.72 | 0.327 |
| CCL25 | 6.60 ± 0.55 | 6.69 ± 0.53 | 0.365 |
| CX3CL1 | 6.18 ± 0.38 | 6.21 ± 0.38 | 0.574 |
| TNFRSF9 | 6.19 ± 0.44 | 6.41 ± 0.47 | <0.001 |
| NT-3 | 1.01 ± 0.37 | 1.02 ± 0.30 | 0.605 |
| TWEAK | 9.40 ± 0.29 | 9.37 ± 0.31 | 0.502 |
| CCL20 | 4.74 ± 0.98 | 5.09 ± 1.20 | 0.002 |
| ST1A1 | 2.01 ± 0.80 | 2.05 ± 0.84 | 0.556 |
| STAMBP | 2.99 ± 0.32 | 3.04 ± 0.37 | 0.125 |
| ADA | 3.80 ± 0.41 | 3.84 ± 0.35 | 0.282 |
| TNFβ | 3.82 ± 0.48 | 3.93 ± 0.44 | 0.007 |
| CSF-1 | 7.72 ± 0.23 | 7.79 ± 0.22 | 0.010 |

Data are given as median and $25^{th}/75^{th}$ percentiles. The *P* values for the comparison of cases and non-cases were derived from logistic regression analysis (likelihood ratio tests comparing models with the respective variable, age and sex as independent variables to models with age and sex only). All analyses were adjusted for age and sex. Please see Supplementary Table 1 for the extended biomarker names.

**Supplementary Table 4 – Age and sex-adjusted associations between biomarker levels and incident DSPN**

| Variable | OR (95% CI) | *P* | $P_{B-H}$ |
|---|---|---|---|
| IL-8 | **1.80 (1.24; 2.60)** | **0.002** | **0.010** |
| VEGF-A | 1.16 (0.76; 1.77) | 0.497 | 0.571 |
| MCP-3 | **1.99 (1.38; 2.86)** | **<0.001** | **<0.001** |
| GDNF | 0.92 (0.47; 1.81) | 0.818 | 0.818 |
| CDCP1 | **1.64 (1.19; 2.26)** | **0.002** | **0.010** |
| CD244 | **2.16 (1.20; 3.90)** | **0.011** | **0.033** |
| IL-7 | 1.15 (0.76; 1.76) | 0.507 | 0.571 |
| OPG | **2.44 (1.22; 4.87)** | **0.012** | **0.033** |
| LAP TGFβ1 | 1.16 (0.64; 2.09) | 0.633 | 0.651 |
| uPA | **2.09 (1.11; 3.95)** | **0.023** | 0.053 |
| IL-6 | **1.43 (1.08; 1.91)** | **0.013** | **0.034** |
| IL-17C | 1.40 (0.98; 2.01) | 0.063 | 0.118 |
| MCP-1 | **1.61 (1.02; 2.55)** | **0.043** | 0.090 |
| CXCL11 | **1.37 (1.04; 1.79)** | **0.023** | 0.053 |
| Axin-1 | 1.13 (0.73; 1.74) | 0.590 | 0.625 |
| TRAIL | 1.47 (0.70; 3.08) | 0.306 | 0.406 |
| CXCL9 | **1.66 (1.27; 2.15)** | **<0.001** | **<0.001** |
| CST5 | 0.77 (0.50; 1.21) | 0.259 | 0.375 |
| OSM | **1.58 (1.14; 2.19)** | **0.006** | **0.022** |
| CXCL1 | 1.24 (0.80; 1.93) | 0.342 | 0.434 |
| CCL4 | 1.17 (0.84; 1.63) | 0.350 | 0.436 |
| CD6 | **1.98 (1.29; 3.06)** | **0.002** | **0.010** |
| SCF | 0.92 (0.52; 1.62) | 0.767 | 0.778 |
| IL-18 | 1.22 (0.86; 1.73) | 0.265 | 0.376 |
| SLAMF1 | **2.05 (1.32; 3.19)** | **0.001** | **0.010** |
| TGFα | **1.62 (1.09; 2.40)** | **0.017** | **0.043** |
| MCP-4 | **1.44 (1.00; 2.07)** | **0.049** | 0.099 |
| Eotaxin | 1.17 (0.72; 1.92) | 0.526 | 0.584 |
| TNFSF14 | 1.32 (0.90; 1.95) | 0.159 | 0.252 |
| FGF-23 | 1.46 (1.00; 2.14) | 0.051 | 0.101 |
| FGF-5 | 1.53 (0.76; 3.09) | 0.235 | 0.348 |
| MMP-1 | 1.11 (0.84; 1.46) | 0.463 | 0.548 |
| LIF-R | **2.68 (1.31; 5.50)** | **0.007** | **0.024** |
| FGF-21 | 1.14 (0.94; 1.40) | 0.189 | 0.292 |
| CCL19 | **1.39 (1.12; 1.73)** | **0.003** | **0.012** |
| IL-15RA | **3.19 (1.50; 6.80)** | **0.003** | **0.012** |

