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Associations of cells from both innate and adaptive immunity with lower nerve conduction velocity: the Maastricht Study

Haifa Maalmi ,[1,2] Kristiaan Wouters,[3,4] Hans H C M Savelberg,[5,6] Jeroen H P M van der Velde,[5,6] Jos P H Reulen,[7] Werner Mess,[7] Casper G Schalkwijk,[3,4] Coen D A Stehouwer,[3,4] Michael Roden ,[1,2,8] Dan Ziegler ,[1,2,8] Christian Herder ,[1,2,8] Nicolaas C Schaper[3,4]

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HM and KW contributed equally. CH and NCS contributed equally.

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For numbered affiliations see end of article.

Correspondence to
Professor Christian Herder; Christian.Herder@ddz.de

ABSTRACT

Introduction Distal sensorimotor polyneuropathy (DSPN) is common in people with diabetes but is also found in pre-diabetes. Peripheral nerve myelin damage, which can be assessed by reduced nerve conduction velocity (NCV), is an essential feature of DSPN. Emerging evidence indicates that the development of DSPN may involve the activation of the immune system. However, available studies have mainly investigated circulating immune mediators, whereas the role of immune cells remains unclear. Therefore, we aimed to test whether leukocyte subsets are associated with NCV.

Research design and methods This cross-sectional study analyzed data from 850 individuals (of whom 252 and 118 had type 2 diabetes and pre-diabetes, respectively) of the Maastricht Study. NCV was measured in the peroneal and tibial motor nerves and the sural sensory nerve and summed to calculate a standardized NCV sum score. Associations between percentages of leukocyte subsets and NCV sum scores were estimated using linear regression models adjusted for demographic, lifestyle, metabolic and clinical covariates.

Results After adjustment for covariates, higher percentages of basophils and $CD4^+$ T cells were associated with lower NCV (p=0.014 and p=0.005, respectively). The percentage of $CD8^+$ T cells was positively associated with NCV (p=0.022). These associations were not modified by glucose metabolism status (all $p_{interaction}$ >0.05). No associations were found for monocytes, eosinophils, neutrophils, lymphocytes, total T cells, Treg cells and B cells.

Conclusions The associations of basophils, $CD4^+$ and $CD8^+$ T cells with NCV suggest that cell types from both innate and adaptive immunity may be implicated in the development of DSPN.

Significance of this study

What is already known about this subject?

- The mechanisms underlying distal sensorimotor polyneuropathy (DSPN) are complex and incompletely understood.
- Studies have implicated activation of the immune system in its pathogenesis, but data on the role of the cellular components of the immune system in the development of DSPN are not available.

What are the new findings?

- Higher percentages of $CD4^+$ T cells and basophils were associated with a lower nerve conduction velocity (NCV), while higher percentages of $CD8^+$ T cells are positively associated with NCV.
- These associations were independent of multiple confounders and not modified by glucose metabolism status.
- These findings corroborate the notion that cells from both the innate and the adaptive immune system might be implicated in lower NCV and the development of DSPN.

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

- Our results allow novel hypotheses about the communication between the immune system and the peripheral nervous system in the context of DSPN.

INTRODUCTION

Distal sensorimotor polyneuropathy (DSPN) is a common complication of diabetes affecting up to 50% of patients with type 2 diabetes after a disease duration of 10 years or more and is associated with an increased risk of foot ulceration, amputation and mortality.[1] Emerging evidence indicates an increased prevalence of DSPN even among individuals with pre-diabetes,[2–4] pointing to the high importance of including people with pre-diabetes when assessing risk factors for DSPN. Both large-fibre nerve myelin damage and small-fibre damage are important features of subclinical DSPN, which precedes the clinical diagnosis of DSPN.[5] Nerve conduction velocity (NCV) measurement is an objective, sensitive and specific method to detect early nerve myelin dysfunction in large-fibre nerves.

The mechanisms underlying DSPN are complex and incompletely understood. Even though DSPN is usually not considered an inflammatory neuropathy, many studies have implicated the activation of the immune system in its pathogenesis.[3 6 7] Cross-sectional studies investigating soluble immune mediators found higher systemic levels of acute-phase proteins and inflammatory cytokines that reflect innate immune activation in patients with DSPN compared with those without.[8–14] Recent prospective analyses within the population-based KORA F4/FF4 cohort showed that serum levels of two proinflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), were positively associated with incident DSPN[15] and identified multiple additional biomarkers including chemokines as predictors of DSPN. Pathway analyses based on these findings suggested that the development of DSPN may involve both innate and adaptive immunity and their cross-talk.[16] However, supporting data about associations of immune cells with DSPN are scarce and have been limited to two studies investigating the neutrophil-to-lymphocyte ratio[17 18] and one study suggesting the potential involvement of dendritic cells expressing the autoantigen proinsulin.[19] Thus, the role of the cellular component of the immune system in the development of DSPN remains unclear.

Therefore, we aimed to evaluate the cross-sectional associations of leukocyte subsets from both the innate and the adaptive immune system with NCV in a population-based observational cohort enriched by individuals with type 2 diabetes. We hypothesized that higher percentages of both innate and adaptive immune cells are associated with reduced NCV after controlling for potential confounders.

MATERIALS AND METHODS

Study design and participants

The Maastricht Study is an observational prospective population-based cohort study that focuses on the etiology, pathophysiology, complications and comorbidities of type 2 diabetes. The study is characterized by an extensive phenotyping approach and an oversampling of patients with type 2 diabetes for reasons of efficiency. Further details of the Maastricht Study were reported elsewhere.[20] Briefly, individuals living in the southern region of the Netherlands and aged between 40 and 75 years are eligible to participate. Participants are recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings. Enrolment started in 2010 and is still ongoing, aiming to include 10 000 participants. For each participant, data were collected within 3 months from enrolment, and all measurements were conducted in the Maastricht Study Center. Standardized questionnaires and protocols were used for data collection and measurement.

The present report includes data from 1098 participants recruited between 2 April 2013 and 14 January 2014, for whom data on immune cell subsets are available. Overall, this sample represents approximatively 85% of all participants gathered during the indicated recruitment period. Lack of immune cell subsets data in the non-included participants was mainly caused by logistic issues.

Measurement of immune cell subsets

Leukocytes were measured in fresh venous blood samples of fasting participants using two methods. First, lymphocytes, monocytes and granulocytes (basophils, eosinophils and neutrophils) were counted using automated blood cell count (Sysmex XE5000, Kobe, Japan), and results were reported as percentages of the total leukocyte count. Second, lymphocytes and their subsets (total T cells, $CD4^+$ T cells, $CD8^+$ T cells, Treg cells and total B cells) were quantified in whole heparinized blood using flow cytometry. The staining cocktail included the following antibodies from BD Biosciences (Erembodegem, Belgium): CD3-V500 (BD 561416), CD4-APC-H7 (BD 560158), CD8-PerCP-Cy5.5 (BD 560662), CD25-PE-Cy7 (BD 335824), CD127-FITC (BD 560549) and CD19-PerCP-Cy5.5 (BD 332780). From single blood cells (based on side scatter parameters), monocytes and lymphocytes were gated based on forward and side scatters plots. As shown in online supplemental figure 1, B cells were identified based on CD19 expression and T cells based on CD3 expression. From total T cells, $CD8^+$ T cells were identified based on CD8 expression, while $CD4^+$ T cells were identified based on CD4 expression. From $CD4^+$ T cells, regulatory T cells (Tregs; $CD127^{low}$ $CD25^{high}$) were identified as described previously.[21] To standardize measurements, antibodies from a single batch were used during the whole study. On arrival, antibody solutions were aliquoted into small volumes to avoid frequent temperature changes or contamination. Furthermore, cytometer tracking beads were used biweekly to define a baseline and standardize daily measurements of the cytometer. Results were expressed as percentages based on the corresponding parent gate (total lymphocytes-monocytes as parent gate for B and total T cells, total T cells as parent gate for $CD4^+$ and $CD8^+$ T cells, and $CD4^+$ T cells as parent gate for Treg cells).