| | | | |
|---|---|---|---|
| IL-10RB | **2.87 (1.42; 5.82)** | **0.003** | **0.012** |
| IL-18R1 | **2.01 (1.20; 3.37)** | **0.008** | **0.026** |
| PD-L1 | 1.26 (0.81; 1.98) | 0.309 | 0.406 |
| Beta-NGF | **2.01 (1.09; 3.68)** | **0.024** | 0.053 |
| CXCL5 | 1.36 (1.00; 1.85) | 0.053 | 0.102 |
| TRANCE | 1.25 (0.88; 1.77) | 0.208 | 0.314 |
| HGF | **3.11 (1.76; 5.50)** | **<0.001** | **<0.001** |
| IL-12B | **1.53 (1.10; 2.12)** | **0.012** | **0.033** |
| MMP-10 | **1.51 (1.06; 2.13)** | **0.021** | 0.051 |
| IL-10 | 1.42 (0.87; 2.31) | 0.160 | 0.252 |
| CCL23 | 1.27 (0.82; 1.97) | 0.283 | 0.386 |
| CD5 | **2.26 (1.33; 3.87)** | **0.003** | **0.012** |
| MIP-1α | **1.86 (1.27; 2.73)** | **0.002** | **0.010** |
| Flt3L | **1.66 (1.02; 2.71)** | **0.042** | 0.090 |
| CXCL6 | 1.42 (0.97; 2.09) | 0.075 | 0.133 |
| CXCL10 | **1.50 (1.21; 1.86)** | **<0.001** | **<0.001** |
| EIF4EBP1 | **1.86 (1.25; 2.77)** | **0.002** | **0.010** |
| SIRT2 | 1.44 (0.94; 2.21) | 0.093 | 0.157 |
| CCL28 | 1.22 (0.71; 2.10) | 0.462 | 0.548 |
| DNER | 2.05 (0.90; 4.64) | 0.086 | 0.149 |
| EN-RAGE | **1.53 (1.16; 2.01)** | **0.002** | **0.010** |
| CD40 | **2.95 (1.55; 5.63)** | **0.001** | **0.010** |
| FGF-19 | 1.25 (0.98; 1.59) | 0.074 | 0.133 |
| MCP-2 | 1.15 (0.86; 1.54) | 0.331 | 0.427 |
| CCL25 | 1.19 (0.81; 1.75) | 0.366 | 0.448 |
| CX3CL1 | 1.16 (0.69; 1.98) | 0.573 | 0.616 |
| TNFRSF9 | **2.53 (1.60; 4.00)** | **<0.001** | **<0.001** |
| NT-3 | 1.16 (0.65; 2.07) | 0.605 | 0.632 |
| TWEAK | 0.79 (0.39; 1.59) | 0.502 | 0.571 |
| CCL20 | **1.35 (1.12; 1.62)** | **0.002** | **0.010** |
| ST1A1 | 1.08 (0.84; 1.39) | 0.556 | 0.607 |
| STAMBP | 1.60 (0.88; 2.94) | 0.125 | 0.206 |
| ADA | 1.33 (0.79; 2.22) | 0.282 | 0.386 |
| TNFβ | **1.87 (1.18; 2.96)** | **0.007** | **0.024** |
| CSF-1 | **3.32 (1.33; 8.27)** | **0.010** | **0.031** |

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 biomarker levels (in NPX). Model 1: adjusted for baseline age (years) and sex.

**Supplementary Table 5 – Fully adjusted associations between biomarker levels and incident DSPN**

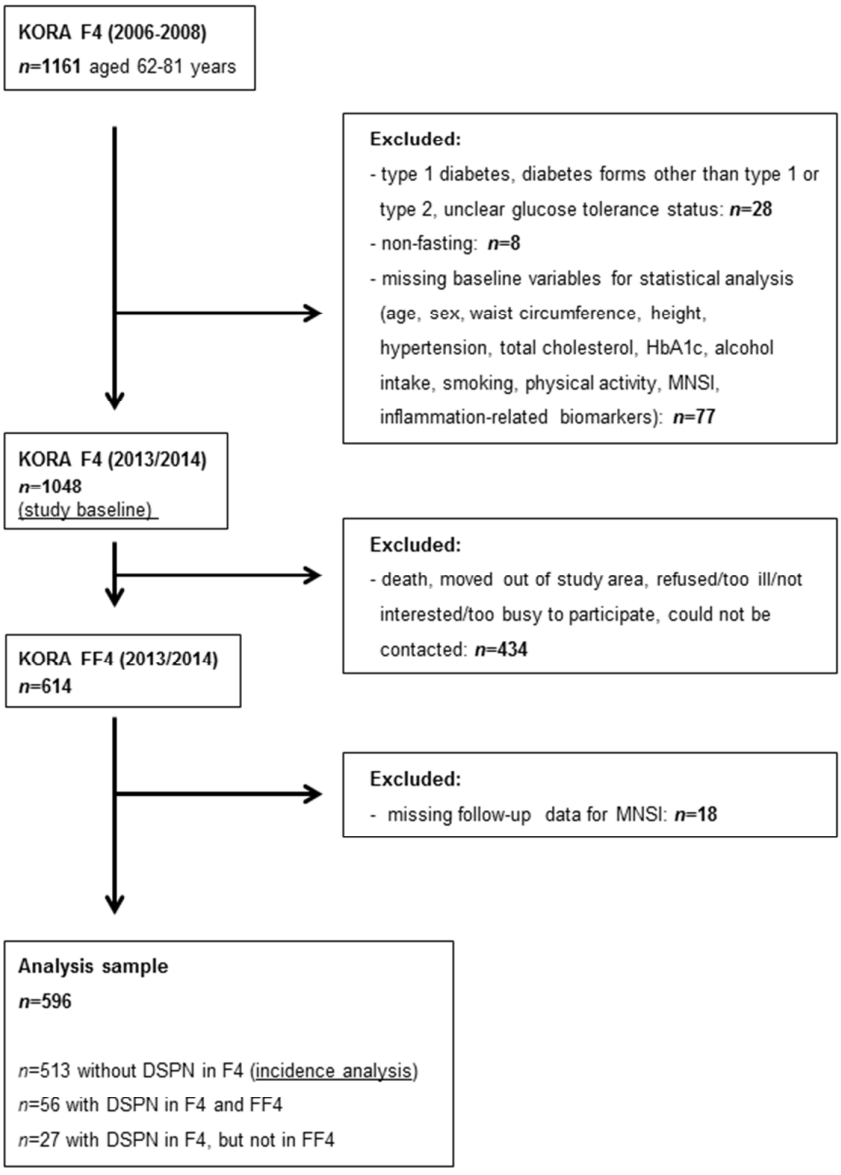
| Variable | OR (95% CI) | *P* | $P_{B-H}$ |
|---|---|---|---|
| IL-8 | 1.47 (0.99; 2.18) | 0.055 | 0.145 |
| VEGF-A | 0.96 (0.60; 1.54) | 0.878 | 0.903 |
| MCP-3 | **1.84 (1.24; 2.73)** | **0.003** | **0.043** |
| GDNF | 0.67 (0.31; 1.42) | 0.294 | 0.426 |
| CDCP1 | **1.50 (1.06; 2.12)** | **0.022** | 0.105 |
| CD244 | **2.01 (1.07; 3.80)** | **0.031** | 0.105 |
| IL-7 | 1.16 (0.73; 1.82) | 0.536 | 0.656 |
| OPG | **2.69 (1.26; 5.73)** | **0.010** | 0.078 |
| LAP TGFβ1 | 0.85 (0.44; 1.62) | 0.614 | 0.716 |
| uPA | **2.45 (1.21; 4.95)** | **0.013** | 0.084 |
| IL-6 | 1.21 (0.89; 1.64) | 0.226 | 0.349 |
| IL-17C | 1.45 (0.98; 2.15) | 0.061 | 0.149 |
| MCP-1 | 1.52 (0.92; 2.51) | 0.101 | 0.205 |
| CXCL11 | 1.34 (0.99; 1.82) | 0.057 | 0.145 |
| Axin-1 | 1.13 (0.70; 1.82) | 0.615 | 0.716 |
| TRAIL | 1.17 (0.51; 2.68) | 0.702 | 0.779 |
| CXCL9 | **1.69 (1.26; 2.28)** | **<0.001** | **<0.001** |
| CST5 | 0.89 (0.54; 1.48) | 0.662 | 0.758 |
| OSM | 1.38 (0.97; 1.96) | 0.072 | 0.167 |
| CXCL1 | 1.04 (0.64; 1.70) | 0.877 | 0.903 |
| CCL4 | 1.05 (0.73; 1.51) | 0.772 | 0.830 |
| CD6 | **1.86 (1.17; 2.97)** | **0.009** | 0.078 |
| SCF | 1.38 (0.73; 2.59) | 0.319 | 0.444 |
| IL-18 | 1.02 (0.68; 1.51) | 0.939 | 0.945 |
| SLAMF1 | **1.79 (1.09; 2.94)** | **0.022** | 0.105 |
| TGFα | 1.47 (0.96; 2.25) | 0.077 | 0.171 |
| MCP-4 | 1.41 (0.95; 2.08) | 0.086 | 0.185 |
| Eotaxin | 1.11 (0.66; 1.89) | 0.687 | 0.774 |
| TNFSF14 | 1.21 (0.80; 1.84) | 0.361 | 0.493 |
| FGF-23 | 1.40 (0.90; 2.20) | 0.138 | 0.237 |
| FGF-5 | 1.69 (0.77; 3.68) | 0.190 | 0.301 |
| MMP-1 | 1.08 (0.80; 1.47) | 0.613 | 0.716 |
| LIF-R | **2.26 (1.04; 4.91)** | **0.039** | 0.107 |
| FGF-21 | 1.01 (0.79; 1.28) | 0.945 | 0.945 |
| CCL19 | **1.32 (1.03; 1.69)** | **0.029** | 0.105 |
| IL-15RA | **3.06 (1.29; 7.23)** | **0.011** | 0.078 |