Nerve conduction study

NCV was measured in the peroneal and tibial motor nerves and the sural sensory nerve using Medelec Synergy electromyography (EMG) apparatus (V.15.0, Viasys Healthcare UK Ltd, Warwick, UK) using surface electrodes. Before testing, feet and lower legs were warmed up in warm water (38°C) for a minimum duration of 10 min to ensure that skin temperature (measured on the dorsal surface of the foot) was >32°C. The three nerves were examined at supramaximal stimulation. The peroneal nerve was assessed on the right leg, at the digitorum brevis muscle with stimulations at the ankle (8 cm proximal from the recording site), below the fibular head and above the fibular head. Peroneal motor NCV were measured from two trajectories: one from 'below vs

above fibular head' and one from 'below fibular head vs ankle'. Tibial motor NCV was recorded on the left leg at the abductor hallucis muscle with stimulations at the ankle and in the popliteal fossa. Sural sensory NCV was recorded on the left leg between the lateral malleolus and the Achilles tendon while stimulating 12 cm proximal to the recording site. Also, the sural sensory nerve action potential (SNAP) amplitude was recorded. Due to strict time limits for the EMG testing, together with our aim to retrieve data from both legs, EMG measurements were split up between both legs.

Because NCV measurements are imprecise in the range below 30 m/s, NCV levels below this threshold indicating advanced DSPN were assigned a value equal to 30 m/s (N=4 for peroneal NCV, N=12 for tibial NCV and N=3 for sural NCV without overlap between these subgroups). Of note, this procedure was not done for individuals with missing data for NCV.

Assessment of covariates

Laboratory assessment of glycated hemoglobin (HbA1c) and total cholesterol was reported elsewhere.[20] To determine the glucose metabolism status, all participants (except those who used insulin) underwent a standardized 75 g oral glucose tolerance test after an overnight fast.[20] Participants were categorized according to the WHO 2006 criteria into normal glucose metabolism, pre-diabetes (impaired fasting glucose and/or impaired glucose tolerance) or type 2 diabetes. Type 1 diabetes was self-reported. Individuals without type 1 diabetes but on glucose-lowering medication were classified as having type 2 diabetes. Body mass index (BMI) was calculated as kilogram per meter squared (kg/m^2). Waist circumference (cm) was measured in an upright position midway between the lowest rib margin and the iliac crest and calculated as the mean of two measurements. Office systolic and diastolic blood pressures were calculated as the average of three measurements on the right arm after 10 min of seated rest, using a non-invasive blood pressure monitor (Omron 705IT, Japan). Use of medication was assessed during a medication interview where the generic name, dose and frequency were registered. Study participants were recorded as hypertensive if systolic blood pressure was ≥130 mm Hg or diastolic blood pressure was ≥85 mm Hg or the use of antihypertensive drugs was reported.

Questionnaires were used to collect information on age (years), sex, alcohol consumption (non-consumer, low-consumer (women ≤7 glasses per week; men ≤14 glasses per week) or high-consumer (women >7 glasses per week; men >14 glasses per week)), smoking behavior (non-smoker, former smoker or current smoker), cardiovascular disease history (defined as a history of any of the following conditions: myocardial infarction, cerebrovascular infarction or hemorrhage, percutaneous artery angioplasty of, or vascular surgery on, the coronary, abdominal, peripheral or carotid arteries). Estimated glomerular filtration rate (eGFR; in $mL/min/1.73\,m^2$) was calculated with the Chronic Kidney Disease Epidemiology equation based on serum creatinine.

Statistical analysis

Continuous variables are reported as mean (SD) or median (25th and 75th percentiles) depending on whether the distribution was normal, while categorical variables are expressed as percentages.