| | | | |
|---|---|---|---|
| IL-10RB | **2.53 (1.14; 5.61)** | **0.023** | 0.105 |
| IL-18R1 | 1.50 (0.84; 2.68) | 0.170 | 0.281 |
| PD-L1 | 1.33 (0.79; 2.24) | 0.287 | 0.426 |
| Beta-NGF | 1.74 (0.87; 3.47) | 0.119 | 0.222 |
| CXCL5 | 1.29 (0.93; 1.81) | 0.129 | 0.229 |
| TRANCE | 1.15 (0.77; 1.70) | 0.500 | 0.634 |
| HGF | **1.99 (1.06; 3.75)** | **0.033** | 0.105 |
| IL-12B | **1.51 (1.03; 2.21)** | **0.035** | 0.105 |
| MMP-10 | 1.40 (0.94; 2.08) | 0.095 | 0.198 |
| IL-10 | 1.51 (0.87; 2.62) | 0.140 | 0.237 |
| CCL23 | 1.58 (0.96; 2.61) | 0.073 | 0.167 |
| CD5 | **1.95 (1.07; 3.56)** | **0.028** | 0.105 |
| MIP-1α | **1.57 (1.03; 2.04)** | **0.037** | 0.105 |
| Flt3L | 1.53 (0.89; 2.63) | 0.122 | 0.222 |
| CXCL6 | 1.22 (0.79; 1.87) | 0.371 | 0.497 |
| CXCL10 | **1.47 (1.17; 1.86)** | **0.001** | **0.018** |
| EIF4EBP1 | **1.62 (1.03; 2.54)** | **0.036** | 0.105 |
| SIRT2 | 1.45 (0.91; 2.32) | 0.115 | 0.222 |
| CCL28 | 1.35 (0.75; 2.45) | 0.316 | 0.444 |
| DNER | **3.88 (1.56; 9.66)** | **0.004** | **0.047** |
| EN-RAGE | **1.43 (1.06; 1.92)** | **0.018** | 0.105 |
| CD40 | **3.39 (1.64; 7.04)** | **0.001** | **0.018** |
| FGF-19 | **1.36 (1.03; 1.78)** | **0.029** | 0.105 |
| MCP-2 | 1.23 (0.90; 1.68) | 0.191 | 0.301 |
| CCL25 | 1.07 (0.69; 1.66) | 0.749 | 0.818 |
| CX3CL1 | 1.21 (0.66; 2.22) | 0.531 | 0.656 |
| TNFRSF9 | **2.46 (1.47; 4.13)** | **0.001** | **0.018** |
| NT-3 | 1.29 (0.69; 2.43) | 0.425 | 0.559 |
| TWEAK | 1.09 (0.05; 2.37) | 0.820 | 0.869 |
| CCL20 | **1.34 (1.09; 1.65)** | **0.006** | 0.061 |
| ST1A1 | 1.11 (0.84; 1.47) | 0.473 | 0.611 |
| STAMBP | 1.68 (0.88; 3.21) | 0.118 | 0.222 |
| ADA | 1.35 (0.77; 2.35) | 0.290 | 0.426 |
| TNFβ | **1.72 (1.03; 2.88)** | **0.037** | 0.105 |
| CSF-1 | **3.19 (1.13; 8.99)** | **0.028** | 0.105 |