For each participant, composite scores that combine information about NCV were constructed by summing z-scores of available NCV in each nerve [(z-score of the peroneal nerve+z-score of the tibial nerve+z-score of the sural nerve)/(number of available NCV measurements)], where z-scores were calculated by subtracting the mean value of NCV in the study population from the value in the individual and dividing the result by the SD. Three NCV sum scores were constructed based on 1, 2 or 3 available NCV measurements, and one NCV sum score was constructed for the two motor nerves. Compared with NCV in individual nerves, the use of an NCV sum score allows for a more comprehensive assessment of nerve function in an individual. This approach was previously suggested as a criterion for the diagnosis of DSPN[22] and used in an analysis of the association between biomarkers of inflammation, NCV and DSPN in people with diabetes.[14]

In the primary analysis, we considered the NCV sum score that combines information on at least one nerve as the primary outcome because it ensures the largest sample size. In secondary analyses, we considered: (1) the NCV sum scores that combine information on 2 or 3 available NCV measurements, (2) the NCV sum score that was constructed for the two motor nerves, (3) the quantitative measurement of NCV for the three individual nerves and (4) the sural SNAP amplitude. For all analyses, associations between percentages of leukocyte subsets and the NCV sum score or quantitative NCV (dependent variable) were estimated using linear regression models. Three models with an increasing level of complexity using a priori defined covariables were built. Model 1 was adjusted for sex and age. Model 2 was additionally adjusted for glucose metabolism status. Model 3 was further adjusted for BMI, HbA1c, total cholesterol, triglycerides, eGFR, history of hypertension, history of cardiovascular diseases, smoking, alcohol consumption and medication use (glucose-lowering drugs, lipid-lowering drugs and non-steroidal anti-inflammatory drugs). Results of multiple linear regressions are reported as β estimates with their 95% CIs and p values.

In a first sensitivity analysis, potential effect modification by glucose metabolism status of the association between percentages of leukocyte subsets and NCV was assessed by repeating model 3 but including an additional term for the multiplicative interaction of each immune cell subset and glucose metabolism status. Furthermore, these associations were assessed stratified by glucose metabolism status. In a second and a third sensitivity analyses, model 3 was repeated with adjustment for waist circumference

instead of BMI and with adjustment for age categories (<50, 50 to <60, 60 to <70 and ≥70 years) instead of age as a continuous variable.

All analyses were performed with SAS V.9.4. P<0.05 indicated statistical significance.

RESULTS

Study population

Among the 1098 eligible participants, 850 individuals had NCV data in at least one nerve. The demographic and clinical characteristics of the study population are reported in table 1.

Slightly more than half of the participants were women. Median age was 61.0 years (IQR 54.0–67.0), median BMI was 26.2 kg/m2 (IQR 24.0–29.5) and median HbA1c was 37 mmol/mol (IQR 34–44). With respect to glucose metabolism, 56.1% of the study participants were classified as having normal glucose tolerance, 13.9% had pre-diabetes, 29.8% had type 2 diabetes and the remaining study participants (0.2%) had other diabetes types. General characteristics of the study population who contributed data to the other NCV sum scores are provided in online supplemental table 1. NCV data for at least two nerves, all three nerves and the motor nerves were available for 786, 558 and 739 individuals, respectively.

Associations between leukocyte subsets and NCV

Results of the primary analysis investigating the associations between percentages of leukocyte subsets and NCV sum score are reported in table 2. After adjustment for age and sex (model 1), higher percentages of basophils and $CD4^+$ T cells were associated with lower NCV. A positive association was found between the percentage of lymphocytes and $CD8^+$ T cells and NCV sum score. Results of model 1 remained significant for basophils, $CD4^+$ and $CD8^+$ T cells but not for lymphocytes after additional adjustment for glucose tolerance status (model 2). In model 3, adjusting for all covariates of interest, higher percentages of basophils and $CD4^+$ T cells were associated with lower NCV, and the percentage of $CD8^+$ T cells remained positively associated with NCV. No associations were found for monocytes, eosinophils, neutrophils, lymphocytes, total T cells, Treg cells and B cells.

Secondary analyses performed on other NCV sum scores as well as quantitative measurement of NCV in the three nerves are reported in online supplemental table 2. Significant associations were still observed between percentages of basophils, $CD4^+$ T cells and $CD8^+$ T cells and NCV sum scores based on two or three velocities and the NCV sum score based on the motor nerves. Also, a significant inverse association was detected between the percentage of B cells and NCV sum score based on three velocities. When individual NCV were assessed, percentages of basophils, $CD4^+$ T cells and $CD8^+$ T cells were similarly associated with peroneal and/or tibial NCV, but not with sural NCV. Secondary analyses performed on the sural SNAP amplitude are reported in online supplemental table 3. The results did not show any association between leukocyte subsets and sural SNAP amplitude.

Table 1 Characteristics of the study population (N=850)

Characteristic	N (%)/mean (SD)/median (Q1; Q3)
Sex, female	440 (51.9)
Age, years	61 (54; 67)
BMI, kg/m^2	26.2 (24.0; 29.5)
Waist circumference, cm	94.5 (85.9; 103.1)
HbA1c, mmol/mol	37 (34; 44)
Glucose metabolism status	
Normal	475 (56.1)
Pre-diabetes	118 (13.9)
Type 2 diabetes	252 (29.8)
Other diabetes types	2 (0.2)
Total cholesterol, mmol/L	5.1 (1.1)
Triglycerides, mmol/L	1.2 (0.9; 1.7)
eGFR, mL/min/1.73 m^2	84.1 (73.3; 93.1)
Hypertension	581 (68.6)
History of CVD	162 (19.2)
Smoking	
Never	324 (38.3)
Former	397 (46.9)
Current	125 (14.8)
Alcohol consumption*	
None	187 (22.1)
Low	469 (55.4)
High	190 (22.5)
Glucose-lowering drugs	191 (22.5)
Lipid-lowering drugs	304 (35.9)
Antihypertensive drugs	334 (39.4)
NSAIDs	74 (8.7)
NCV	
Tibial nerve, m/s	43.4 (4.9)
Sural nerve, m/s	47.6 (5.6)
Peroneal nerve, m/s	45.4 (4.7)
Sural SNAP amplitude (µV)	10.6 (6.7)

Continuous data are given as mean (SD) or median (IQR). Categorical variables are given as numbers and percentages (%).
Missing values: sex (n=3); age (n=3); BMI (n=3); waist circumference (n=3); HbA1c (n=3); glucose metabolism status (n=3); total cholesterol (n=3); triglycerides (n=3); hypertension (n=3); history of CVD (n=5); smoking (n=4); alcohol consumption (n=4); glucose-lowering drugs (n=3); lipid-lowering drugs (n=3); antihypertensive drugs (n=3); NSAIDs (n=3); tibial NCV (n=79); sural NCV (n=242); peroneal NCV (n=35); sural SNAP amplitude (n=235).
Missing values were excluded from percentage calculations and from calculations of mean (SD) or median (IQR).
*None defined as 0 glasses/week; low defined as ≤7 glasses/week for women and ≤14 glasses/week for men; high defined as >7 glasses/week for women and >14 glasses/week for men.
BMI, body mass index; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; NCV, nerve conduction velocity; NSAIDs, nonsteroidal anti-inflammatory drugs; SNAP, sensory nerve action potential.

Table 2 Association of leukocyte subsets with nerve conduction velocity (NCV) sum score (N=850)

		Model 1			Model 2			Model 3		
Leukocyte subset		**β**	**95% CI**	**P value**	**β**	**95% CI**	**P value**	**β**	**95% CI**	**P value**
Innate immunity	Monocytes	−0.004	(−0.029 to 0.020)	0.745	−0.010	(−0.034 to 0.015)	0.450	−0.007	(−0.032 to 0.017)	0.539
	Basophils	−0.096	(−0.190 to −0.003)	0.042	−0.115	(−0.208 to −0.022)	0.015	−0.116	(−0.210 to −0.023)	0.014
	Eosinophils	−0.005	(−0.031 to 0.020)	0.670	−0.006	(−0.032 to 0.020)	0.642	−0.008	(−0.033 to 0.017)	0.538
	Neutrophils	−0.005	(−0.011 to 0.001)	0.072	−0.004	(−0.011 to 0.002)	0.172	−0.002	(−0.009 to 0.003)	0.399
Adaptive immunity	Lymphocytes	0.007	(0.001 to 0.014)	0.036	0.006	(−0.001 to 0.013)	0.076	0.004	(−0.003 to 0.011)	0.225
	T cells	0.001	(−0.004 to 0.007)	0.565	0.002	(−0.004 to 0.008)	0.441	0.002	(−0.003 to 0.008)	0.326
	$CD4^+$ T cells	−0.007	(−0.011 to −0.002)	0.001	−0.006	(−0.011 to −0.002)	0.006	−0.006	(−0.010 to −0.001)	0.005
	$CD8^+$ T cells	0.006	(0.001 to 0.011)	0.007	0.006	(0.001 to 0.011)	0.023	0.005	(0.001 to 0.010)	0.022
	T reg cells	−0.009	(−0.036 to 0.017)	0.499	−0.014	(−0.041 to 0.012)	0.293	−0.011	(−0.038 to 0.015)	0.394
	B cells	−0.001	(−0.011 to 0.008)	0.827	−0.001	(−0.011 to 0.009)	0.777	−0.002	(−0.012 to 0.007)	0.585