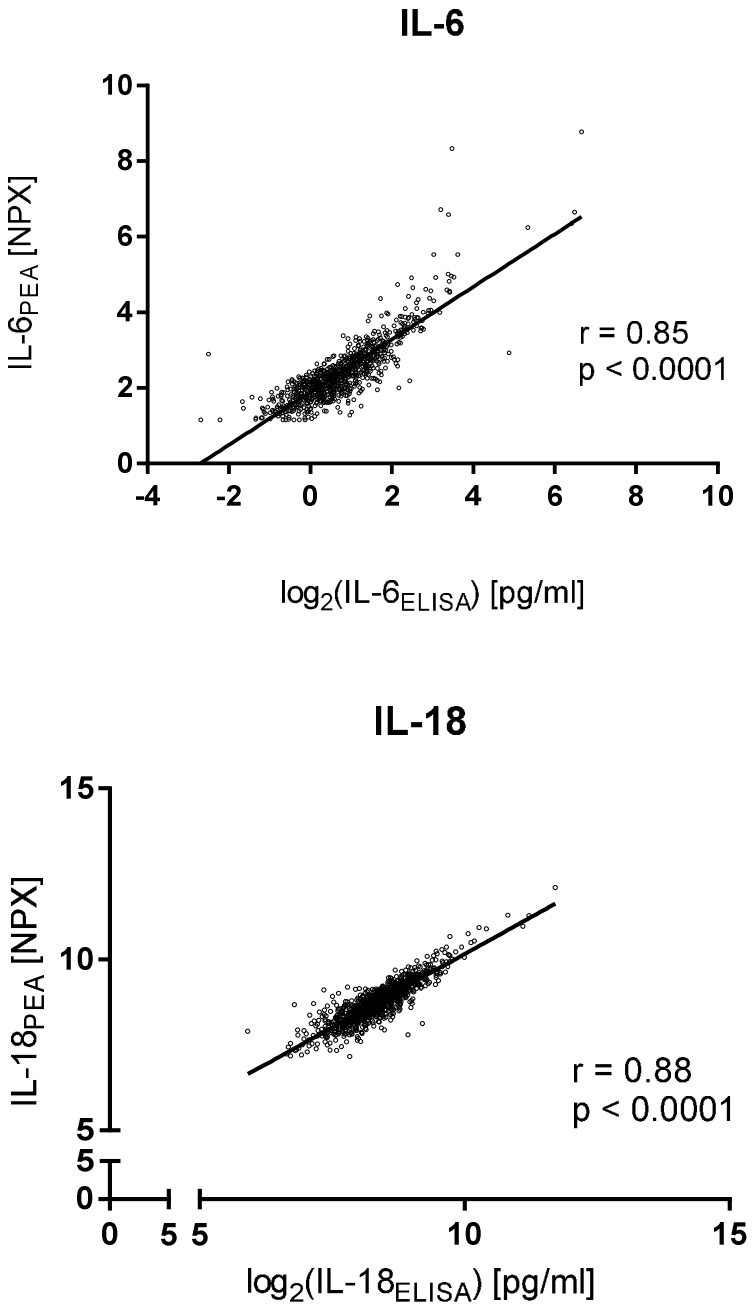
OR (95% CI), corresponding *P* values and Benjamini-Hochberg corrected *P* values ($P_{B\text{-}H}$) values for incident DSPN are given for a 1-unit increase in biomarker levels (in NPX).

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), HbA1c (%), alcohol intake (none/moderate/high), smoking (never/ex/current), physical activity (active/inactive), use of lipid-lowering drugs (yes/no), use of non-steroidal anti-inflammatory drugs (NSAIDs) (yes/no), estimated glomerular filtration rate (eGFR; ml/min per $1.73m^2$), prevalent myocardial infarction and/or stroke (yes/no) and neurological conditions that might cause nerve damage (yes/no).