NCV sum score is defined as a continuous variable calculated based on NCV available for at least one nerve.
Model 1: adjusted for sex and age (based on 850 participants).
Model 2: adjusted for model 1+glucose metabolism status (based on 847 participants).
Model 3: adjusted for model 2+BMI, HbA1c, total cholesterol, triglycerides, eGFR, history of hypertension, history of CVD, glucose-lowering drugs, lipid-lowering drugs, non-steroidal anti-inflammatory drugs, smoking and alcohol consumption (based on 845 participants).
BMI, body mass index; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c.

Sensitivity analyses

When a potential effect modification of glucose metabolism status on the association between percentages of leukocyte subsets and NCV sum score was assessed, no evidence for interaction was found (p values for all interaction terms were ≥0.367). Effect estimates for the associations stratified by glucose metabolism status are shown in online supplemental table 4. Moreover, the results of the fully adjusted model (table 2, model 3) remained unchanged when analyses were repeated with adjustment for waist circumference instead of BMI or with adjustment for age categories instead of age as a continuous variable (data not shown).

DISCUSSION

In a population-based sample of people with normal glucose metabolism, pre-diabetes and diabetes, we found that higher percentages of basophils and $CD4^+$ T cells were associated with lower NCV, whereas the association for $CD8^+$ T cells pointed into the opposite direction. These findings were independent of age, sex, demographic, lifestyle and clinical characteristics and were not modified by glucose metabolism status.

Associations between leukocyte subsets and DSPN

To the best of our knowledge, this study is the first one to systematically investigate associations between leukocyte subsets in peripheral blood and NCV. Our data implicate that both innate immunity (basophils) and adaptive immunity (T cells) may be linked to reduced NCV reflecting peripheral nerve dysfunction. No associations were found between leukocyte subsets and sural SNAP as a marker of axon degeneration. Overall, our findings corroborate data from the population-based KORA F4/FF4 cohort that identified multiple biomarkers of inflammation that were related to incident DSPN and that clustered in pathways suggesting a cross-talk between innate and adaptive immunity.[16] The main novelty of this study and the aforementioned KORA F4/FF4 study lies in the associations of components of the adaptive immune system with NCV and DSPN.

Our results relate a higher percentage of $CD4^+$ T cells to lower NCV. Early histological studies demonstrated an abundant presence of T cells in nerves of patients with DSPN,[23–25] but it remained unclear to what extent this observation would be relevant to the pathophysiology of DSPN. In this context, it is important to note that we previously identified the proinflammatory cytokines IL-6 and TNF-α as independent predictors of DSPN.[15] Although both cytokines are closely related to the activation state of the innate immunity, IL-6 also promotes the differentiation of naïve $CD4^+$ T cells, and TNF-α can be produced by activated $CD4^+$ T cells. In addition, both cytokines activate antigen-presenting cells, which in turn contribute to T cell activation. Therefore, evidence linking higher systemic levels of IL-6 and TNF-α to DSPN is in line with the association between higher percentages of $CD4^+$ T cells and lower NCV in our study. In contrast to neuropathies with autoimmune etiologies,[26] DSPN is considered to be mainly associated with long-term hyperglycemia, aging and obesity.[27–30] To be involved in the pathogenesis of DSPN, naïve $CD4^+$ T cells should be activated upon recognition of antigens presented by antigen-presenting cells such as dendritic cells, macrophages or B cells through class II major histocompatibility complexes (MHC-II). Indeed, recent in vitro and in vivo studies showed that in peripheral nerves, naïve $CD4^+$ T cells can be activated by Schwann cells, which can express MHC-II under inflammatory conditions.[31 32]

Interestingly, minor injuries caused by diagnostic sural nerve biopsies have been reported to result in transient autoreactive T and B cell responses.[33] A small study also suggested that patients with diabetes and DSPN may more frequently show cell-mediated autoimmunity than diabetes patients without DSPN.[34] However, this study did not clarify to what extent this autoimmune reactivity may be related to diabetes type.

Unlike $CD4^+$ T cells, a higher percentage of $CD8^+$ T cells was associated with faster NCV in our study. Given previous histological and experimental studies on DSPN and the implication of $CD8^+$ cells in other neuropathic conditions,[25 35 36] opposing roles of $CD4^+$ and $CD8^+$ T cells in the impairment of peripheral nerve function are difficult to reconcile. With our analyses, we assessed qualitative shifts in T cell populations, meaning that a rise in the percentage of $CD4^+$ T cells inevitably would implicate a decrease in the percentage of $CD8^+$ T cells relative to the total $CD3^+$ T cell population in the parent gate as shown in online supplemental figure 1. In any case, it is important to keep in mind that both $CD4^+$ and $CD8^+$ T cells are highly heterogeneous so that further investigations are necessary to identify which subpopulations could be related to peripheral nerve dysfunction. Our secondary analysis showed significant associations of $CD4^+$ and $CD8^+$ T cell percentages only with motor NCV, whereas early DSPN is strongly affected by both impaired sensory and motor NCV.[37] However, effect estimates for the individual nerves were very similar so that our study does not provide evidence that motor and sensory NCV may be affected differently.

Finally, any discussion of a T cell component of DSPN immediately provokes the question which antigen(s) may be responsible. Candidates are endogenous antigens such as neuronal, Schwann cell, myelin and nodal antigens. One hypothesis that may merit further investigation is that cellular nerve damage due to metabolic stress related to hyperglycemia, oxidative stress, obesity or aging-related stressors may trigger low-level cell-mediated autoreactivity that could perpetuate or even exacerbate the development of DSPN driven by metabolic and/or age-related risk factors. It is also possible that cell death due to microvascular dysfunction contributes to immune activation. It is tempting to speculate that this may partially explain the low efficacy of interventions to reduce metabolic stress in nerves so that anti-inflammatory interventions appear as an avenue that should be explored.[38]

Our study suggests that a higher percentage of B cells among lymphocytes may also be associated with lower NCV. However, this association was only found using the NCV sum score based on three nerves and after full adjustment for covariates. Many experimental studies have implicated B cells in the regulation of glucose metabolism and the development of type 2 diabetes.[39] The link between B cells and type 2 diabetes is most likely mediated by the capacity of B cells to act as antigen-presenting cells to T cells by their release of proinflammatory cytokines (among them IL-6 and TNF-α), which have been related to incident DSPN[15] and by their antibody production.[39] Direct cell contact between B cells and peripheral nerves appears unlikely given the absence of B cells in sural nerve biopsies in histological studies.[25] However, existing nerve damage may lead to danger signals being transferred via lymphatics to draining lymph nodes, where B cells capture and present free-floating antigen coming from the tissue and where B cell/T cell interaction mainly happens. With respect to cytokine release and antibody production, it was shown that B cells are involved in the pathophysiology of different inflammatory neuropathies,[26 40] but whether similar processes operate in DSPN is unclear. As mentioned above, one study pointed towards autoreactive B cells after diagnostic sural nerve biopsy.[33] Other studies found associations between peripheral neuropathy in patients with diabetes and autoantibodies against glycolipids (ganglioside) or antinuclear antibodies.[41 42] Altogether, these data appear too preliminary to decipher to what extent autoreactive B cells may exist in DSPN, but further studies in this field should be of interest.