**Supplementary Figure 1 – Description of the study design and population**

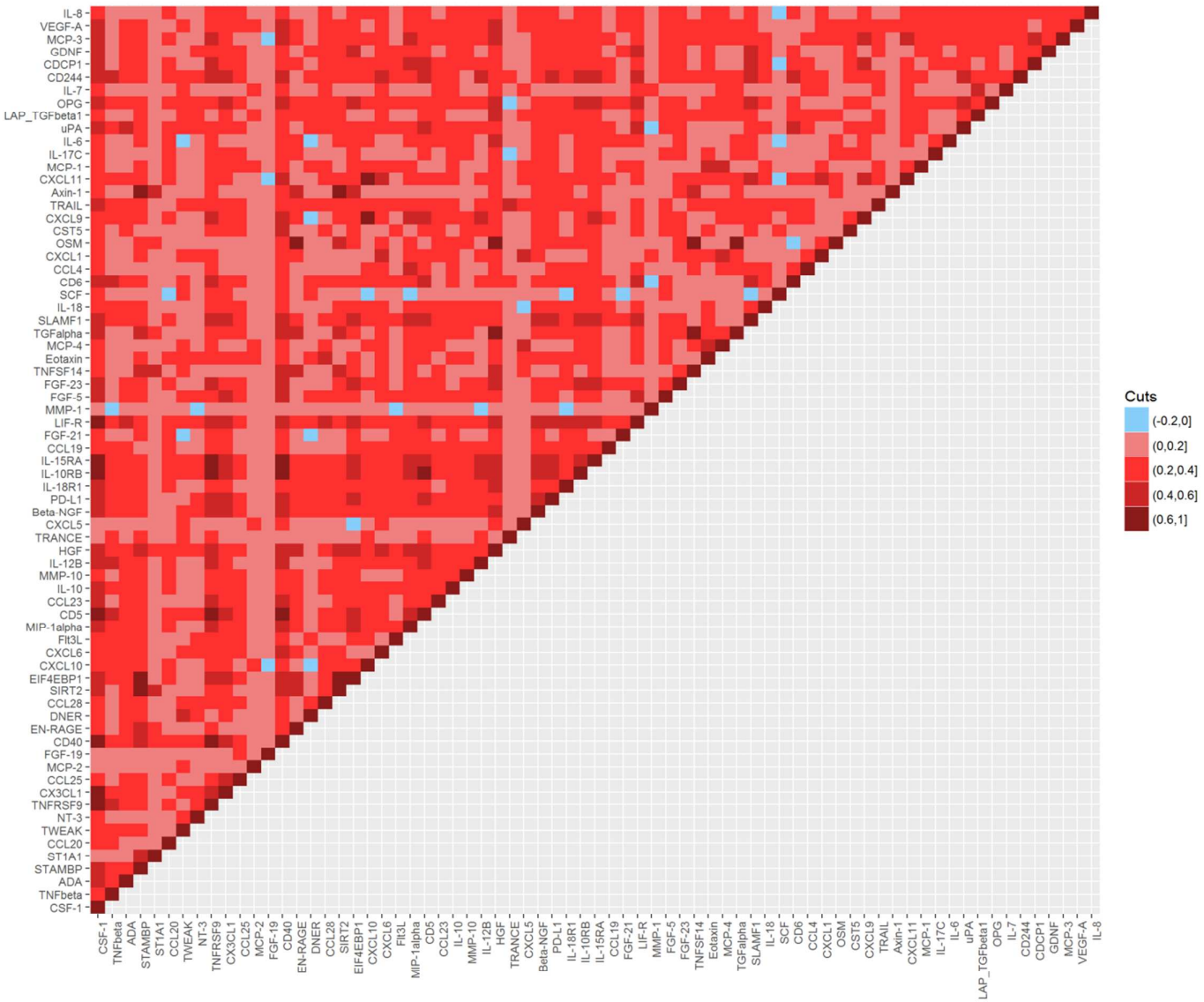


**Supplementary Figure 2 – Correlation of IL-6 and IL-18 levels measured with ELISA and primer extension assay (PEA) technology**



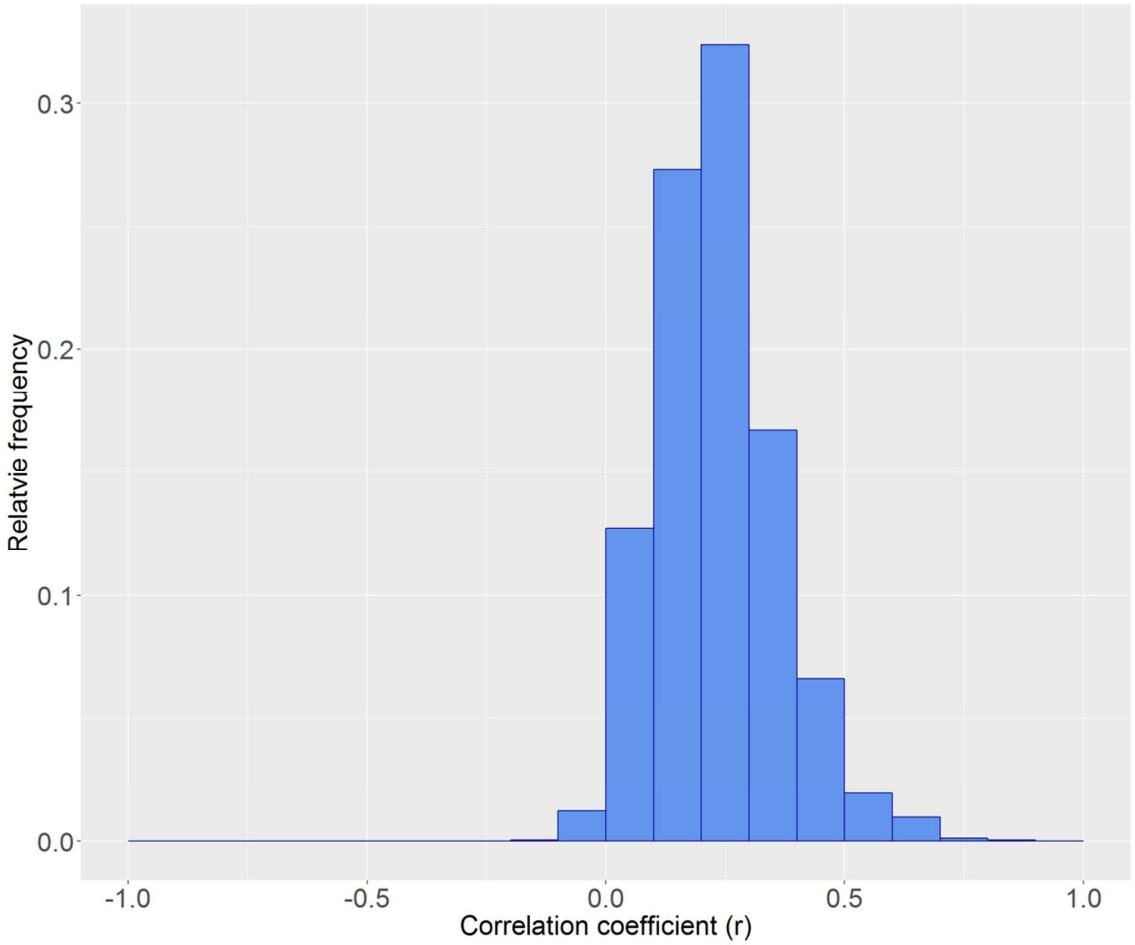

The analysis is based on the 1048 samples from the study baseline. Correlations are estimated using Pearson correlation coefficients (*r*) and corresponding *P* values.