Our study demonstrated also an inverse association between the basophil count and NCV. So far, data on granulocytes (neutrophils, basophils, eosinophils and mast cells) and DSPN are extremely limited. Available evidence from epidemiological studies exploring the involvement of granulocytes in DSPN is scarce, and two Chinese studies showed a positive association between the neutrophil-to-lymphocyte ratio and DSPN in patients with diabetes.[17 18] Since neither neutrophil nor lymphocyte counts were related to NCV in our fully adjusted model, we did not analyze this ratio. Additionally, it is interesting that 'granulocyte adhesion and diapedesis' was the top pathway emerging in the in silico analysis based on biomarkers of inflammation related to incident DSPN in the aforementioned KORA F4/FF4 cohort.[16] Although we are not aware of studies linking basophils to impaired NCV, it is noteworthy that one of their main products to be released on activation is histamine, which has been implicated as an important mediator in the development of neuropathic pain.[43] Our secondary analysis indicated that the inverse association between basophils and NCV was driven by the results for motor NCV, whereas early DSPN is strongly affected by impaired sensory NCV. This unexpected finding cannot be analyzed in more detail with the current dataset and merits corroboration in other cohorts.

Of note, we did not observe associations between circulating monocytes and NCV although previous studies implicated monocytes and macrophages in the development of DSPN and neuropathic pain in animal models of diabetes.[44–46] It is possible that monocytes and macrophages may be more relevant to the pathophysiology of DSPN at later stages of the disease, but this needs to be explored in longitudinal studies in humans.

Strengths and limitations

Our study has specific strengths. First, by exploring a panel of cells from both the innate and the adaptive immune system, our study offsets the limitation of previous studies and adds evidence on the role of the cellular component of the immune system in DSPN. Second, our study was based on NCV measurements, which represent a highly sensitive and specific test for DSPN even in the early disease stages in the absence of signs and symptoms. Third, the comprehensive phenotyping of participants of the Maastricht Study allowed us to control for multiple potential confounders in our linear regression analyses.

This study also has limitations that require consideration. It has a cross-sectional design so that the temporal relationship between immune activation and possible nerve damage cannot be inferred. Epidemiological studies like this one are also not able to address the issue of causality that may underlie the observed associations. Because of the potential for type I error due to multiple comparisons, our findings should be interpreted as exploratory. We only had data for immune cell counts in peripheral blood but no data on potential antigen specificity of lymphocyte clones or functional lymphocyte assays. Further analyses investigating immune cell function, cellular responses to immunological stimuli and analyses of cytokine and chemokine profiles will be required to gain further insights into the potential role of immune cells for NCV and DSPN. We also had no nerve or skin biopsies, so that histological studies were not possible. Finally, the Maastricht Study included participants from a specific region within the Netherlands, so that generalizability of our findings beyond European populations may be limited.

CONCLUSION

This study shows that higher percentages of $CD4^+$ T cells and basophils are associated with a lower NCV, while higher percentages of $CD8^+$ T cells are positively associated with NCV. These associations are independent of multiple confounders and not modified by glucose metabolism status. Overall, the results suggest that cells from both the innate and the adaptive immune system might be related to reductions in NCV and potentially to the development of DSPN. Although the scarcity of data in this field means that potential explanations for these associations must remain speculative, our results give rise to novel hypotheses about the communication between

the immune system and the peripheral nervous system in the context of DSPN.

Author affiliations
[1]Institute for Clinical Diabetology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine University Düsseldorf, Düsseldorf, Germany
[2]German Center for Diabetes Research (DZD), Partner Düsseldorf, Düsseldorf, Germany
[3]Department of Internal Medicine, Maastricht University Medical Centre+ (MUMC+), Maastricht, The Netherlands
[4]Cardiovascular Research Institute Maastricht (CARIM), Maastricht, The Netherlands
[5]Department of Nutrition and Movement Sciences, Maastricht University, Maastricht, The Netherlands
[6]NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, The Netherlands
[7]Department of Clinical Neurophysiology, Maastricht University Medical Center+, Maastricht, The Netherlands
[8]Division of Endocrinology and Diabetology, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany

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ORCID iDs
Haifa Maalmi http://orcid.org/0000-0002-2910-1142
Michael Roden http://orcid.org/0000-0001-8200-6382
Dan Ziegler http://orcid.org/0000-0001-8956-3552
Christian Herder http://orcid.org/0000-0002-2050-093X

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