**Supplementary Figure 3 – Correlation matrix of all 71 analysed biomarkers of subclinical inflammation**



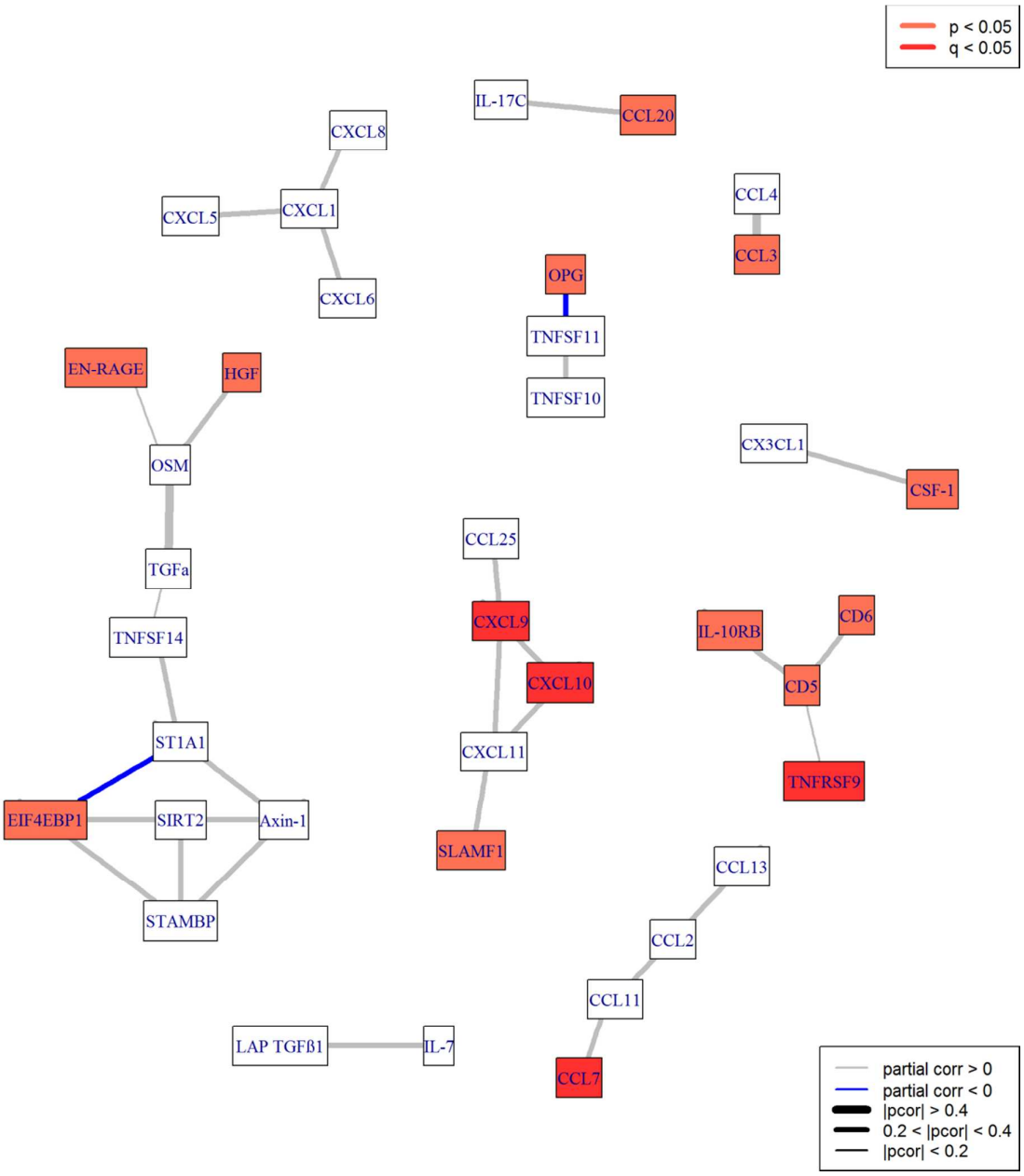

Data are based on *n*=1048 (baseline sample).

**Supplementary Figure 4 – Histogram of correlations among all 71 analysed biomarkers of subclinical inflammation**




The histogram shows relative frequencies of Pearson correlation coefficients *r* for pairwise correlations among all 71 biomarkers (baseline sample).

**Supplementary Figure 5 – Gaussian graphical model (GGM) showing the conditional dependence structure between biomarkers of inflammation and their associations with incident DSPN**




The GGM calculates pairwise partial correlations between the 71 biomarkers of inflammation adjusted for all other biomarkers. Thirty-eight biomarkers showed a significant partial

correlation with at least one other biomarker as shown in the figure, whereas the remaining 33 biomarkers were independent (not shown). Positive and negative partial correlations are given using gray and blue lines, respectively. The strength of the correlation is indicated by the respective line width. Associations between biomarkers and incident DSPN in model 2 (fully adjusted) are highlighted by light red ($P<0.05$) or dark red (Benjamini-Hochberg adjusted $Q<0.05$).

**Reference**

1. Herder C, Kannenberg JM, Huth C, Carstensen-Kirberg M, Rathmann W, Koenig W, Heier M, Püttgen S, Thorand B, Peters A, Roden M, Meisinger C, Ziegler D. Proinflammatory cytokines predict the incidence and progression of distal sensorimotor polyneuropathy: KORA F4/FF4 Study. Diabetes Care 2017;40:569-576